A COMPARATIVE ANALYSIS OF THE INFLUENCE OF DEFENSIVE VENOM SPITTING ON THE COMPOSITION AND FUNCTION OF COBRA VENOMS

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By

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ABSTRACT

Snake venoms are a complex cocktail of toxin proteins, the evolution of which is driven foremostly by the need to swiftly incapacitate or immobilise prey. Spitting cobras possess a defensive use for their venom, unique to these snakes, in the ability to deploy their venoms as a spray projectile into the eyes of an assailant, and this ability has evolved three times in elapid snakes (venomous, bearing fixed, posteriorly-located fangs). Spitting cobras show differences to non-spitting cobras in fang morphology and in clinical pathology following bites to humans, suggesting that there are underlying toxin composition differences between spitting and non-spitting cobras. This work represents the first of its kind; a large-scale and multidisciplinary comparative analysis incorporating the true cobras (genus: *Naja*) and its spitting sister taxa, the rinkhals/ring-necked spitting cobra (*Hemachatus haemachatus*), with a focus on examining patterns of venom evolution associated with the ability to spit venom.

To do this, analysis of 17 species (14 species of *Naja*, *Hemachatus haemachatus*, and the outgroup elapid snakes *Walterinnesia aegyptia* and *Aspidelaps scutatus*), covering all lineages of spitting and non-spitting cobra, was undertaken at the transcriptome, proteome and functional levels and these put into evolutionary context by using phylogenetic statistical analyses. Additionally, the composition of spat venom versus venom extracted manually (simulating venom injected via biting) was compared to identify any control mechanisms at the morphological level that may alter venom composition when spitting.

The data here show that, while there are no differences in venom composition between spitting and non-spitting cobras at the transcriptome level, African spitters show evidence of a phospholipase A_2 (PLA_2) gene duplication. Additionally, spitting cobras have a significantly higher abundance of PLA_2 toxins than non-spitting cobras at the proteome level (Phylogenetic Generalized Least Squares, $t = 4.24$, $p = 0.0007$). Furthermore, cobra venom PLA_2 proteins group by lineage based on amino acid composition, with African spitting cobras and *Hemachatus haemachatus* forming one group, Asian cobras another, and African non-spitters a third.

These findings suggest that spitting cobra lineages have convergently upregulated PLA_2s in their venoms following divergent selection on PLA_2 amino acid composition between African and Asian cobras. African spitting cobras have upregulated the abundance of PLA_2 in the venom proteome through gene duplication, while Asian spitting cobras have upregulated PLA_2 abundance through posttranslational means. These data provide the first evidence that defensive uses of venom can drive the evolution of snake venom composition, and that the convergent emergence of a unique behavioural adaptation can result in convergent molecular evolution.

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I would like to thank my primary supervisor, Professor Nicholas Casewell, for overseeing my project and providing encouragement, advice and support throughout my degree. Additional gratitude is expressed to Dr. Laura-Oana Albuescu and Dr. Stuart Ainsworth for their eternal patience in educating me on laboratory protocols, and Dr. Kevin Arbuckle for access to his wealth of knowledge on statistical analysis in biology. I thank my family and friends, as well as the rest of my colleagues at the Centre for Snakebite Research and Interventions for being a continuing source of guidance, morale and companionship during my work. Finally, I would like to acknowledge the late Elizabeth Artin Kazandjian for enabling my studentship to be funded. Without your generous gift, this would not have been possible.

PUBLICATIONS ARISING FROM THIS WORK

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2. Slagboom, J., Mladić, M., Xie, C., Kazandjian, T. D., Vonk, F., Somsen, G. W., … Kool, J. (2020). High throughput screening and identification of coagulopathic snake venom proteins and peptides using nanofractionation and proteomics approaches. PLoS Neglected Tropical Diseases, 14(4), e0007802. https://doi.org/10.1371/journal.pntd.0007802

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ABREVIATIONS

3FTX - Three-finger toxin
ACN - Acetonitrile
AUC - Area under the curve
CRISP - Cysteine-rich secretory protein
CTL - C-type lectin
CTX - Cytotoxic three-finger toxin/Cytotoxin
ESI - Electrospray ionization
CGR - Centre for Genomics Research
Kunitz - Kunitz-type serine protease inhibitor
LC-MS/MS - Liquid chromatography–tandem mass spectrometry
LTX - Long neurotoxin
mAUCC - Mean area under the concentration curve
MS/MS - Tandem mass spectrometry
MTX – Muscarinic toxin
NGF - Nerve growth factor
NLP – Neurotoxin-like protein
NP - Natriuretic peptide
PC - Principal component
PCA - Principal component analysis
PCoA - Principal coordinate analysis
PGLS - Phylogenetic generalized least squares
pI - Isoelectric point
PLA_2 - Phospholipase A_2
PSE - Post-spitting extraction
RIN - RNA Integrity Number
SDS - Sodium dodecyl sulfate
SDS Page - Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SEM - Standard error of the mean
SP - Serine protease
STX – Short neurotoxin
SVMP - Snake venom metalloproteinase
TEMED - Tetramethylethylenediamine
TFA - Trifluoroacetic acid
TRIS - Tris(hydroxymethyl)aminomethane
UNK - Unknown
WHO - World Health Organisation
WTX – Weak neurotoxin

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1. INTRODUCTION

1.1. Snake venoms

Venom has evolved in a multitude of animal groups, including cephalopods, Cnidaria, fish, arthropods, gastropods, reptiles and even mammals (see fig 1.1.). It can be defined as a chemical secretion produced by a specialised gland, which causes disruption to metabolic processes when injected into a target or inflicted through an open wound. Venom can be used in prey acquisition or to defend against predation, and a number of morphological adaptations have emerged as venom delivery systems, including beaks, spines, barbs and fangs (Casewell et al., 2013).

The origin of venom in squamates is a complex issue that currently centres around two main theories. The first is that venom toxin genes have a single origin at the base of the "toxicofera"; a proposed monophyletic group formed of snakes (Serpentes) and the lizard suborders Anguimorpha and Iguania (Fry et al., 2009; Fry et al., 2006), with venoms being further shaped through recruitment and/or loss of different toxin families (Fry et al., 2012). The contrary argument is that there was no single venom-using ancestor and that venom toxins have evolved multiple times throughout the squamate tree (Hargreaves et al., 2015; Hargreaves et al., 2014). One argument for the single-origin hypothesis has been that the above lizard groups appear to have homology in both the dental secretory glands and the toxin genes expressed in the salivary transcriptome (Fry et al., 2009; Fry et al., 2006, Koludarov et al., 2012), however this has been disputed by the presence of toxin gene families being expressed in body tissues of both toxicoferan and non-toxicoferan squamates (Hargreaves et al., 2014). The issue may then lie in the definition of a truly "venomous" squamate, as salivary proteins from varanids and anguimorphs have been shown to have pharmacological effects (Fry et al., 2006) but their primary method of prey incapacitation appears to be through direct force rather than envenomation (Sweet, 2016). Whatever the origin or origins of squamate venom, it is generally agreed upon that their venom systems evolve through the recruitment of non-toxic genes from other body tissues into specialised secretory glands, with these genes eventually undergoing modification into toxic forms.

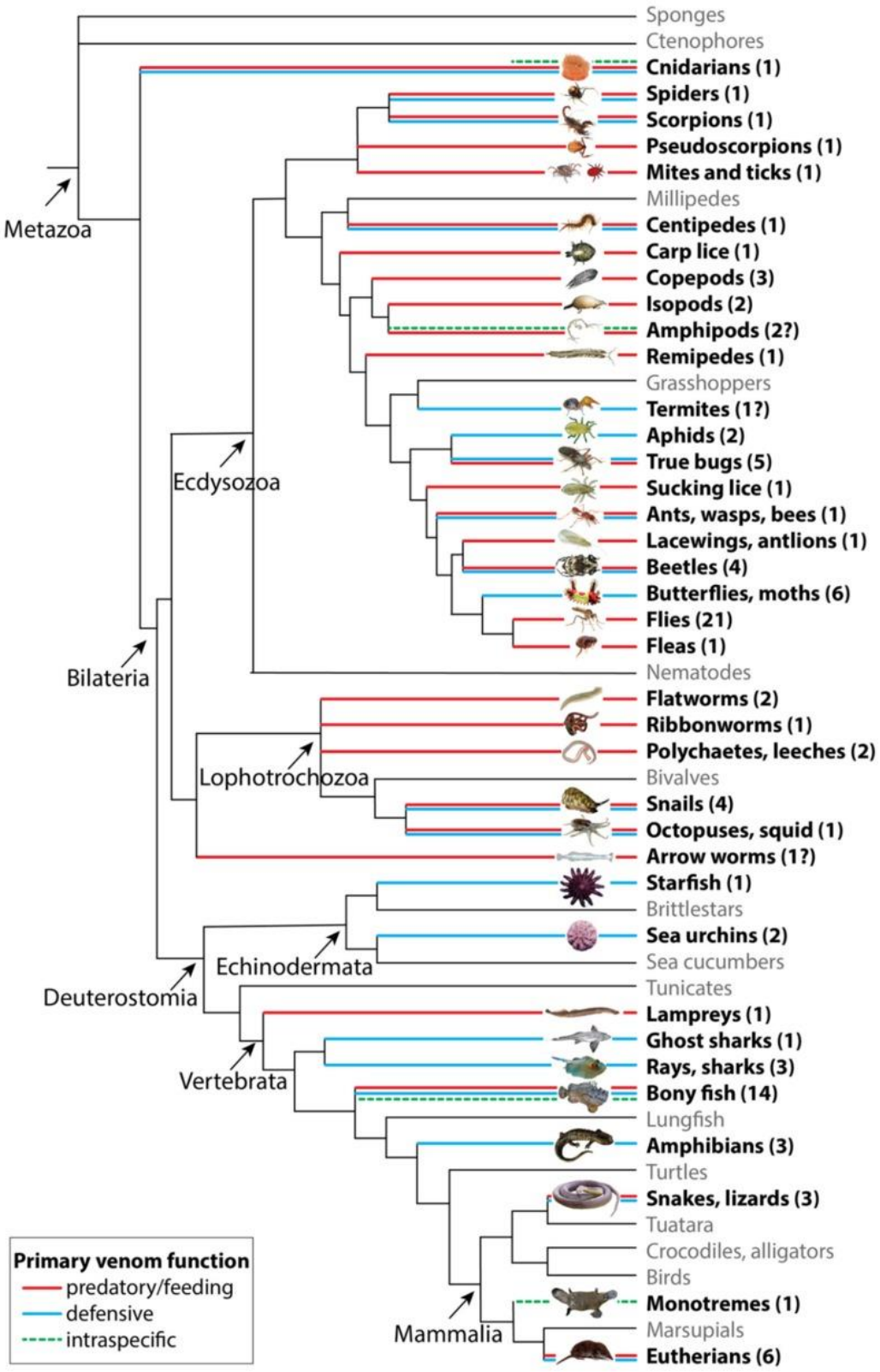


Fig. 1.1. A schematic tree representing the evolution of venom across animal taxa. Lines highlighted in red indicate predatory uses of venom, blue, defensive uses of venom, and green for intraspecific competition. Source: Schendel et al., 2019.

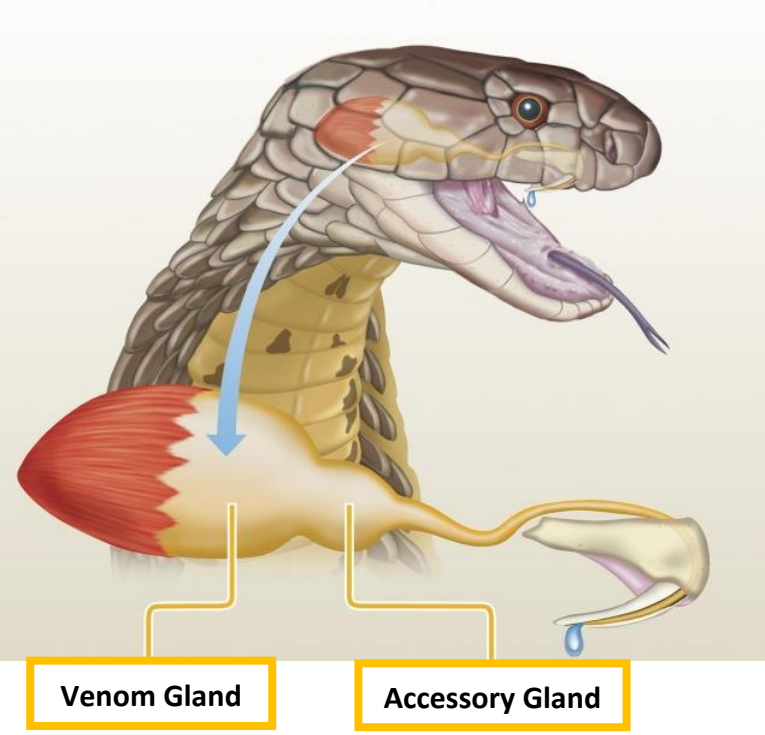


Figure 1.2. Basic outline of the snake venom system. Image modified from Vonk et al. 2013.

Venomous snakes belong to the several families of the monophyletic group Endoglyptodonta, with the most well-known venomous families of this group being Viperidae, Elapidae and Colubridae (Burbrink et al., 2020; Zaher et al., 2019). Venoms from these snakes are used mainly for the purpose of prey acquisition. However, the threat of envenomation through biting can be used as a deterrent against aggressors, which is seen in the case of snakebite in humans. Snake venoms have evolved to become a complex cocktail of toxins with the purpose of subduing prey as quickly as possible to avoid prolonged confrontation and potential injury. It is typically produced in the venom glands, which are located posteriorly to the eye at either side of the head, however toxin-encoding transcripts have also been found in the accessory glands of some snakes (Vonk et al., 2013). For many snakes, venom is expelled from the venom glands via muscular compression, forcing the venom down the venom duct, through an accessory gland (in elapid and viperid snakes, see Fig. 1.2) and then into the fangs. In colubrid snakes the venom gland is occasionally referred to as the Duvernoy's gland, as it was previously believed to be distinct from

the venom glands in the advanced venomous snakes, however this was found to be a false distinction as the venom glands of vipers and elapids both independently evolved from this gland (Fry et al., 2003) and thus the term 'venom gland' can be used to accurately define the homologous venom-secreting glands of all caenophidian snakes.

Venom has been shown to be metabolically costly to produce, with metabolic activity increasing significantly in snakes that have just been milked (McCue, 2006) and rattlesnakes have been shown to carefully meter their venom depending on the size of prey (Hayes, 1995). However, the cost of replenishment is suggested to be minimal in comparison to those of shedding and digestion (Pintor et al., 2010) and the ongoing metabolic costs of the venom delivery systems and replacement of fangs is unknown. Snakes have adapted their dentition in a highly variable manner to deliver their venom, of which 3 fang types are the most prominent: opisthoglyphous, proteroglyphous and solenoglyphous (Fig. 1.3.). In opisthoglyphous snakes the venom-delivering fangs (or modified grooved teeth) are located at the back of the mouth, and it is for this reason that they are referred to as 'rear-fanged snakes', and snakes that typically display this type of delivery system are in the colubrid family. Venom is injected into the target through a low-pressure chewing motion (Kardong & Lavin-Murcio, 1993). Proteroglyphous snakes are a type of front-fanged snakes that have hollow, fixed fangs positioned at the anterior end of the maxilla (Bogert, 1943). This system of venom delivery is unique to the elapid snakes. Solenoglyphous snakes are also front-fanged, however their fangs are not fixed in position, and the maxilla is highly reduced, with no other teeth present (Bogert, 1943). When the mouth is closed, the fangs lay flat against the roof of the mouth. When the mouth is opened, the maxilla pushes the fangs forward and out, allowing the snake to bite deeply. This highly-specialised fang morphology is unique to vipers. Two less common delivery systems are that of the genus *Atractaspis* and Xenodontine snakes. *Atractaspis* are technically 'front-fanged' snakes, however, they have fangs that can be projected laterally from the mouth when closed, that have evolved as a result of the constraints of a subterranean lifestyle (Shine et al. 2006; Underwood & Kochva, 1993). Front-fanged systems have therefore evolved three times independently

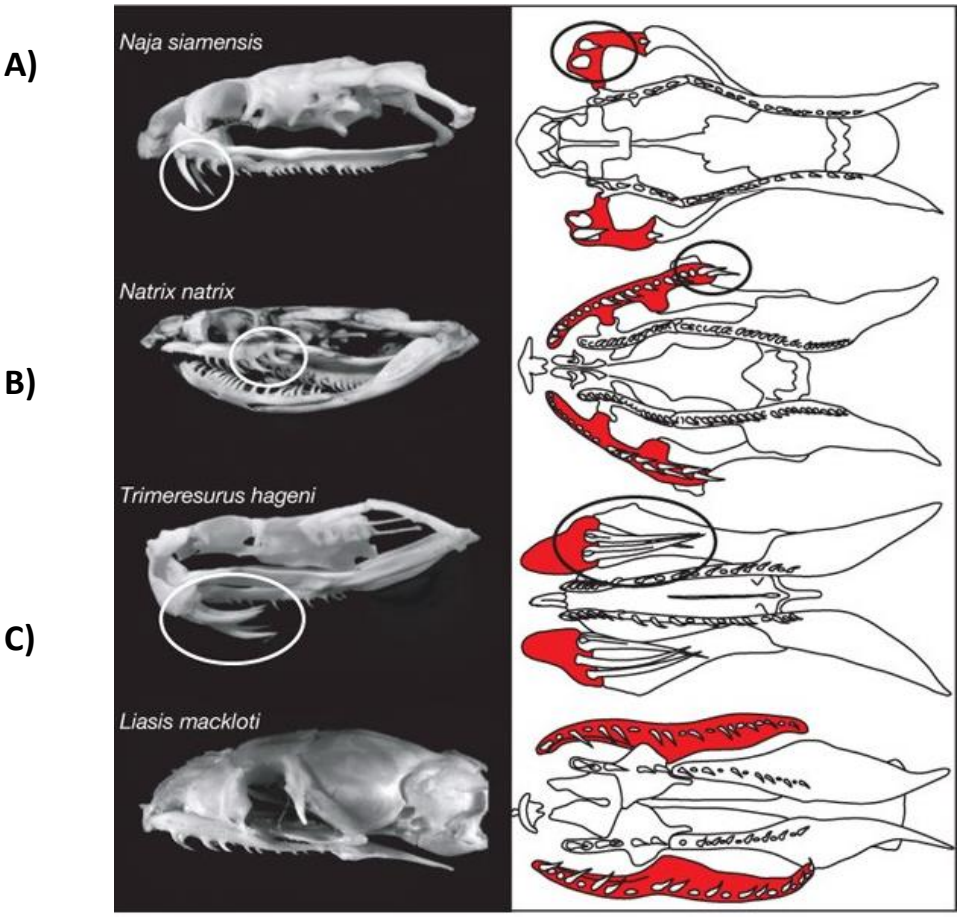


Figure 1.3. The venom delivery systems of snakes. A) the proteroglyphous front-fanged elapid, *Naja siamensis* **B)** the rear-fanged/opisthoglyphous colubrid, *Natrix natrix*, **C)** the solenoglyphous front-fanged viper, Trimeresurus hageni. Image modified from Vonk et al., 2008.

(Vidal, 2002; Vidal & Hedges, 2002), however they all develop from the same tissues during embryonic development and are therefore homologous structures (Vonk et al. 2008). This could imply that the structure and development of the snake maxilla preadapts it to developing more advanced venom delivery systems, though there is evidence to suggest that the venom system has evolved to become more specialised in conjunction with the recruitment of venom toxins (Fig. 1.4.), with each front-fanged snake group having at least one toxin recruitment event. Xenodontine snakes are rear-fanged but, unlike venomous colubrids, their fangs are sharply ridged and blade-like and venom is secreted into the oral cavity before being drained into the open wound caused by these fangs (de Olivera et al. 2016). In summary, in rear-fanged snakes, the fangs develop from an independent dental tissue and retain their

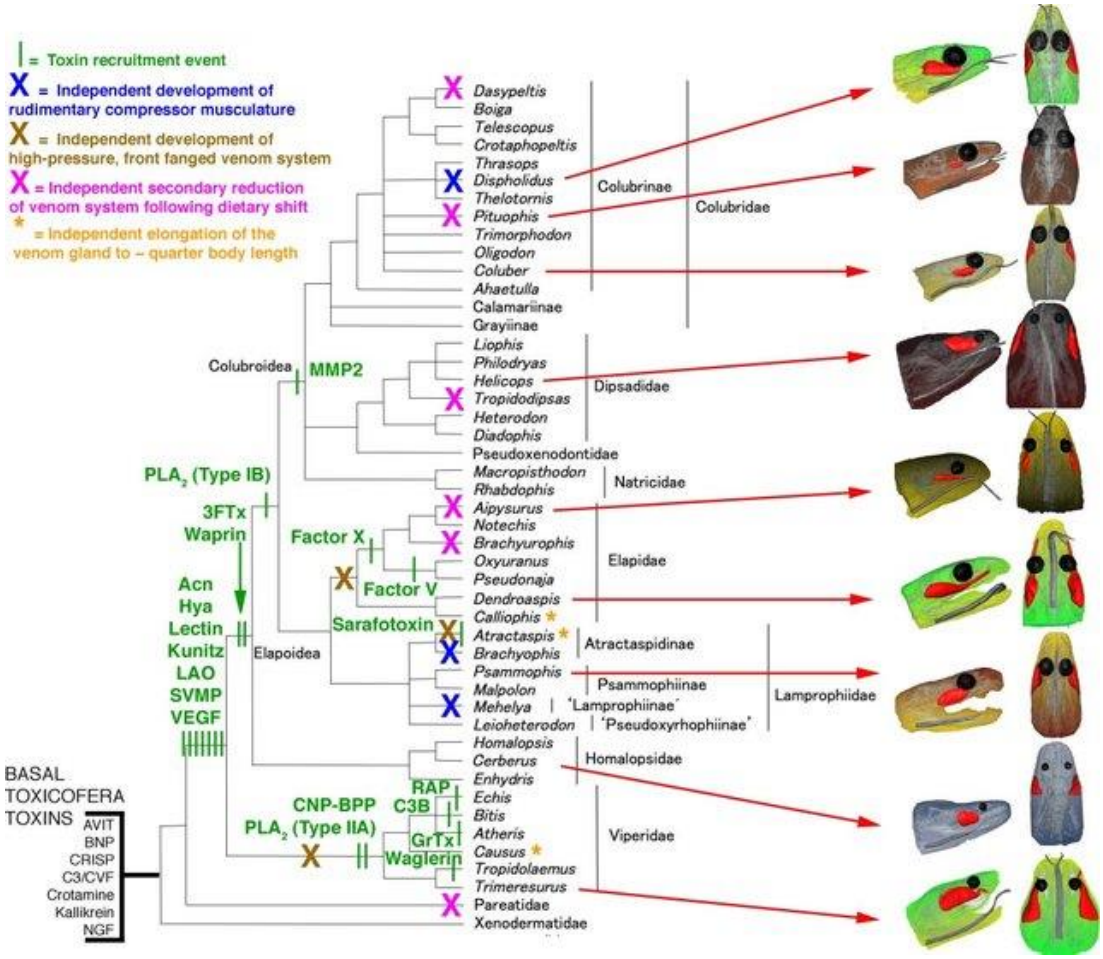


Figure 1.4. Cladogram showing the toxin recruitment events and development of the venom delivery system in advanced snakes. Image from Fry et al. 2008.

posterior position in the mouth post development, whereas the fangs migrate anteriorly during embryonic development in the front-fanged species (Vonk et al., 2008). Of the venomous snakes, the majority that are medically relevant to humans belong to front-fanged families, with only a few non-front fanged species capable of causing lethality (e.g. boomslang, *Dispholidus typus*; twig snakes, *Theletornis* spp.; the keelbacks, *Rhabdophis* spp.). It may be that the evolution of a more advanced venom delivery system promotes the evolution of more deadly venom, or that venom is simply injected deeper into tissues and at higher quantities in front-fanged snakes.

1.2. The Global impact of snakebite

As alluded to above, snakebite can have severe medical consequences for envenomed people. There are estimated to be a minimum of 421,000 snakebites and 20,000 resulting human deaths each year (Kasturiratne et al., 2008), with some estimates suggesting a more realistic figure of 1,841,000 envenomings and 125,000 deaths a year (Chippaux, 1998; Kasturiratne et al., 2008). Reliable estimates of snakebite cases are difficult to attain due to data being reliant on household surveys and hospital statistics (Chippaux, 1998). Individuals can be reluctant to divulge information on snakebite and even hospitals sometimes fail to record the occurrences and fatalities caused by snakebite (Fox et al., 2006). As a result, estimates are likely to be higher than predicted, and recent estimates place approximately 519 million people as being at high risk of snakebite and associated mortality (Longbottom et al., 2018). It is due to these factors that snakebite is now considered by the World Health Organisation (WHO) to be a neglected tropical disease.

Snakebite is often described as a disease of poverty, having been demonstrated to particularly affect areas of the world in which there is limited medical aid, little education around snakes and snakebite and a lack of suitable tools and clothing for working in areas that may contain venomous snakes (Harrison et al., 2009). This is reflected in the high incidence and fatality rates in Asia, Latin America and Sub-Saharan Africa (see Table 1.1. & Fig. 1.5.), whose high population densities also compound the issue of snakebite. In South Asia, there is a huge disparity in the time to receive treatment for snakebite, and over 50% of first aid practices for snakebite are incorrect, potentially causing further damage (Alirol et al., 2010). Additionally, up to 80% of people seek medical aid from traditional practices before resorting to modern medicine (Chippaux, 1998), causing further delay in treatment times. The issue of snakebite is worsened by a now critically low level of available antivenom, and antivenom that is available is usually highly expensive and, in the case of some antivenoms, is not effective at neutralising envenomation across species (Félix-Silva et al., 2017; Harrison et al., 2017; Leong et al., 2015; Maduwage et al., 2016), which can lead to a distrust of medical services and a higher number of people resorting to traditional medicine or local ‘healers’. As around half of all bite victims are farmers,

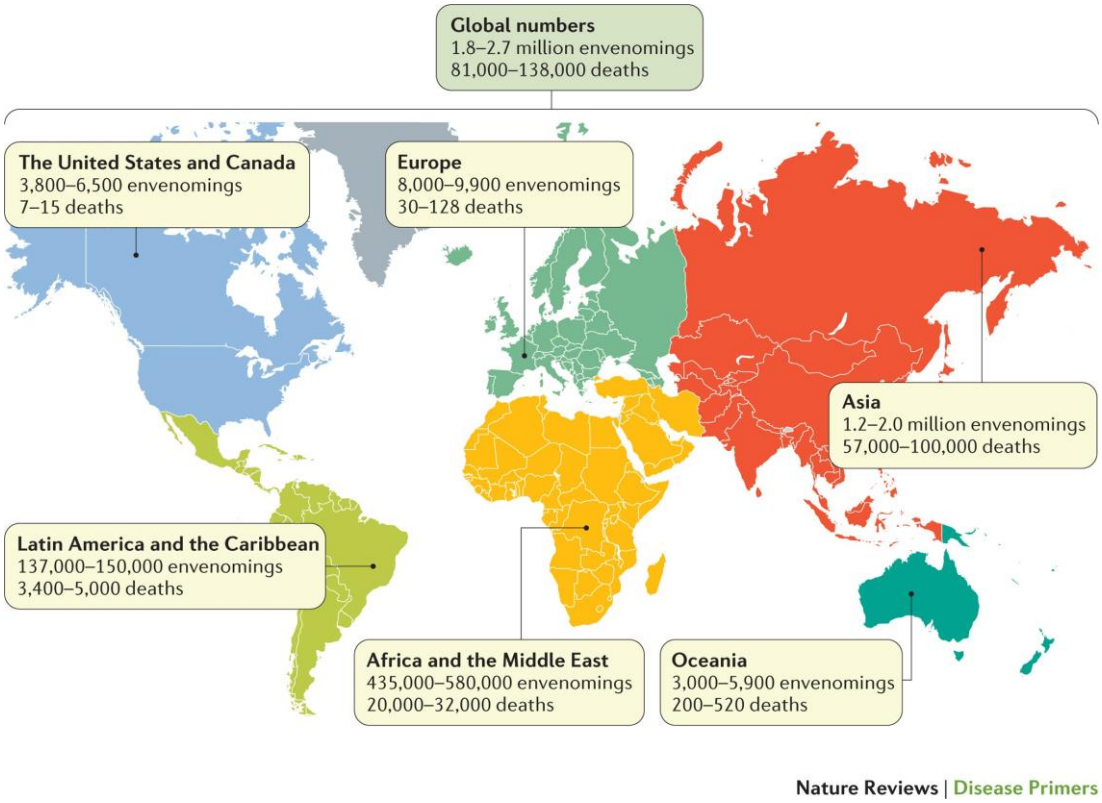


Figure 1.5. An estimate of snakebite morbidity across the globe. Taken from Gutiérrez et al. 2017.

Table 1.1. Global snakebite statistics from the year 2007. Modified from the most recently-available information from the knoema database (https://knoema.com/ueecokg/global-snakebite-statistics-may-2014).

Geographical Region	Number of cases (average)	Number of deaths (average)
Asia	503843	36519
Sub Saharan Africa	255132	17824
Latin America	58721	1243
Caribbean	4568	634
Oceania	2498	371.5
Europe	6822.5	86.8
North Africa/Middle East	41713	61.3
North America	3271	5.9
Australasia	1179.5	2.95
World	1133425	56916

the majority male and between the ages of 10-40 (Alirol et al., 2010; Sharma et al., 2003), snakebite can have a significant effect on resource production in poorer areas. The study of snake venom evolution can have implications for the way antivenoms are synthesised as well as the treatment of snakebite victims and thus is a medically-important undertaking. With an improved understanding of snake venom composition and evolution, more effective antivenoms can be synthesised and administered and better hospital treatments advised.

The majority of severe bites are caused by viperid and elapid snakes, for example, in India, which suffers the greatest burden of snakebite, 4 snakes responsible for the majority of envenomings are members of these families; *Naja naja* and *Bungarus caeruleus* (elapids), and *Daboia russelii* and *Echis carinatus* (vipers). Not all snake bites result in envenomation, and those in which venoms are not injected are known as 'dry bites', though such bites are rare (Bucaretchi et al., 2006; Spano et al., 2013). The symptoms of snakebite envenoming can vary extensively depending on the species of snake responsible for the bite. The severity of envenomation also relies on socioeconomic factors such as the capability and proximity of medical assistance and the availability of antivenom treatments. In general, bites from vipers cause extensive local swelling, haemorrhaging, coagulopathies and necrosis, and can occasionally induce cardiac arrest through cardiotoxic means. Elapids tend to have neurotoxic symptoms (Gutiérrez et al., 2017), via venom toxins perturbing the neuromuscular junctions, resulting in paralysis, which can be fatal if the respiratory organs become paralysed. The medically-important colubrids (boomslang, twig snakes, keelbacks) cause disturbances in haemostasis (Slagboom et al., 2017). The extensive variation in symptoms of envenoming are fundamentally the result of differences in the toxin composition of different snake species, which in turn is the result of variable evolutionary processes acting on snake venom toxins in different snake lineages.

1.3. Venom composition and evolution

In snakes, the proteins that form the toxic biological mixtures known as venoms are believed to have been recruited into the salivary glands from non-toxic forms expressed in other body tissues. As the salivary glands gradually evolved into what

we recognise today as venom glands, the co-opted proteins are thought to have adapted, via accelerated rates of sequence evolution, to increase their toxicity and neofunctionalise (Brust et al., 2013). Moreover, many snake venom toxins are thought to have diversified by the birth and death process of gene evolution, by which frequent gene duplication events have seemingly generated a suite of related toxin genes that may act synergistically to exert toxic effects (Fry *et al.*, 2003; Casewell *et al.*, 2013).

Following their recruitment for a role in venom, the structure of some proteins has changed to such an extent that they form different protein groups. An example of this is the snake venom metalloproteinase (SVMP) family of snake venoms, which evolved from ADAM (a disintegrin and metalloprotease) and ADAM-TS (ADAM with thrombospondin motifs) proteins by losing the thrombospondin, EGF-like, transmembrane and cytoplasmic domains (Casewell et al., 2015). The SVMPs are further split into 3 groups: P-III SVMPs, which retain all subdomains, P-II SVMPs, which have lost the cysteine-rich domain, and P-I SVMPs, which have lost both the the cysteine-rich domain, and the disintegrin, leaving only the prodomain and the metalloproteinase domains. P-III SVMPs can be found in vipers, elapids and colubrids but P-II and P-I SVMPs are only found in vipers, with P-Is restricted to a small handful of viper groups. Different venom toxins can have vastly different pathological effects; SVMPs are associated with the disruption of haemostasis (Moura-da-Silva et al., 2007), causing blood clots, local necrosis and haemorrhaging, with groups P-III and P-II being haemorrhagic and fibrinolytic and P-I fibrinolytic only (Bernardoni et al., 2014). It is for this reason that vipers bites are generally associated with necrosis and clotting.

Elapids and colubrids are more neurotoxic due to the abundance of three finger toxins (3FTXs) in their venoms. 3FTXs are non-enzymatic proteins that are comprised of 60-74 amino acids (Kini & Doley, 2010), containing 4-5 disulphide bridges, of which 4 are conserved and form the hydrophobic core from which the 3 finger-like loops branch off (Tsetlin, 1999). The original 3FTXs were isolated from the many-banded krait (*Bungarus multicinctus*) in 1963 (Chang & Lee, 1963) and were found to be strongly neurotoxic, causing paralysis and ptosis in human victims, and 3FTXs are mainly confined to the Elapid family of venomous snakes. These

neurotoxins, referred to as α-neurotoxins, bind to nicotinic acetylcholine receptors (nAChRs) and can be classified into “short” or “long” depending on whether the amino acid chain contains a fifth disulphide bridge (Barber et al., 2013). Elapids have experienced a radiation of the 3FTX family, resulting in the development of a cytotoxic/cardiotoxic group and the diversification of the neurotoxic group into different subgroups (Fry et al., 2003; Kini & Doley, 2010), such as muscarinic toxins, which bind to distinct types of muscarinic acetylcholine receptors (mAChRs; Adem *et al.*, 1988) and fasciculins that bind to and inhibit acetylcholinesterase (Cervenansky et al., 1991). This diversity is due to multiple gene duplications and subsequent radiation of the 3FTX family (Fry et al., 2003), and this is thought to result from accelerated evolution due to exon switching (Doley et al., 2008). This radiation has even resulted in several types of 3FTXs that are genera-unique such as the 3FTX dimer Hemexitin, an inhibitor of the blood coagulation factor VIIa that is found uniquely in Hemachatus haemachatus (Banerjee et al., 2007).

One of the remarkable results of the 3FTX radiation is the neofunctionalization of a cytotoxic subfamily (Dufton & Hider, 1988). These go by many names, including “cardiotoxins”, “cytotoxins”, “Direct Lytic Factors” and “Cobramines” (Lee et al., 1972), though these last two have been largely forgone in modern literature. As the names suggest, these proteins have cytotoxic and cardiotoxic effects (Dufton & Hider, 1988; Sun & Walker, 1986) with cytotoxic effects being caused by cationic residues on each of the three loops (Kini, 2011). CTXs are homologous to short neurotoxins (Dufton & Hider, 1983), suggesting gene duplication and neofunctionalization of this 3FTX group, and the emergence of cytotoxic activity has been linked to the evolution of hooding in elapids (Panagides et al., 2017). Attempts have been made to further resolve cytotoxins (CTXs) into type I and type II CTXs (Fry et al., 2003), with type II being unique to Hemachatus haemachatus, however no functional difference between subgroups has thus far been identified.

Another large snake venom family is Phospholipase A_2 (PLA_2s); a group of enzymes that catalyse phospholipid hydrolysis (Dawson, 1963). PLA_2s are common constituents of elapid venoms but can be found in all groups of venomous snakes to some degree (Mackessy, 2010; Tasoulis & Isbister, 2017). Snake venom PLA_2s

catalyse the hydrolysis of phospholipids to form a fatty acid and lysophospholipid (Dawson, 1963). Elapid PLA_2s, also known as group IA PLA_2s, are 115-120 amino acids long, contain 7 disulfide bridges and are distinguished from viperid PLA_2s by a distinctive "elapid loop" (Danse et al., 1997). These PLA_2s have a plethora of functions due to their rapid evolution (Sunagar et al., 2015a) but many act as presynaptic neurotoxins (Ranawaka et al., 2013). It therefore may be due to this functional plasticity that expression in the transcriptome is so varied, with some species using primarily 3FTXs as their neurotoxic agent and others using PLA_2s, or PLA_2s in conjunction with 3FTXs to attack prey nerve systems (Bicher et al., 1965) or cause cytotoxicity. Group II PLA_2s can be found in vipers and these proteins have diverse pharmacological effects, including anticoagulation, necrosis, myotoxicity, cytotoxicity and neurotoxicity (Sunagar et al., 2015b). There are other toxins responsible for causing pathological effects (see Table 1.2.) but these are often less prominent than the families of toxins described above.

The expression and diversity of these toxin proteins can differ extensively in venomous snakes. As described previously, toxins can differ between taxonomic clades, with viper groups possessing more derived SVMPs, group II PLA_2s and no 3FTXs, elapids possessing primitive SVMPs, group I PLA_2s and highly derived and advanced 3FTxs, and venomous colubrids having the more primitive 3FTXs, PLA_2s and SVMPs (Tasoulis & Isbister, 2017). Venom composition can also vary between species (Pla et al., 2017) and subspecies (Calvete et al., 2010). Differences in expression of venom toxins and composition of venom can even differ within a species, between geographically-isolated populations (Ali et al., 2013; Williams et al., 1988). Additionally, ontogenetic changes in venom are not uncommon; in *Naja kaouthia*, juveniles were found to have more toxin proteins of higher molecular mass than adults, and there was a significant difference in phospholipase A_2, phosphodiesterase and acetylecholinase activities between juvenile and adult specimens (Modahl et al., 2016), and ontogenetic changes in venom toxicity for different prey items has been found in *Bothrops insularis* (Zelanis et al., 2008). This may be due to differences in prey type and availability between adults and juveniles, which has shown to cause differences in venom variability in adults of some species (Daltry et al., 1996), and in the *Echis* genus toxicity of venoms was found to be strongly associated with the

percentage of diet made up by arthropods (Barlow et al., 2009). Contrarily, in littermates of *Bothrops jaracara* there are differences in venom composition between sexes (Menezes et al., 2006), which presumably would feed on similar prey as hatchlings.

Due to the large number of factors that can affect venom composition and pathology, the question arises of whether similarities in these characteristics can be seen between taxonomic groups of venomous snakes; whether evolution has selected for convergence in venom composition.

1.4. Cobras and the subdivision of Naja

Cobras are a monophyletic group of proteroglyphous (fixed front-fanged) venomous snakes that consist of several genera plus the "true cobras", genus *Naja* (Slowinski & Keogh, 2000), with *Aspidelaps* and *Walterinnesia* making up the most basal cobra

Table 1.2. The types and functions of some clinically important snake venom toxins. Adapted from Warrell (2010).

	Example of toxin	Snake	Function
Three-finger-fold polypeptide toxins	α bungarotoxins	*Bungarus* spp (other *Elapidae, Colubridae*)	Paralysis by blocking nicotinic acetylcholine receptors
Angiotensin-converting enzyme inhibitors and bradykinin-potentiating peptides	..	*Viperidae*	Hypotension
Acetylcholinesterase	..	*Elapidae*	Paralysis by destroying acetylcholine
Anticholinesterase	Fasciculins	*Dendroaspis* spp	Paralysis (with dendrotoxins) by depolarising neuromuscular block
Disintegrin and metalloproteinase (ADAM)	Haemorrhagins (atrolysins, jararhagin); procoagulants (fibrolase, ecarin, Russell's viper venom factor-X activator)	*Viperidae, Elapidae*	Endothelial damage, bleeding, necrosis
AVIT sequence cysteine-rich proteins	Mamba intestinal toxin (prokineticin)	*Dendroaspis polylepis*	Painful gut spasm, hyperalgesia, CNS effects
Cobra venom factor, complement C3	Cobra venom factor	*Elapidae, Viperidae*	Tissue damage
Small basic myotoxic peptides	Crotamine and crotasin	*Crotalus durissus* subspecies (some circumscribed geographical populations)	Muscle necrosis and spasm
Calcium dependent-type galactose-binding lectins	Rhodocytin	*Calloselasma rhodostoma* (and other *Viperidae, Elapidae*)	Platelet effects
Cysteine-rich secretory proteins	..	*Elapidae, Viperidae, Colubridae*	Smooth muscle inhibition
Cysteine proteinase inhibitors	Cystatin	*Viperidae, Elapidae*	Inhibit metalloproteinases
Endothelins	Sarafotoxins	*Atractaspis* spp	Hypertension, myocardial effects
Factor-V, factor-X activators	..	*Viperidae*, Australasian *Elapidae*	Coagulopathy
Kallikrein (kininogenase) serine proteases	..	*Viperidae*	Hypotension
Kunitz-type proteinase inhibitors	Dendrotoxins	*Dendroaspis* spp (and other *Elapidae*)	Depolarising neuromuscular block (inhibition of circulating serine proteases)
L-amino oxidase	..	All	Apoptosis
Natriuretic peptides	..	*Elapidae*: atrial-type and brain-type; *Viperidae*: C-type	Hypotension
Nerve growth factor	..	Many	Not known
Phospholipases A_2	β bungarotoxins	*Bungarus* spp (many phospholipases A_2 in venoms of most snakes)	Paralysis by presynaptic block and destruction of nerve terminals, myotoxicity, haemolysis, inflammation, necrosis, platelet effects
Vascular endothelial growth factor (VEGF)	VEGF-homologous potent hypotensive factor	*Viperidae*	Endothelial damage, permeability, oedema, hypotension

Table: Some groups of snake venom proteins and peptides of scientific and clinical importance

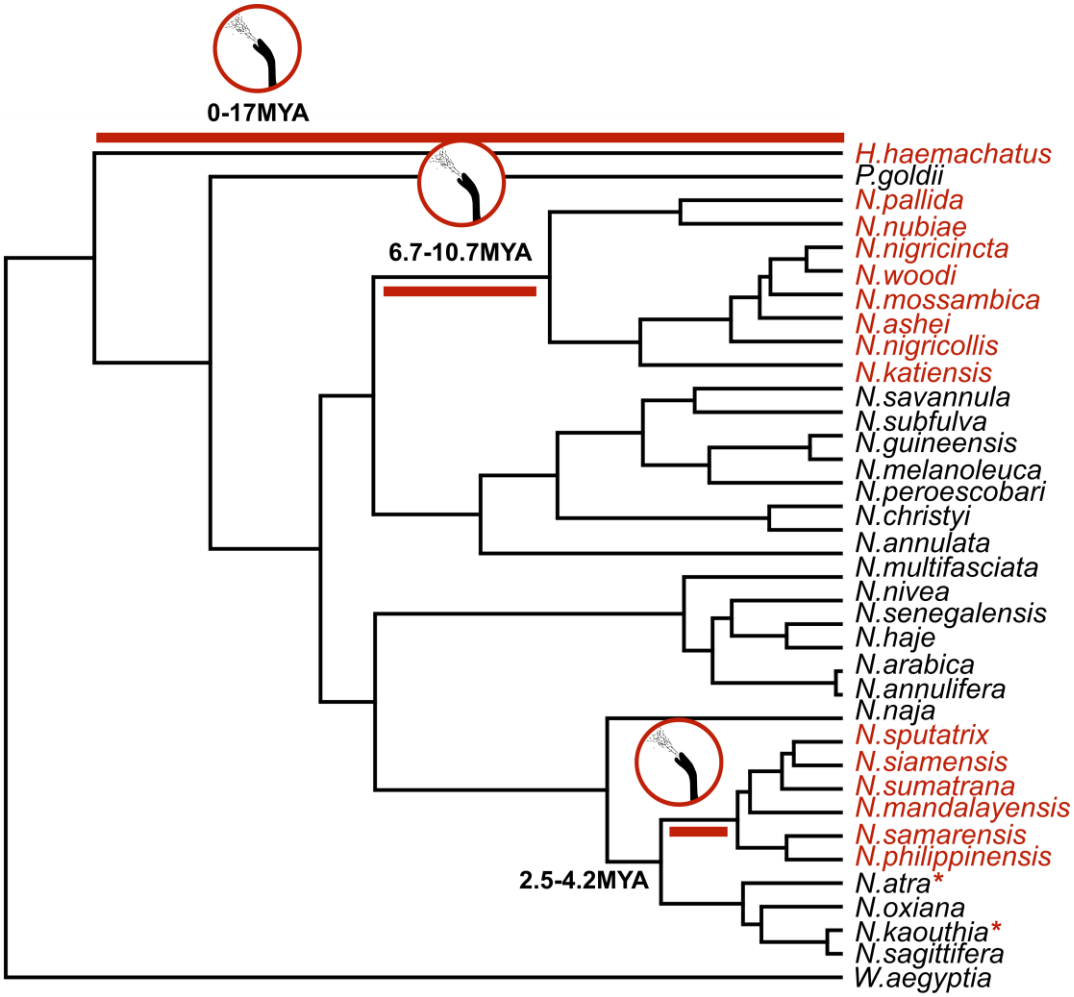


Figure 1.6. A phylogenetic species tree of the cobras. Boldened text indicates species that were sampled in this study and red text highlights spitting species. Red arrows indicate origin points for spitting behaviour (not to time scale). Species tree pruned from a larger elapid species tree provided by Wüster. *There are some reports of spitting in these species (Paterna, 2008; Santra et al. 2017) but they are generally considered to be non-spitting.

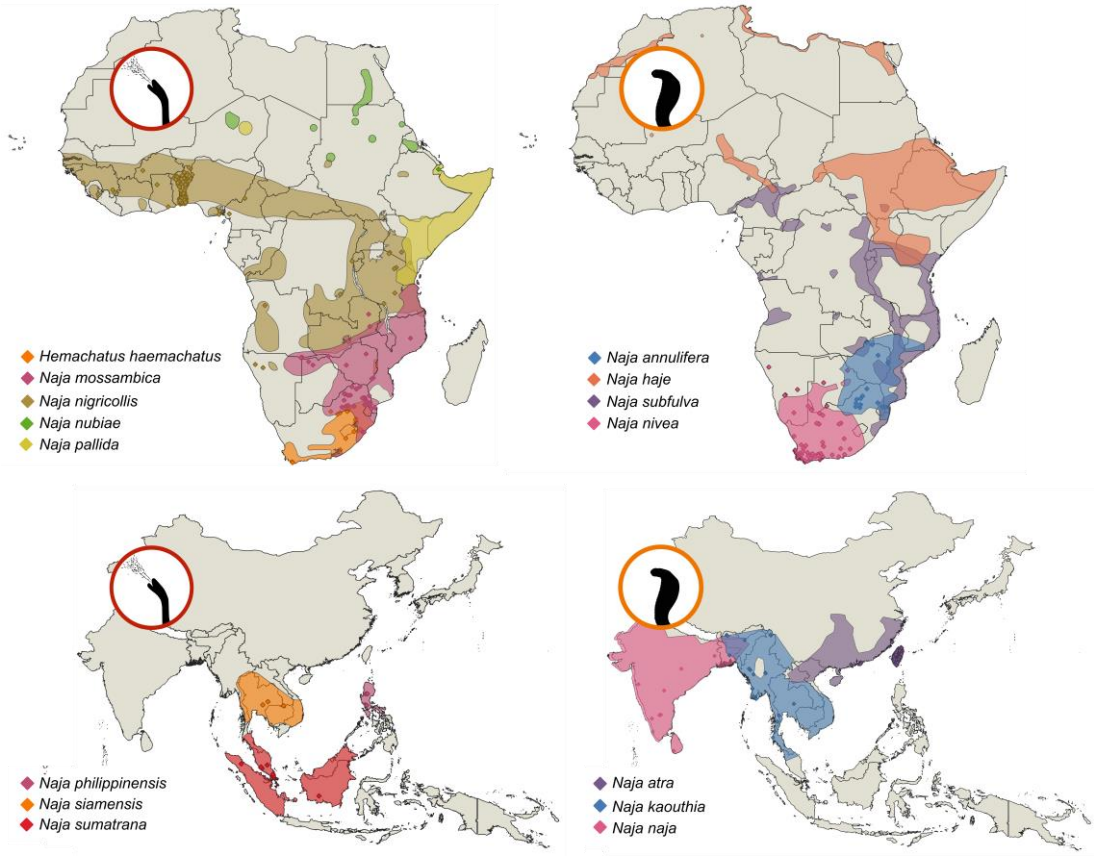


Figure 1.7. The global distribution of the true cobras (*Naja*) and the spitting cobra, rinkhals (*Hemachatus haemachatus*), used in this study. Distribution data was acquired from the following databases: the Global Biodiversity Information Facility (GBIF), Biodiversity Information Serving Our Nation (BISON), iNaturalist, ecoengine and VertNet and were overlaid on global shapefiles using the QGIS software. Hand-drawn sections were undertaken using the IUCN and World Health Organization databases.

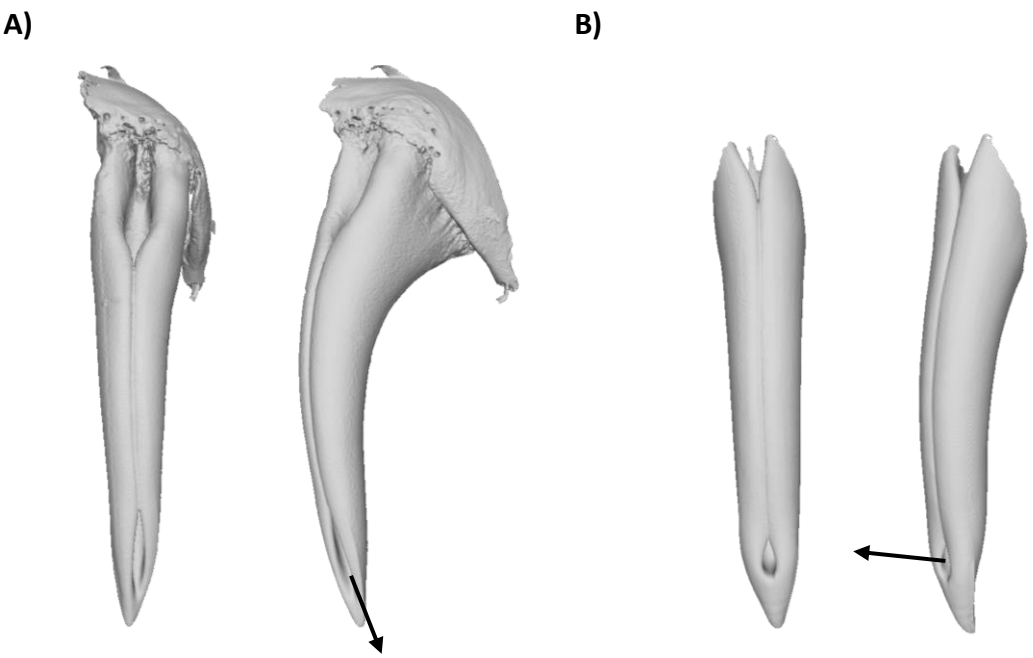


Figure 1.8. CT scans showing the differences in morphology between the fangs of spitting and non-spitting cobras. Arrows represent directional flow of venom. **A)** *Naja nivea*, a non-spitting cobra. **B)** *Naja nubiae*, a spitting cobra. The fangs used were shed fangs from animals at the Centre for Snakebite Research and Interventions Herpetarium. CT scans were provided by Chris McCabe of the University of Bristol.

groups (Wüster et al., 2007) (see Fig. 1.6.). The *Naja* genus currently contains 33 species which are distributed throughout much of Africa and Asia (see Fig. 1.7.). The African spitting cobras diverged from other members of the genus around 16 million years ago (Wüster et al., 2007), whereas the Asian group diverged later.

When encountering an aggressor, the primary defence of a cobra is to rear up and expand the ribs posteriorly to the head to form a distinctive hood with its skin. This display makes the cobra appear larger and perhaps more threatening to help deter a would-be predator. However, if this display is not sufficient to repel an aggressor, there is the last resort of striking and biting, resulting in a possible envenomation. Cobras deploy venom through muscular pressure on the venom glands, which sends venom through the venom ducts and into hollow-tipped, front-facing fangs via the discharge orifice (Bogert, 1943). However, spitting cobras can eject venom by forcing it through small holes at the front of each fang (Fig. 1.8.),

causing it to emerge as a jet or spray, which can be angled at the face and eyes (Westhoff et al., 2004). Spitting cobra fangs have smaller discharge orifices than non-spitting cobras (Bogert, 1943; Wüster & Thorpe, 1992), and this may aid in forcing venom through at a high pressure. In the Asiatic cobras, fang morphology changes geographically and can be described in their levels of preadaptation for spitting (Bogert, 1943).

Snakes have been shown to have a variety of defensive adaptations, other than the physical deterrence of biting, which have evolved for the purposes of dissuading aggressors; auditory defences, such as the noises produced by rattlesnakes through the specially-adapted tail rattle or by the saw-scaled vipers through rubbing together striated lateral scales; visual defences, such as mimicry in the coral snake complex, hooding in cobras, playing dead in the *Heterodon* and *Hemachatus* genera, and neck swelling in some colubrid species (Rand & Ortleb, 1969). However, in the elapid group known commonly as cobras there is a unique chemical defence that involves spitting venom into the eyes of an aggressor, sometimes from up to 2.5 metres away (Rasmussen et al., 1995). This causes extreme pain, inflammation and occasionally blindness (Chu et al. 2010; Warrell & Ormerod, 1976), allowing a cobra to escape. Spitting has evolved three times in cobras (fig 1.5.); twice in the *Naja* genus and once in the genus *Hemachatus* (Wüster et al., 2007), a monospecific African genus. There are other reptile groups that have evolved chemical defences; the thorny devil, which ejects a stream of blood from its eyes (Sherbrooke & Middendorf, 2001; Sherbrooke, 2000), and the gecko genus *Strophurus*, which can produce a sticky, foul-smelling substance from glands that run along the underside of the tail (Rosenberg, Russell, & Kapoor, 1984). Defensive ejection of fluids has also been shown in a multitude of species across the animal kingdom, such as the bombardier beetle, which has been shown to eject a stream of fluid from the abdomen when threatened (Eisner, 1958). Additionally, there has been one case of a rattlesnake reportedly spitting venom one meter into the face of a victim (Troutman & Wilson, 1989), however occurrences like this are rarely if ever documented, are subject to the accuracy of witness reports and are generally not considered to be a true phenomenon. However, while chemical defences are not uncommon across animal taxa, spitting cobras are unique in their ability to precisely

eject venom at a target location (namely the eyes of an aggressor) and in addition have been shown to be able to track these targets and oscillate their heads to spit in anticipation of the target's movement (Berthé et al., 2010; Young, Boetig, & Westhoff, 2009). This makes venom spitting in cobras a unique phenomenon in snakes and raises the question of why such an advanced chemical defence has evolved in this single group when all other snakes use venom defensively through biting or the direct threat of biting. Venom can be costly to produce and replenish (Pintor et al., 2010). It is therefore a distinct possibility that, in order to prevent expending a large amount this potentially expensive and valuable substance, in a defensive scenario, the fangs of spitting cobras have evolved to effectively eject small amounts of venom long distances. Alternatively, it has previously postulated that spitting might have evolved to prevent becoming trampled by large herding animals (Barbour, 1922) but this has since been disputed due to the timings of spitting cobra radiations occurring much earlier than that of grazing undulates (Wüster et al., 2007). If it is true that spitting cobra venom has evolved to become more effective at repelling aggressors through pain, this may mean a larger number of genes or higher expression of genes coding for proteins that are known to cause pain or dehabilitation. However, it may also be the case that any venom, no matter its composition, is effectively painful enough when in contact with the eye of an aggressor that there is in fact little to no selection pressure on venom composition or variability in spitting cobras, and it has been shown that rattlesnake venom also causes extreme pain to the eye (Troutman & Wilson, 1989). However, if this is the case, why has spitting only evolved in the cobras?

One possible answer is preadaptation. When cobras hood, they lift the front section of their body off the ground, which can enable them to display at an aggressor's eye-level; an optimal position to spit venom from if the eyes are the intended target. It may be then that the evolution of the primary defence (the flattened dorsal hood) of cobras has pre-adapted them for spitting capabilities. Another possibility is the advantage in venom metering. *Naja kaouthia* has been shown to expend a huge amount of venom when biting defensively (Hayes et al., 2002). However, the red spitting cobra (*Naja pallida*) has been shown to have a distinctive control over the volume of venom that is spat at an aggressor, with each

spit constituting around 1.7% of the venom gland, allowing them to spit over 40 times consecutively (Cascardi et al., 1999), and in *Naja nigricollis*, the mean mass of venom used for biting was over 7.5x the amount of venom used for spitting (Hayes et al., 2008). This control over defensive venom metering means that spitting cobras are likely to use less of their expensive venom in a defensive encounter, potentially giving them a predation advantage over non-spitting cobras that must defensively bite and expend more venom.

1.5. Global impact of cobra envenomation

Many species of cobra are medically important, with 21 of the 26 species of *Naja* being listed as category 1: highest medical importance by the WHO database (http://apps.who.int/bloodproducts/snakeantivenoms/database/), and in some cases existing antivenoms have differing efficacies in their ability to neutralize cobra venoms due to differences in venom composition at the species, population and even individual level (Ali et al., 2013; Sintiprungrat et al., 2016). In the case of spitting cobras, ocular contact with venom can cause severe pain, ophthalmia, conjunctivitis and even blindness (Siraj & Joshi, 2012; Warrell & Ormerod, 1976). This is especially significant given the geographic distribution of spitting cobras lies within regions of particularly poor income, where people rely heavily on their ability to work in order to provide a living wage and food to sustain themselves. It is therefore important in the development of antivenoms and suitable medical care to improve our understanding of the evolution and diversity cobra venoms. Additionally, it has been found that oral ingestion of venoms from *Naja naja* is just as toxic as injected venoms in rats (Malleswari et al. 2014). As ejected venom is aimed at the facial region of humans it is more likely to be ingested, and if an equal toxicity of injection and ingestion is also the case for spitting species of the *Naja* genus then it makes them a high medical priority. Additionally, snake venom has been shown to have medically beneficial effects such as the inhibition of bacterial and leukemic cell growth (Debnath et al., 2010; Gâz Florea et al., 2016), and venoms can be stored for decades without losing their clinical activities (Jesupret et al., 2014), which makes them practical as medical treatments.

1.6. Cobra venom composition

The venoms of the *Naja* genus are rich in 3-finger toxins (3FTXs) and Phopholipase A_2s (PLA_2s) (Petras et al., 2011), which are involved in nicotinic and muscarinic acetylcholine receptor binding and the release of arachidonic acid from the plasma membrane of phospholipids (Fry et al., 2003; Fry, 2005). 3FTX can make up to nearly 85% of a cobra's venom composition (Shan et al., 2016), with unique long-chain 3FTXs called 'α-cobrotoxins' (Utkin, 2013). Neurotoxic fragments within cobra venom have been shown to be the main inducers of fatalities in mice (Lauridsen et al., 2017). It is for these reasons that neurotoxicity is generally the primary clinical activity of cobra venoms (Faiz et al., 2017). However, cobras have been shown to have haemorrhagic (Chanda, Sarkar, & Chakrabarty, 2016; Doley & Mukherjee, 2003; Sakurai et al., 2001) cytotoxic and necrotic (Debnath et al., 2010; Faiz et al., 2017; Reid, 1964; Rivel et al., 2016) and cardiotoxic activities (Lachumanan et al., 1998), and while the African non-spitters are mainly neurotoxic, the African spitting cobras have venoms that cause a more cytotoxic pathology (Warrell, 2008a). The Asiatic cobras are unusual in that the extent to which they spit can vary between species (Fig. 1.6.), however it is known that at least *Naja philippinensis* venom results in neurotoxicity from envenomation. *Naja* PLA_2s have also been shown to cause disruptions in haemostasis (Doley & Mukherjee, 2003) and inhibit thrombin and Factor Xa clotting factors (Mukherjee et al., 2014). Thus far, only a potentially inflammatory and wound-healing disruptive function has been found in cobra cysteine rich secretory proteins (CRISPs), in the venom of *Naja* (Wang et al., 2010), and in *Naja* and *Hemachatus*, Kunitz toxins have been found to inhibit trypsin (Hokama et al., 1976) and chymotrypsin (Zhou et al., 2004), enzymes both associated with digestion.

Cobras inspire local myth and culture wherever they inhabit, yet they remain relatively understudied, with 2 new species (*Naja ashei* and *Naja nubiae*) being described in the last 15 years (Wüster & Broadley, 2007, 2016) and *Naja melanoleuca* recently split up into 5 distinct species (Wüster et al., 2018). Evolutionarily they are fascinating, being highly neurotoxic in their venom pathology and having both primary (visual) and secondary (chemical, in the case of spitting cobras) defences. Currently, it is believed that prey acquisition is the primary driving force of venom

evolution in snakes, with defence rarely (if ever) considered. It is therefore important to investigate any possible molecular convergence in spitting and non-spitting cobras in order to identify whether defence can also be a driver of venom evolution. Previous evidence has shown that there appears to be a high degree of conservation in venom composition between the African spitting cobras (Petras et al., 2011) but without further evidence it can only be speculated that the pain inflicted by the venom of spitting cobras has evolved as a defensive measure and not as a beneficial side-function to its original predatory purpose.

1.7. Project Outline

In this project, I perform large-scale comparative analysis on the transcriptomic, proteomic and functional data of several species of spitting and non-spitting cobras of the genus *Naja* and *Hemachatus hemachatus*. This is supplemented with new data from the Elapid outgroup *Walterinnesia aegyptia* and previously-generated data from the outgroup *Aspidelaps scutatus* (Whiteley et al., 2019). This is done with the purpose of understanding whether venom composition between spitting and non-spitting cobras differs significantly, if there are signs of molecular convergence or unique patterns of gene expression in the venom composition of spitting cobras and, in the event that this is the case, if these differences are due to the increased transcription of proteins that affect the defence function of venom. This data will be used to analyse and discuss if the molecular composition of spitting cobra venom has evolved as a result of defence or is simply a by-product of its original purpose of prey acquisition.

2. COMPARATIVE ANALYSIS OF VENOM GLAND TRANSCRIPTOMES FROM SPITTING AND NON-SPITTING COBRAS

2.1. Introduction

The transcriptome represents a localised snapshot of DNA transcription. As DNA expression can differ spatially within an organism (Kilchher et al., 1986), characterising the transcriptome of a specific tissue or region of an organism allows the identification of tissue-specific RNA. This in turn facilitates gaining an understanding about function through the presence of ribozymes, functional RNAs and the prediction of proteins resulting from RNA translation. In venomous snakes, venom gland transcriptomes can be used as a predictor of venom composition and function.

Analysing venom gland transcriptomes primarily lets us quantify the toxin expression there. This allows for the prediction of toxins that may be in the venom itself and the subsequent pharmacological properties of that venom, aiding in the design and synthesis of new treatments (Leão, Ho, & Junqueira-de-Azevedo, 2009; Wagstaff et al., 2006). It has also aided in gaining information on the venom composition of snakes that are difficult to extract venom from for proteomics, such as rear-fanged snakes (colubrids) (Pla et al., 2018; Pla et al., 2015), which produce venom through a consistent chewing action, and burrowing asps (*Atractaspis* sp.) (Terrat et al., 2013), which have laterally-protruding fangs that make them exceptionally dangerous to handle using standard procedures. In some cases, transcriptomics can reveal novel toxin genes (Fry et al., 2012), such as β-Cardiotoxin from *Ophiophagus hannah* (Rajagopalan et al., 2007), a subfamily of three-finger toxins (3FTXs) that causes a decrease in heart rate in rats. Comparative analyses of toxic sequences at the species and genus level can allow us to identify the similarities and differences of transcriptionally-active regions and, on a larger scale, analyse the evolution of venom toxin families through time (Fry et al., 2008) or under the influence of various external factors. Indeed, venom gland transcriptome research has provided evidence that venom genes can be influenced by changes in diet (Li et al., 2005a), geographic isolation (Doley et al., 2017), age (Durban et al., 2017; Zelanis

et al., 2012), and even climate (Zancolli et al., 2018). Additionally, transcriptomics can allow us to follow the evolution of specific toxins through families or genera (Brust et al., 2013; Kashima et al., 2004; Sunagar et al., 2013).

As of September 2020, there are around 60 papers that describe snake venom gland transcriptomes, covering 43 genera and 79 species (Fig 2.1.) (Ainsworth et al., 2018; Aird et al., 2017; Amorim et al., 2017; Bénard-Valle et al., 2020; Cardoso et al., 2010; Casewell et al., 2014; Ching et al., 2012; Chong et al., 2019; Cidade et al., 2006; Corrêa-Netto et al., 2011; Doley et al., 2008; Durban et al., 2011, 2017, 2018; Francischetti et al., 2004; Fry et al., 2012; Gonçalves-Machado et al., 2016; Harrison et al., 2007; Hofmann et al., 2018; Jia et al., 2008; Jiang et al., 2011; Junqueira-de-Azevedo et al., 2016; Junqueira-De-Azevedo et al., 2015; Kashima et al., 2004; Kini et al., 2010; Leão et al., 2009; Li et al., 2005a; Margres et al., 2013, 2014, 2015; Modahl et al., 2018; Neiva et al., 2009; Pla et al., 2018; Pla et al., 2017; Qinghua et al., 2006; Rokyta et al., 2011, 2017; Siang et al., 2010; Tan et al., 2015; Tan et al., 2017; Terrat et al., 2013; Vaiyapuri et al., 2011; Whiteley et al., 2019; Xu et al., 2017; Zelanis et al., 2012; Zhang et al., 2019; Zhang et al., 2015). Currently, the only way to generate a transcriptome from a snake venom gland requires euthanising an animal, as the trace messenger RNA present in secreted venom is insufficient for sequencing library preparation (Whiteley et al., 2016). This raises the ethical issue of whether the life of a snake is worth more than the potential benefit of its transcriptomic data, along with the additional difficulty of sourcing the desired species and localities required for various studies. Transcriptomic research is therefore heavily reliant on the transcriptome sequences generated from one or two specimens per species or genus to minimise loss of life, and, in the case of snake venom, these studies have focused predominately on medically-important species to aid antivenom research. As both venom gland transcriptomes and venom proteomes have been shown to exhibit both interspecific (Ali et al., 2015; Casewell et al., 2009; Méndez et al. 2011; Petras et al., 2011; Pla et al., 2017) and intraspecific variation (Currier et al., 2010; Gibbs et al., 2011; Gonçalves-Machado et al., 2016; Huang et al., 2015; Modahl et al., 2016; Sintiprungrat et al., 2016; Tan et al., 2015; Williams et al., 1988; Zelanis, Travaglia-Cardoso, & Furtado, 2008), the extrapolation of inferences based on the venom gland

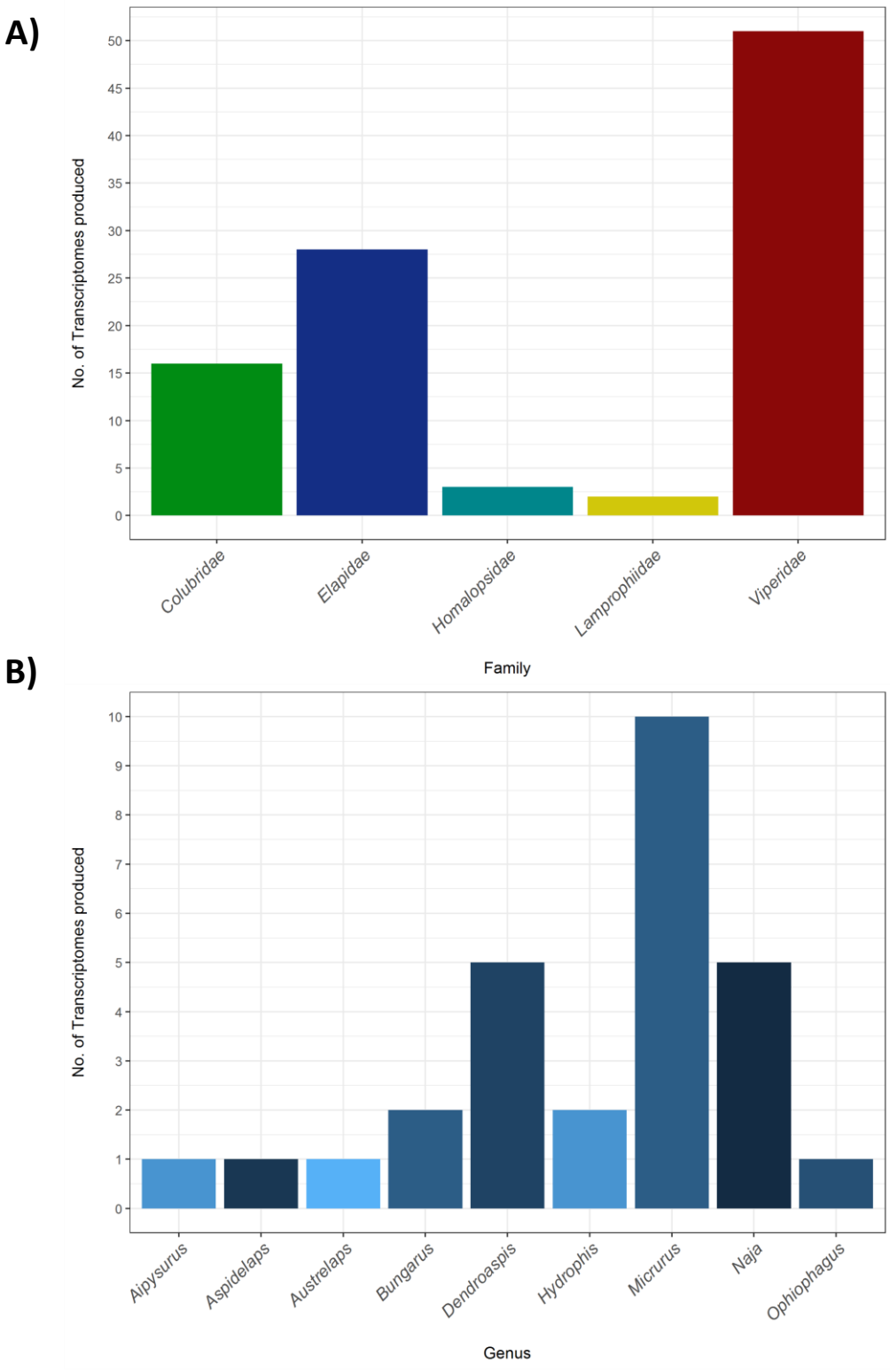


Figure 2.1. The number of venom gland transcriptomes with available expression data currently available in the literature. A) by snake family and **B)** by genera of the family Elapidae. The latter are shaded by their phylogenetic proximity to the genus *Naja* (darker = more closely related) based on Zaher et al., 2019.

from a single individual to the whole species is problematic. In addition, the information that can be inferred from the venom gland transcriptome alone is limited; not all detected mRNA will necessarily be translated into proteins, such as that observed with several species of the genus *Echis*, *Bitis arietans* and *Cerastes cerastes* (Casewell et al., 2014). Additionally, this approach cannot predict translational or post-translational processes that could occur, such as the differential proteolytic cleavage of many different precursors in to distinct active toxins, as observed for example in natriuretic peptides (Jackson et al., 2013) or snake venom metalloproteinases (Casewell et al., 2011). Despite these limitations, transcriptomics are a useful tool in the analysis of snake venom toxin evolution, particularly when combined with proteomic and functional approaches, as it provides a direct indication of what toxins (and other genes) are being transcribed in the venom gland.

Many recent publications describing snake venom gland transcriptomes have focused on viperid snakes (Fig. 2.1A.). This may be due to ease of availability or access to these snakes, the medical importance of the species, or potentially decreased ethical or legal concern regarding the sacrificing of viper species for medical research. A renowned example of the decreased value of snake life is the annual Rattlesnake roundups in the South and Midwest of the USA, where rattlesnakes of many kinds are rounded up in their thousands for the purpose of entertainment and slaughter. In contrast, it is unlikely that anyone would be willing to sacrifice a specimen of the monotypic Fiji burrowing snake (*Ogmodon vitianus*, family Elapidae) due to its highly endangered status (IUCN), subterranean lifestyle and endemism to the Fiji area. However, generally speaking vipers are common snakes and are often medically important in terms of snakebite. The results of transcriptome studies on vipers have demonstrated that their venom glands consistently exhibit high expression of the snake venom metalloproteinases (SVMPs), serine proteases (SPs) and phospholipases A_2 (PLA_2) toxin families (Aird et al., 2013; Amorim et al., 2017; Cardoso et al., 2010; Cidade et al., 2006; Yee et al., 2018). These toxins are commonly associated with haemorrhagic, coagulopathic and necrotic activities (Doley, Zhou, & Kini, 2010; Gutiérrez & Escalante, 2010; Phillips et al., 2010).

Contrastingly, there are fewer venom gland transcriptomes described from the other group of medically important venomous snakes, the Elapidae (Fig. 2.1A.).

Of the 27 elapid venom gland transcriptomes described to date for which toxin expression data is available, the majority of these relate to snakes of the genera *Micrurus* (coral snakes) (Fig. 2.1B.), possibly due to the high medical importance of these snakes across their geographical distributions (Warrell, 2005). From the available elapid transcriptomes, the toxin-encoding components can comprise of as much as 75.6% of the whole venom gland (Ainsworth et al., 2018), showing that these glands are highly specialised for toxin secretion. Non-toxin genes usually represent 'house-keeping' or physiological genes and other such regulatory elements, with a small number of these being unidentifiable in terms of functional identification.

Of the toxin-encoding transcripts detected in elapid venom gland transcriptomes, a major feature is the often-extreme expression levels of three finger toxins (3FTXs) (Table 2.1.), ranging from 19.23% to 97.03% of the toxinome (total toxin expression). The 3FTXs are predominately neurotoxic (see 1.3.) (Nirthanan & Gwee, 2004; Utkin, 2013), and the high expression levels of 3FTXs in detected in elapid transcriptomes is therefore likely to be the main factor responsible for their notoriously neurotoxic venoms. 'Cytotoxins' are a unique neofunctionalized sub-group of 3FTXs, capable of causing widespread cell and muscle damage (see 1.3.) (Gasanov, 2014; Lomonte & Gutiérrez, 1989). Due to the distinct functional activities between neurotoxic 3FTXs and cytotoxic 3FTXs (CTXs), I have decided to partition the 3FTX gene family into CTXs and 'Other 3FTXs', which can generally be defined as neurotoxic. The literature demonstrates that relatively few species of elapid snakes express CTXs in their venom glands, although those that do express CTXs, tend to express them at high levels, such as *Aspidelaps scutatus* (51.30%), *Naja atra* (66.90%) and *Naja kouthia* (57.83-81.90%). The high expression levels of CTXs in the venom glands of these species suggests that bites by these species may result in cytotoxic/necrotic symptomology, which is reflective of several clinical studies on *Naja atra* and *Naja kaouthia* (Faiz et al., 2017; Wang et al., 2014; Wong et al., 2010) but not *Aspidelaps scutatus*, which causes predominately neurotoxic symptoms in the few existing bite reports (Barton et al., 2019; Zaltzman et al., 1984). The king cobra (*Ophiophagus hannah*) venom gland transcriptome also contains CTXs but these are not highly expressed. Additionally, only a single CTX has been identified in

Table 1. Overview of toxin expression from elapid venom gland transcriptome literature from which sequence expression data was made available. Toxin families are represented as the percentage of toxin transcripts, not the entire venom gland transcriptome. 3FTX – three-finger toxin, CTX – cytotoxic three-finger toxin, SVMP – snake venom metalloproteinase, kunitz – kunitz-type serine protease inhibitor, CRISP – cysteine rich secretory protein, PLA_2 – phospholipase A_2, CTL – c-type lectin, NP – natriuretic peptide, Ves – vespryn, LAAO – L-amino acid oxidase, NGF – nerve growth factor, Prokin – prokinase.

Species	3FTX		SVMP	Kunitz	CRISP	PLA_2	CTL	NP	Ves	LAAO	NGF	Prokin	Other
	CTX	Other											
Aipysurus eydouxii[1]	0	19.23	0	0	0	80.77*	0	0	0	0	0	0	0
Aspidelaps scutatus[2]	51.30	14.00	0.97	2.50	1.34	23.80	0	1.08	0.2	0.045	0.66	0	4.11
Austrelaps labialis[3]	45.00		3.00	9.00	8.00	33.00	2.00	0	0	0	0	0	0.00
Bungarus flaviceps[4]	0	74.17	0	21.19	0.44	3.31	0.44	0.44	0	0	0	0	0.01
Bungarus multicinctus[5]	0	89.80	0	0.30	0.70	3.20	3.80	1.30	0.40	0.10	0.10	0	0.30
Dendroaspis angusticeps[6]	0	71.40	3.38	14.49	0.44	0.13	0	5.93	0	0	0	3.98	0.25
Dendroaspis jamesoni jamesoni[6]	0	80.26	0.48	15.12	0	0.12	0	2.61	0	0	0	0.50	0.91
Dendroaspis jamesoni kaimosae[6]	0	65.81	2.96	15.25	0	0.20	0	1.86	0	0	0	13.91	0.01
Dendroaspis polylepis[6]	0	45.13	1.76	48.87	0	0	0	3.82	0	0	0	0	0.42
Dendroaspis viridis[6]	0	77.72	2.69	15.24	0.32	0.08	0	2.29	0	0	0	1.28	0.38
Hydrophis platurus[7]	0	88.57	0.04	0.009	0	10.68	0	0	0	0	0	0	0.70
Hydrophis platurus[7]	0	87.03	0.05	0.006	0	11.40	0.01	0	0	0	0	0	1.50
Micrurus altirostris[8]	0	86.40	0.60	3.80	0	4.90	1.30	1.90	0	0	0	0	1.10
Micrurus browni browni[9]	0	35.09	0.24	6.83	0	52.67	0.56	3.92	0.22	0.05	0.21	0	0.21
Micrurus corallinus[10]	0	52.20	0.60	0.50	0	33.30	10.40	2.2	0	0.15	0.07	0	0.58
Micrurus corallinus[11]	0	20.3	2.1	0.16	0.02	37.72	3.35	29.5	0.01	0.84	1.12	0	4.85
Micrurus fulvius[12]	0	25.16	2.95	2.15	0	64.85	0.80	0.7	0	0.88	0.58	0	1.93

Table 1. cont.

Species	3FTX		SVMP	Kunitz	CRISP	PLA_2	CTL	NP	Ves	LAAO	NGF	Prokin	Other
	CTX	Other											
Micrurus lemniscatus carvalhoi[11]	0	72.59	0.22	10.65	0	15.29	0.13	0.41	0	0.1	0.23	0	0.38
Micrurus lemniscatus lemniscatus[11]	0	67.22	1.12	7.15	0.24	17.28	0.29	0.2	1.66	0.73	1.12	0	2.99
Micrurus paraensis[11]	0	68.56	0.34	0.76	0	24.91	1.87	0.82	0.01	0.15	0.26	1.93	0.39
Micrurus spixii[11]	0	62.55	0	0.13	0	36.44	0	0	0.24	0	0	0	0.64
Micrurus surinamensis[11]	0	60.24	0.14	1.07	0	26.23	0.17	2.67	0.01	0.05	7.88	0	1.54
Naja atra[5]	66.90	28.90	0.40	0.10	0.70	1.20	0.60	0	0.50	0.10	0.20	0	0.40
Naja kaouthia[13]	29.01	57.83	1.62	0.63	4.03	1.80	0.31	0.56	0.12	0.12	1.04	0	2.93
Naja kaouthia[13]	20.54	76.49	0.18	0.25	0.31	1.54	0.12	0.12	0.11	0.02	0.1	0	0.22
Naja kaouthia[14]	0.10	81.90	1.10	0.10	0.00	13.60	0.50	0.00	0.00	0.20	0.30	0.00	2.20
Naja sumatrana[15]	72.83	18.27	0.17	0.01	0.33	7.42	0.22	0.05	0.25	0.02	0.32	0	0.11
Ophiophagus hannah[16]	0.03	84.92	3.68	1.84	2.12	2.16	0.01	0.48	0.58	1.47	0.2	0	2.51

*** In this paper, these toxins were simply referred to as "phospholipases", so it may be possible that not all of these are phospholipase A_2.**

1. Li, Fry, & Kini, 2005, 2 . Whiteley et al., 2019, 3. Doley et al., 2008, 4. Siang et al., 2010, 5. Jiang et al., 2011, 6. Ainsworth et al., 2018, 7. Durban, Sasa, & Calvete, 2018, 8. Corrêa-Netto et al., 2011, 9. Bénard-Valle et al., 2020, 10. Leão, Ho, & Junqueira-de-Azevedo, 2009, 11. Aird et al., 2017, 12. Margres et al., 2013, 13. Tan et al., 2017, 14. Tan et al., 2017, 15. Chong et al.,, 2019, 16. Tan et al.,2015.

the venom gland transcriptome of *O. hannah* and, was described as a "β-cardiotoxin" that shares approximately 55% sequence identity with CTXs identified from the genus *Naja* (Rajagopalan et al., 2007). However, phylogenetic analysis has shown that this *O. hannah* CTX is phylogenetically distinct from the 'core' CTX group (Sunagar et al., 2013). It is therefore currently unknown whether CTXs have evolved independently on multiple occasions in elapid snakes, or whether some species have lost CTX 3FTXs.

While 3FTXs appear to be the major toxin components detected in most elapid venom gland transcriptomes, there are exceptions. PLA_2s are common constituents of elapid venoms (Tasoulis & Isbister, 2017) but their representation in elapid venom gland transcriptomes can vary extensively, from 0% in *Dendroaspis polylepis* to 80.77% in *Aipysurus eydouxii* (though it should be noted that this species is unusual in that it is an egg-eating snake with atrophied venom glands, so may not undergo the same selection pressures as other elapids). In several species of *Micurus* particularly it can be the major venom component, making up as much as 64.85% of the toxinome in *M. fulvius*. PLA_2s have a variety of functions (see 1.3.), including neurotoxicity, cytotoxicity, myotoxicity and anticoagulant effects (Gutiérrez & Lomonte, 2013; Kini, 2003).

There are several other, typically less abundant toxin families that are often recovered in Elapid venom gland transcriptomes. The kunitz-type serine protease inhibitors (kunitz) are a particularly abundant toxin family in *Dendroaspis* transcriptomes (Ainsworth et al., 2018), where their "dendrotoxin" class of kunitz toxins act as sodium, calcium and potassium channel blockers (Inagaki, 2017). These toxins, in combination with neurotoxic 3FTXs, are responsible for causing the systemic neurotoxicity observed following bites by these species. However, for most other elapid snakes, the abundance of these toxins in the venom gland transcriptome represents less than ten percent of all toxins (Table 2.1.). The remaining frequently detected toxin families include: (i) SVMPs, which can contribute to haemorrhage and local tissue envenoming (Casewell et al., 2015; Moura-da-Silva et al., 2007), (ii) cysteine rich secretory proteins (CRISPs), which display a wide range of pharmacological activities such as blocking ion channels and preventing smooth muscle contraction (Heyborne & Mackessy, 2010; Yamazaki & Morita, 2004) (iii) C-type lectins, which can contribute to coagulopathy (Du & Clemetson, 2010). Despite

these toxin families being commonly detected, their abundances are typically low (<5% of total toxins) across elapid species (Table 2.1.).

Despite cobras (snakes of the genus *Naja*) representing a large genus containing 33 species, only three species have published venom gland transcriptomes; *Naja atra*, *Naja kaouthia* and *Naja sumatrana* (Table 2.1.) (Chong et al., 2019; Jiang et al., 2011; Tan et al., 2017; Xu et al., 2017). These prior studies have demonstrated that the toxins expressed in the venom gland of these three Asian cobras are dominated by 3FTXs - representing ~96% of all toxins in *N. atra* (Jiang et al., 2011), and 82%, 87% and 97% of all toxins in different geographical variants of *N. kaouthia* (China, Malaysia and Thailand, respectively) (Tan et al. 2017; Xu et al. 2017). At initial glance, these findings suggest that these cobras likely have highly neurotoxic venoms. However, upon annotation of the 3FTXs into CTXs and 'Other 3FTXs', 66.90% of the *N. atra* toxin transcripts are actually made up of CTXs (Jiang et al., 2011), 72.83% of the *N. sumatrana* toxinome is made up of CTXs (Chong et al., 2019) and two of the three *N. kaouthia* geographical variants also exhibit moderate CTX expression (29.01%, 20.54% and 0.10% for the Malaysian, Thai and Chinese localities respectively)(Tan et al., 2017; Xu et al., 2017). This conflicts with the idea of cobras (and elapids in general) having solely neurotoxic venoms but does fall in line with the mixed clinical symptomology of seen following snakebites by these species in that they can cause either local swelling and necrosis or neurotoxicity (Faiz et al., 2017; Wang et al., 2014; Wong et al., 2010). As with other elapid transcriptomes, the expression levels of PLA_2 toxins were found to vary considerably, including intra- and inter-specifically, with 13.60% of the total toxin expression in the Chinese *N. kaouthia,* compared with around 2% in the Thai and Malaysian variants of this species (Tan et al., 2017; Xu et al., 2017), 7.42% in *N. sumatrana* (Chong et al., 2019) and 1.2% in *N. atra* (Jiang et al., 2011). Also consistent with other elapid venom gland transcriptomes were the consistently low expression levels of SVMPs (between 0.4% and 1.1%), while all other toxin types had total toxin expression levels of less than 1%, with the exception of CRISPs in the Malaysian *N. kaouthia* (4% of total toxins).

While these findings provide us with an initial insight into the venom gland composition of snakes from the genus *Naja*, the limited number of species studied make it difficult to extrapolate the results to other cobras. Firstly, both *N. atra* and

N. kaouthia are related Asian cobras, whereas many species of *Naja* inhabit African regions, with these two groups diverging from one another over 16 million years ago (Wüster et al., 2007). In addition, both *N. atra* and *N. kaouthia* are generally considered to be non-spitting cobras, although there is some evidence that individuals or populations from these species may be capable of spitting to some extent (Fung et al., 2009; Santra & Wüster, 2017). Consequently, both these species are atypical in the context of most members of the genus *Naja*, which either definitely spit or do not. Therefore, should the evolution of this behavioural characteristic be associated with shifts in venom composition, the venom gland transcriptome profiles of *N. atra* and *N. kaouthia* may not show similarities to those definitively spitting or, perhaps, non-spitting species, particularly since there is much variation identified in the two species studied to date.

To redress this knowledge gap, in this chapter, I analysed and performed comparative analysis of the venom gland transcriptomes of 14 species of the genus *Naja*, including multiple representatives from Africa and Asia, and spitting and non-spitting lineages. In addition, I incorporated analyses of the venom gland transcriptomes from near relatives to the cobras, the rinkhals *Hemachatus haemachatus*, a venom-spitting snake from southern Africa, and the desert black snake *Walterinnesia aegyptia*, a non-spitting snake from the Middle East. Due to its close relationship to the cobras and near relatives, and identical transcriptomic approach applied, I also included one recently published transcriptome in to my comparative analysis, from the African shield nosed snake (*Aspidelaps scutatus*) (Whiteley et al., 2019). In addition to characterising the venom toxin profile of these various medically-important snake species and adding 16 new Elapid transcriptomes to the literature, I investigated whether the expression of different toxin-encoding gene families differs between spitting and non-spitting cobras to determine whether there are transcriptomic patterns associated with the evolution of defensive venom-spitting behaviour. The transcriptomic data described herein will be reconciled with proteomic and functional data in subsequent chapters to provide a thorough overview of the composition, evolution and functional activity of cobra venoms.

2.2. Methods

2.2.i. Construction of venom gland transcriptomes

The sixteen assembled and batch-annotated unanalysed venom gland transcriptomes were provided to me by my supervisor Professor Nicholas Casewell, as they had been constructed prior to me commencing my PhD. The snake species and their geographical origins are listed in Table 2.2., and venom gland transcriptomes were constructed using previously validated in-house procedures (Ainsworth et al., 2018; Pla et al., 2017; Whiteley et al., 2019). For each species, venom glands were dissected from a single specimen, which was euthanised with an overdose of pentobarbitone and the dissected venom glands immediately stored in liquid nitrogen until use. In the case of *N. kaouthia* and *N. sumatrana*, historical venom glands that had been kept in cryostorage for over a decade were used to avoid sacrificing further animals. For all other animals, venom glands were used within one year of dissection. For RNA extraction, venom glands were homogenised under liquid

Table 2.2. The characteristics of the snakes used to generate the venom gland RNA libraries analysed in this study. All snakes were maintained at the Alistair Reid Herpetarium prior to euthanisation. RIN – RNA Integrity Number.

Species	Spitter?	Species Distribution	Wild-caught origin/Captive Bred (CB)	RIN
Hemachatus haemachatus	Yes	Africa	South Africa	8.1
Naja annulifera	No	Africa	CB	7.4
Naja atra	No	Asia	CB	8.6
Naja haje	No	Africa	Uganda	6.3
Naja kaouthia	No	Asia	CB	4.5
Naja mossambica	Yes	Africa	Tanzania	7.7
Naja naja	No	Asia	CB	5.6
Naja nigricollis	Yes	Africa	Nigeria	6.6
Naja nivea	No	Africa	South Africa	6.8
Naja nubiae	Yes	Africa	CB	6.7
Naja pallida	Yes	Africa	Tanzania	8.6
Naja philippinensis	Yes	Asia	CB	6.8
Naja siamensis	Yes	Asia	CB	6.7
Naja subfulva	No	Africa	Cameroon	8.0
Naja sumatrana	Yes	Asia	CB	5.2
Walterinnesia aegyptia	No	Africa/Asia	CB	6.3
Aspidelaps scutatus scutatus	No	Africa	CB	7.4

nitrogen using a pestle and mortar, before the TRIzol Plus RNA Purification System (Thermofisher) was used according to the manufacturer's instructions. The resulting RNA samples were then DNAse treated using On-Column PureLink DNAase (Life Technologies) and total RNA eluted in 30 µL of nuclease free water. Total RNA then underwent two rounds of poly(A) selection for mRNA using the Dynabeads mRNA DIRECT purification kit protocol (Life Technologies). The RNA-Seq libraries were constructed from 50 ng of this mRNA enriched sample using the Script-Seq v2 RNA-Seq Library preparation kit (epicentre), following 12 cycles of amplification. The libraries were then purified using AMPure XP beads (Agencourt), quantified using the Qubit dsDNA HS Assay kit (Life Technologies) and the size distribution assessed using a Bioanalyser (Agilent). The RNA Integrity Number (RIN) was calculated using the Bioanalyser-generated algorithm of RNA marker ratios. Sequencing libraries were then multiplexed and sequenced on an Illumina MiSeq instrument (six libraries per lane) with 2 × 250 bp paired-end sequencing technology (Centre for Genomics Research [CGR], University of Liverpool). Following sequencing, the ensuing read data was quality processed according to CGR's standard protocols (Ainsworth et al., 2018; Pla et al., 2017; Whiteley et al., 2019), resulting in the removal of adapter sequences using Cutadapt (Martin, 2011) and low quality based using Sickle (Joshi & Fass, 2011). The resulting reads were then assembled into contigs using the in-house venom gland transcriptome assembly program VTBuilder (Archer et al., 2014) with the following parameters: minimum transcript length 150 bp, minimum read length 150 bp, min. isoform similarity 96%. As part of this process, the VTBuilder algorithm generates relative transcript expression data for each sequence as the number of reads mapping to that transcript normalized by the length of the transcript. Assembled contigs were batch annotated with BLAST2GO Pro v3 (Conesa et al., 2005) using the BLASTx-fast algorithm with a significance threshold of 1e-5 against the NCBI non-redundant (NR) protein database (41 volumes, Nov. 2015).

2.2.ii. Transcriptome analysis and toxin identification

Resulting BLAST2GO annotations were inspected manually and preliminary annotation was performed by assigning contigs as 'toxins' (those showing BLAST annotations with known venom toxins), 'non-toxins' (those showing annotations not

associated with toxins) and 'unknowns' (those with no significant BLAST hit) based on the output given by BLAST2GO. As BLAST2GO occasionally resulted in mixed or "NA" annotations, Secondary annotation was then performed using BLASTx searches against the Non-redundant protein sequences (nr) database of NCBI on all contigs with 0.1% expression in the total transcriptome or higher. These were classified into the same three groups based on their BLASTx annotations: (i) 'toxins', where they exhibited annotations consistent with known venom toxins or toxin families previously published in the literature, (ii) 'non-toxins', where annotations were consistent with physiological proteins not previously described as venom toxins, or non-toxic genes from potentially toxic gene families (distinguished from toxic genes by matches with sequences from body tissues or non-venomous organisms) and (iii) 'unknown', where contigs displayed no matching annotation or were described as 'hypothetical' by BLASTx annotation. Subsequently, the number of contigs and the percentage expression of those contigs were calculated for each of the three classification groups.

2.2.iii. Venom toxin curation

Transcriptome contigs that were identified as 'toxins' were then subjected to in depth analysis. First, toxins were preliminarily assigned to specific toxin families based on their BLAST annotations. The BLASTx result for each of these contigs was visually inspected to confirm toxin family identity, before contigs for each gene family exhibiting summed expression equating to >1% of that of the total transcriptome in at least one species, were used to generate sequence alignments in MEGA v7 (Kumar et al., 2016). Consequently, sequence alignments were produced for nine toxin families: three-finger toxins (3FTX), cysteine-rich secretory proteins (CRISP), c-type lectins (CTL), kunitz-type serine protease inhibitors (kunitz), nerve growth factors (NGF), natriuretic peptides (NP), phospholipases A_2 (PLA_2), serine proteases (SP) and snake venom metalloproteinases (SVMP). 3FTXs were also separated into cytotoxins (CTXs) and 'other 3FTXs', for previously explained purposes (see p26). As basic PLA_2s appear to be more pharmacologically-active in *Naja* (Bhat & Gowda, 1989; Kerns et al., 1999; Tan et al., 2019), full-length PLA_2 sequences were further disaggregated into "acidic" or "basic" based on their isoelectric point, estimated by the Compute

pI/Mw tool of the Expasy Bioinformatics Resources Portal (Bjellqvist et al., 1994; Bjellqvist et al., 1993; Gasteiger et al., 2003). For all toxin families, each contig was analysed for the identification of the correct open reading frame in MEGA v7 (Kumar et al., 2016), facilitated by manual inspection of BLASTx matches and the online ExPASy Translate portal (Gasteiger et al., 2003).

As a quality control process for assessing the venom gland transcriptome assemblies, and in order to produce high quality toxin sequence alignments, the following parameters were implemented with toxins discarded from downstream analysis if they did not meet any of these conditions: i) sequence length was less than 100bp long, ii) 50% or more of the sequence did not match the target protein in a BLASTx search, iii) the first 50% of the sequence was interrupted by a stop codon (indicating missed base calling, misassembly or pseudogenic contig), iv) the sequence was made up either of two exonic regions interspersed by an intron (indicative of genomic DNA contamination) or two distinct sequences, based on annotation, that were attached end to end (indicative of chimeric misassembly). Following this manual inspection data curation step it was apparent that 'underclustering' of some toxins had occurred during transcriptome assembly, which is a common by-product of assembling isoform-rich gene data (Archer et al., 2014). Underclustering is, however, more desirable than the alternative scenario of overclustering, because underclustered contigs can be manually merged (both in terms of overlapping sequence and expression), whereas overclustering results in chimeric contigs that cannot be easily disassociated from one another. To resolve the issue of highly similar, often identical, overlapping contigs in the same transcriptome assembly, I applied the following rule to merge underclustered contigs: two or more contig sequences were merged if they exhibited $\geq$98% sequence similarity over an overlapping region of $\geq$50bp. As outlined above, once this rule was met, sequences were merged both in terms of the sequence itself and their expression levels, which were combined. Following data curation, the finalised datasets were used to update toxin family contig numbers and expression levels, before sequences were trimmed to the open reading frame (i.e. start methionine and stop codon) in MEGA v7 (Kumar et al., 2016). The MUSCLE algorithm (Edgar, 2004) was then implemented, using standard parameters, to align each of the toxin family sequence datasets in amino

acid space, before manual visual inspection for error correction. The transcriptome data have been deposited in the SRA and TSA databases of NCBI and are associated with the BioProject accession number PRJA506018

2.2.iv. Venom toxin analysis

To determine whether spitting cobras exhibit significantly different expression levels of different toxin families in their venom glands compared to non-spitting cobras, the expression data and number of contigs were compared for the following toxin families: PLA_2s, cytotoxic 3FTXs (CTXs), other 3FTXs, kunitz, NGFs, SVMPs, natriuretic peptides (NPs), CRISPs, CTLs and SP. To examine both the differences between spitting and non-spitting cobras and the influences of geography at the transcriptome level, a two-way ANOVA was performed on all data meeting the assumptions of normal distribution and equal variance. To ensure that data fell within the assumptions of a two-way ANOVA, normal distributions were determined using qq-plots and equality of variance of each group (African spitters, African non-spitters, Asian spitters and Asian non-spitters, with *Hemachatus haemachatus* being grouped with African spitting cobras) were estimated using Levene's Test for Homogeneity of Variance in the 'car' package (Fox & Weisberg, 2019) in RStudio. Data found to be in violation of the assumptions were analysed by the non-parametric factorial analysis ARTool R package (Wobbrock et al., 2011). Bonferroni corrections were then applied to the outputs of these tests, treating each factor (group, geography, group+geography) as a model set.

To further test whether transcriptomic toxin composition formed distinct groups based on geography (e.g. Africa vs Asia), venom-spitting (spitting vs non-spitting snakes) or lineage (African spitters, African non-spitters, Asian spitters, Asian non-spitters and *H. haemachatus*), a Principal Component Analysis (PCA) was performed using the same toxin families. The input data consisted of summed toxin family expression levels for each species for the nine toxin families (stated above) used to produce sequence alignments. PCA was performed using the prcomp() function in R with the centre and scale arguments of the function set to 'TRUE'. The output of the analysis was visualised with the ggplot2 package (Wickham, 2016).

2.3. Results

2.3.i. Overview of Transcriptome Composition

Toxin-encoding sequences comprise over 50% of the venom gland transcriptomes in the majority of the studied species (13 of 17 species), with the exceptions being *N. haje*, *N. subfulva*, *N. sumatrana* and *N. kaouthia* (Fig. 2.2.). Two of these species, *N. haje* and *N. subfulva*, exhibit toxin encoding sequences approaching 50% (47% and 38%, respectively). These findings of >35% toxin expression in the venom gland are highly consistent with many prior studies (Corrêa-Netto et al., 2011; Doley et al., 2008; Jiang et al., 2011; Leão et al., 2009; Siang et al., 2010; Tan et al., 2017, 2015; Xu et al., 2017), although it is worth noting that for some of the species analysed here, their toxin expression levels were particularly high (e.g. >70% of total expression; Fig. 2.2.). In contrast, the venom gland transcriptomes of *N. sumatrana* and *N. kaouthia* exhibited much lower expression levels of toxin-encoding sequences (10% and 19%, respectively). These findings may be the result of the venom glands of these species being maintained in cryostorage for prolonged periods of time, likely resulting in increased degradation of RNA. In addition, the number of contigs encoding 'non-toxins' in these transcriptomes are far more numerous than for all other species sampled, with the exception of *N. subfulva* (Fig. 2.3.), and thus it is possible that the dissection of these tissues was suboptimal compared to those performed more recently by different individuals. Despite these observations, the number of toxin-encoding sequences identified in these two species (76 for *N. kaouthia* and 83 for *N. sumatrana*) was highly consistent with all other species studied here (range of 49-160; Fig. 2.3.), which suggests that while expression levels are affected, toxin recovery is not. Summarising the data across all species sampled here reveals that, while the number of toxin-encoding contigs are greatly outnumbered by non-toxins in each of the venom gland transcriptomes, the mean expression levels of the toxin encoding contigs are >60 fold higher (Table 2.3.). Comparisons between spitting and non-spitting species revealed that spitting cobras have a higher mean expression of non-toxin contigs (33%, compared to 29%), but this is not statistically significant (Analysis of Variance of Aligned Rank Transformed Data,

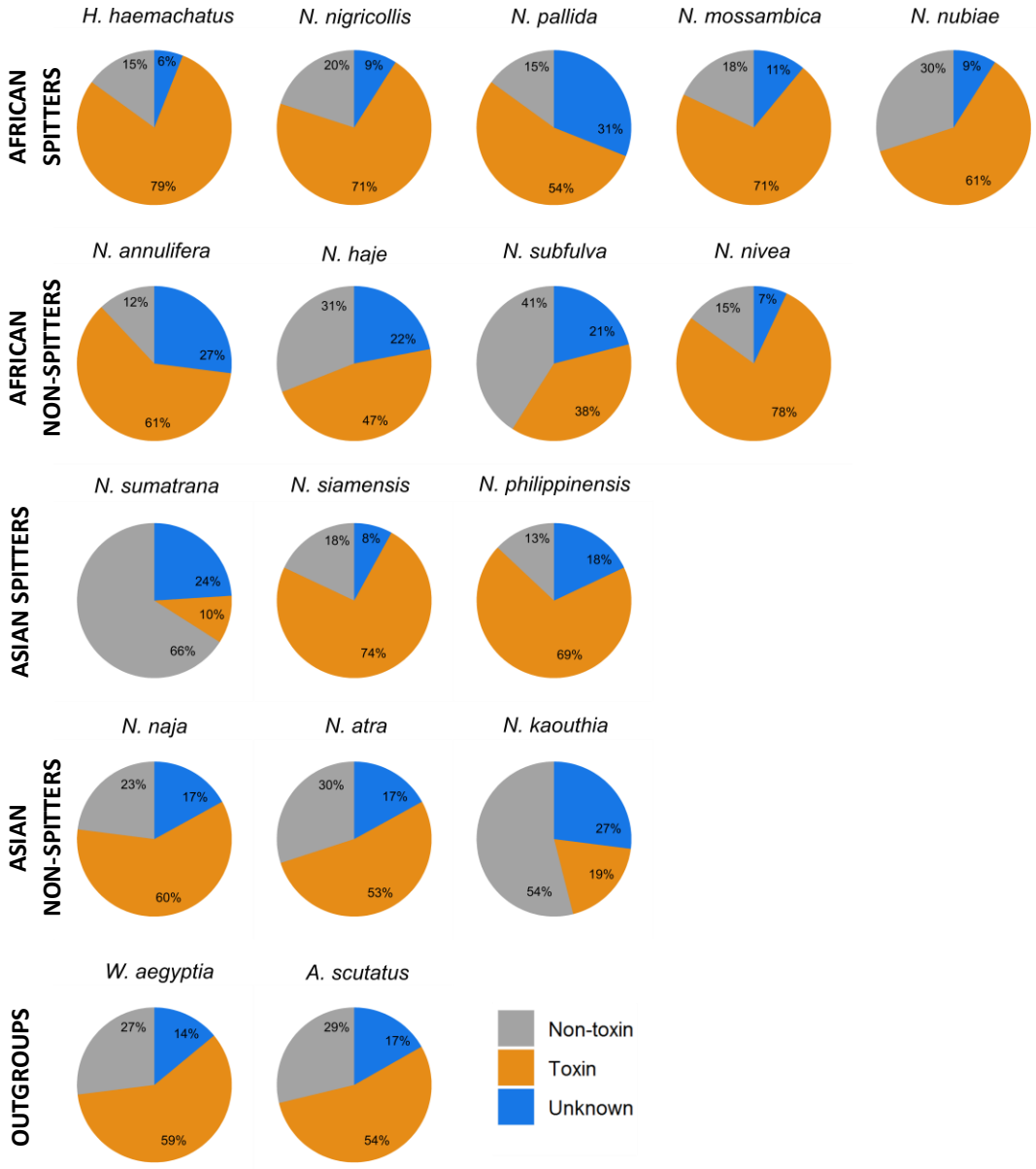


Figure 2.2 An overview of gene expression in the venom gland transcriptomes of cobras and their near relatives.

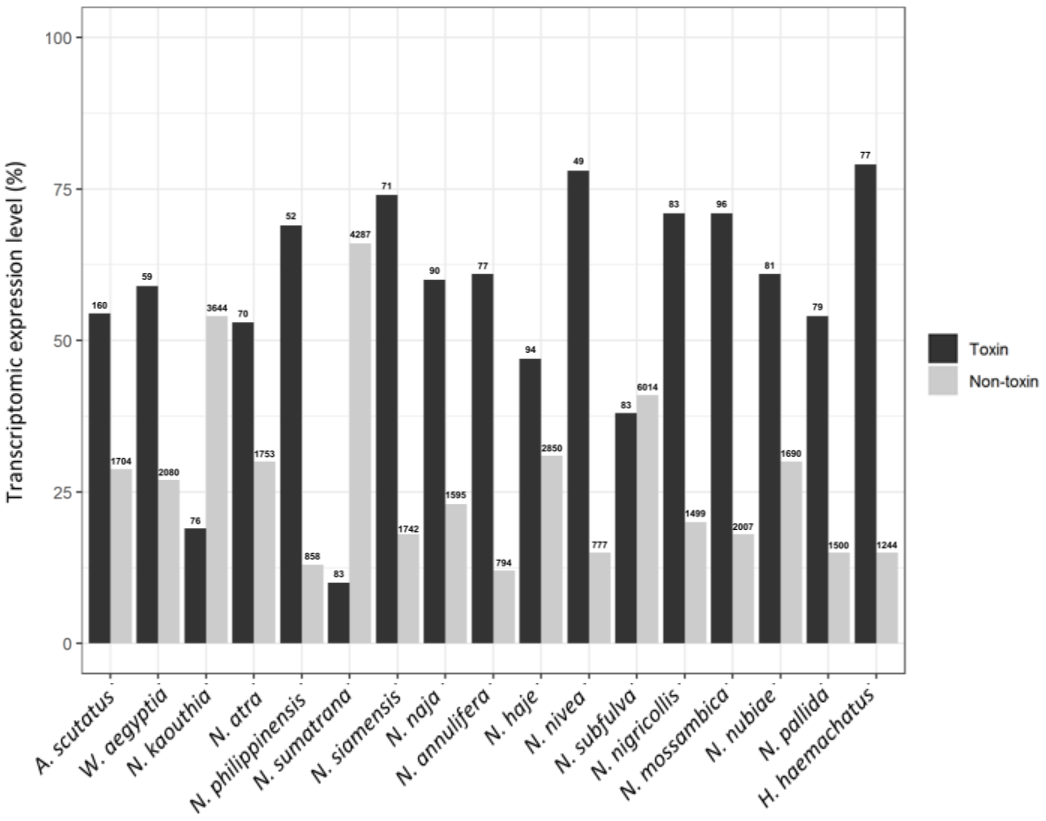


Figure 2.3. Comparisons of the numbers and expression levels of contigs annotated as 'toxin' and 'non-toxin' in each of the venom gland transcriptomes. Bars represent the summed expression levels for each category, and the numbers above each bar represent the number of contigs contributing to that classification. Toxins classified as 'unknowns' were excluded from the figure.

Table 2.3. The average number and expression level of contigs annotated as toxins and non-toxins in the venom gland transcriptomes (n=17).

Protein family	Average number of sequences	Average expression per sequence (%)	Range of the mean expression per sequence
Toxin	68	0.886	0.2% - 1.6%
Non-toxin	1978	0.014	0.009% - 0.018%

F = 0.30, df = 1, p = 0.60) (Table 2.4.). Similarly, spitting species also had a higher mean toxin expression in the venom gland transcriptomes than non-spitting species (53% and 51% respectively), which again is not significantly different (Analysis of Variance of Aligned Rank Transformed Data, F = 1.21, df = 1, p = 0.30) (Table 2.4.). By default therefore, non-spitting cobras had a higher mean expression of sequences classed as 'unknown' by BLAST annotation (20%, compared to 15%), but once again this is not statistically significant (Two-way ANOVA, F = 1.29, df = 1, p = 0.28) (Table 2.4.). In addition to these spitting vs non-spitting comparison, statistical analyses found no influence of geographic origin or the interaction of geography and spitting ability on the relative expression of toxin, non-toxin or unknown sequences (Table 2.4.), as anticipated.

2.3.ii. Overview of the Venom Gland Toxinomes

The toxin encoding portion of venom gland transcriptomes is often referred to as the "toxinome". Comparisons of the species-level toxinomes of the venom gland transcriptomes studied here can be found in Fig. 2.4., while Fig. 2.5. provides additional detail by displaying the individual contig expression levels for major toxin families. Table 2.5. summarises the data for all species grouped together. For all members of the genus *Naja*, 3FTXs are the most abundant expressed venom toxin family, as they account for between 44.80 and 91.35% of all toxins. Within the 3FTXs, perhaps surprisingly, cytotoxic 3FTXs (CTX) are the most predominant class observed, except for *N. haje*, *N. atra* and *N. philippinensis*, where expression levels are less than 'other 3FTXs' (Fig. 2.4.). A similar pattern was observed in *Hemachatus haemachatus*, with 3FTXs accounting for 45% of all toxin expression, and CTX the majority of those (35% of total toxin expression), whereas the non-spitting outgroup *Walterinnesia aegyptia* showed a distinct expression profile, being dominated by PLA_2 toxins (35%), with only 25% of expression related to 3FTXs and no evidence of CTX expression at all (Fig. 2.5.), instead having a moderate expression of kunitz contigs (18%). In many of the cobra venom gland transcriptomes, PLA_2s are the second most dominant toxin family after the 3FTXs, although the expression levels of these toxins vary extensively

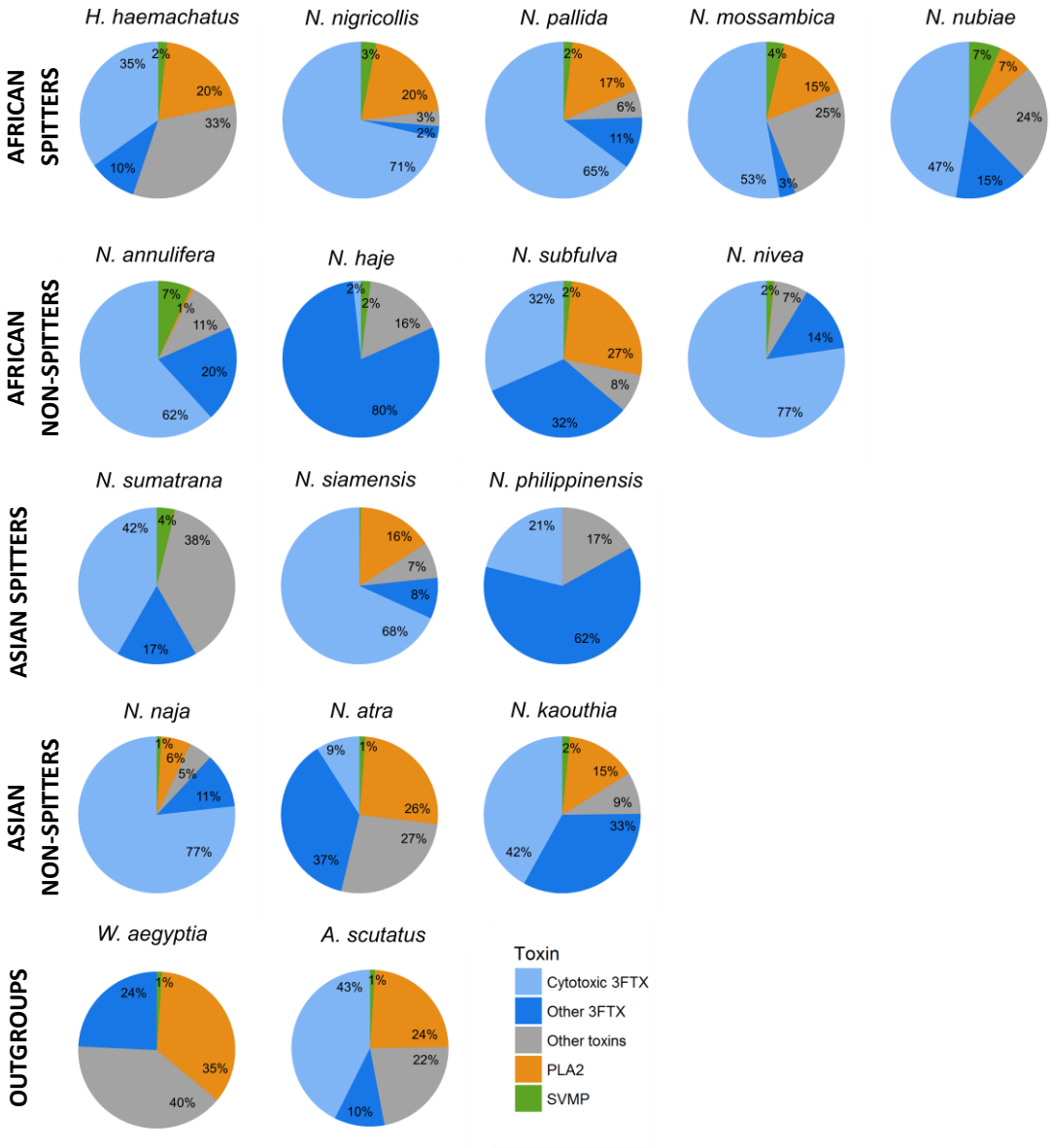


Figure 2.4. An overview of toxin gene expression in the venom gland transcriptomes of cobras and their near relatives. Data presented represents the relative abundance of each toxin family and not absolute abundance.

Table 2.4. Test statistics from ANOVA analyses assessing the influence of spitting, geography, and the combination of both factors on transcriptome expression in *Naja* and *Haemachatus*. The first three variables represent absolute expression in the transcriptome, while the remaining variables represent expression as a percentage of total toxins. Significant values are emboldened, and those that remain significant post Bonferroni-correction are represented by red text. Degrees of freedom = 1.

Test Variable	Test type	Factor	F value	p
Toxin expression	Analysis of Variance of Aligned Rank Transformed Data	Group (spitter/non-spitter)	1.21	0.30
		Geography (African/Asian)	0.26	0.62
		Group + Geography	0.17	0.69
Non-toxin expression	Analysis of Variance of Aligned Rank Transformed Data	Group (spitter/non-spitter)	0.30	0.60
		Geography (African/Asian)	0.99	0.34
		Group + Geography	0.26	0.62
Unknown expression	Two-way ANOVA	Group (spitter/non-spitter)	1.29	0.28
		Geography (African/Asian)	0.45	0.52
		Group + Geography	0.12	0.74
3FTX expression	Two-way ANOVA	Group (spitter/non-spitter)	1.48	0.25
		Geography (African/Asian)	0.01	0.93
		Group + Geography	1.71	0.22
No. of 3FTX sequences	Two-way ANOVA	Group (spitter/non-spitter)	0.01	0.92
		Geography (African/Asian)	0.00	0.98
		Group + Geography	1.44	0.26
CTX expression	Two-way ANOVA	Group (spitter/non-spitter)	0.35	0.57
		Geography (African/Asian)	0.18	0.68
		Group + Geography	0.12	0.74
No. of CTX sequences	Two-way ANOVA	Group (spitter/non-spitter)	4.49	0.06
		Geography (African/Asian)	3.11	0.11
		Group + Geography	3.45	0.09
'Other 3FTX' expression	Analysis of Variance of Aligned Rank Transformed Data	Group (spitter/non-spitter)	2.16	0.17
		Geography (African/Asian)	0.20	0.66
		Group + Geography	0.11	0.75
No. of 'Other 3FTX' sequences	Two-way ANOVA	Group (spitter/non-spitter)	0.01	0.91
		Geography (African/Asian)	0.86	0.37
		Group + Geography	0.44	0.52
SVMP expression	Analysis of Variance of Aligned Rank Transformed Data	Group (spitter/non-spitter)	0.33	0.57
		Geography (African/Asian)	**6.21**	**0.03**
		Group + Geography	0.23	0.64
No. of SVMP sequences	Analysis of Variance of Aligned Rank Transformed Data	Group (spitter/non-spitter)	0.09	0.76
		Geography (African/Asian)	**6.49**	**0.03**
		Group + Geography	2.09	0.18

Table 2.4. Continued

Test Variable	Test type	Factor	F value	p
PLA_2 expression	Two-way ANOVA	Group (spitter/non-spitter)	0.08	0.79
		Geography (African/Asian)	0.08	0.78
		Group + Geography	3.69	0.08
No. of PLA_2 sequences	Analysis of Variance of Aligned Rank Transformed Data	Group (spitter/non-spitter)	2.21	0.17
		Geography (African/Asian)	0.95	0.35
		Group + Geography	0.41	0.53
Kunitz expression	Analysis of Variance of Aligned Rank Transformed Data	**Group (spitter/non-spitter)**	**7.04**	**0.02**
		Geography (African/Asian)	**7.47**	**0.02**
		Group + Geography	4.09	0.07
No. of Kunitz sequences	Two-way ANOVA	**Group (spitter/non-spitter)**	**15.97**	**0.002**
		Geography (African/Asian)	**31.67**	**0.0002**
		Group + Geography	5.54	**0.04**
NGF expression	Two-way ANOVA	Group (spitter/non-spitter)	1.06	0.33
		Geography (African/Asian)	0.80	0.39
		Group + Geography	0.33	0.58
No. of NGF sequences	Analysis of Variance of Aligned Rank Transformed Data	Group (spitter/non-spitter)	0.39	0.55
		Geography (African/Asian)	0.09	0.77
		Group + Geography	0.97	0.35
NP expression	Analysis of Variance of Aligned Rank Transformed Data	Group (spitter/non-spitter)	2.32	0.16
		Geography (African/Asian)	3.02	0.11
		Group + Geography	0.45	0.52
No. of NP sequences	Two-way ANOVA	Group (spitter/non-spitter)	7.61	**0.02**
		Geography (African/Asian)	2.20	0.17
		Group + Geography	0.70	0.42
CRISP expression	Two-way ANOVA	Group (spitter/non-spitter)	8.53	**0.01**
		Geography (African/Asian)	0.00	0.95
		Group + Geography	3.35	0.09
No. of CRISP sequences	Two-way ANOVA	**Group (spitter/non-spitter)**	**16.75**	**0.002**
		Geography (African/Asian)	0.40	0.54
		Group + Geography	0.00	0.98
CTL expression	Analysis of Variance of Aligned Rank Transformed Data	Group (spitter/non-spitter)	0.03	0.87
		Geography (African/Asian)	1.81	0.21
		Group + Geography	0.01	0.91
No. of CTL sequences	Analysis of Variance of Aligned Rank Transformed Data	Group (spitter/non-spitter)	0.87	0.37
		Geography (African/Asian)	0.20	0.66
		Group + Geography	1.05	0.33
SP expression	Analysis of Variance of Aligned Rank Transformed Data	Group (spitter/non-spitter)	0.02	0.89
		Geography (African/Asian)	0.30	0.59
		Group + Geography	0.39	0.54

Table 2.4. Continued

Test Variable	Test type	Factor	F value	p
No. of SP sequences	Analysis of Variance of Aligned Rank Transformed Data	Group (spitter/non-spitter)	0.26	0.62
		Geography (African/Asian)	0.09	0.77
		Group + Geography	0.00	0.97
Acidic PLA_2 expression	Analysis of Variance of Aligned Rank Transformed Data	**Group (spitter/non-spitter)**	**28.34**	**0.0002**
		Geography (African/Asian)	4.32	0.06
		Group + Geography	3.93	0.07
No. of Acidic PLA_2 sequences	Analysis of Variance of Aligned Rank Transformed Data	Group (spitter/non-spitter)	3.16	0.10
		Geography (African/Asian)	0.38	0.55
		Group + Geography	4.12	0.07
Basic PLA_2 expression	Two-way ANOVA	**Group (spitter/non-spitter)**	**4.99**	**0.05**
		Geography (African/Asian)	0.36	0.56
		Group + Geography	4.43	0.06
No. of Basic PLA_2 sequences	Analysis of Variance of Aligned Rank Transformed Data	**Group (spitter/non-spitter)**	**6.06**	**0.03**
		Geography (African/Asian)	3.36	0.09
		Group + Geography	1.72	0.22
PC1	Analysis of Variance of Aligned Rank Transformed Data	Group (spitter/non-spitter)	5.00	**0.05**
		Geography (African/Asian)	0.12	0.73
		Group + Geography	0.12	0.74
PC2	Analysis of Variance of Aligned Rank Transformed Data	Group (spitter/non-spitter)	0.06	0.81
		Geography (African/Asian)	4.71	0.053
		Group + Geography	1.65	0.23
PC3		Group (spitter/non-spitter)	0.93	0.36
		Geography (African/Asian)	0.60	0.46
		Group + Geography	1.24	0.29

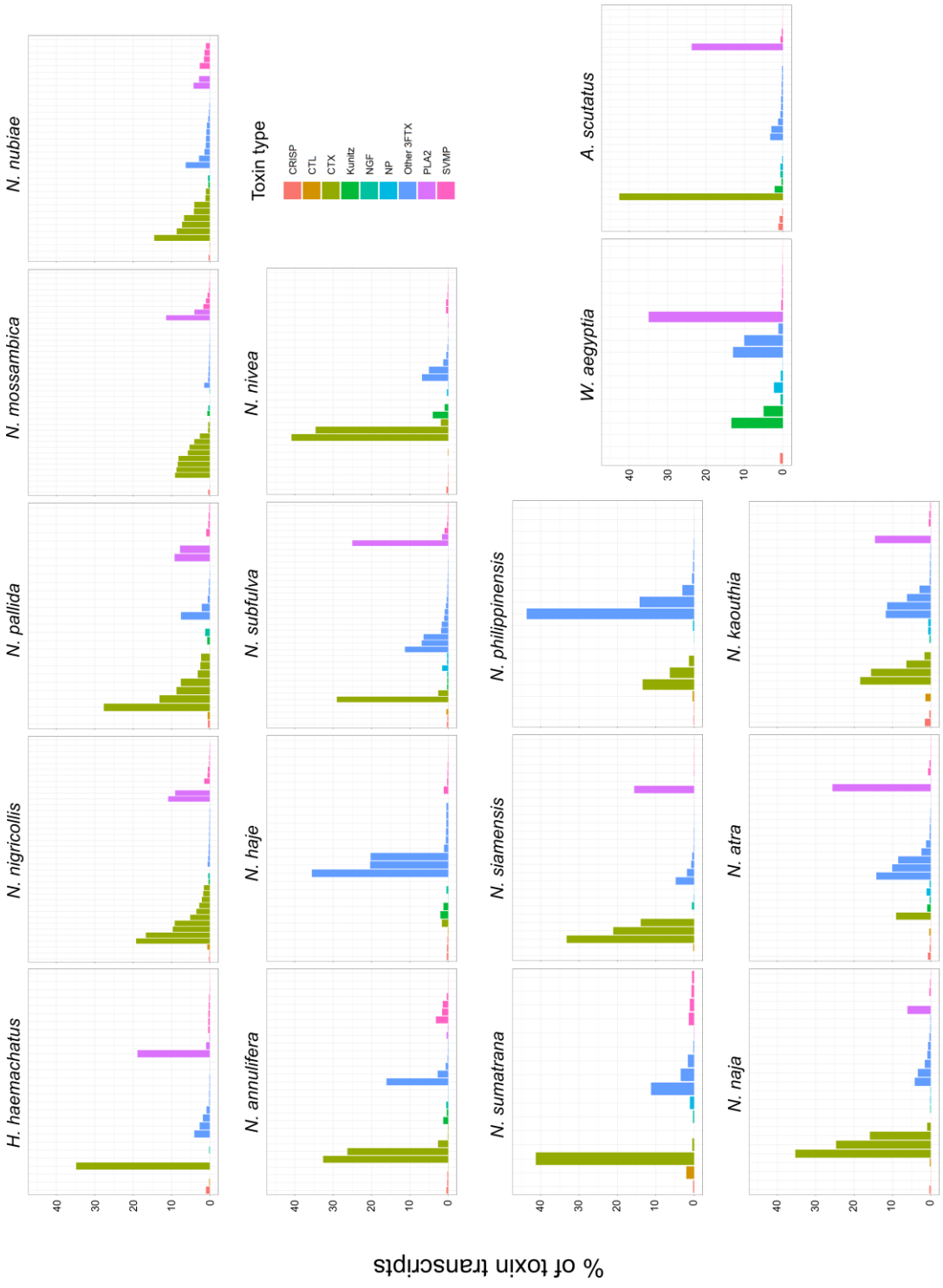


Figure 2.5. An overview of the expression of individual toxin sequences in cobras. Each bar represents an individual contig in the toxic transcriptome.

Table 2.5. The average number and expression levels of toxin encoding contigs identified in the various venom gland transcriptomes (n = 17).

Toxin family	Average number of contigs	Average summed expression (% of toxins)	Average expression per contig	Range of the mean expression per contig
3FTX	19	45.30	2.51	0.90-7.10%
SVMP	13	1.74	0.21	0.03-1.50%
PLA_2	4	8.69	1.92	0.01-5.80%
CRISP	3	0.40	0.11	0.00-0.20%
Kunitz	1	0.59	0.32	0.00-1.90%
CTL	2	0.20	0.14	0.04-0.50%

from 27% of total toxin expression in *N. subfulva* to less than 1% in *N. haje* and *N. nivea*. The SVMP toxin family are the third most expressed toxin family for all species studied here, with the exception of *W. aegyptia*, as described above, although the expression levels of SVMPs never accounted for more than 7% of the total toxin expression in any of the studied species.

2.3.iii. Three-finger Toxins (3FTXs)

Across the dataset, the 3FTX family exhibit the highest average expression at both the toxin family and contig level, as well as the most variation in expression per contig (Table 2.5.). In addition, 3FTXs have consistently high expression across the sampled taxa and make up the highest number of sequences in the toxinome (Fig. 2.4. and 2.6A.) with only *W. aegyptia*, *H. haemachatus* and *N. atra* exhibiting toxinome expression levels of less than 50%. When excluding the outgroup species, non-spitting cobras were found to have the same mean number of 3FTX-encoding contig as spitting species, but a considerably higher level of 3FTX expression (average of 75.4% and 66.3%, respectively), although this difference was not statistically significant (Two-way ANOVA, $F = 0.01$, $df = 1$, $p = 0.92$; $F = 1.48$, $df = 1$, $p = 0.25$, respectively) (Table 2.4.). To identify any regional influences on 3FTX expression, data was additionally separated by continent (Fig. 2.6B.). African cobras were found to have a lower level of 3FTX expression than Asian cobras (average of 68.8% and 73.3%, respectively), but a higher number of 3FTX contigs (13 compared to 10). However,

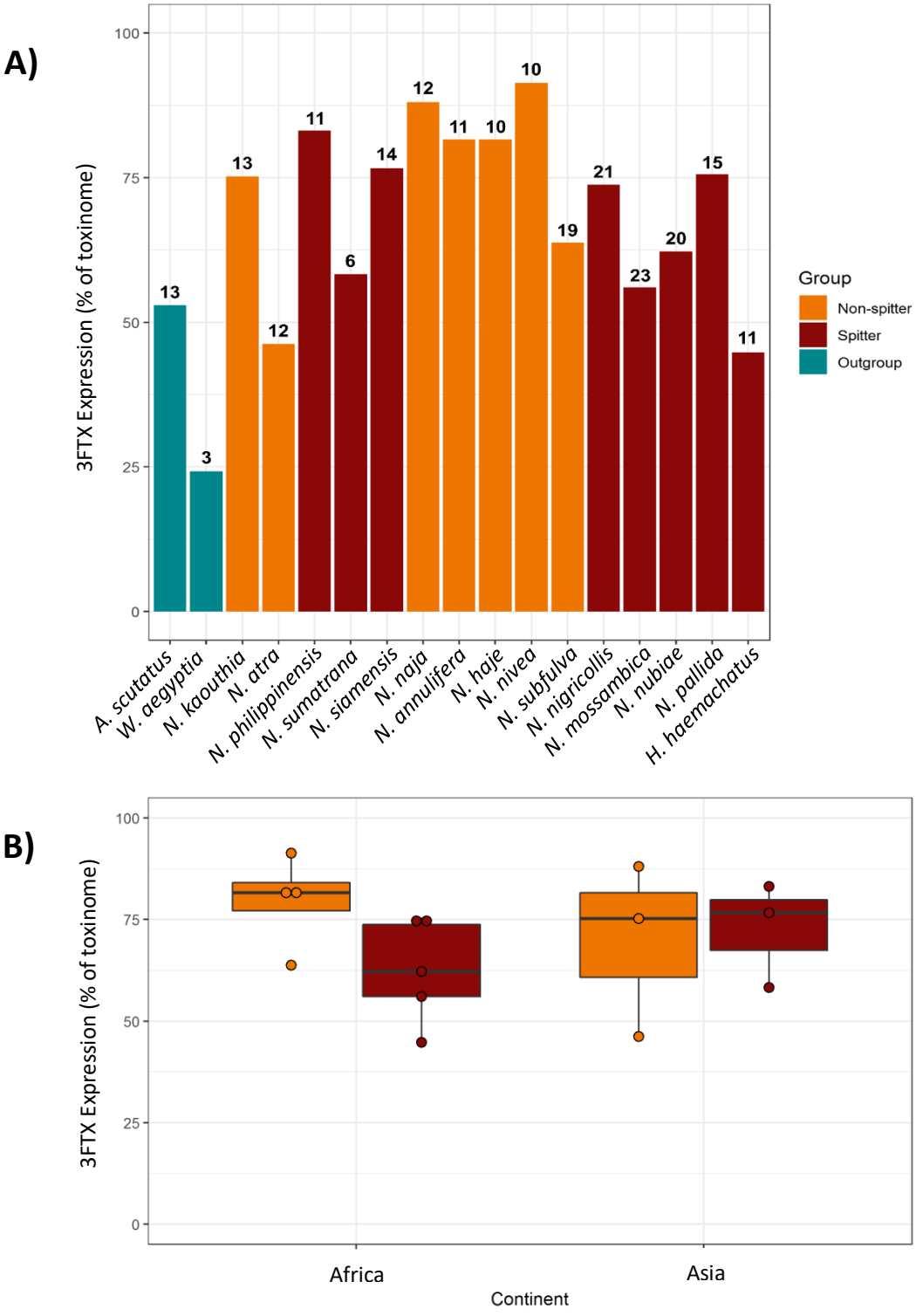


Figure 2.6. Comparisons of 3FTX expression in cobra venom gland transcriptomes. A) 3FTX expression and number of 3FTX contigs (numbers above each bar) detected in the toxinome of the sampled species. **B)** Comparisons of 3FTX expression between spitting and non-spitting cobras (excluding outgroups), separated by continent.

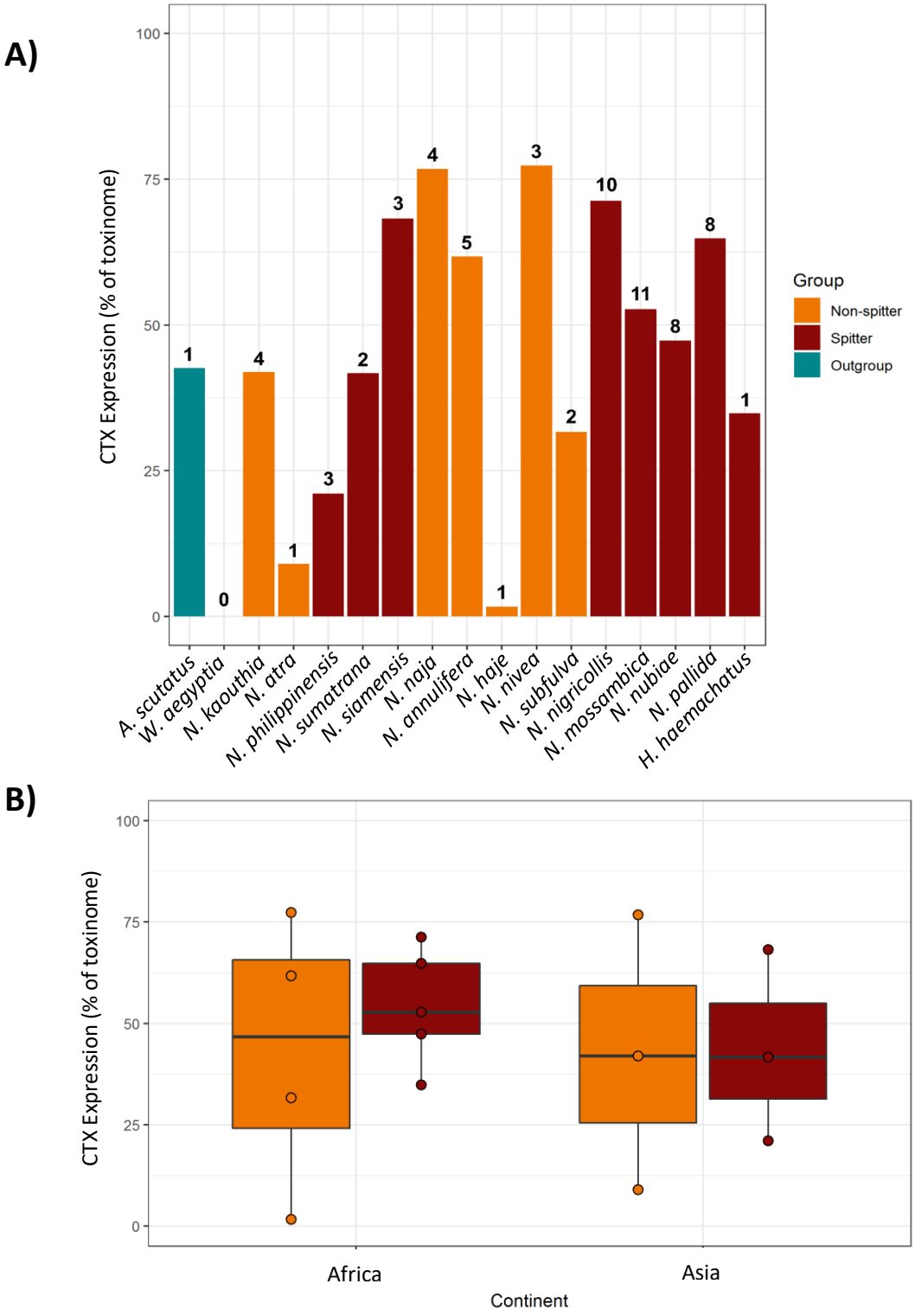


Figure 2.7. Comparisons of CTX expression in cobra venom gland transcriptomes. A) CTX expression and number of CTX contigs (numbers above each bar) detected in the toxinome of the sampled species. **B)** Comparisons of CTX expression between spitting and non-spitting cobras (excluding outgroups), separated by continent.

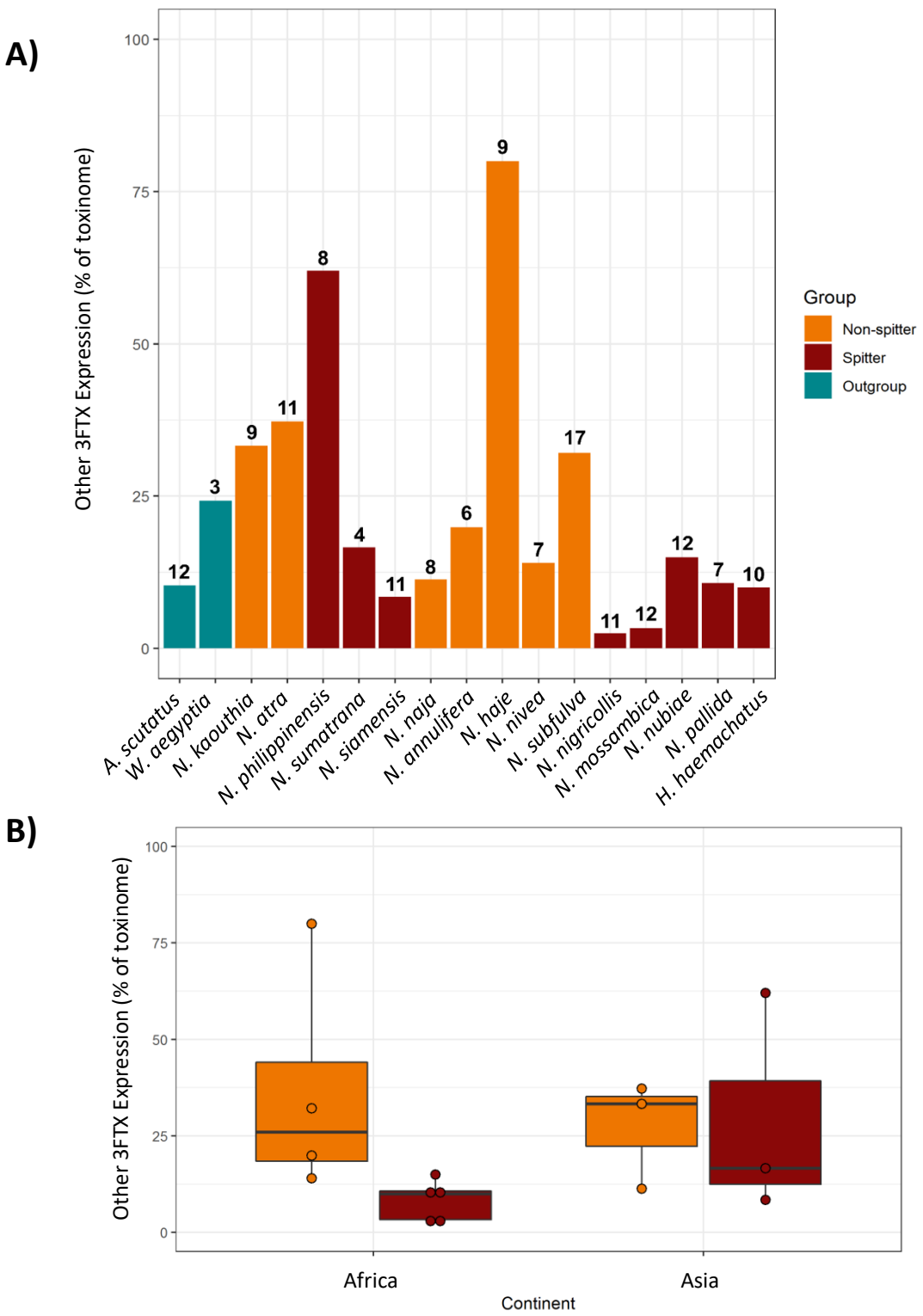


Figure 2.8. Comparisons of 'other 3FTX' (non-cytotoxin) expression in cobra venom gland transcriptomes. A) 'Other 3FTX' expression and number of 'other 3FTX' contigs (numbers above each bar) detected in the toxinome of the sampled species. **B)** Comparisons of 'other 3FTX' expression between spitting and non-spitting cobras (excluding outgroups), separated by continent.

neither of these observed differences are statistically significant (Two-way ANOVA, F = 0.01, p = 0.93; F = 0.00, df = 1, p = 0.98, respectively) (Table 2.4.).

BLAST annotation and sequence analyses facilitated separating the functionally diverse 3FTXs into two distinct categories. For simplicity, here the 3FTXs were partitioned into those identified as CTXs and all other 3FTXs, the majority of which are assumed to have neurotoxic properties. Perhaps surprisingly, the majority of species exhibited a much higher CTX expression than all other 3FTXs in the venom gland transcriptomes (Fig. 2.4.). Non-spitting cobras had a much larger range of CTX expression (1.7-75.8%) than spitting cobras (21.1-71.3%), and a lower mean number of CTX-encoding contigs (3, compared to 6 in spitting cobras) (Fig. 2.7.). However, likely due to the large spread of this data, no significant differences in CTX expression or contig numbers were detected between spitting and non-spitting cobras (Two-way ANOVA, F = 0.35, df = 1, p = 0.57; F = 4.49, df = 1, p = 0.06, respectively) (Table 2.4.). African cobras have a higher average expression of CTXs (54.4%) and number of CTX sequences (6) than Asian cobras (34.6% and 2 sequences), but this was also not statistically significantly (Two-way ANOVA, F = 3.11, df = 1, p = 0.11) (Table 2.4.). While CTXs typically accounted for the majority of 3FTX expression, all non-outgroup species have more contigs encoding for 'other 3FTXs' than CTXs. However, of the ingroup species, only *N. philippinensis* and *N. haje* exhibited expression levels of these 'other 3FTXs' of greater than 50% of the toxinome (Fig. 2.4. and 2.8.), with the range for the genus being between 2.45 and 79.97%. While non-spitting cobras have a higher average expression of 'other 3FTXs' (33.2%) than spitting cobras (16.1%), and a slightly higher mean number of contigs encoding these toxins (10, compared to 9), neither of these differences are statistically significant (Analysis of Variance of Aligned Rank Transformed Data, F = 2.16, df = 1, p = 0.17; Two-way ANOVA, F = 0.01, df = 1, p = 0.91, respectively) (Table 2.4.). In addition, no significant effect of geography on 'other 3FTX' expression or contig number was detected (Analysis of Variance of Aligned Rank Transformed Data, F = 0.20, df = 1, p = 0.66; Two-way ANOVA, F = 0.86, df = 1, p = 0.37, respectively) (Table 2.4).

2.3.iv. Snake venom metalloproteinases (SVMPs)

SVMPs are the third-most abundant toxin family in the studied venom gland transcriptomes, with the exception of the outgroups *Aspidelaps scutatus* and *Walterinnesia aegyptia* (Fig. 2.4. and 2.5.). All SVMPs that could be classified to the sub-class level (e.g. P-I, P-II or P-III SVMPs) were found to be P-III metalloproteases, which is consistent with the literature describing elapid snakes not containing P-I or P-II SVMPs in their venom (Tasoulis & Isbister, 2017). However, many of the SVMPs found could not be classified due to their partial length preventing accurate detection of the N-terminal domains that permit sub-class classification. These short length SVMP contigs are commonplace in venom gland assemblies, as this toxin class are notoriously difficult to assemble due to their large size (up to ~1800 bp) and multiple isoforms, thus typically resulting in numerous partial length sequences due to under assembly, unless sequence coverage is very high (Archer et al., 2014). Likely because of this, the number of SVMP contigs presented here is an exaggeration of the genuine number. Both spitting and non-spitting cobras have a similar range (0.95-6.82%, non-spitters; 0.00-6.54%, spitters) and mean (2.26%, non-spitters; 2.63% in spitters) of SVMP expression in the venom gland transcriptomes (Fig. 2.9.), and as anticipated, these differences are not statistically significant (Analysis of Variance of Aligned Rank Transformed Data, $F = 0.33$, $df = 1$, $p = 0.57$) (Table 2.4.). Moreover, there are no differences in SVMP contig number either (Analysis of Variance of Aligned Rank Transformed Data, $F = 0.09$, $df = 1$, $p = 0.76$) (Table 2.4.), although variable contig inflation due to under-assembly, as discussed above, likely renders this comparison of questionable value. Similarly, there is no significant difference in SVMP expression or contig number between geographic locations (Analysis of Variance of Aligned Rank Transformed Data, $F = 6.21$, $df = 1$, $p = 0.96$; $F = 6.49$, $df = 1$, $p = 0.96$, respectively) (Table 2.4.).

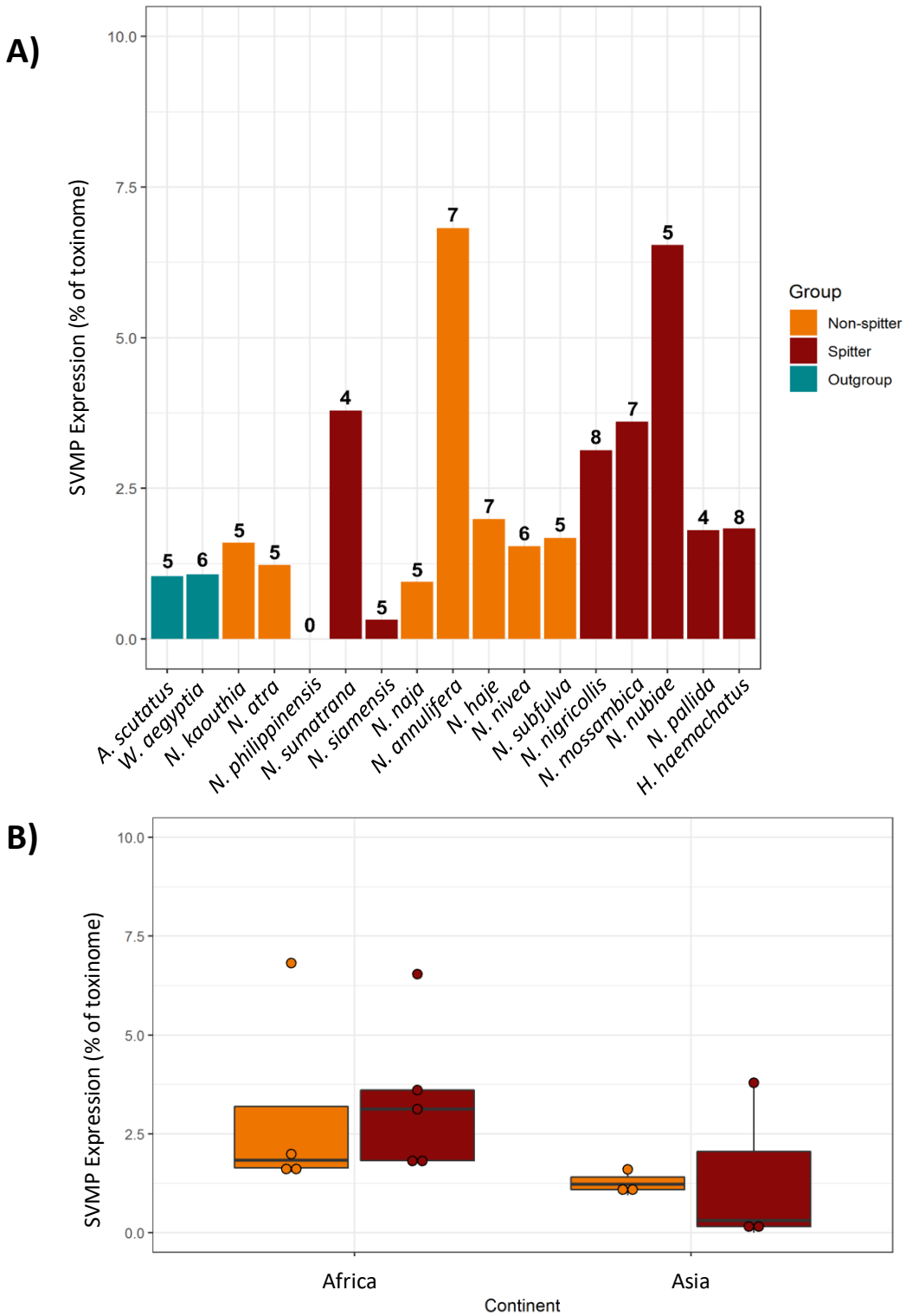


Figure 2.9. Comparisons of snake venom metalloproteinase (SVMP) expression in cobra venom gland transcriptomes. A) SVMP expression and number of SVMP contigs (numbers above each bar) detected in the toxinome of the sampled species. **B)** Comparisons of SVMP expression between spitting and non-spitting cobras (excluding outgroups), separated by continent.

2.3.v. Phospholipase A_2s (PLA_2s)

The PLA_2 gene family exhibits considerable variation in venom gland expression levels across cobras and their near relatives, with non-spitting cobras exhibiting expression levels that range from 0.00% to 26.61% and spitting species from 0.01% to 19.9% of all toxins. Despite these large variations in expression levels, the number of contigs encoding PLA_2s detected in the transcriptomes remains low across all species (Fig. 2.10A.). All species have at least one contig annotated as a PLA_2, with the exception of *N. haje*, while the most detected was three PLA_2 encoding contigs, found in three African spitting cobra species and the cobra relative and spitting species *Hemachatus haemachatus*. As a consequence of these low contig numbers, the expression level per contig is fairly high (1.92%) and approaches the levels observed for 3FTXs (2.51%) (Table 2.5.), suggesting that the PLA_2s are functionally important toxins for at least some cobra species. However, the transcriptome data suggests that abundant PLA_2 expression occurs independently of venom spitting or geographical distribution (Fig. 2.10B.). Although spitting cobras have a higher mean expression of PLA_2s than non-spitting cobras (11.9% and 10.5%, respectively) and non-spitting cobras exhibit greater variation in PLA_2 abundance, ranging from complete absence in *N. haje* to over 25% of the toxinome in *N. subfulva* (Fig. 2.10.), statistical comparisons of PLA_2 expression between the various groups tested here revealed no statistically significant differences (Two-way ANOVA, $F = 0.08$, $df = 1$, $p = 0.79$) (Table 2.4.).

While viper PLA_2s have been subjected to extensive study that has facilitated their classification based on the residues found at position 49, which determines their enzymatic or non-enzymatic mode of action (Kini, 2003; Mora-Obando et al., 2014; Xiao et al., 2017), elapid PLA_2s have proven more problematic to subclassify. However, snake venom PLA_2s in general exhibit highly variable isoelectric points, and thus can be facilely characterised as basic or acidic. Consequently, the isoelectric point (pI) of PLA_2 contigs detected in the various venom gland transcriptomes was predicted, and the resulting data is displayed in Table 2.6. Only full-length PLA_2s were used for this analysis, as missing amino acids from incomplete sequences can potentially interfere with accurate pI estimations. This analysis revealed that non-spitting cobras have a much higher expression level (mean 10.21%) and expression range (0.00-25.61%) of acidic PLA_2 toxins in comparison to spitting cobras (mean

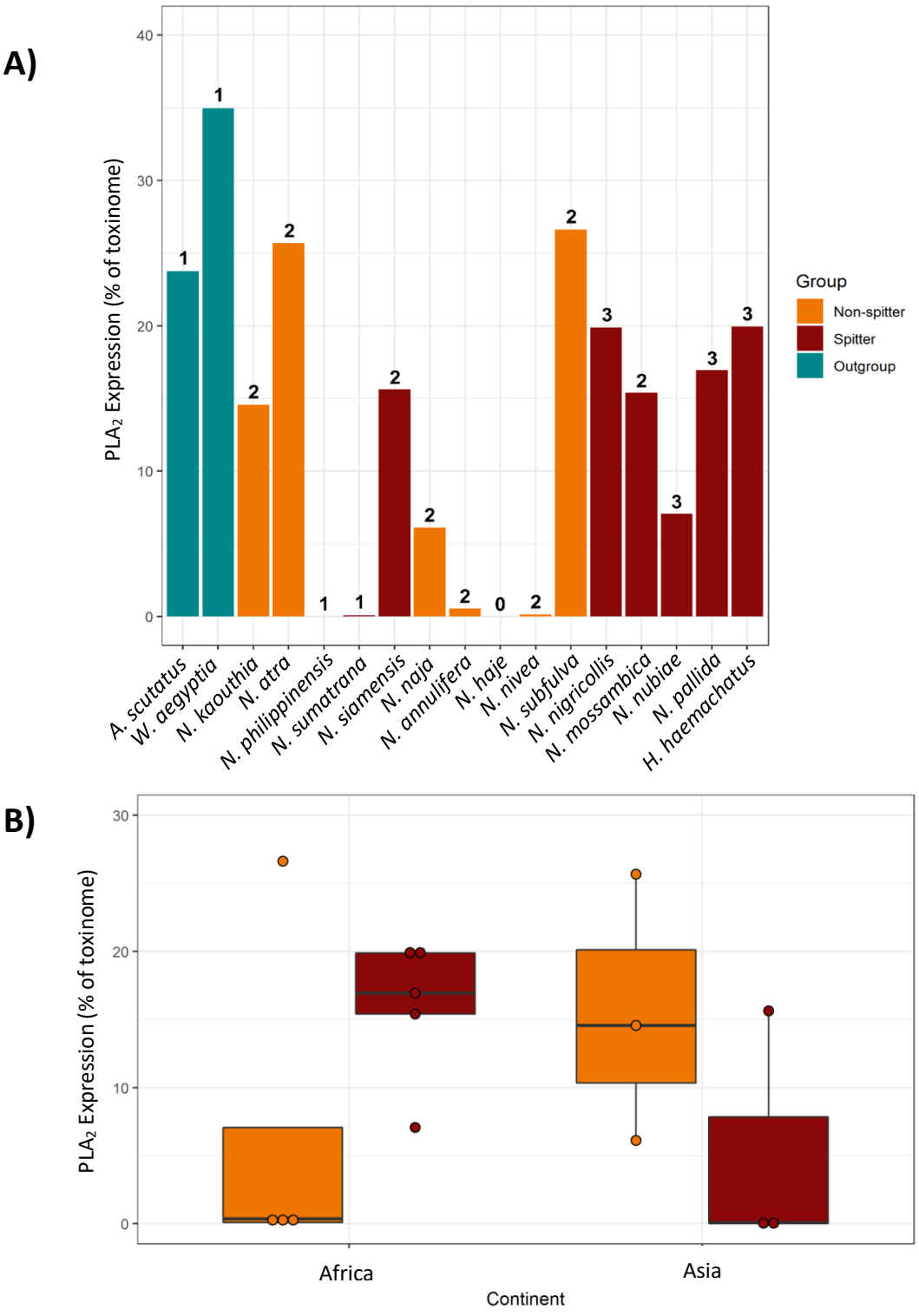


Figure 2.10. Comparisons of phospholipase A_2 (PLA_2) expression in cobra venom gland transcriptomes. A) PLA_2 expression and number of PLA_2 contigs (numbers above each bar) detected in the toxinome of the sampled species. **B)** Comparisons of PLA_2 expression between spitting and non-spitting cobras (excluding outgroups), separated by continent.

Table 2.6. A summary of PLA2 isoelectric point-based classification in the venom gland transcriptomes.

Species	Sequence ID	BLAST descriptor	Expression (%)	Predicted isoelectric point (Pi)
A. scutatus	T2474…	Acidic phospholipase A2 CM-II	23.77	5.24
H. haemachatus	T1867…	Basic phospholipase A2 DE-1	18.84	7.56
N. annulifera	T0585…	Acidic phospholipase A2 1	0.43	6.68
N. annulifera	T1304…	Notechis scutatus PLA-3 precursor	0.13	5.24
N. atra	T0869	Phospholipase A2 GL16-1	19.74	8.37
N. atra	T1375…	Acidic phospholipase A2 1	25.61	5.7
N. kaouthia	T1080	Acidic phospholipase A2 2	14.52	4.93
N. kaouthia	T4307…	Acidic phospholipase A2 HTe	0.04	5.28
N. mossambica	T3199…	Naja atra partial phospholipase A2	4.95	8.18
N. mossambica	T2086…	Naja naja (clone 2) phospholipase A2 (pla2)	11.38	8.76
N. naja	T0142…	N.naja mRNA for phospholipase A2	6.09	5.19
N. naja	T0547	Phospholipase A2 GL16-1	0.03	5.18
N. nigricollis	T1049…	Phospholipase A2 "basic"	9.04	9.02
N. nigricollis	T0927…	Naja naja (clone 2) phospholipase A2 (pla2)	10.82	7.56
N. nigricollis	T0833	Phospholipase A2 GL16-1	0.02	5.06
N. nivea	T0584	Naja naja (clone 2) phospholipase A2 (pla2)	0.05	7.96
N. nivea	T0525	Phospholipase A2 GL16-1	0.09	5.18
N. nubiae	T0061…	Naja naja (clone 2) phospholipase A2 (pla2)	2.79	6.3
N. nubiae	T0697…	Acidic phospholipase A2 1	4.23	8.85
N. nubiae	T1052	Phospholipase A2 GL16-1	0.04	5.18
N. pallida	T0443	Basic phospholipase A2 CM-III	9.16	8.52
N. pallida	T0808…	Naja naja (clone 2) phospholipase A2 (pla2)	7.77	8.2
N. pallida	T1879	Phospholipase A2 GL16-1	0.01	5.08
N. philippinensis	T0988	Naja naja (clone 3) phospholipase A2 (pla2)	0.01	8.45
N. siamensis	T1090…	Naja naja (clone 2) phospholipase A2 (pla2)	15.61	7.53
N. subfulva	T8777…	phospholipase A2 [Naja naja]	24.99	6.08
N. subfulva	T2707	Acidic phospholipase A2 2	1.62	8.65
N. sumatrana	T2210	Phospholipase A2 GL16-1	0.07	5.17
W. aegyptia	T3262…	Acidic phospholipase A2 PL-II	34.96	4.79

0.37% and range of 0.00-2.83%) (Fig. 2.11.). These differences in expression level are statistically significant (Analysis of Variance of Aligned Rank Transformed Data, F = 28.34, df = 1, p = 0.0002) (Table 2.4.), despite no difference in the number of acidic PLA_2 contigs (Analysis of Variance of Aligned Rank Transformed Data, F = 3.16, df = 1, p = 0.10) (Table 2.4.). Conversely, spitting cobra venoms have a higher mean expression level of basic PLA_2 toxins (11.48%) than non-spitting cobras (3.06%), which is statistically significant (Two-way ANOVA, F = 4.99, df = 1, p = 0.05) (Table 2.4.). Additionally, there was no evidence for the effect of geography on the variable expression of acidic or basic PLA_2s, with all statistical comparisons resulting in a lack of significance (Table 2.4.).

2.3.vi. Other toxins

Of the remaining toxin families consistently detected in the venom gland transcriptomes, the next most abundant was kunitz, particularly in *W. aegyptia*, in which this toxin makes up a substantial proportion of the total toxin expression (20.28%). This does, however, contrast extensively with the other outgroup species

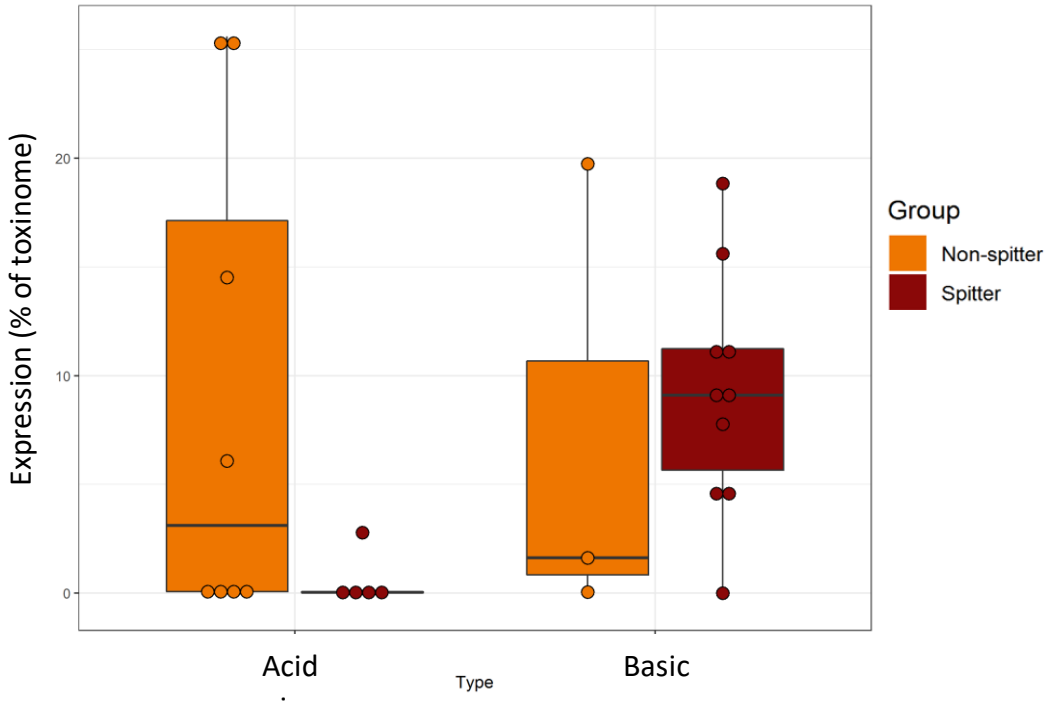


Figure 2.11. Comparisons of the expression of acidic and basic phospholipase A_2s (PLA_2s) in the toxinome between spitting and non-spitting cobras. PLA_2s were determined to be acidic if pI of < 6.5 or basic if having a pI of >7.5.

analysed (*A. scutatus*, 1.04%), and the ingroups of *Naja* and *Hemachatus*, where an average expression level of 1.65% (range 0.00-4.98%) was detected in the non-spitting species and 0.26% (range 0.00-0.67%) in the spitting species. There is a significant difference in the expression of kunitz toxins between spitting and non-spitting species (Analysis of Variance of Aligned Rank Transformed Data, F = 7.04, df = 1, p = 0.02) (Table 2.4.), and between geographical locales (Analysis of Variance of Aligned Rank Transformed Data, F = 7.47, df = 1, p = 0.02), and non-spitting cobras have significantly more kunitz contigs (Two-way ANOVA, F = 31.67, df = 1, p = 0.002) (Table 2.4.).

The expression levels of NP, CRISP, CTL and NGF toxins detected in the venom gland transcriptomes were all low, suggesting that these toxin families may not contribute extensively to the prey capturing ability or defensive (whether by spitting or biting) potency of the venoms. For NPs, mean toxinome expression levels of 0.8% and 0.2% were detected in non-spitting and spitting cobras, respectively. There were no significant differences in NP expression or contig number between spitting and non-spitting cobras (Table 2.4.). The expression levels of CRISPs were also higher in the venom gland transcriptomes of non-spitting cobras (0.98% vs 0.41%, respectively), which was statistically significant (Two-way ANOVA, F = 8.53, df = 1, p = 0.01) (Table 2.4.). The transcriptomic expression level of CTLs ranged from 0.06-1.41% of all toxins in non-spitting species and 0.00-2.05% in spitting species. No significant differences were detected in these expression levels or the numbers of CTL encoding contig between these groups (Analysis of Variance of Aligned Rank Transformed Data, F = 0.03, df = 1 p = 0.87; Transformed Data, F = 0.87, df = 1, p = 37, respectively) (Table 2.4.). Similarly, no differences in expression level or contig numbers were detected between spitting and non-spitting cobras for the NGF toxin family (Two-way ANOVA, F = 1.06, df = 1, p = 0.33; Analysis of Variance of Aligned Rank Transformed Data, F = 0.39, df = 1, p = 0.55, respectively), which exhibited low expression levels across both groups (0.00-0.53% and 0.05-1.22% of all toxins, respectively) or SPs (Analysis of Variance of Aligned Rank Transformed Data, F = 0.02, df = 1, p = 0.89; F = 0.09, df = 1, p = 0.62, respectively), which also exhibited low expression across both groups (0.00-1.20% and 0.00-0.22% of all toxins, respectively).

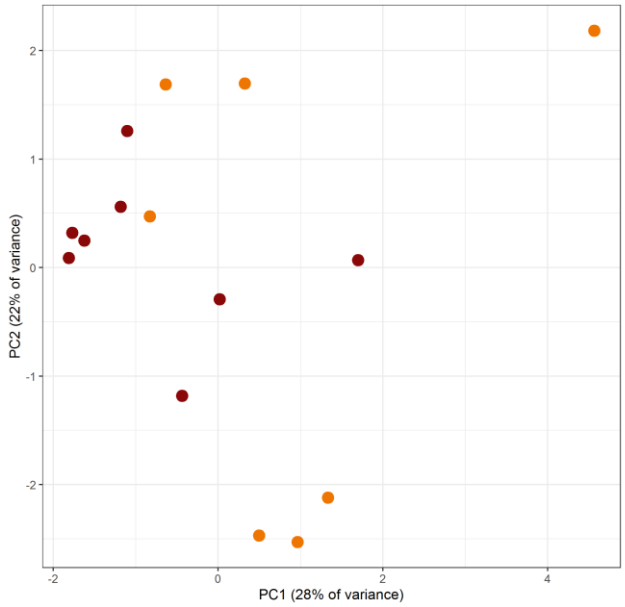
2.3.vii. Principal Component Analysis (PCA) of the venom gland transcriptomes

In order to see if a combination of toxins contribute to differences between spitting and non-spitting cobras, a PCA was performed using the expression data from all toxin families described in the section above. The first three Principal components (PCs) generated represent 28.4%, 22.4% and 12.0% of the transcriptome variation, respectively. The factors that positively contributed to PC1 are kunitz, NP, CRISP, 'Other 3FTX' and SP expression, while PLA_2, CTX, NGF, SVMP and CTL expression are the negatively contributing factors (see figs. 2.12. & 2.13). The factors that positively contribute to PC2 are CTX, 'Other 3FTX' (though at a low level), kunitz, NGF, SVMP and SP expression, while PLA_2, NP, CRISP and CTL expression are the negatively contributing factors (see figs. 2.12. & 2.13.). The factors that positively contribute to PC3 are PLA_2, 'Other 3FTX', NGF and SP expression, while CTX, kunitz, SVMP, NP, CRISP and CTL expression are the negatively contributing factors (see Fig. 2.13.).

The results of the PCA analyses show that the transcriptomic profiles of cobras do not cluster together based on their spitting or non-spitting capability (Fig. 2.12A.), nor by their lineage (e.g. African spitting cobras, African non-spitting cobras, Asian spitting cobras, Asian non-spitting cobras) (Fig. 2.12B.), when the first two principal components (representing 51% of the variation) are considered. The African spitting cobras do cluster more closely together than any other individual group, although a representative of both the Asian non-spitting and spitting groups are also found plotted adjacent to these data points. Most spitting cobras have a PC1 value of less than 1, and all spitting species have a PC1 value of less than 2 (Fig. 2.12A.). This suggests that spitting cobras are inclined to have higher CTX expression and lower SP and 'Other 3FTX expression' (Fig. 2.13A.). African spitting cobras have a particularly low PC1 score (Fig. 2.13A.), always being below 0, suggesting more exaggerated features of this component, and spitting cobras show a significantly lower PC1 score than non-spitters (Analysis of Variance of Aligned Rank Transformed Data, $F = 5.00$, $df = 1$, $p = 0.05$) (Table 2.4.).

The spread of PC2 results are highly variable, with the highest mean score observed in African non-spitters and the lowest mean score in Asian non-spitters,

A)



Factor	PC1	PC2
PLA2	-0.157	-0.426
CTX	-0.483	0.199
Other 3FTX	0.557	0.004
Kunitz	0.248	0.364
NGF	-0.208	0.077
SVMP	-0.176	0.227
NP	0.142	-0.553
CRISP	0.265	-0.223
CTL	-0.016	-0.399
SP	0.454	0.272

B)

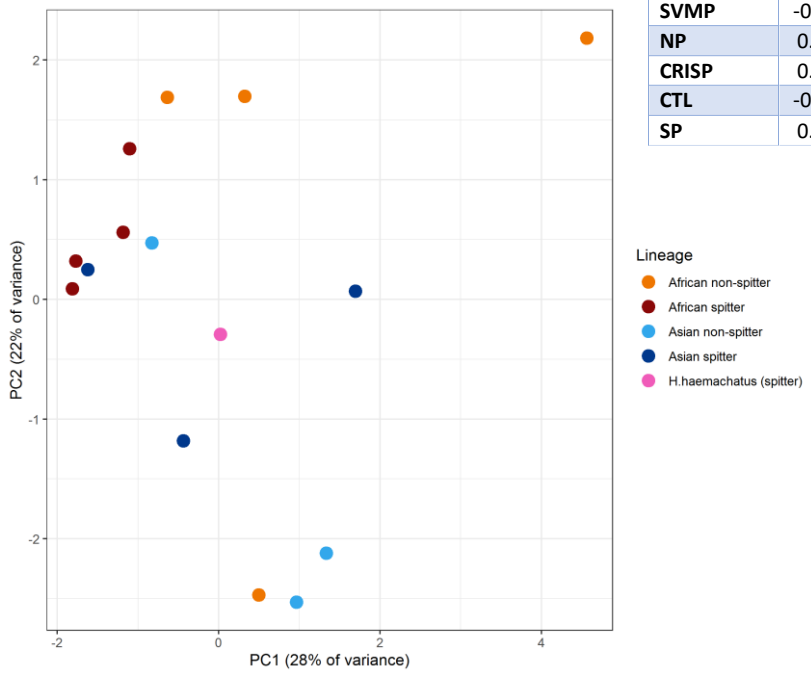
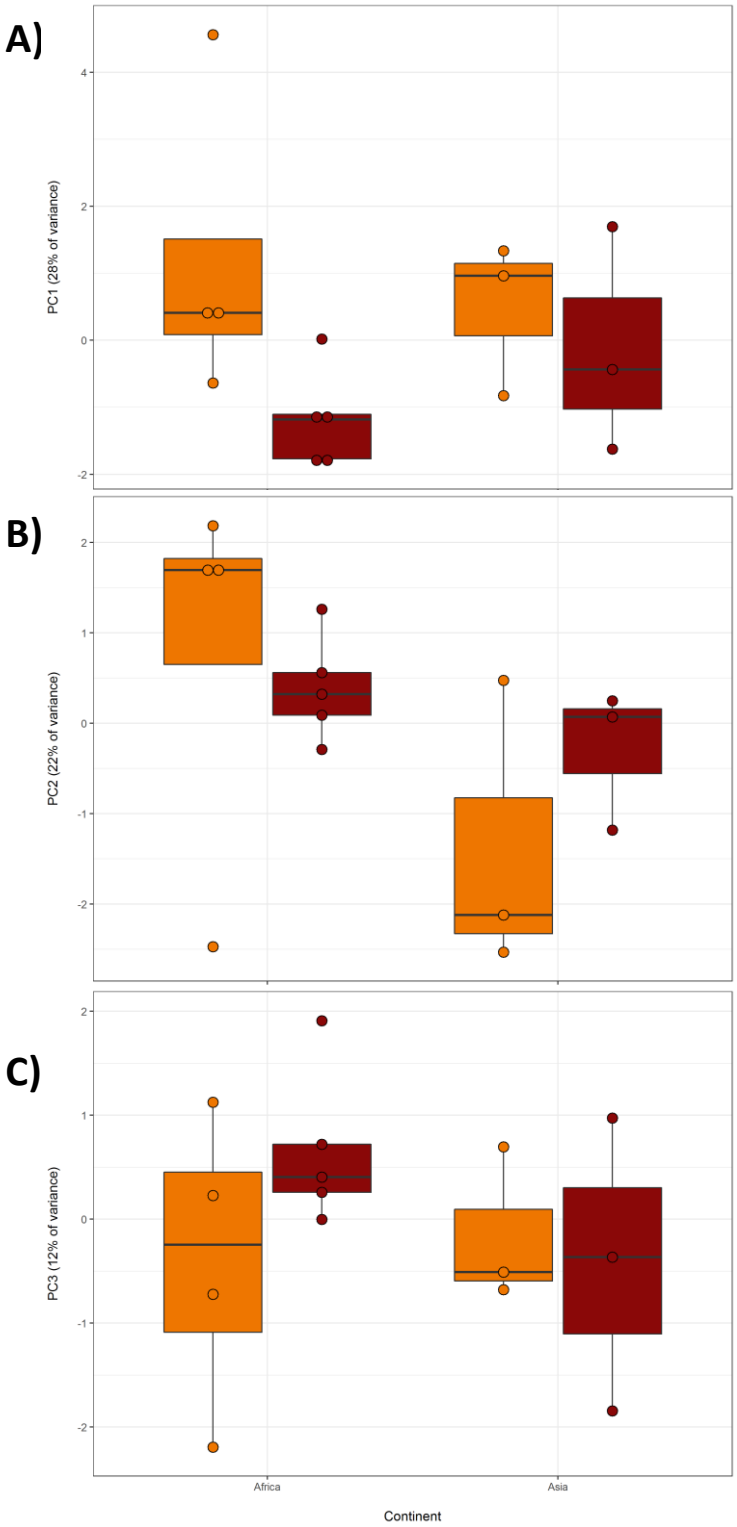


Figure 2.12. Scatter plot of the first two principle components (representing 50% of total variation) of the principal component analysis (PCA) performed on toxin family expression at the transcriptome level. Coloured by **A)** spitting or non-spitting and **B)** lineage. PC scores represent the correlation between component and the variable.



Factor	PC1
PLA2	-0.157
CTX	-0.483
Other 3FTX	0.557
Kunitz	0.248
NGF	-0.208
SVMP	-0.176
NP	0.142
CRISP	0.265
CTL	-0.016
SP	0.454

Factor	PC2
PLA2	-0.426
CTX	0.199
Other 3FTX	0.004
Kunitz	0.364
NGF	0.077
SVMP	0.227
NP	-0.553
CRISP	-0.223
CTL	-0.399
SP	0.272

Factor	PC3
PLA2	0.511
CTX	-0.228
Other 3FTX	0.078
Kunitz	-0.251
NGF	0.583
SVMP	-0.179
NP	-0.224
CRISP	-0.070
CTL	-0.342
SP	0.272

Figure 2.13. Box plots comparing the principal component analysis (PCA) scores of spitting and non-spitting cobras, separated by continent. A) PC1, **B)** PC2, **B)** PC3. The Tables next to each plot detail the representation of each toxin family in each component. Highlighted values show families strongly associated with the component (> 0.5 or < -0.5). PC scores represent the correlation between component and the variable.

and the net effect of this resulted in non-spitters having, on average, a lower PC2 score than those of spitting species (-0.2 and 0.1, respectively). However, because of this disparity among non-spitting species, the differences in PC2 observed between spitters and non-spitters was not found to be statistically significant (Analysis of Variance of Aligned Rank Transformed Data, F = 0.06, df = 1, p = 0.81) (Table 2.4.). The mean PC3 score resulting from the PCA showed extensive overlap across the two groups (-1.8-1.9 in spitting species, -2.2-1.1 in non-spitting species), and consequently no significant differences were detected between spitting and non-spitting cobras (Two-way ANOVA, F = 0.93, df = 1, p = 0.36) (fig 2.13C. and Table 2.4.). Finally, no statistically significant differences were observed when testing the effect of geographical distribution on any of the three different sets of PC scores (Table 2.4.).

2.4 Discussion

2.4.i. Overview

The objectives of this chapter were to compare toxin gene expression across the venom gland transcriptomes of various cobra species and analyse potential differences that might be associated with venom spitting. The comparative transcriptomic analysis revealed that cobra venom glands are dominated by the expression of two major toxin families: PLA_2s and 3FTXs, of which the 3FTX subfamily of CTXs are generally the most abundantly expressed. However, no significant differences between the expression levels of these major toxin families were observed between spitting and non-spitting cobras, although a significant difference in the subclass of PLA_2s was found between these groups when these toxins were separated by isoelectric point into acidic and basic forms. Spitting cobras exhibited significantly lower expression levels of acidic PLA_2s and significantly higher expression levels of basic PLA_2s than their non-spitting counterparts (Table 2.4.). In addition, spitting cobras were found to have significantly lower expression of CRISP and kunitz toxins, although the general low expression levels of these two toxin families makes it difficult to interpret how functionally relevant these detected differences might be. When global analyses of transcriptomic toxin expression levels

were performed via Principal Component Analysis, it was found that there were no differences in the first 3 principal components between spitting and non-spitting cobras (Table 2.4.).

The finding that cobras (genus *Naja*) have high expression levels of 3FTX toxins in their venom glands was perhaps not surprising, given that this toxin family is typically the most abundant type of toxins found in the venom glands of elapid snakes (Tasoulis & Isbister, 2017). The frequently observed high numbers of 3FTX isoforms and their correspondingly high expression levels are thought to underpin the predominately neurotoxic functional activities of many elapid venoms (Utkin et al., 2015). However, in the case of the cobras, it is CTXs rather than neurotoxins that appear to be the most expressed sub-class of 3FTXs (Fig. 2.4.). This is particularly interesting since CTXs are a class of 3FTXs that are said to be unique to cobras (Dufton & Hider, 1988). The main function of these toxins is to induce cytolysis through the formation of pores in the cell lipid membrane (Bilwes et al., 1994). In high concentrations, CTXs have been demonstrated to induce cardiac arrest in toads, cats and guinea pigs (Debnath et al., 2010; Dufton & Hider, 1988). Thus, the high CTX expression detected here across all cobra species suggests that their venoms are likely to cause cytotoxic and/or necrotic pathologies, perhaps in addition to neurotoxic effects, in snakebite victims. These findings therefore likely explain why the majority of cobra venoms cause cytotoxicity in cell models (Panagides et al., 2017) and why ocular contact following venom-spitting can cause extensive cell damage (Warrell & Ormerod, 1976). Moreover, a number of both African and Asian cobras have been described to cause severe local effects following snakebite envenoming, resulting in inflammation and necrosis (Faiz et al., 2017; Wang et al., 2014; Wong et al., 2010).

Many species of cobra show substantial expression levels of PLA_2 toxins, with this toxin family representing as much as 26.6% of all toxin transcripts in the venom gland transcriptome (fig 2.10.). This toxin class appears most prominently in the African spitting cobras, the Asian non-spitting cobras and the two outgroup species studied (*W. aegyptia* and *A. scutatus*). The elapid group I class of PLA_2s are generally considered to exert neurotoxic functions (Doley et al., 2010; Gutiérrez & Lomonte, 2013), but some PLA_2s isolated from the genus *Naja* have been shown to have

cytotoxic or antiplatelet effects (Rosenberg, 1997). Indeed, PLA_2s are generally considered to be multifunctional toxins (Ferraz et al., 2019). While PLA_2s might be expected to vary between spitting and non-spitting cobras, due to the potential for their lipase activities to promote cytotoxic effects, statistical comparisons of PLA_2 transcriptomic expression levels between these groups found no significant difference. However, when PLA_2s were classified into acidic and basic subclasses, a stark pattern emerged, with non-spitting species predominately expressing acidic PLA_2s (mean expression 10.2%), while expression by spitting cobras was dominated by basic PLA_2s (mean expression 11.5%). Previous work on viper PLA_2 toxins has shown that basic PLA_2s have myonecrotic, cytolytic and haemolytic functions which are absent in acidic PLA_2s (Jiménez-Charris et al., 2016). While the elapid PLA_2s studies here represent a different class of toxins to those of vipers, these findings hint that these detected differences could potentially impact upon the cytotoxic functional activities of these venoms. Further supporting this hypothesis is evidence from the BLAST2GO annotations recovered during transcriptome annotation. While the majority of the PLA_2 annotations recovered matched previously characterised PLA_2s that exhibited evidence of cytotoxic function via the hydrolysis of phospholipids, annotation matches to two PLA_2s that have known neurotoxic activity (Acidic phospholipase A2 HTe and Acidic phospholipase A2 1) were predominately found in the transcriptomes of non-spitting species (Table 2.7.).

The SVMP toxin family was generally found to exhibit moderate to low expression levels across the various cobra species sampled, yet this toxin family has the second-highest number of contigs of all toxin families detected. While this may, initially at least, suggest a considerable diversity of toxin isoforms within this toxin family, it is worth stressing that SVMPs are highly susceptible to underassembly due to their large sequence length, which is almost certainly inflating the number of SVMP-encoding contigs detected across the species. All of the SVMP genes expressed in the cobra transcriptomes that could be identified to sub-class level are P-III SVMPs, which represent the plesiotypic form of this toxin family. P-III SVMPs consist of metalloprotease, disintegrin-like and cysteine-rich domains, with the main function

Table 2.7. The BLAST annotations and functional activities associated with PLA_2 toxins matching those recovered from the venom gland transcriptomes. The species that the matches were identified in are displayed in the third column.

BLAST descriptor	Function(s)	Species found in
Acidic phospholipase A2 CM-II	Hydrolysis of phospholipids	*A. scutatus*
Basic phospholipase A2 DE-1	Hydrolysis of phospholipids	*H. haemachatus*
Acidic phospholipase A2 1	• Binds to muscarinic acetylcholine receptors (Huang et al. 2008) • Weak anticoagulant (Kini & Evans, 1987)	*N. annulifera, N. atra, N. nubiae*
Phospholipase A2 GL16-1	Hydrolysis of phospholipids	*N. atra, N. naja, N. nigricollis, N. nivea, N. nubiae, N. pallida, N. sumatrana*
Acidic phospholipase A2 2	Hydrolysis of phospholipids	*N. kaouthia, N. subfulva*
Acidic phospholipase A2 HTe	• Blocks neuromuscular transmission (Francis et al. 1995) • Hydrolysis of phospholipids	*N. kaouthia*
Acidic phospholipase A2 PL-II	• Hydrolysis of phospholipids • Possible haemostatic activity	*W. aegyptia*

seemingly being to cause haemorrhage (Gutiérrez & Escalante, 2010). However, the contribution of these toxins to the functional activities of elapid venoms remains largely unknown, and it seems unlikely that cobras are distinct from other elapid snakes in this regard – the expression levels of SVMPs detected here (0-7% of all toxins) are highly in line with the proteomic abundance of these toxins observed in the venoms of many other elapid snakes (0-12% of all toxins) (Tasoulis & Isbister, 2017).

The remaining toxins analysed each represented less than 5% of the total toxins recovered from the various venom gland transcriptomes studied, with the exception of kunitz toxins, which showed high expression levels in the outgroup *Walterinnesia aegyptia* (20.3%). While this abundance means kunitz are the third most dominantly expressed toxin type found in the venom gland of this species, expression levels for kunitz were much lower across the cobras (0.00-4.98%) (Fig.

2.5.). However, spitting cobras were found to have significantly lower expression levels of kunitz and CRISP than non-spitting cobras. However, given that the expression levels of these two toxin families are small (< 5% for kunitz and < 2% for CRISP), they represent minor toxins, and it is therefore difficult to surmise that these differences would have a large biological impact of the functional consequences of the venom. Prior research has shown that kunitz toxins isolated from the genera *Naja* and *Hemachatus* are capable of inhibiting enzymes associated with digestion (Hokama et al., 1976; Zhou et al., 2004), but to date, there is no evidence of such toxins inducing major or clinically-relevant symptomology in models of envenoming. Finally, since these toxin expression levels are reduced in spitting lineages, there is no evidence that these toxins might contribute towards the effectiveness of defensive venom spitting.

2.4.ii. Venom spitting and Geography

As discussed above, the transcriptomic analysis revealed no significant associations between the spitting characteristic or geography with the expression levels or number of contigs encoding the two dominant toxin families, 3FTXs and PLA_2s (Table 2.4.). However, when the venom transcriptome expression data is holistically analysed via Principal Component Analysis, spitting cobras were found to have a significantly lower PC1 score than non-spitting cobras. This component is most strongly associated with negative CTX expression and a positive 'Other 3FTX' and SP expression. This suggests that spitting cobras group together by having a combination of high CTX and low 'Other 3FTX' and SP expression, and this is most notable in the African spitting cobras, which have the lowest mean PC1 score (-1.2) and cluster more closely together than any other lineage (Fig. 2.12B.). The results of this analysis could potentially simultaneously explain the predominantly cytotoxic and necrotic symptoms, and frequent lack of neurotoxic symptoms, resulting from bites from African spitting cobras in comparison to their non-spitting counterparts (Warrell, 2008c). Neither PC2 or PC3 were found to be significantly associated with spitting. Both of these components are associated with PLA_2 and CTL expression (fig 2.13B. & fig 2.13C.), suggesting that these toxin families do not play a role in spitting in cobras.

There are very few associations between the geographical locale of the sampled species and the transcriptomic expression of toxins in the cobra venom glands, with the only significant associations being in SVMP and Kunitz expression (Table 2.4.). There can be extensive intraspecific variation in transcriptome expression resulting from geographic location (Tan et al., 2017), which makes it unusual that there are so few significant variations at the genus level. This may be due to conservation of toxin genes across cobras but it is more likely that this is an issue with using continent-level geography as a factor, given that African cobras do not form a monophyletic group. African non-spitters *Naja nivea*, *N. haje* and *N. annulifera* being more closely related to Asian cobras than to other African cobras, and *Hemachatus haemachatus* does not form a monophyletic group with African spitting *Naja* (see Fig. 1.6.). It is therefore probable that phylogenetic structuring may obscure any geographical differences in cobras at the transcriptome level.

2.4.iii.Comparisons to the literature

The current literature only describes the venom gland transcriptomic profiles for three Asian cobra species, *N. kaouthia*, *N. atra* and *N. sumatrana* (Chong et al., 2019; Jiang et al., 2011; Tan et al., 2017; Xu et al., 2017). Consequently, comprehensive comparisons between prior cobra transcriptomes and those produced here are inherently limited. However, when comparing the venom gland compositions of these three species, it is apparent that transcriptome expression can vary dramatically between sampled individuals (Fig. 2.14.). For example, for *N. atra*, the PLA_2 expression levels differ from 1.2% of all toxins for the Chinese specimen previously described by Jiang et al. (2011), to 25.7% detected in the captive bred individual sampled herein. Consequently, 3FTXs differ proportionally too, ranging from 46.3% (9% of this being CTX) in this study, to 95.8% (66.9% of this being CTX) in Jiang et al. (2011). In contrast, the venom gland transcriptome of the captive bred *N. kaouthia* studied here shows high levels of similarity with that of the Chinese specimen described by Xu et al. (2017), with 3FTXs and PLA_2s dominating the venom gland transcriptomes of both individuals (3FTX: 75.2% and 84.9%; PLA_2: 14.6% and 13.6%, respectively), however in the Chinese individual there is the notable absence of CTXs, forming only 0.10% of the toxinome. More variations are observed when

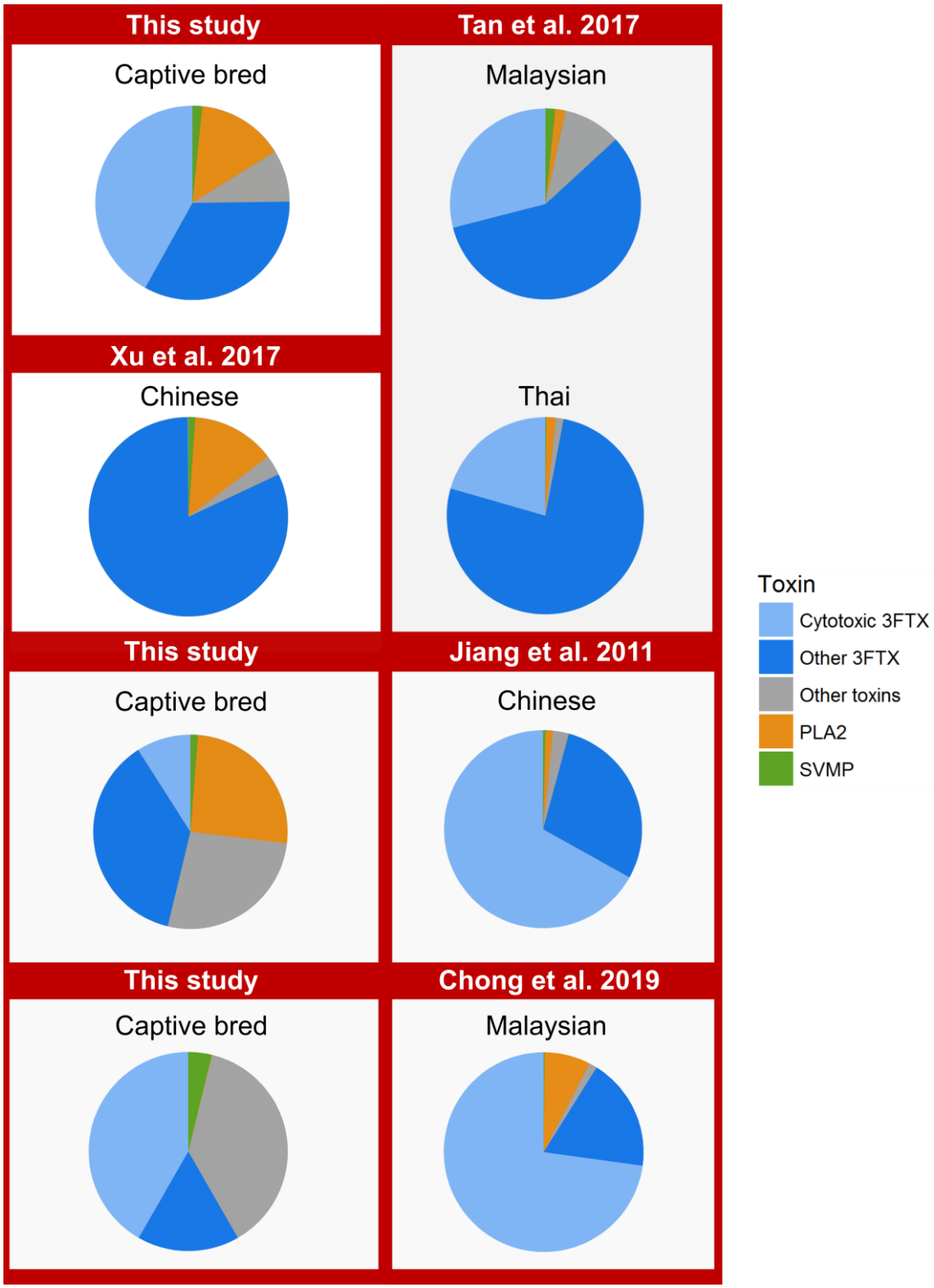


Figure 2.14. Comparisons of venom gland transcriptome toxin expression described in this study with those in the literature. A) *Naja kaouthia*. **B)** *Naja atra* **C)** *Naja sumatrana*.

comparing toxin expression with the Malaysian and Thai individuals sampled by Tan et al. (2017), both of which show increased 3FTX abundance (86.8% and 97.0%, respectively) and reductions in PLA_2 expression levels (1.8% and 1.5%, respectively) compared to the data generated here (Fig. 2.4. and 2.14.) and by Xu et al. (2017). Perhaps the most wildly-disparate results are between the *N. sumatrana* from this study and the Malaysian individual used in Chong et al. (2019). The latter individual has vastly more CTX expression and PLA_2 expression (72.83% and 7.42%, respectively, compared to 41.72% and 0.07%). Indeed, a large proportion of toxins from the individual used in this study are made up of other toxins (37.85%), which make up a tiny proportion of the toxinome in the Malaysian cobra. These observations reinforce the concept that snake venom variation can be extensive even at the intraspecific level (Oguiura et al., 2009; Halassy et al., 2011; Sunagar et al., 2014; Tan et al., 2015; Rautsaw *et al.*, 2019). Despite these disparities, there are many broad patterns of similarity across the various cobra venom gland transcriptomes described here and previously; for instance, all have very high 3FTX expression, and a very low expression level of C-type lectins, SVMPs, kunitz and many other minor toxin families.

2.4.iv. Limitations

While this study provides the first 'genus-wide' overview of venom gland transcriptomic toxin expression for the medically-important genus *Naja*, there are a number of technical and biological challenges associated with interpreting the transcriptomic data described here. The first relates to the samples used in this study and the potential for RNA degradation during cryostorage. Tissue sample storage could not be avoided in this study, but does not appear to have affected the transcriptomic profiles of the majority of the species sampled (e.g. for most storage was a maximum of two years, and typically <1 year), as their proportion of 'toxins', 'non-toxins' and 'unknown' contigs are highly consistent with the literature on snake venom gland transcriptomes (Corrêa-Netto et al., 2011; Doley et al., 2008; Jiang et al., 2011; Leão et al., 2009; Siang et al., 2010; Tan et al., 2017, 2015; Whiteley et al., 2019; Xu et al., 2017). However, in the case of *N. kaouthia* and *N. sumatrana* venom glands, which were kept in cryostorage for over a decade, a major shift from dominant 'toxin' expression to 'non-toxin' expression was observed, and could

possibly be the result of differential degradation of the most abundant mRNAs (Fig. 2.2.). Such differences in gene expression variation have been demonstrated *in vitro* (Romero et al., 2014), and it is well known that the size and secondary structures of mRNA can affect their rate of degradation (Higgins, 1991). Thus, there is potential for long term cryostorage to skew toxin family representation in the toxinome for these two species. However, it has been suggested that biological differences in expression far outweigh those introduced by RNA degradation (Opitz et al., 2010) so it may be that the data here is largely uncompromised. Comparisons of the data suggest this to be the case. For example, despite reduced overall toxin expression in both *N. kaouthia* and *N. sumatrana*, the toxinomes of these two species provide highly comparable contig numbers to those of their congeners (figs. 2.6.–2.10.), suggesting that RNA degradation has not impacted upon toxin recovery. In addition, the expression levels of these toxins within the toxinome are highly consistent with those of the other cobra species (Fig. 2.4. and 2.5.). Finally, as described in the section above, our venom gland toxinome for *N. kaouthia* resulted in a toxin expression profile highly similar to the study of Xu et al. (2017), thus providing confidence that the toxin data recovered in this study remains robust. It may therefore be the case that rather than RNA degradation being a major factor, the quality of the gland dissection may have impacted on the resulting data. It is possible that additional non-venom gland tissue was dissected along with the glands from these historical samples, which could in turn explain the reduction of overall toxin expression levels compared with non-toxins. One solution is to generate an RNA Integrity Number (RIN) (Schroeder et al., 2006) for each species and weight the data analysis accordingly. RIN values from the transcriptome sequences generated in this study indicate RNA degradation from *N. sumatrana* and *N. kaouthia* (Table 2.2.), which was expected due to their extensive cryostorage duration, though the value for *N. naja* was unusually low as well (5.6). RINs are not reflective of all RNA degradation however (Schroeder et al., 2006), and this must be borne in mind when considering the quality of the subsequent transcriptome sequences produced.

Another limitation with this study is that the venom gland transcriptomes described here represent a single snapshot in time of the gene expression profile of the venom glands of a single animal. Thus, utilising these transcriptomes as species

representatives is challenging, particularly since it is apparent that venom gland transcriptomes profiles can vary extensively between individuals of the same species (Fig. 2.14.), and it is possible that the status of the individual itself (e.g. recently-fed or full, sick or healthy) shortly before time of death may also contribute to changes in toxin expression. Ideally, to circumvent issues with intraspecific venom variation (Ali et al., 2013; Barlow et al., 2009; Menezes et al., 2006; Modahl et al., 2016; Williams et al., 1988; Zelanis et al., 2008), multiple individuals from many different geographical localities, ages and sexes would be utilised to characterise the toxinome profiles of venomous snakes. Unfortunately, because of the requirement to euthanise animals to extract venom gland tissue to yield sufficient RNA, ethical and resource constraints currently prevent such an approach. It is worth noting that alternate means of RNA extraction that do not require euthanasia, such as the isolation of mRNAs directly from venom, have been described (T. Chen et al., 2002; Modahl & Mackessy, 2016; Whiteley et al., 2016). These studies demonstrated that full-length sequences encoding toxin genes can be successfully amplified and sequenced via PCR approaches for both front and rear-fanged snakes, but that total RNA yields are of insufficient quantity and quality for the construction of next-generation sequencing libraries. However, further optimisation of this process, coupled with continual reductions in the amount of RNA material required for DNA sequencing approaches, provides confidence that such approaches may be feasible in the future.

Another limitation is that all of the Asian cobra species used in this study are captive bred in origin. With this there is the risk that, due to a lack of ecological factors that they would experience in the wild (e.g. diet, climate), transcriptional modifications could result in differences in venom composition. There is the additional possibility that RNA transcription of toxins in the venom gland may change over time in captivity, though evidence suggests that differences between the venoms of wild-caught and long-term captive individuals are minimal (Freitas-De-Sousa et al., 2015). Due to the difficulty in attaining wild-caught specimens of many Asian snakes due to strict export laws the individuals here were seen as the best possible references for the species in this study.

Finally, all sequencing, assembly and annotation approaches associated with de novo transcriptomics have their limitations. In the case of multi-isoform toxin family-rich data derived from venom glands, one of the major challenges relates to de novo assembly, as a number of assembly programs result in the merging of related, but distinct, contigs into chimeric sequences (Archer et al., 2014). To combat this challenge here, the custom-designed venom gland assembler VTBuilder was used, which has previously been shown to outperform standard popular assembly programs such as Trinity (Archer et al., 2014; Grabherr et al., 2013). However, even such optimised assembly methods can result in a degree of underclustering, whereby identical or near identical sequences are separated into multiple contigs. These challenges can be overcome via manual downstream analysis, such as that described herein. The remaining challenge with the assembly approach undertaken here is that VTBuilder requires 250 bp reads, and has a maximum limit of 10 million reads for inputting for assembly. Thus, while the assemblies are robust, the transcriptomes described here are of lower coverage to a number in the literature that use Illumina HiSeq sequencing technology (Rokyta et al., 2012) in preference to the MiSeq used here. One option, given sufficient time and resources, would be to combine both long and short read sequencing technologies (Durban et al., 2017; Margres et al., 2013; Pla et al., 2018; Rokyta et al., 2011) and transcriptome assembly approaches, to benefit from increased sequence coverage and robust assembling (Rautsaw et al., 2019), although this would have been particularly challenging from a resource perspective for the number of samples assembled in this study.

2.4.v. Concluding remarks

In summary, this chapter provides a broad, yet comprehensive, analysis of the venom gland composition of toxin encoding genes found in cobras and their near relatives. In this study, I comparatively analysed the venom gland transcriptomes of 14 *Naja* species, alongside data from three close relatives, making this study the most speciose comparison of snake venom gland transcriptomes to date and adding 16 new elapid transcriptomes to the field. My data demonstrates that cobra venom glands are dominated by the 3FTX family, both in terms of expression levels and numbers of putative genes. Interestingly, the major sub-class of 3FTXs appears to be

the CTXs for the majority of these species, supporting recent work suggesting that cytotoxic venom activity may be commonplace across the genus, despite differences in pathology being observed following cases of cobra snakebite, particularly in Africa (Panagides et al., 2017; David A. Warrell, 2008c). The expression levels of PLA_2 toxins indicates that these toxins are typically the second most abundant toxin family in the venom glands of cobras, although their representation varies extensively, particularly among African spitting and non-spitting species. Despite these observations, I observed no significant differences among toxin representation, for any toxin family, between spitting and non-spitting cobras. However, significant differences in the representation of acidic and basic PLA_2s in the venom glands of non-spitting and spitting species, suggests that PLA_2s require further investigation in the context of the evolution of this defensive character. In particular, I will address the phylogenetic context of venom toxin evolution in chapter 4. The next chapter will analyse whether the proteomic composition of secreted venoms from the same cobra species is reflective of the gene expression data observed in the venom gland transcriptomes. This is important because of the limitations described above (i.e. transcriptomes represent a snap shot in time), and because a number of previous studies have demonstrated that differential post-transcriptional regulation can influence the protein composition of secreted venom (Calvete et al., 2011; Casewell et al., 2014).

3. PROTEOMIC ANALYSIS OF COBRA VENOM COMPONENTS

3.1. Introduction

Snake venom proteomics, often referred to as 'venomics' (Calvete, 2017), identifies the protein composition of venom extracted directly from a snake. Such an approach facilitates identification of the composition of the biologically-relevant sample itself, as well as the detection of post-translational mechanisms that may underpin differences between toxin expression at the transcriptome level and toxin abundance at the protein level. The field of snake venom proteomics is much more diverse than that of venom gland transcriptomics, due in part to the relative ease and less ethically-restrictive mode of sample acquisition compared with venom gland transcriptomics (e.g. venom from a live snake, not requiring euthanasia, and often such samples are available commercially). Moreover, for proteomic analyses, an individual animal, or many individuals from the same species, can be maintained and their venom extracted multiple times to assess ontogenetic changes (Mackessy et al., 2018; Modahl et al., 2016; Pla et al., 2017; Zelanis et al., 2008) and the venoms from multiple individuals can also be pooled to capture population-level differences or broad toxin composition within a species and thus offset the sample-size issue typically observed in transcriptome work (Herrera et al., 2012; Oh et al., 2017; Rey-Suárez et al., 2011; Kae Yi Tan et al., 2015). Proteomic approaches also facilitate the identification of toxins responsible for pathology seen in pre-clinical and clinical studies, particularly when combined with toxin isolation approaches, thereby providing potential neutralisation targets for research focused on creating more effective and efficient treatments to snakebite (Albulescu et al., 2019; Calvete et al., 2010; Lomonte & Calvete, 2017; Sintiprungrat et al., 2016). However, it has become apparent that species-relevant transcriptomic data is of great importance for ensuring the accuracy of proteomic-derived toxin annotations, and thus more recently it has been advocated that a combination of transcriptomics and proteomics is the most desirable approach to characterising snake venom composition (Ainsworth et al., 2018; Gutiérrez et al., 2017; Pla et al., 2017).

The literature addressing the proteomic composition of viper venoms currently consists of over 80 papers, and reveals that the toxin composition of these species tends to be dominated by phospholipase A_2 (PLA_2s), serine protease (SPs), snake venom metalloproteinase (SVMPs) and C-type lectin (CTLs) toxins, although a variety of other toxin types such as natriuretic peptides (NPs), L-amino acid oxidases (LAAOs), cysteine-rich secretory proteins (CRISPs), Kunitz-type serine protease inhibitors (kunitz) and cystatins have also been detected (Tasoulis & Isbister, 2017). These cocktails of toxin components tend to result in haemorrhagic, coagulopathic and/or necrotic pathologies in snakebite victims (Gutiérrez et al., 2017). These are far fewer studies describing the proteomic composition of venoms from colubrid snakes, most likely due to the very small number of species which are considered of human medical importance. However, the literature to date suggests that their venoms are predominantly composed of SVMPs or 3FTXs (Barua et al., 2019; Junqueira-de-Azevedo et al., 2016, Peichoto et al., 2017; Weldon & Mackessy, 2010) or snake venom matrix metalloproteinases (SVMMPs) (Ching et al., 2012), which have a similar binding site to SVMPs (Bode et al., 1993). Bites by colubrids therefore tend to be haemorrhagic or induce local swelling (Gutiérrez et al., 2017).

While not as numerous as those of vipers, to date 41 publications have described the proteome composition of various elapid venoms, covering in total 50 species (see Table 3.1.). Similar to those findings on toxin expression in venom gland transcriptomes (see Chapter 2), elapid proteomes tend to be dominated by three-finger toxins (3FTXs), with some exceptions. For example, the black mamba (*Dendroaspis polylepis*) from the 2015 study has venom predominantly composed of Kunitz toxins (Laustsen et al., 2015), while kraits (*Bungarus* spp.) and coral snakes (*Micrurus* spp.) have variable compositions, with some exhibiting predominantly 3FTX-based venoms, and others predominantly PLA_2-based. Similarly, the venoms of the sea snakes *Aipysurus laevis* and *Hydrophis curtus* are also predominately composed of PLA_2 toxins, as are those of the Australian elapids *Notechis scutatus*, *Oxyuranus scutellatus* and *Pseudechis papuanus*. From this review of the literature,

Table 3.1. Overview of the toxin families found in elapid proteomes in the literature, modified from Tasoulis & Isbister, 2017, rounded to one decimal place where able. Numbers represent the percentage of toxin proteins in the venom of the sampled individual(s). Papers that use only SDS-page-based methods to estimate protein abundance were excluded. 3FTX – three-finger toxin, SVMP – snake venom metalloproteinase, Kunitz – kunitz-type serine protease inhibitor, CRISP – cysteine-rich secretory protein, PLA_2 – phospholipase A_2, CTL – C-type Lectin, LAAO – L-amino acid Oxidase, ACE – acetylcholinesterase, SP – Serine Protease, Ves – Vespryn/Ohanin.

Species	3FTX		SVMP	Kunitz	CRISP	PLA_2	CTL	LAAO	ACE	SP	Ves	Other
	CTX	Other										
Aipysurus laevis[1]	0	25.3	0	0	2.5	71.2	0	0	0	0	0	1.0
Aspidelaps lubricus cowlesi[2]	4.9	71.2	5.1	8.6	3.5	4.9	0	1	0	0	0	0.8
Aspidelaps lubricus lubricus[2]	2.1	75.7	4	5.5	5.2	5.7	0	1	0	0	0	0.8
Aspidelaps scutatus intermedius[2]	33.7	49	2.9	1	4.9	6.1	0	0	0	0	0	2.4
Bungarus caeruleus[3]	0	19	1.3	4.4	5.5	64.5	0	0	1.6	0	0	3.7
Bungarus caeruleus[4]	0	61.2	0	0	0	37.6	0	0	0	0	0	1.2
Bungarus candidus[5]	0	30.1	4.9	12.7	3.9	25.2	0	5.9	4.9	3.9	2	6.5
Bungarus fasciatus[6]	0	1.3	3.5	1.8	0.4	66.8	0.2	7.1	5.1	0	0	13.8
Bungarus fasciatus[5]	0	17.4	4.7	10.7	1.2	44.2	0	5.8	4.7	5.8	0	5.5
Bungarus fasciatus[7]	0	6.7	2.4	24.7	0	63.4	0	1.0	1.6	0	0	0.2
Bungarus fasciatus[7]	0	7.3	2.5	21.3	0	65.8	0	0	0	0	0	3.1
Bungarus fasciatus[7]	0	15.5	2.4	49.0	0	29.5	0	1.7	1.7	0	0	0.2
Bungarus fasciatus[7]	0	16.9	2.6	1.5	0	76.2	0	0	0.2	0	0	2.6
Bungarus fasciatus[7]	0	2.8	3.6	1.7	0	91.9	0	0	0	0	0	0
Bungarus flaviceps[8]	0	22.3	< 3	19	< 3	11.9	3.3	< 3	12.6	< 3	< 5	~ 20
Bungarus multicinctus[9]	0	32.6	0.1	0.5	0	66.4	0	0.2	0	0	0	0.2
Bungarus multicinctus[10]	0	27.5	0.8	2.3	2.4	15.3	0.4	2.1	1.1	0.1	0.2	47.8

Table 3.1. Continued.

Species	3FTX		SVMP	Kunitz	CRISP	PLA_2	CTL	LAAO	ACE	SP	Ves	Other
	CTX	Other										
Bungarus sindanus[10]	0	50.3	0.5	13.3	2.6	32.6	0	0.1	0.2	0	0.3	0.1
Calliophis intestinalis[11]	15.9*	5.1	5.8	14.3	1.7	43.8	0	6.8	0	0	0	22.5
Dendroaspis angusticeps[12]	0	69.2	6.7	16.3	2.0	0	0	0	0	0	0	5.8
Dendroaspis angusticeps[13]	0	64.2	0	4.9	0	0	0	0	0	0	0	30.9
Dendroaspis jamesoni jamesoni[13]	0	65.5	0	16.3	0	2.2	0	0	0	0	0	16.0
Dendroaspis jamesoni Kaimosae[13]	0	74.8	0	9.6	0	1.8	0	0	0	0	0	13.8
Dendroaspis polylepis[14]	0	31.0	3.2	61.1	0	0	0	0	0	0	0	4.7
Dendroaspis polylepis[13]	0	40.0	0	39.0	0	0	0	0	0	0	0	21.0
Dendroaspis viridis[13]	0	76.7	0	5.6	0	0.2	0	0	0	0	0	17.5
Hemachatus haemachatus[15]	53.5	9.8	7.1	1.5	4.1	22.8	0	0	0	0	0.2	1.0
Hydrophis curtus[16]	0	26.3	0.36	0	9	62	0	0.2	0	0	0	2.14
Hydrophis curtus[17]	0	30.4	0	0	2.5	66.7	0.1	0	0	0	0	0.3
Hydrophis curtus[17]	0	40.4	0	0	5.0	54.5	0	0	0	0	0	0.1
Hydrophis curtus[18]	0	54.0	0.28	0	2.7	37.8	0.1	0	0	0.1	0	5.0
Hydrophis cyanocinctus[18]	0	69.0	0.20	0	0.5	22.2	0	0	0	0	0	8.1
Hydrophis cyanocinctus[19]	0	81.1	0	0	0	18.9	0	0	0	0	0	0
Hydrophis platurus[20]	0	49.9	0.9	0	9.1	32.9	0	0	0	0	0	7.2
Hydrophis schistosus[21]	0	70.5	0.5	0	1.3	27.5	0	0.2	0	0	0	0
Laticauda colubrina[22]	0.3	66.1	0	0	0.1	33.3	0	0	0	0	0	0.2
Micropechis ikaheka[23]	0	9.2	7.6	0.7	1.8	80	0	0.4	0	< 0.1	0.3	0
Micrurus alleni[24]	0	77.3	1.2	0	0	10.9	1.8	3.0	0	0	0.2	5.6
Micrurus altirostris[25]	0	79.5	0.9	2.1	0.1	13.7	< 0.1	1.2	0	0	0	2.5
Micrurus browni browni[26]	0	31.3	6.7	2.7	0	47.6	1.6	1.8	0	0.9	0.2	7.2

Table 3.1. Continued.

Species	3FTX		SVMP	Kunitz	CRISP	PLA_2	CTL	LAAO	ACE	SP	Ves	Other
	CTX	Other										
Micrurus corallinus[27]	34.8		10.4	0.5	8.5	38.5	0	5.4	0	0	0	1.9
Micrurus dumerilii[28]	0	28.1	1.8	9.0	0	52.0	1.1	3.1	0	1.9	0	3.0
Micrurus frontalis[29]	0	42.4	1.0	1.3	0.7	49.2	0.1	0.4	0	0	0	4.9
Micrurus lemniscatus carvalhoi[27]	2.3		6.3	12.6	0	48.6	2.6	2.0	0.2	2.1	21.5	1.8
Micrurus lemniscatus lemniscatus[27]	34.3		4.9	11.7	0	19.4	2.6	3.5	0.3	11.4	9.3	2.6
Micrurus mipartitus[30]	0	61.1	1.6	1.9	0	29.0	1.1	4.0	0	1.3	0	0
Micrurus mipartitus[30]	0	83.0	3.6	1.9	0	8.2	0	3.2	0.3	0	0	0
Micrurus mosquitensis[24]	0	22.5	2.6	9.8	0	55.6	1.3	2.8	0	0.5	0	4.9
Micrurus nigrocinctus[31]	0	38.0	4.3	0	0	48.0	2.2	2.3	0	0.7	3.8	0.7
Micrurus ruatanus[34]	46.4		0	10.6	0	29.8	2.0	3.4	0	1.2	0.4	6.2
Micrurus paraensis[27]	12.3		8.0	3.4	0	65.9	5.0	3.1	0	0	0	2.3
Micrurus pyrrhocryptus[33]	0	27	12	5	2	17	7	5	1	7	2	15
Micrurus spixii spixii[27]	9.5		6.6	0.7	0	64.0	7.4	4.8	0.2	0	3.7	3.1
Micrurus spixii spixii[29]	0	56.5	0	1.0	0	37.4	0	5.1	0	0	0	0
Micrurus surinamensis[27]	90.1		0.1	2.6	0	6.6	0.3	0.2	0	0	0	0.1
Micrurus surinamensis[27]	36.8		1.2	25.3	0	33	1.7	0.3	0	0	0	1.7
Micrurus surinamensis[29]	0	95.4	0	0	0	4.2	0	0.4	0	0	0	0
Micrurus tschudii[34]	0	95.2	0	1.6	0	4.1	0	0.7	0	0	0	0
Naja annulifera[35]	73.5	4.9	11.2	0	0.6	0	0	5.0	1.4	0	0.2	3.2
Naja atra[36]	52.9	23.5	0	1.0	2.4	16.8	0	0.2	0	0	0	3.2
Naja atra[36]	59.4	20.5	0	0.7	2.2	14	0	0.2	0	0	0	3
Naja atra[9]	65.3	19.0	1.6	0	1.8	12.2	0	0	0	0	0	0.1
Naja haje[37]	54	6	9	1.9	10	4	0	1	1	0	0	13.1

Table 3.1. Continued.

Species	3FTX		SVMP	Kunitz	CRISP	PLA_2	CTL	LAAO	ACE	SP	Ves	Other
	CTX	Other										
Naja kaouthia[38]	45.7	18.0	3.3	0.5	4.3	23.5	0.2	1.1	0	0	0.3	3.1
Naja kaouthia[38]	27.6	50.7	2.6	0	2.3	12.2	0.4	1.0	0	0	0.7	2.5
Naja kaouthia[38]	44.9	31.5	1.6	< 0.1	0.8	17.4	0	0.5	0	0	0.2	3.1
Naja kaouthia[39]	27.9	28.6	1.1	0	5.4	26.9	0	0	0	0	9.2	0
Naja katiensis[40]	62.7	4.4	3.3	0	0.2	29	0	0	0	0	0	0.4
Naja melanoleuca[41]**	25.2	31.9	9.7	3.8	7.6	12.9	< 0.6	0	0	0	< 0.5	8.9
Naja mossambica[40]	67.7	1.6	2.6	0	0	27.1	0	0	0	0	0	1.0
Naja naja[42]	32	26	5	2	0	19	0	5	0	0	2	9
Naja naja[43]	63.8		1	0.4	2.1	11.4	0	0.8	6.3	0.3	0	13.9
Naja naja[44]	69.3	4.8	0.9	0.1	2.5	21.4	0	0	0	0	0	1.0
Naja naja[44]	71.6	8.9	0.9	0.3	3.7	14	0	0	0	0	0	0.6
Naja naja[45]	46.9	28.4	1.5	0.9	0.7	14.2	0	1.7	0	0	3.4	2.3
Naja nigricollis[40]	72.8	0.5	2.4	0	0.2	21.9	0	0	0	0	0	2.2
Naja nubiae[40]	58.3	12.6	2.6	0	0	26.4	0	0	0	0	0	0.1
Naja pallida[40]	64.9	2.8	1.6	0	0	30.1	0	0	0	0	0	0.6
Naja philippinensis[46]	21.3	45.3	3.9	0	1.5	22.9	0	0.4	0	0.4	0.1	4.2
Naja sputatrix[47]	48.1	16.1	1.3	0.2	0	31.2	0	0.1	0	0.4	0.1	2.5
Notechis scutatus[48]	0	5.6	0	6.9	0.3	74.5	0	1.4	1.4	5.9	0.3	3.7
Ophiophagus hannah[49]	0	31	25	3	9	5	1	0	1	1	1	23
Ophiophagus hannah[50]	9	55.2	11.9	3.3	6.5	2.8	0	0.5	0	0	1.1	9.7
Ophiophagus hannah[51]	0.5	42.5	24.4	1	8.7	4	0	5.7	0.1	0.7	5.7	6.7
Oxyuranus scutellatus[52]	0	4.2	8.9	13.0	0.8	68.3	0.6	0	0	0	0	4.2
Oxyuranus scutellatus[52]	0	1.5	5.2	7.8	0.6	79.4	0	0	0	0.6	0	4.9

Table 3.1. Continued.

Species	3FTX		SVMP	Kunitz	CRISP	PLA_2	CTL	LAAO	ACE	SP	Ves	Other
	CTX	Other										
Pseudechis papuanus[53]	0	3.1	2.8	0	2.3	90.2	0	1.6	0	0	0	0
Toxicocalamus longissimus[19]	0	92.1	1.4	0	0	6.5	0	0	0	0	0	0

* Upon Blast searching the relevant sequences of this paper, these were found to have very little sequence identity (if any) to cytotoxin sequences so are unlikely to be genuine cytotoxins.

** This study occurred before the splitting of *N. melanoleuca* into 5 distinct species. The venom from this study may therefore be from *N. melanoleuca* or *N. subfulva*, based on locality of the venom sourced and distribution data from Wüster et al. 2018.

1 - Laustsen, et al. 2015, 2 - Whiteley et al. 2019, 3 - Oh et al. 2017, 4 - Patra, Chanda, and Mukherjee 2019, 5 - Rusmili et al. 2014, 6 - Ziganshin et al. 2015, 7 – Hia et al. 2020, 8 - Chapeaurouge et al. 2018, 9 - Shan et al. 2016, 10 - Oh et al. 2019, 11 – Tan et al. 2019, 12 - Lauridsen et al. 2016, 13 - Ainsworth et al. 2018, 14 – Laustsen et al. 2015, 15 - Sánchez et al. 2018, 16 - Tan et al. 2019, 17 - Neale et al. 2017, 18 – Wang et al. 2020, 19 - Calvete et al. 2012, 20 - Lomonte et al. 2014, 21 - Tan et al. 2015, 22 - Tan et al. 2017, 23 - Paiva et al. 2014, 24 - Fernández et al. 2015, 25 - Corrêa-Netto et al. 2011, 26 - Bénard-Valle et al. 2020, 27 - Aird et al. 2017, 28 - Rey-Suárez et al. 2016, 29 - Sanz et al. 2019, 30 - Rey-Suárez et al. 2011, 31 - Fernández et al. 2011, 32 - Lippa et al. 2019, 33 - Olamendi-Portugal et al. 2018, 34 - Sanz et al. 2016, 35 - Tan et al. 2020, 36 - Huang et al. 2015, 37 - Malih et al. 2014, 38 - Tan et al. 2015, 39 - Xu et al. 2017, 40 - Petras et al. 2011, 41 - Lauridsen et al. 2017, 42 - Asad et al. 2019, 43 - Dutta et al. 2017, 44 - Sintiprungrat et al. 2016, 45 - Wong et al. 2018, 46 - Tan et al. 2019, 47 - Tan, Wong, and Tan 2017, 48 - Tan, Tan, and Tan 2016, 49 - Kunalan et al. 2018, 50 - Petras et al. 2015, 51 - Tan et al. 2015, 52 - Herrera et al. 2012, 53 - Pla et al. 2017.

it is clear that the major toxins in elapid snake venoms are 3FTXs and PLA_2s. Both these toxin families are known to contain isoforms that exert neurotoxic functions (see Chapter 1), with 3FTXs typically acting post-synaptically on nicotinic acetylcholine receptors (nAChRs) (Kini & Doley, 2010) and PLA_2s acting presynaptically (Kini, 2003), while the specialised kunitz toxins of mambas, known as dendrotoxins, can also cause neurotoxicity through the blocking of sodium, calcium and potassium channels (Inagaki, 2017). Consequently, it has been suggested that the primary function of elapid venom is to cause the death of prey through neuromuscular paralysis, resulting in paralysis of the respiratory system (de Vries & Condrea, 1971; Lee, 1970). In many cases, elapid venoms seem likely to contain multiple isoforms of different neurotoxins, both within and between different toxin classes, that work synergistically, and in a variable manner inter-specifically, to cause neurotoxicity (Lee, 1970; Xiong & Huang, 2018). Indeed, variations have also been observed within the same species, with *Bungarus multicinctus* venom, for example, capable of being either 3FTX- or PLA_2-dominant depending on the individual or population sampled (Shan et al., 2016; Ziganshin et al., 2015).

A number of other toxins have been detected in elapid proteomes with >5% of total toxin abundance (Table 3.1.). As mentioned previously, kunitz are highly abundant in *Dendroaspis polylepis* but have also been detected in varying amounts in the venoms of *Bungarus* and *Micrurus* spp., ranging from complete absence in the venoms of *Micrurus alleni*, *Micrurus nigrocinctus* and one *Micrurus surinamensis* individual (Fernández et al., 2011; Fernández et al., 2015; Sanz et al., 2019) to 25.3% of all toxins detected in the venom of a different *Micrurus surinamensis* specimen (Aird et al., 2017). Thus, the abundance of these toxins cover the inter- and intra-specific variability that can occur in the venom toxin composition of snakes. Kunitz toxins can act as enzyme-inhibitors and ion channel-blockers (Eng et al., 2015) (see Table 1.3), and thus have been implicated in both neurotoxicity and perturbing enzymes involved in haemostasis. Cysteine-rich secretory proteins (CRISPs) are another variably abundant toxin family detected in elapid venoms, although no species sampled to date exhibits a higher abundance than the 10% of all toxins detected in *Naja haje* (Malih et al., 2014). Similarly to kunitz toxins, CRISPs can act as

neurotoxins by blocking ion channels (Sunagar et al., 2015a) (see 1.3). SVMPs, which cause haemorrhage (Casewell et al., 2015; Moura-da-Silva et al., 2007), are generally present at very low abundance in elapid venom, however there is moderate to high expression (5-15%) in several of the *Micrurus* species (Aird et al., 2017; Olamendi-Portugal et al., 2018; Rey-Suárez et al., 2016), *Micropechis ikaheka* (Paiva et al., 2014), *Oxyuranus scutellatus* (Herrera et al., 2012), *Naja haje* and *N. melanoleuca* (Lauridsen et al., 2017; Malih et al., 2014), and the Indonesian *Ophiophagus hannah* (Petras et al., 2015), and SVMPs make up nearly a quarter of the Malaysian *Ophiophagus hannah* venom (Tan et al., 2015). This suggests the potential for the venoms of many elapid species to cause coagulant disruptions, and indeed this is common in Australasian elapids (Gulati et al., 2013). Vespryn, a highly under-studied toxin that induces hyperalgesia (Fry et al., 2015), is largely absent in elapids but has a substantial presence in the venom of *Micrurus lemniscatus carvalhoi* (21.5%)(Aird et al., 2017), and is moderately present in the venoms of *Micrurus lemniscatus lemniscatus* (9.3%)(Aird et al., 2017), the Chinese *Naja kaouthia* (9.2%)(Xu et al., 2017) and Malaysian *Ophiophagus hannah* (5.7%)(Fung, et al., 2015), which suggests bites from these species may be particularly painful.

A number of other minor toxin families, which exhibit less than 5% mean toxin abundance, have been detected across the various elapid proteomes described to date (Table 3.1). These include C-type lectins (CTLs), L-amino acid oxidases (LAAOs), acetylcholinesterases (ACEs), serine proteases (SPs) and Natriuretic peptides (NPs). Some of the functions of these toxins can be found in 1.3, though they are understudied and thus still poorly understood (Fry et al., 2015).

The venoms of snakes from the genus *Naja* genus appear, at first glance at least, to be similar to those of other elapids in that they are dominated by 3FTXs (see Table 3.1). Unlike other elapids, these 3FTXs are mostly made up of the CTX subfamily, with the exception of the *Naja kaouthia* from Thailand (Tan et al., 2015), *N. melanoleuca* (Lauridsen et al. 2017) and *N. philippinensis* (Tan et al., 2019), which have a greater abundance of neurotoxic 3FTXs. Cobras possess a subfamily of 3FTXs known as cytotoxins (CTXs), which have cytotoxic and cardiotoxic effects in prey (see 1.3). This subfamily is largely understudied and exist only in *Naja*, *Aspidelaps*, *Ophiophagus* and (potentially) *Micrurus*. These occur in high abundance in the *Naja*

genus, often in higher abundance than neurotoxic 3FTXs (Table 3.1.). This is somewhat consistent with clinical symptoms of bites by *N. philippinensis* and *N. kaouthia*, which cause strongly-neurotoxic and a mix of neurotoxic and necrotic symptoms, respectively (Bernheim et al., 2001; Faiz et al., 2017; Reid et al., 1988) (see Table 5.1.). Likewise, the high abundance of cytotoxins in African spitting cobras reflects the predominately local pathologies, including swelling, blistering and necrosis, seen in bites by these snakes (Warrell, 2008c). Like many other elapids, the second most abundant toxin family detected in *Naja* proteomes are the PLA_2s, especially in the African spiting cobras, in which these toxins may comprise as much as a third of the total venom proteome (Petras et al., 2011; Tan et al., 2017). Overall, the literature suggests that spitting cobras have a higher PLA_2 abundance than non-spitting cobras (mean of 26.9% from 7 individuals, compared to 15.6% from 8 individuals, respectively), which contrasts with the venom gland transcriptomic expression levels described in Chapter 2, Table 2.1. The SVMP and CRISP toxin families are the next most abundant toxin types found in *Naja* proteomes, with abundances for each ranging from complete absence to ~10%. The Egyptian cobra *N. haje* and the forest cobra *N. melanoleuca* have the highest abundance of both these toxin types, suggesting the potential for haemorrhagic pathology to occur following bites, although thus far no such symptoms have been reported for either of these species (see Table 5.1.), as well as increased neurotoxic capacity, which is indicative of clinical reports for bites from African non-spitting cobras (Warrell, 2008c). The venom of *N. haje* was also found to have an unusually high abundance (~5%) of nerve growth factor (NGF) (mean abundance for the genus = 0.8%); a toxin responsible for cell growth and apoptosis of sensory neurons (Fry et al., 2015). Some other unusual patterns of toxin abundances include the large amount of ACE in Indian *N. naja* venom (Dutta et al., 2017) and the high abundance of Vespryn in the venom of Chinese *N. kaouthia* (Xu et al., 2017).

In contrast to the relative dearth of data available for venom gland transcriptomic profiles of cobras, a total of 12 cobra species, including 7 spitting species (*N. katiensis*, *N. mossambica*, *N. nigricollis*, *N. nubiae*, *N. pallida*, *N. philippinensis* and *N. sputatrix*) have been proteomically characterised. These species include representation of both African and Asian species. However, the majority of

these studies did not perform comparative analysis of venom proteomes, with only the study of Petras et al. (2011) providing a direct comparison of the proteomic composition of African spitting cobra venoms via bottom up proteomic approach consisting of high performance liquid chromatography (HPLC) followed by tandem mass spectrometry (MS-MS). Crucially, a wide-scale comparative analysis of the genus that includes representatives from both spitting and non-spitting species, including the multiple different lineages of each of these groups, has not yet been performed.

To redress this data gap, in this chapter I describe the comparative analysis of venom proteomic data from 16 cobras and near relatives, including 7 species which have not yet had proteomic characterisation. Specifically, a large-scale comparative analysis on the genus *Naja* (n=14), and their two close relatives; the spitting rinkhals (*Hemachatus haemachatus*) and the non-spitting desert black snake (*Walterinnesia aegyptia*) was performed. The species utilised were identical to those for which venom gland transcriptomics were performed in Chapter 2. In addition, and as with Chapter 2, comparative data recently published from the African shield nosed snake (*Aspidelaps scutatus*) (Whiteley et al., 2019) was incorporated into this comparative analysis due to its close relationship to the *Naja* genus. The resulting dataset was interrogated to analyse differences between spitting and non-spitting cobras and to elucidate how toxin composition has evolved in the context of the emergence of spitting in this group.

3.2. Methods

3.2.i. Venom

Venoms were collected from 16 species of elapid snakes maintained at the Herpetarium of the Liverpool School of Tropical Medicine, UK (Table 3.2.). For many species, venom samples were pooled from multiple specimens to incorporate potential intraspecific variation into the analyses. The number of individuals incorporated into these pooled samples ranged from 1 for *Naja sumatrana* and *Aspidelaps scutatus scutatus* to 6 for *Naja haje*, with the modal number of individuals

being 2. Proteome data for *Aspidelaps scutatus scutatus* was taken from Whiteley et al. 2019.

3.2.ii. SDS-PAGE Gel Electrophoresis

To gain a general overview of any obvious potential differences in the venom composition of spitting and non-spitting cobras, precast (Novex™ WedgeWell™ 4-20% Tris-Glycine Gel with 12 1.0mm wells) SDS page gels were run. Samples were prepared by adding 12µl of 1mg/mL to 12µl loading dye (diluted from a 2x mixture of 3.55mL H_20, 1.25mL 0.5M TRIS [pH6.8], 2.5mL glycerol, 2.0mL 10% Sodium dodecyl sulfate [SDS] and 1.5mL saturated bromophenol blue). To samples intended to make reduced gels, 150µl of of B-mercaptoethanol was added to the 2x loading dye before dilution, and the venom-dye mixture was allowed to denature for 10 minutes in a heating block at 95 degrees. A ThermoFisher Mini Gel Tank (catalogue number A25977) was filled to the loading point with SDS page buffer, diluted from a 5x stock solution (made with 151g Tris-base, 720g glycine, 50g SDS and deionised H_20 to make

Table 3.2. The species characteristics of the venoms used for the proteomic and functional analyses of this study.

Species	Spitter?	Species Distribution	Wild-caught origin/Captive Bred (CB)	No. of individuals in Venom Sample
Hemachatus hemachatus	Yes	Africa	South Africa	2
Naja annulifera	No	Africa	CB	2
Naja atra	No	Asia	CB	2
Naja haje	No	Africa	Uganda	6
Naja kaouthia	No	Asia	Unknown	3
Naja subfulva	No	Africa	Cameroon	2
Naja mossambica	Yes	Africa	Tanzania	4
Naja naja	No	Asia	CB	2
Naja nigricollis	Yes	Africa	Nigeria	3
Naja nivea	No	Africa	South Africa	3
Naja nubiae	Yes	Africa	CB	3
Naja pallida	Yes	Africa	Tanzania	2
Naja philippinensis	Yes	Asia	CB	2
Naja siamensis	Yes	Asia	CB	2
Naja sumatrana	Yes	Asia	CB	1
Walterinnesia aegyptia	No	Africa & Asia	CB	2
Aspidelaps scutatus scutatus	No	Africa	CB	2

a 10L solution) using deionised water. Into the first well, 5µL of Promega Broad Range Protein Molecular Marker (10–225kDa; reference number: V849A) was pipetted, followed by 20µL of each sample into their respective wells. Gels were run at 110V, 44mA for around 25 minutes followed by 200V for around 15 minutes. The gels were then removed from the tanks and submerged in bromethyl blue dye and placed on a rocker at 15rpm overnight. The next day, the dye was drained off and replaced with destaining solution, and the gels were placed back on the rocker for a few hours, with the solution being replaced as needed.

3.2.iii. Top-Down Proteomics

Top-down proteomic analyses were performed and raw data provided to me by project collaborator Dr. Daniel Petras (University of California, San Diego) using the following methods. First, venom samples were dissolved in ultrapure water to a concentration of 10 mg/mL, then centrifuged at 12,000 x g for 5 min. 10 µL of venom solution were mixed with 10 µL of 0.5 M tris(2-carboxyethyl) phosphine (TCEP), and 30 µL of 0.1 M citrate buffer (pH 3) to reduce the disulphide bonds in each protein, before being incubated at 65 °C for 30 minutes. Samples were then mixed with 50 µL of acetonitrile/formic acid/H_2O (prepared at a ratio of 10:1:89) and centrifuged at 12,000 x g for 5 min. For Liquid chromatography–tandem mass spectrometry (LC-MS/MS) analyses, 5 µL of resulting sample supernatant were eluted with a gradient of water with 0.1% formic acid and 0.1% FA in acetonitrile (ACN) and injected in replicates of two, with MS/MS performed on a Vanquish ultra-high-performance liquid chromatography (UHPLC) system coupled to a Q-Exactive hybrid quadrupole orbital ion trap (Thermo Fisher Scientific, Bremen, Germany) and liquid chromatography separation on a Supelco Discovery Biowide C18 column (300Å pore size, 2 x 150 mm column size, 3 µm particle size) at a temperature of 30 °C and a flow rate of 0.5 mL/min. Gradient elution started with 5% ACN for 0.5 min, followed by a linear increase to 40% for 50 min, then 40-70% for 60 min. Afterwards, the column was washed with 70% ACN for 5 min before being re-equilibrated at 5% for 5 min. Electrospray ionization (ESI) settings of the mass spectrometer were adjusted as follows: 18 L/min auxiliary gas, 53 L/min sheath gas, spray voltage 3.5 kV, S lens RF level 90 V, capillary voltage 63 V, and capillary temperature 350 °C. MS/MS spectra

were obtained in data dependent acquisition mode and were acquired with 1 micro scan and 1000 ms maximal C-trap fill time. AGC targets were set to 1E6 for MS1 full scans and to 3E5 for MS/MS scans. All scans were performed with a mass resolution of 140,000 at m/z 200. The three ions with both the highest abundance and having a known charge were selected for Higher-energy C-trap dissociation (HCD) at the apex of a peak within 15 seconds from their first occurrence. The default charge state was set to z = 6, and the activation time to 30 msec. The mass window for precursor ion selection was set to 3 m/z. A window of 3 m/z was set for dynamic exclusion within 30 sec. Any ions with unknown charge as well as isotope peaks were excluded. For initial data analysis and annotation, extracted ion chromatograms of intact proteins were generated of deconvoluted of multiple charged spectra with XTRACT of the Xcalibur Qual Browser v2.2 (Thermo, Bremen, Germany). Chromatograms of mono-isotopic deconvoluted LC-MS runs were performed with MZmine 2 v2.2, using a signal intensity threshold of 1.0E4, mass tolerance of 10 ppm and a mass alignment minimum peak width and height of 30 seconds and 3.0E4 respectively. Maximum peak width was set to 10 min. The same signal intensity threshold was used for the baseline cutoff algorithm for chromatographic deconvolution. Feature alignment was performed with 10 ppm mass accuracy and 0.5 min retention time tolerance. Before protein spectrum matching could be performed, multiple charged MS/MS spectra were deconvoluted using MS-Deconv v0.8.0.7370 under the following settings: maximum charge 30, maximum mass 50,000, signal-to-noise threshold 2, m/z tolerance 10 ppm. Protein spectrum matching was performed against the NCBI protein database and venom gland-specific transcriptomic derived protein sequence database using TopPIC v1.1.0 (Kou et al., 2016), which had mass error tolerance set to 10 ppm and a 1% false discovery rate cut-off was used. Pairing of MS/MS derived protein ID from TopPic with Extracted ion chromatograms was performed by mass matching through the use of an R script (available from the following website: https://github.com/DorresteinLaboratory/match_ables_by_exact_mass.). Mass spectrometry data and database search results for top-down and bottom-up proteomic experiments are publicly available in the MassIVE repository under accession number MSV000081885 and in proteomXchange with accession number PXD008597.

3.2.iv. Curation of Identified Proteins

The resulting annotated top-down proteomic profiles generated by this collaborator were provided to me for further analysis. First, profiles were passed through a manual curation step to confirm identity by confirming them through protein BLAST (BLASTp) of each amino acid sequence against the NCBI non-redundant protein sequences database. Next, the protein sequences from the various datasets were partitioned into toxin families and independently imported into MEGA v7 (Kumar et al., 2016), and a pairwise comparison analysis was performed to determine the number of amino acid site differences between each sequence and to identify identical proteins. Where identical proteins were detected, their identities were merged, and their abundances summed. As the 3FTX and PLA_2 toxin families are the most abundant and isoform diverse of those found in elapid venoms, additional curation was performed to further discriminate these toxin families by functional annotation and physicochemical properties, respectively. For the reasons outlined in Chapter 2, 3FTXs were separated into cytotoxins (CTXs) and 'Other 3FTXs' based on BLASTp annotation, with any protein matching "cytotoxin", "cardiotoxin" or "direct lytic factor" classed as a CTX. PLA_2s were characterised into "acidic" or "basic" based on their isoelectric point, which was estimated by the Compute pI/Mw tool of the Expasy Bioinformatics Resources Portal (Bjellqvist et al., 1994, 1993; Gasteiger et al., 2005). This categorisation was performed to explore whether the significant differences observed between the transcriptomic expression of acidic and basic PLA_2s for spitting and non-spitting cobras (Fig. 2.11.) was also detected with the proteomic data.

3.2.v. Comparative Analysis of Identified Proteins

In order to determine whether spitting cobras exhibit significantly different abundance of toxin proteins in the venom glands compared to non-spitting cobras, the abundance data for proteins were summed for each toxin family and compared in *Naja* and *Hemachatus*. Due to the differences in number of individuals used for pooled venoms, number of proteins of each toxin family were not analysed. To examine both the differences between spitting and non-spitting cobras and the

influences of geography at the proteome level, a two-way ANOVA was performed on all data meeting the assumptions of normal distribution and equal variance. To ensure that data fell within the assumptions of a two-way ANOVA, normal distributions were determined using qq-plots and equality of variance of each group (African spitters, African non-spitters, Asian spitters and Asian non-spitters, with *Hemachatus haemachatus* being grouped with African spitting cobras) were estimated using Levene's Test for Homogeneity of Variance in the 'car' package (Fox, J. and Weisberg, 2019) in RStudio. Data found to be in violation of the assumptions were analysed by the non-parametric factorial analysis ARTool R package (Wobbrock et al., 2011). The statistical outputs can be found in Table 3.3. Bonferroni corrections were then applied to the outputs of these tests, treating each factor (group, geography, group+geography) as a model set.

To further test whether venom protein composition separates into distinct groups based on geography (e.g. Africa vs Asia), venom-spitting (spitting vs non-spitting species) or lineage (African spitters, African non-spitters, Asian spitters, Asian non-spitters and *H. haemachatus*), the top-down proteomics methods described above allowed a Principal Coordinate Analysis (PCoA) to be performed. First, a pairwise distance matrix was firstly conducted on the toxin amino acid sequences using the JTT matrix-based model (Jones et al., 1992) in MEGA v.7. The rate variation among sites was modelled with a gamma distribution (shape parameter = 0.8657) for PLA2s and uniform rates for 3FTX subfamilies. A Euclidian dissimilarity matrix was then generated from the pairwise distance matrix for all toxin proteins using the Daisy() function from the R package "Cluster" (Maechler et al., 2019). Classical multidimensional scaling was then applied to this matrix using the cmdscale() function in RStudio (RStudio, 2016).

3.3. Results

3.3.i. Comparative Overview of Venom Proteomes

The SDS-PAGE gel electrophoretic profiles revealed that the venom of all cobras show distinct and intensely-stained protein bands at around the 10 kDa mark (Fig. 3.1.), which are indicative of the abundant presence of three-finger toxins (3FTXs). *Naja*

subfulva, the African spitting cobras, the Asian cobras (with the exception of *N. philippinensis*) and outgroup species all show a strong band at ~14-15 kDa, which corresponds with the molecular weight of PLA_2 toxins (Fig. 3.1.). The outgroup species (*Walterinnesia aegyptia* and *Aspidelaps scutatus*) and *N. philippinensis* exhibit an intense protein band at ~22 kDa, and a similar sized protein also appears in the venom of the African spitting cobras and *N. siamensis*, although at much lower abundance (Fig. 3.1A.). This protein band is absent from the corresponding reduced gel (Fig. 3.1B.), suggesting that it consisted of a hetero- or homo-dimer of toxins, with the molecular weight indicating mostly likely a dimer of 3FTXs. The Asian cobras and outgroups show some evidence of higher molecular weight proteins in their venom, with faint protein bands detected in the 50-150 kDa range, the most consistent of which is found at ~50 kDa (Fig. 3.1A.), and likely represents a PIII-SVMP toxin.

Because similar molecular weight toxins are indistinguishable from one another by gel electrophoresis, and to gain a more thorough and comprehensive characterisation of the identity of the toxins found in the venom of the various *Naja* and related species studied here, I next curated and analysed venom proteomes for 16 species of elapid snake generated by top down proteomics. The curated venom proteomes (Fig. 3.2.) showed several similarities with the transcriptome overview provided in Chapter 2 (see Fig. 2.4.). Firstly, 3FTXs were the most abundant toxins across the various proteomes, representing between 55.06 and 98.56% of all proteins detected in venom (Fig. 3.2.). Moreover, upon disaggregation of proteins detected at the toxin family level, in all *Naja* species, individual 3FTX proteins showed the highest abundances when compared with members of any other toxin family (Fig. 3.3.). Notably, cytotoxic 3FTXs (CTXs) were the most dominant subclass of 3FTXs detected in the majority of these species (Fig. 3.2. and Fig. 3.3.), with summed abundances ranging from 19.15-89.26%, with a mean of 57.62%. The second most abundant toxin family typically detected in all venom proteomes were the PLA_2s (mean 15.23%), with the exceptions being the African non-spitting cobras *N. haje*, *N. annulifera* and *N. nivea*, where PLA_2 toxins represented <3% of the total venom proteome (Fig. 3.2.). Next was kunitz, having a mean of 1.74%, though this was largely due to the abnormally-high abundance of kunitz toxins in *W. aegyptia* (20.28%, mean kunitz abundance drops to 0.59 when *W. agyptia* is excluded). NGF never contributed

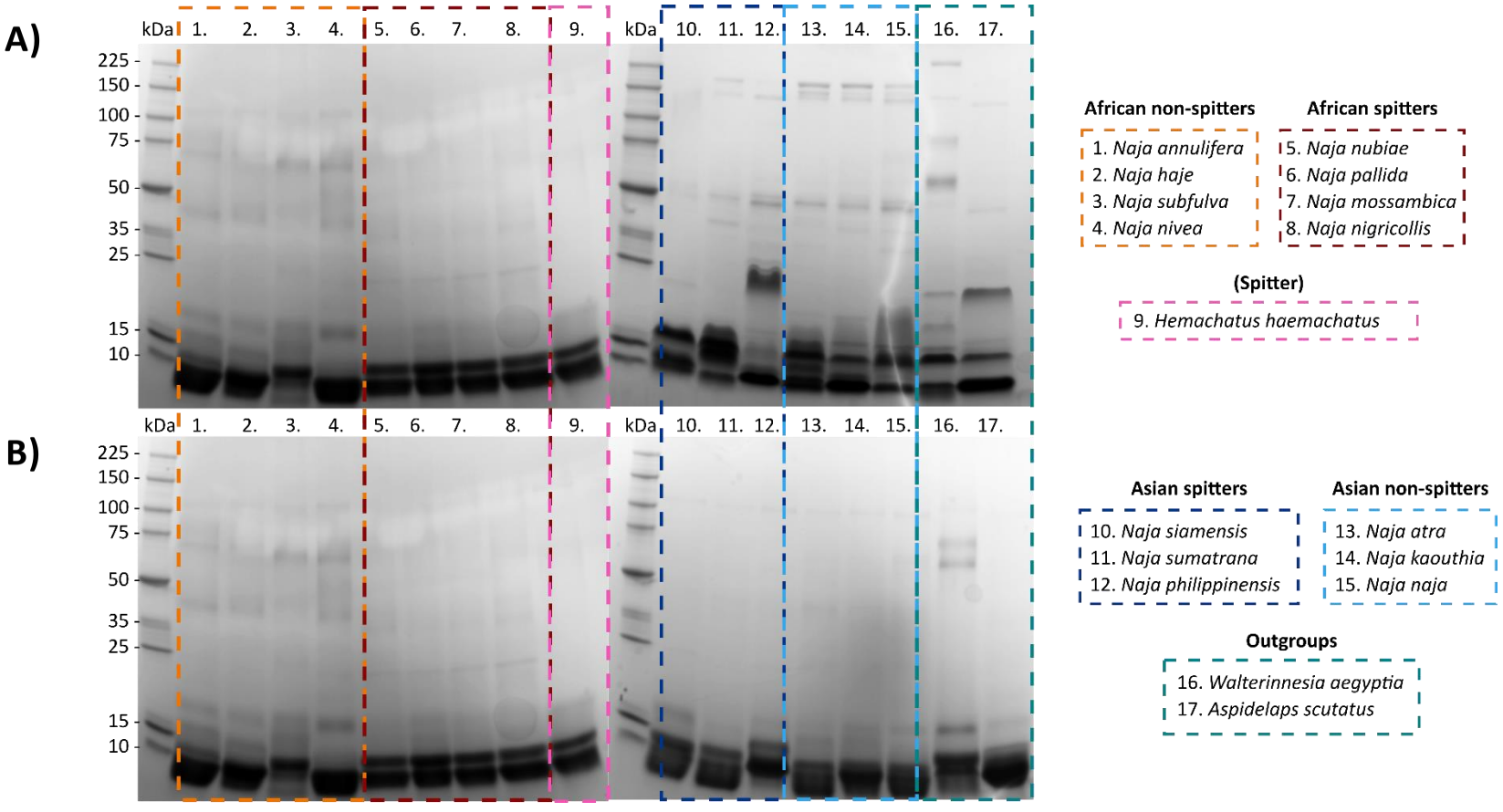


Figure 3.1. SDS-PAGE gel electrophoretic profiles of venom from *Naja* and *Hemachatus* species, treated under non-reducing A) and reducing B) conditions.

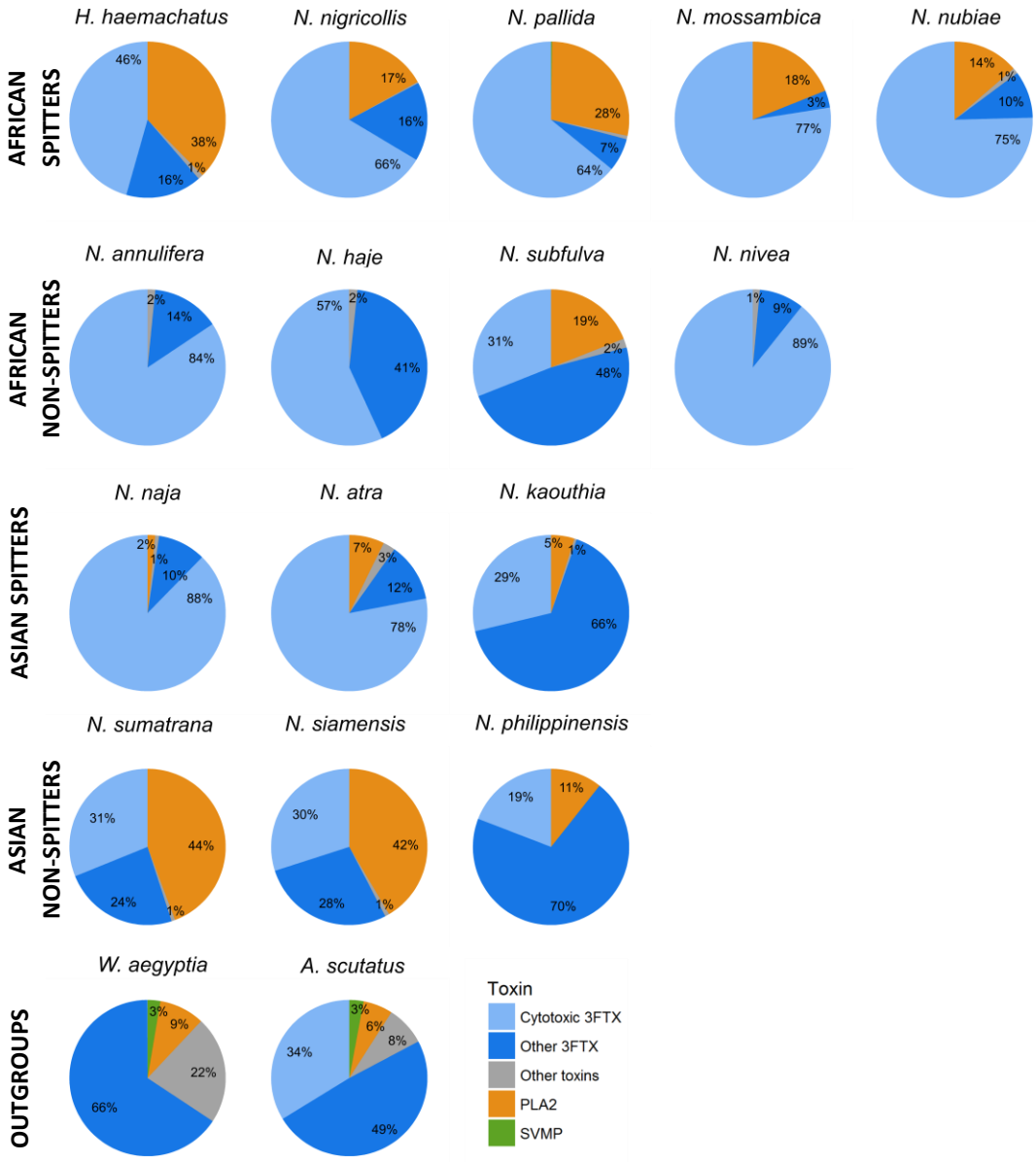


Figure 3.2. An overview of toxin protein abundance in the venom proteomes of cobras and their near relatives.

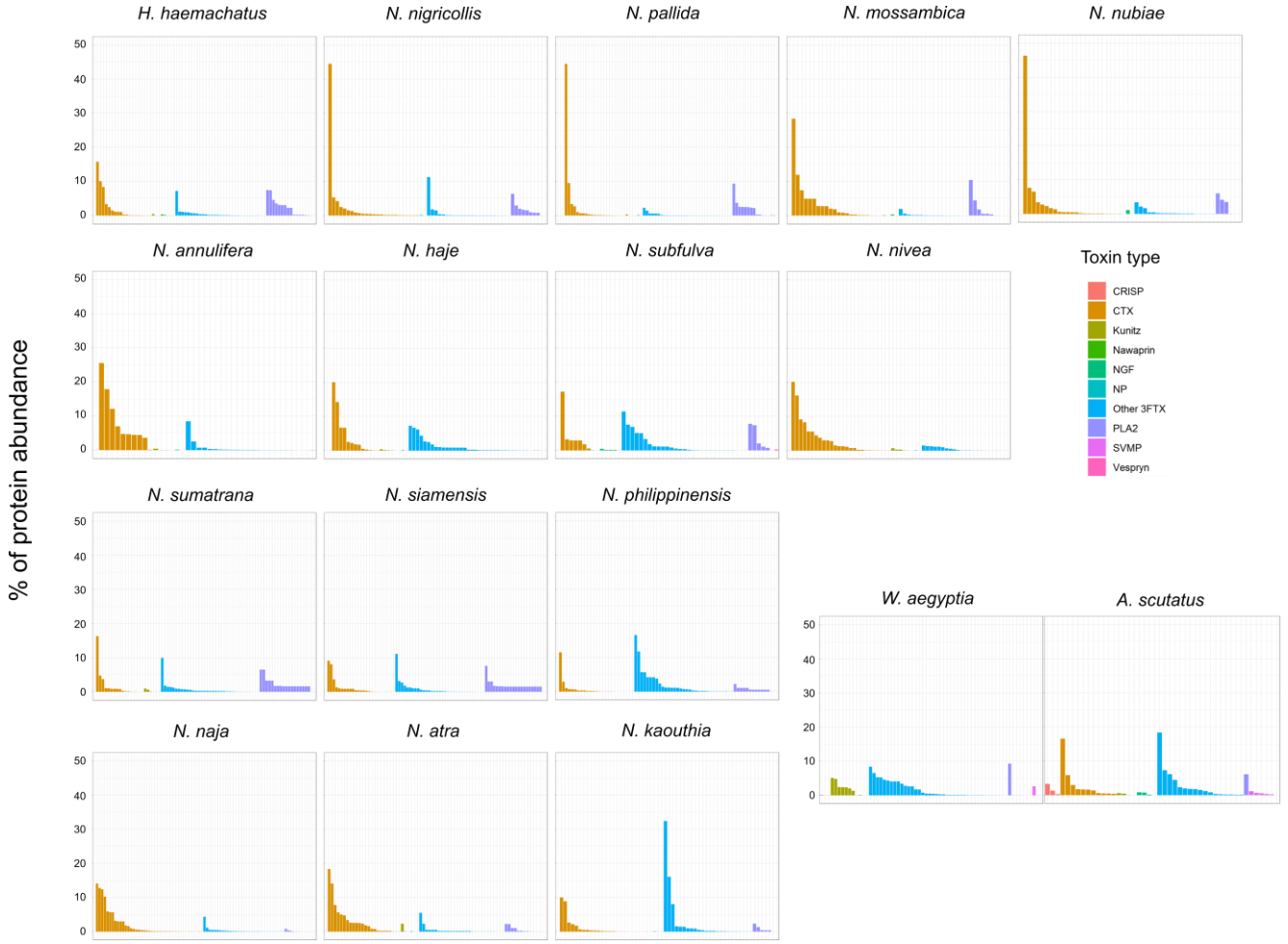


Figure 3.3. An overview of the abundance of individual toxin proteins in cobras, represented by coloured bars.

more than 1.87% of the total proteome in any particular species (Fig. 3.2. and 3.3.), and in the "ingroup" species (*Naja* & *Hemachatus*), SVMPs never exceeded 0.20%. Contrastingly, the venom composition of the outgroups, *W. aegyptia* and *A. suctatus* displayed much higher amounts of SVMPs (around 3% of the proteome for both species).

A high-level comparison between the toxin abundances detected here in the venom proteomes with gene expression data elucidated from the venom gland transcriptomes (Chapter 2), revealed several differences. For example, PLA_2s show considerably higher representation in the proteomes of Asian spitting cobras (range 10.66-44.27%, mean 32.14%) than in their corresponding transcriptomes (range 0.01-15.62%, mean 5.23%). This is perhaps best evidenced by PLA_2 toxins consisting nearly half of the proteomes of *Naja sumatrana* and *N. siamensis*, yet only 0.07% and 15.62% (respectively) of the total toxin expression detected in the venom gland trancriptomes (Fig. 2.4.). Contrastingly, PLA_2 toxins are of much lower abundance in the venom of Asian non-spitting species (range 1.56-7.26%, mean 4.55%) than their corresponding transcriptomes (range 6.12-25.68%, mean 15.45%). In addition, the relative proportion of SVMPs detected are considerably reduced across the ingroup species sampled from the transcriptome to corresponding proteome (range 0-6.82% and mean 2.45% in the transcriptome, dropping to 0-0.2% and mean 0.01% in the proteome), while the abundances of the 'other toxin' families are also typically reduced; these comprised less than 5% of the proteomes of the *Naja* spp. and *Hemachatus*, and decrease from around 40% to 22% in *W. aegyptia* and around 22% to 8% in *A. scutatus*. Finally, the representation of CTXs in *N. haje* and *N. atra* venom is dramatically higher in the proteomes than predicted from the venom gland transcriptomes - making up more than half of the total proteome composition, compared to 1.67% and 9.04% respective expression levels detected in the transcriptome (Fig. 2.4.). In general, there is an overrepresentation of toxins in the proteome (Fig. 3.4A.), with the main toxins contributing to this pattern of overrepresentation being 3FTXs and kunitz (Fig. 3.4B.), while SVMPs, PLA2s and other toxins have a fairly equal representation in both transcriptome and proteome.

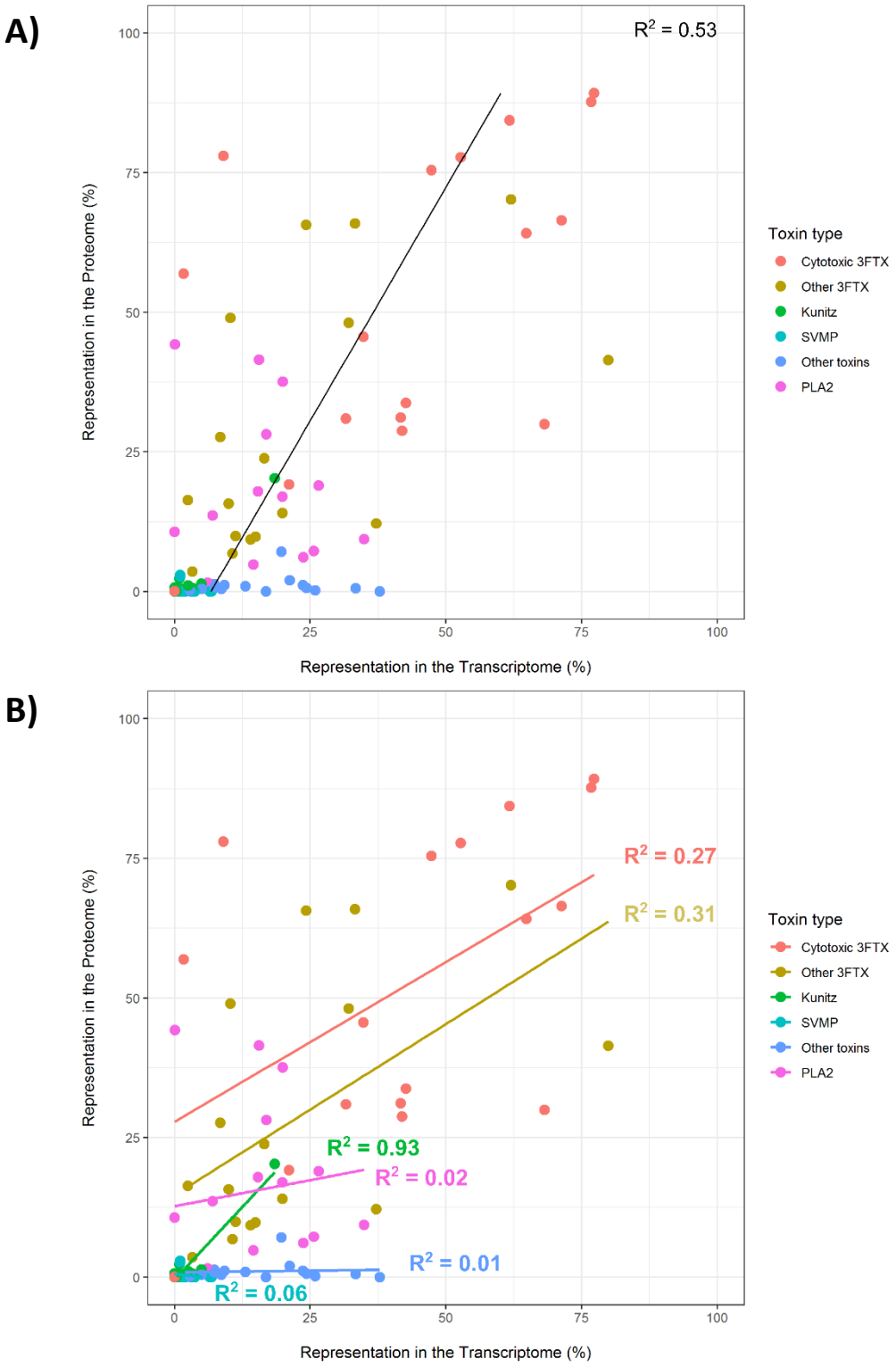


Figure 3.4. Scatterplot representing the expression in the transcriptome verses the abundance in the proteome of toxin families in cobra venoms. Linear regression is plotted as a solid line for **A)** all toxins **B)** each toxin family.

3.3.ii. Three-finger Toxins (3FTXs)

As described above, 3FTXs are the most abundantly represented toxin family found across the various cobra species. Overall, non-spitting cobras have the highest proteomic abundances of 3FTXs (Fig. 3.5.), resulting in a mean of 93.89%, compared to 72.96% in spitting cobras, and this difference is statistically significant (Two-way ANOVA, F = 12.56, df = 1, p = 0.005) (Table 3.3.). The exception to this general trend is *Naja subfulva*, which despite being a non-spitting species, exhibits a 3FTX abundance (79.07%) closer to that of African spitting cobras than African non-spitters. When comparing across geographical locales, African and Asian cobras have very similar patterns of 3FTX abundance (Fig. 3.5B.), and this is reflected by a lack of significance between these abundances following statistical testing (Two-way ANOVA, F = 0.52, df = 1, p = 0.48) (Table 3.3.).

Cytotoxins (CTXs) are the predominant sub-class of 3FTXs found in the venoms of the species studied here (Fig. 3.6A.), with the exception of the two outgroup species, the non-spitting species *N. subfulva* and *N. kaouthia*, and the Asian spitter *N. philippinensis*. Of the ingroup species, the spitting and non-spitting cobras were found to have similar mean CTX abundances (51.20% and 65.13%, respectively), and consequently there is no significant difference in CTX abundance between the two groups (Two-way ANOVA, F = 1.62, df = 1, p = 0.34) (Table 3.3.). African cobras, whether spitting or non-spitting species, have higher abundances of CTXs (mean 67.89% and 65.88%, respectively), and these levels are also highly comparable with those detected in the venom of Asian non-spitting cobras (mean 64.82%) (Fig. 3.6B.). However, Asian spitting cobras have considerably lower CTX abundance that all other cobras (mean 26.75%), resulting in significantly lower abundances of CTXs in the venom of Asian cobras (overall) compared with their African counterparts (Two-way ANOVA, F = 3.43, df = 1, p = 0.03) (Table 3.3.).

The abundance of the other subclasses of 3FTXs ('other 3FTXs') is highly variable across cobras (range 2.45-70.19%), but is noticeably high in the two outgroup species *W. aegyptia* and *A. scutatus* (65.65% and 49.01%, respectively), the Asian non-spitting cobra *N. kaouthia* (65.91%) and the Asian spitting cobra *N. philippinensis* (70.19%) (Fig. 3.7A.). Contrastingly, the abundances of other 3FTXs in the venoms of African spitting cobras is relatively low (range 3.56-16.34%). Despite

Table 3.3. Test statistics from ANOVA analyses assessing the influence of spitting, geography, and the combination of both factors on toxin protein abundance in Naja and Haemachatus. Significant values are emboldened, and those that remain significant post Bonferroni-correction are represented by red text.

Test Variable	Test type	Factor	F value	p
3FTX abundance	Two-way ANOVA	**Group (spitter/non-spitter)**	**12.56**	**0.005**
		Geography (African/Asian)	0.52	0.48
		Group + Geography	0.65	0.44
CTX abundance	Two-way ANOVA	Group (spitter/non-spitter)	0.99	0.34
		Geography (African/Asian)	**6.70**	**0.03**
		Group + Geography	2.03	0.18
'Other 3FTX' abundance	Analysis of Variance of Aligned Rank Transformed Data	Group (spitter/non-spitter)	1.54	0.24
		Geography (African/Asian)	**6.13**	**0.03**
		Group + Geography	0.78	0.40
PLA_2 abundance	Two-way ANOVA	**Group (spitter/non-spitter)**	**14.03**	**0.003**
		Geography (African/Asian)	0.65	0.44
		Group + Geography	0.65	0.44
Kunitz abundance	Analysis of Variance of Aligned Rank Transformed Data	Group (spitter/non-spitter)	1.81	0.21
		Geography (African/Asian)	0.93	0.35
		Group + Geography	0.08	0.78
NGF abundance	Analysis of Variance of Aligned Rank Transformed Data	Group (spitter/non-spitter)	0.11	0.75
		Geography (African/Asian)	**28.96**	**0.0002**
		Group + Geography	1.24	0.29
Acidic PLA_2 abundance	Analysis of Variance of Aligned Rank Transformed Data	**Group (spitter/non-spitter)**	**6.00**	**0.03**
		Geography (African/Asian)	2.64	0.13
		Group + Geography	2.70	0.13
Basic PLA_2 abundance	Two-way ANOVA	**Group (spitter/non-spitter)**	**28.18**	**0.0002**
		Geography (African/Asian)	**6.75**	**0.02**
		Group + Geography	**5.23**	**0.04**

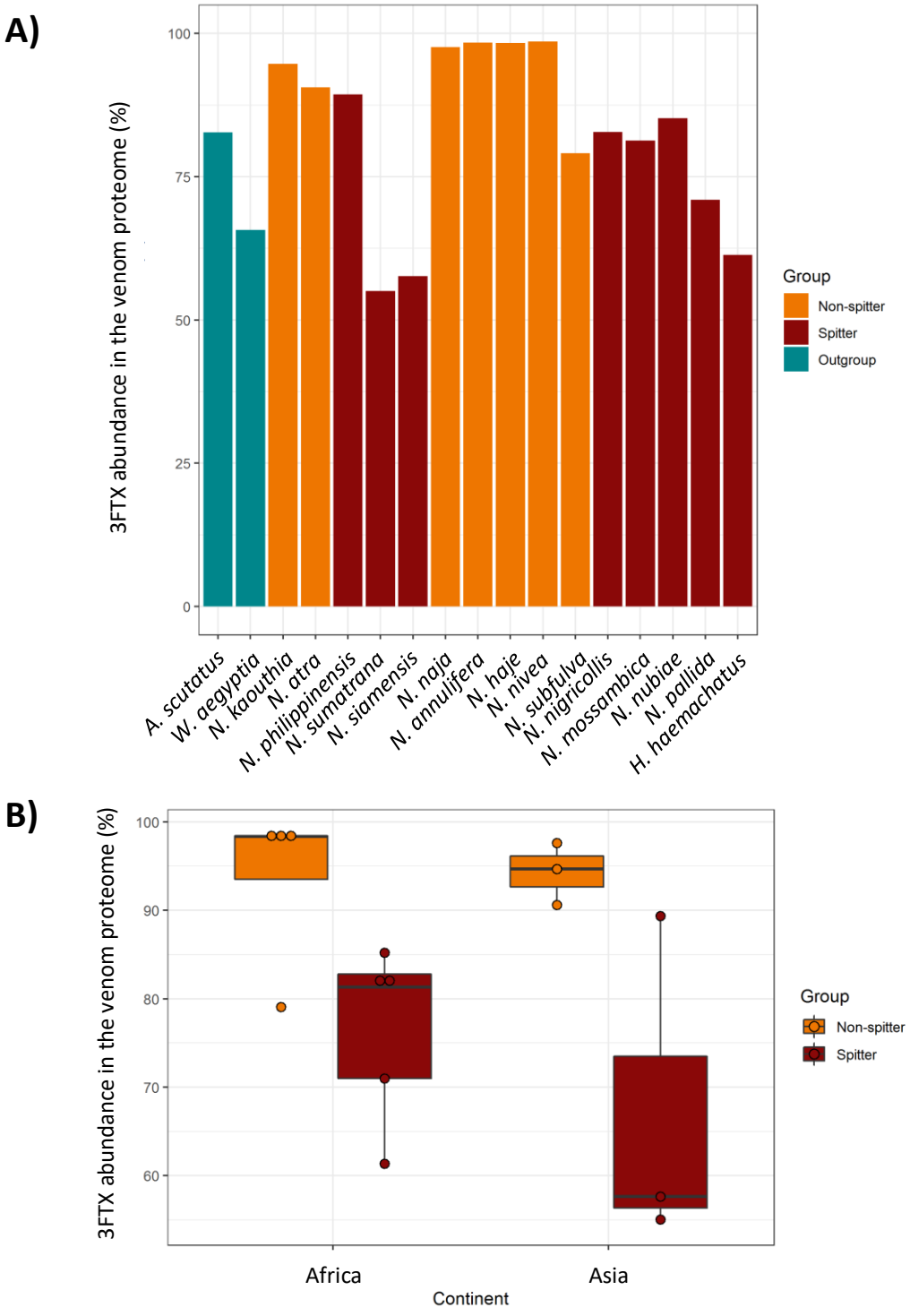


Figure 3.5. Comparisons of three-finger toxin (3FTX) abundance in cobra venom proteomes. A) bar graph of 3FTX abundance by species **B)** box plot comparing 3FTX abundance between spitting and non-spitting cobras (excluding outgroups), separated by continent.

this observation, there is no evidence of venom spitting being associated with the abundances of these toxins (Analysis of Variance of Aligned Rank Transformed Data, F = 0.07, df = 1, p = 0.24) (Table 3.3.), despite differences in their mean abundance of other 3FTX (28.70% and 21.75%, respectively). Nonetheless, Asian cobras as a whole have a mean proteomic abundance of other 3FTXs that is nearly double that of African cobras (34.96% vs 18.35%), which is statistically significant (Analysis of Variance of Aligned Rank Transformed Data, F = 1.24, df = 1, p = 0.03) (Table 3.3.).

3.3.iii. Phospholipase A_2s (PLA_2s)

PLA_2 abundance ranges from 0.00 to 44.27% in all species, with the outgroups *W. aegyptia* and *A. scutatus* showing moderate PLA_2 abundance (9.35% and 6.12%, respectively). Looking at the ingroup species, spitting cobras have considerably higher PLA_2 abundance than non-spitting cobras (ranges 10.66-44.17% and 0.00-19.00%, respectively) (see Fig. 3.8A.), with a mean abundance (26.33%) over 5 times that of non-spitting cobras (4.68%). This is particularly the case for Asian spitting cobras, which have a mean abundance of 32.14%, compared to 22.84% in African spitters. This difference in PLA_2 abundance between spitting and non-spitting cobras is statistically significant (Two-way ANOVA, F = 14.03, df = 1, p = 0.003) (Table 3.3.). *N. subfulva* is an odd exception to the general rule of non-spitting cobras having low PLA_2 abundance (19.00%), being comparable to that of *N.philippinensis*, *N. nubiae*, *N. nigricollis* and *N. mossambica* (10.66%, 13.63%, 16.95% and 17.91%, respectively) (Fig. 3.8.), and over double the next-highest non-spitter value from *N. atra* (7.26%). This is even more unusual in that PLA_2 abundance is particularly low in the other African non-spitters (see Fig. 3.8B.), which all show values close to zero. It is perhaps because of these low values that African cobras have lower PLA_2 abundance than Asian cobras in general (means 14.81% and 18.35%, respectively), though this is no significant (Two-way ANOVA, F = 0.65, df = 1, p = 0.44) (Table 3.3.).

When separated by isoelectric point, the ingroup species have a lower abundance of basic PLA_2 in general (mean 0.35% in non-spitters and 8.22% in spitters, compared to 4.33% and 18.11% in acidic PLA_2s), though this may be to the larger number of acidic PLA_2 proteins in the venom in general (Fig. 3.9.). Additionally, spitting cobras have significantly higher abundance of both acidic and basic PLA_2s

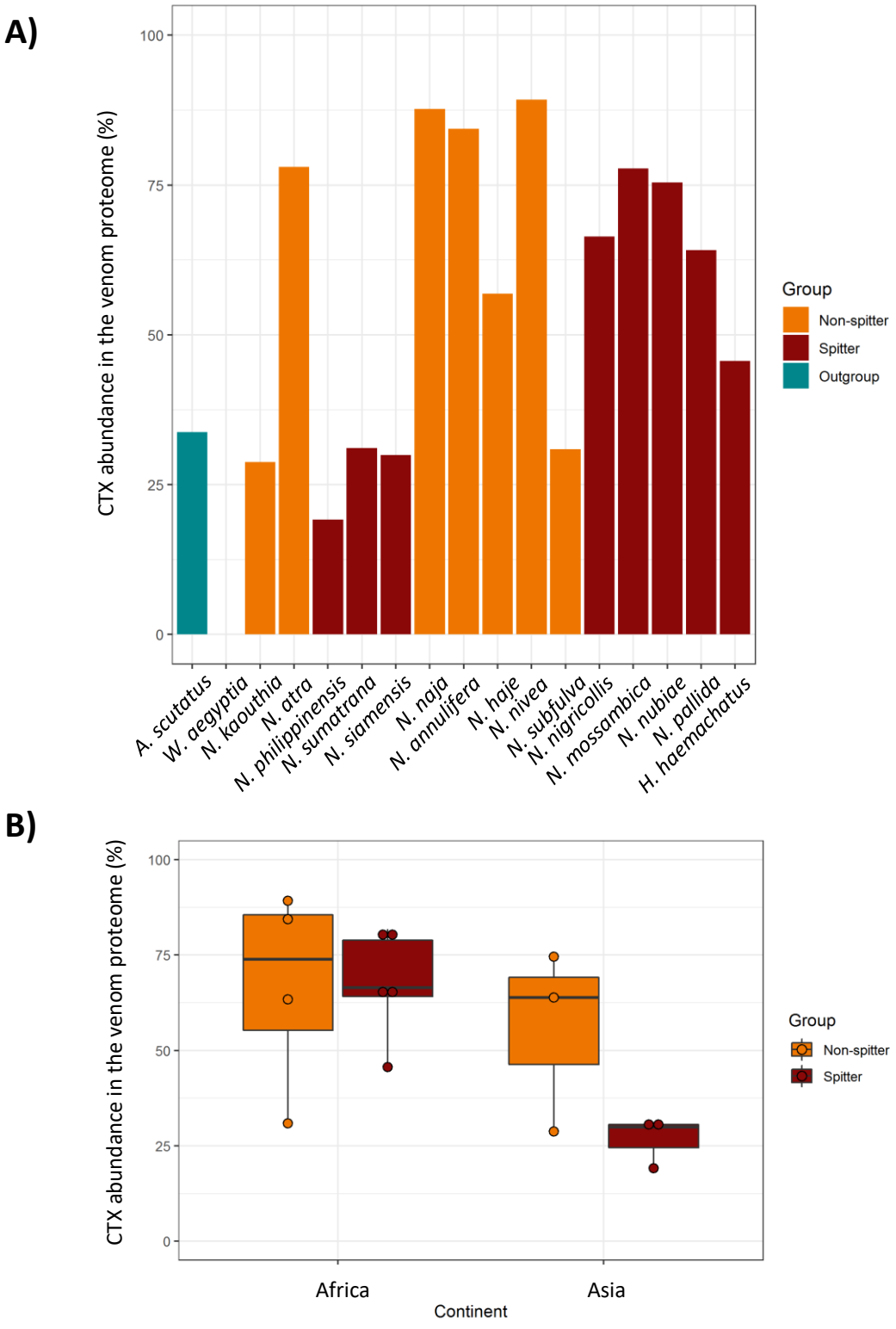


Figure 3.6. Comparisons of cytotoxin (CTX) abundance in cobra venom proteomes. A) bar graph of CTX abundance by species **B)** box plot comparing CTX abundance between spitting and non-spitting cobras (excluding outgroups), separated by continent.

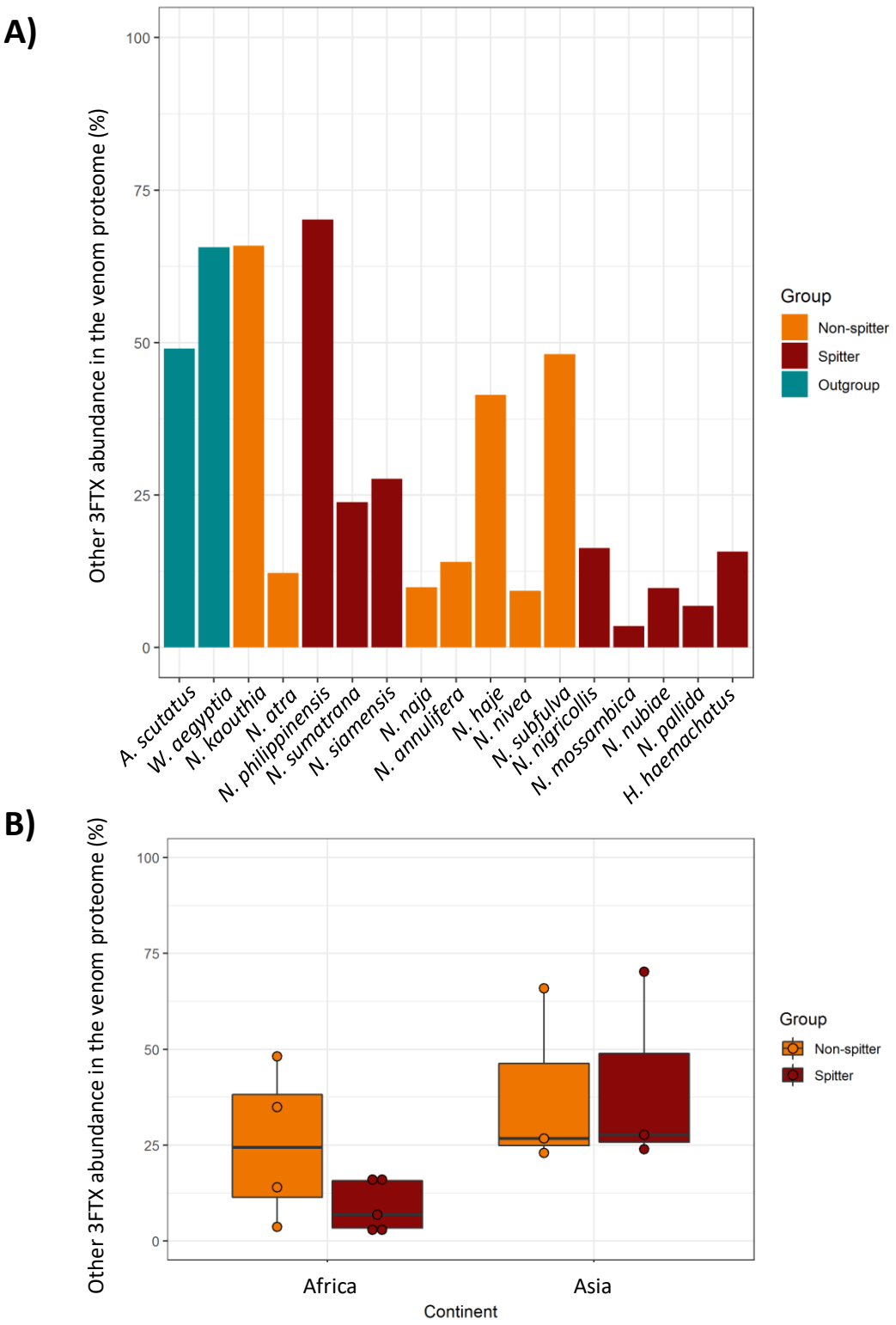


Figure 3.7. Comparisons of 'other 3FTX' (non-cytotoxin) abundance in cobra venom proteomes. A) bar graph of CTX abundance by species **B)** box plot comparing 'other 3FTX' abundance between spitting and non-spitting cobras (excluding outgroups), separated by continent.

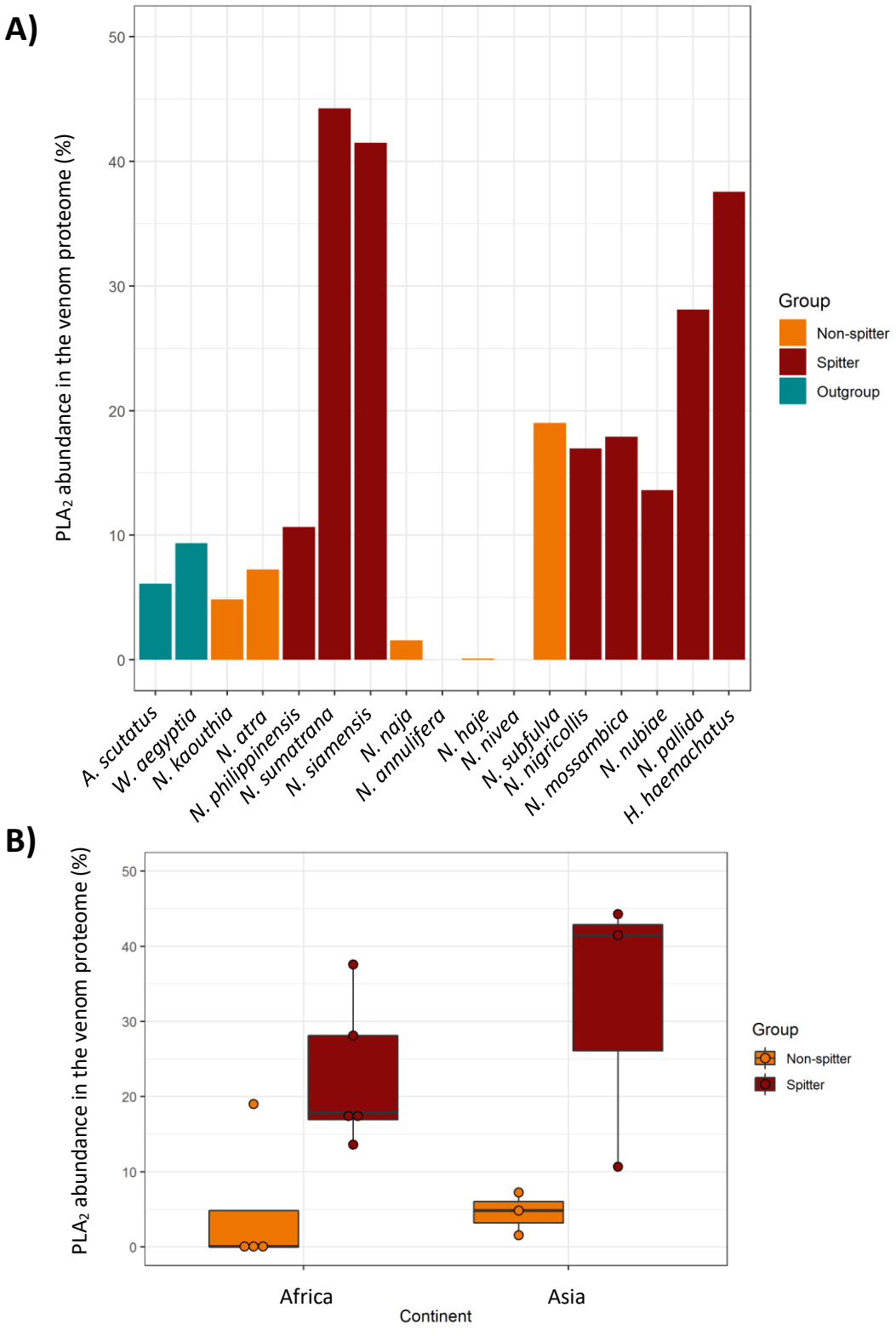


Figure 3.8. Comparisons of phospholipase A_2 (PLA_2) abundance in cobra venom proteomes. A) bar graph of CTX abundance by species **B)** box plot comparing PLA2 abundance between spitting and non-spitting cobras (excluding outgroups), separated by continent.

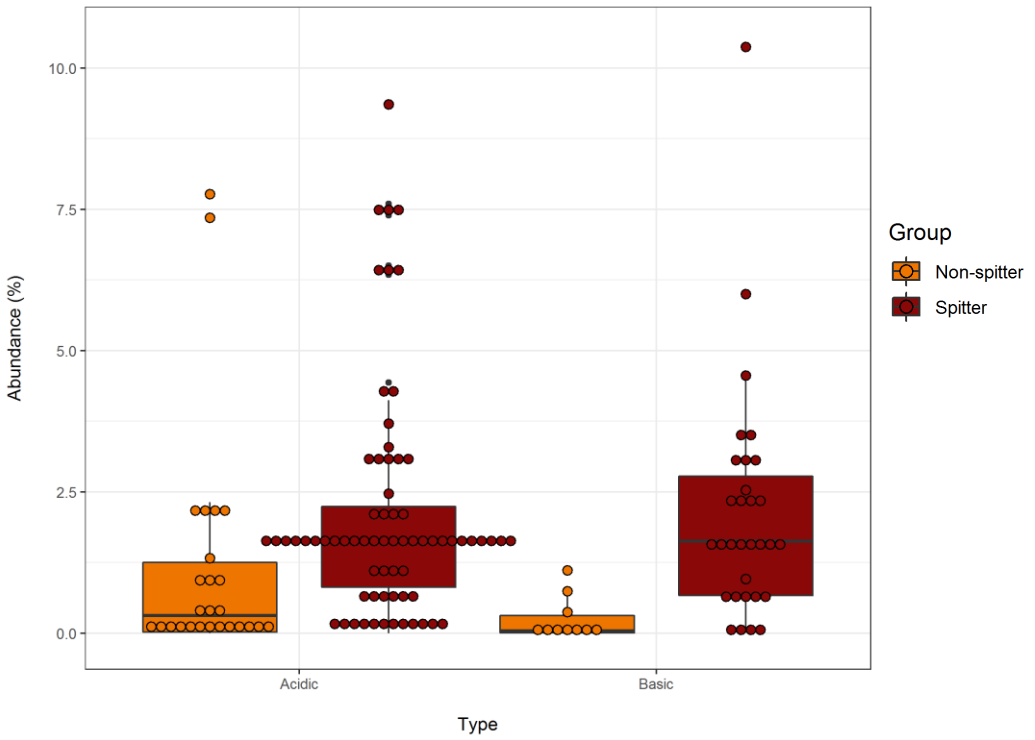


Figure 3.9. Comparisons of the abundance of acidic and basic phospholipase A_2s (PLA_2s) in the proteome between spitting and non-spitting cobras. Individual PLA_2 proteins were determined to be acidic if pI of < 6.5 or basic if having a pI of >7.5.

than non-spitting cobras (Analysis of Variance of Aligned Rank Transformed Data, F = 6.00, df = 1, p = 0.03; Two-way ANOVA, F = 28.18, df = 1, p = 0.0002, respectively) but again this may be due to the sheer number of PLA_2s in spitting cobra venom (fig 3.9.).

3.3.iv. Other toxins

The mean abundances of all other toxin families detected in the venom proteomes of the ingroup species *(Naja* spp. and *H. haemachatus*) represents less than 1% of the total proteome. Indeed, the outgroup species have the highest abundance of such toxin types (see Fig. 3.2.), with 20.28% of the *Walterinnesia aegyptia* proteome represented by Kunitz and a further 2.66% consisting of SVMPs. The *Aspidelaps scutatus* proteome contains reasonable abundances of CRISPs (4.90%), SVMPs

(2.94%), NGF (1.87%) and Kunitz (1.04%). In comparison, the highest protein abundance of Kunitz detected in the venom of cobras species is 2.34% (*Naja atra*), and much less for SVMPs (0.2%, *N. pallida*), CRISPs (0.03%, *N. pallida*) and NGF (1.12%, *N. nubiae*). Nonetheless, since some ingroup species do exhibit >1% abundances of Kunitz and NGF toxin types, comparative statistical analyses were performed for these protein families. The analysis revealed no association between these toxin families and venom spitting, as the abundances of both Kunitz and NGF in the venom proteomes of the various spitting and non-spitting species were not significantly different (Analysis of Variance of Aligned Rank Transformed Data, $F = 1.81$, $df = 1$, $p = 0.21$; $F = 0.11$, $df = 1$, $p = 0.75$, respectively) (Table 3.3.). However, African cobras were found to have significantly higher abundances of NGF than Asian cobras (Analysis of Variance of Aligned Rank Transformed Data, $F = 28.96$, $df = 1$, $p = 0.0002$) (Table 3.3.), although this finding is likely due to the unusually high abundance detected in *N. nubiae* compared to all other species (1.12%, compared with the next highest value of 0.97%).

3.3.v. Principal Coordinate Analysis (PCoA) of the venom proteome

This analysis utilised the ability of the top-down proteomics approach to enable the calculation of the differences between individual amino acid sequences and use this to generate a visualisation in 2D space. The results of this analysis separated cobras into three distinct spitting lineages, representing African spitting cobras, Asian spitting cobras and *H. haemachatus* (Fig. 3.10.). African and Asian cobras separate from one another based on PC1, while *H. haemachatus* separated from both the *Naja* spitting lineages by both PC1 and PC2. In contrast, the non-spitting species were found to cluster close together, although there was some separation between Asian and African non-spitting species, based on PC1 (Fig. 3.10.). The only exception to this general pattern was that of the Asian spitting cobra *Naja philippinensis*, which clustered closely with Asian non-spitting cobras rather than other spitting species (see asterisk in Fig. 3.10.). Nonetheless, these findings suggest that in general each spitting cobra lineage has evolved distinct venom compositional changes in response to the evolution of defensive venom spitting, and thus exhibit differences when compared with their non-spitting counterparts. Furthermore, these findings also

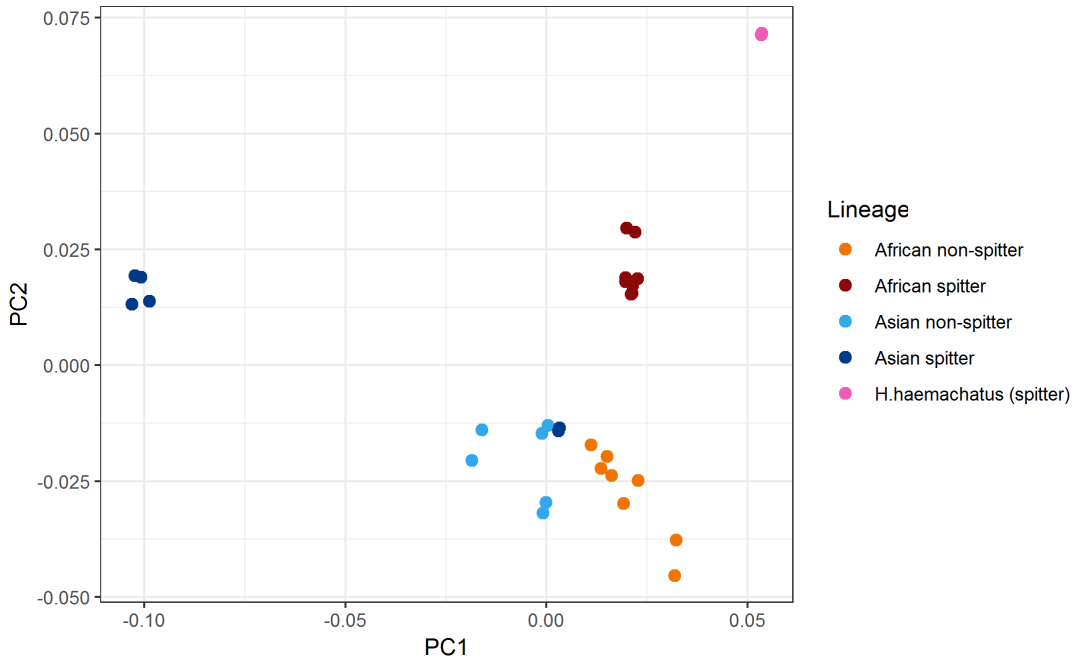


Figure 3.10. Principal Coordinate Analysis (PCoA) of the venom proteomes separates the species studies into distinct spitting and non-spitting lineages. The scatter plot visualises the output of the Principal Coordinate Analysis (PCoA), which represents the distances between venom proteomes calculated from the differences in amino acids between all toxin proteins detected in the venom proteomes (excluding outgroups). Dots represent duplicate samples from each species and are coloured by lineage. The asterisk represents the spitting species *Naja philippinensis* and is highlighted based on its placement among non-spitting cobras.

suggest that the different spitting cobra lineages have not converged upon the same 'solution' for effective venom spitting, suggesting different venom compositional changes in have arisen following the independent origins of this characteristic.

Discussion

3.4.i. Overview

This chapter reveals that the main protein components of cobra venoms are three-finger toxins (3FTXs). For the majority of *Naja* spp. and *H. haemachatus*, it is the

cytotoxic 3FTXs (CTXs) that are the most abundant subclass of 3FTXs detected in their venom; the exceptions being *N. subfulva*, *N. kaouthia* and *N. philippinensis*. These data suggest that CTXs are important venom toxins in the *Hemachatus-Naja* lineage, as they are present in much lower abundance in the outgroup species *Aspidelaps scutatus* and are nearly-completely absent in the proteome of the other outgroup species, *Walterinnesia aegyptia* (Fig. 3.2. & 3.6.). Moreover, CTXs are not highly present (if at all) in the venoms of other elapids (Ainsworth et al., 2018; Aird et al., 2017; Calvete et al., 2012; Corrêa-Netto et al., 2011; Fernández et al., 2011, 2015; Herrera et al., 2012; Lauridsen et al., 2016; Laustsen et al., 2015; Lomonte et al., 2014; Oh et al., 2017, 2019; Olamendi-Portugal et al., 2018; Paiva et al., 2014; Petras et al., 2015; Pla et al., 2017; Rey-Suárez et al., 2016, 2011; Rusmili et al., 2014; Sanz et al., 2019, 2016; Shan et al., 2016; Tan et al., 2015; Tan et al., 2015; Tan et al., 2019; Tan et al., 2016, 2017; Williams et al., 2011; Ziganshin et al., 2015) (Table 3.1.). These data suggest that the ancestors of *Naja* and *Hemachatus* have likely upregulated CTX venom abundance in a unique manner compared with most other elapid snakes, whose venoms are predominately neurotoxin-dominated (Tasoulis & Isbister, 2017).

The second most abundant family of toxins typically detected in the cobra venom proteomes are phospholipases A_2 (PLA_2s), although the abundances of these toxins varied extensively across the different *Naja* spp. Notably though, spitting cobras were found to have significantly higher abundances of PLA_2s than their non-spitting counterparts (Table 3.3.). Despite the often great diversity of toxin families observed in the venom of snakes (Tasoulis & Isbister, 2017), there were very few examples of toxin families other than 3FTXs and PLA_2s contributing more than 1% of the total venom proteomic abundance across any of the studied species. Indeed, summing the abundances of these 'other toxins' across the species results in an average abundance of only 0.86% of the proteome. These data suggest that the venoms of cobras likely only utilise two (or on a few occasions, e.g. African non-spitting cobras, one) toxin families to cause prey (and occasionally human) lethality. Finally, as top-down proteomics methods enable the recovery of full-length amino acid sequences, sequence-based comparative methods of venom toxins were able to be performed; a novelty for the field. Principal Coordinate Analysis (PCoA) on the amino acid composition of the toxins recovered from across the different cobra

venoms clearly separated representatives of the three spitting lineages (African spitting cobras, Asian spitting cobras, and *H. haemachatus*) into three distinct clusters that were separated from one another and also form a broad cluster containing the non-spitting cobras (and the outlier *N. philippinensis*). The combination of these results suggest that cobras that spit their venom have evolved different toxin compositions to those species that never evolved defensive venom spitting. Moreover, it suggests that the overall toxin compositions of the three spitting lineages have diverged away from each other, although elements of convergence can be detected when looking at the comparative data generated for specific toxin classes, such as the seeming convergent upregulation of PLA_2s in the venoms of the spitting lineages.

3.4.ii. Proteomic comparisons of Spitting and Geography

When comparing the proteomic abundances in the context of venom spitting, significant differences in the abundances of the two major toxin families, the 3FTXs (Two-way ANOVA, F = 12.56, p = 0.005; Table 3.2.) and PLA_2s (Two-way ANOVA, F = 14.03, p = 0.003; Table 3.2.), were detected between spitting and non-spitting cobras. As mentioned above, spitting cobras were found to have significantly higher abundances of PLA_2 toxins in their venoms, while in terms of 3FTXs, non-spitting species had significantly higher abundances. However, when disaggregating the 3FTXs into CTXs and 'other 3FTXs' (predominately neurotoxins), significance is lost (Table 3.3.), with no differences detected between the abundances of these 3FTX subclasses and venom spitting. Thus, it is possible that overall differences in 3FTX abundances may simply be an artefact of differences in PLA_2 abundances imposing shifts in proportional venom composition, since these two toxin families account for the vast majority of all toxins detected in the cobra venoms (Fig. 3.2.). The relatively high abundance, and also activity, of PLA_2s in spitting cobras has been noted previously (Petras et al., 2011; Tan et al., 2019), suggesting that the mechanical and behavioural adaptation of venom spitting in cobras has resulted in convergent upregulation of these toxins. This data therefore suggests that PLA_2 toxins may play a role in venom spitting. A number of elapid PLA_2s are known to act as pre-synaptic neurotoxins (Ranawaka et al., 2013), and it is possible that the upregulation of PLA_2s

in the venom of spitting cobras may increase their relative neurotoxicity, given that the majority of 3FTXs detected are CTXs rather than short or long chain neurotoxins. However, this hypothesis is not supported by clinical evidence following snakebite, as spitting cobras are known to cause substantial cytotoxic effects in envenomed victims, compared with their non-spitting counterparts, particularly in African species (Warrell, 2008c). Another possibility is that PLA_2s have been upregulated due to their ability to form toxin complexes in elapid venoms. Notable examples of these are β-bungarotoxins, found in kraits (*Bungarus* spp.), which are formed from two PLA_2s in a complex with a Kunitz toxin, and the heterodimeric Taipotoxin, found in taipans (*Oxyanurus* spp.) (Sunagar, Jackson, & Reeks, 2015). These examples involve PLA_2s forming complexes with other PLA_2s to form potent neurotoxins, however in cobras, PLA_2s have been found in complex with both cytotoxic and neurotoxic 3FTXs to form fast-acting toxins that are up to 30 times more effective at lysing cells (Dutta et al., 2019; Harvey, Hider, & Khader, 1983; Louw & Visser, 1978; Mukherjee, 2010). Moreover, even when not complexed, elapid PLA_2 toxins have been proposed to enhance the cytotoxic effect of CTXs (Dutta et al., 2019; Fletcher et al., 1991). Consequently, the increased abundances of PLA_2s in spitting cobra venoms may have evolved to increase effective cytotoxicity when ejected into the eyes of aggressors.

At a geographic level, African cobras have a significantly higher CTX abundance (Two-way ANOVA, $F = 6.70$, $p = 0.03$) and significantly lower 'Other 3FTX' abundance (Analysis of Variance of Aligned Rank Transformed Data, $F = 6.13$, $p = 0.03$) than Asian cobras (see Table 3.2.), suggesting that Asian cobras may have more neurotoxic venoms. This somewhat falls in line with both the mixed symptoms observed from bites by Asian cobras (Warrell, 2008c) (see Table 5.1.) and the strongly neurotoxic symptomology resulting from *N. philippinensis* bites (Watt et al., 1988) (Table 5.1). While, African cobras were also found to have significantly higher abundances of NGF than Asian cobras (Analysis of Variance of Aligned Rank Transformed Data, $F = 28.96$, $p = 0.0002$; see Table 3.2.), this is likely due to the unusually high abundance found in the African cobras *Naja nubiae* and *N. subfulva*, which have 4-5 times the average abundance of NGF across the *Hemachatus-Naja* lineage. There are no significant differences in kunitz abundance between spitting and non-spitting cobras, or between African and Asian cobras, which is perhaps

anticipated given the relatively low abundances of this toxin class and the seeming use of 3FTXs and PLA_2s as the predominant neurotoxins in these venoms.

The proteomes of the various cobra venoms have many similarities with the venom gland transcriptomes described in Chapter 2. Firstly, the dominance of 3FTXs, particularly cytotoxins, is evident at both levels (see Figs. 2.3. and 3.2.), while the high abundances of 'other 3FTXs' in the venoms of *N. haje*, *N. subfulva* and *N. philippinensis* remains consistent from venom gland to venom. Similarly, the high PLA_2 expression seen in the venom glands of African spitting cobras is also reflected in similarly high PLA_2 abundances in the venom (see Figs. 2.3. and 3.2.). However, there are also some major differences between the various transcriptomes and proteomes. Firstly, toxin family diversity, in terms of the number of putative toxin types detected, is extensively decreased from the transcriptome to the proteome level (see Fig. 3.4.). This is most evident when observing the relative abundances of the 'other toxins' (e.g. minor toxins) in the venom glands of many species decreasing dramatically in the corresponding venom proteomes. This is particularly noticeable in *H. haemachatus*, *N. sumatrana* and *W. aegyptia*, which see comparative reduction of up to 38% of all toxins. However, this general finding is perhaps not surprising – many prior studies on venom gland transcriptomics and venom proteomes have shown decreased diversity of toxin families detected using these distinct methodological approaches (Corrêa-Netto et al., 2011). However, a more intriguing distinction observed between the transcriptomes and proteomes relates to the PLA_2s, which are largely absent in the venom gland transcriptomes of the Asian spitting cobras , yet highly abundant in the venom proteomes, particularly that of *N. sumatrana*, which saw a 44% increase in abundance of this toxin type.

Differences between the transcriptome and proteome profiles of cobras may have several causes. For one, transcriptome profiles represent gene expression captured at a single moment in time and from a single individual, whereas proteome profiles represent the profile of venoms pooled from multiple individuals (with the exception of *Naja sumatrana*; Table 3.2.) from different days and times. As venom profiles can differ extensively between individuals due to factors such as diet (Barlow et al., 2009; Daltry et al., 1996; Li et al., 2005a), climate (Zancolli et al., 2019), age (Gibbs et al., 2011) and even sex (Menezes et al., 2006), pooled venoms may result

in a higher diversity of toxin proteins than venoms taken from a single individual. However, what is seen here is in fact the opposite; proteomic profiles are less diverse than the transcriptome profile. This may be due to increased sensitivity of transcriptomic approaches or it may suggest that the major toxins are upregulated through post-translational mechanisms while other toxins are down regulated, though there is debate as to how much post-translational mechanisms contribute to venom proteome diversity. Rokyta et al. (2015) found that mRNA from snake venom glands and proteins from snake venoms are highly correlated, thus calling the role of post-translational modifications in proteomic variation into question (Rokyta et al., 2015), but conflicting evidence suggests that post-translational modifications can result in large changes in the resulting venom proteome (Casewell et al., 2014). Post-translational modifications may shed light into the plasticity of venoms to become optimised specific pathologies, and it may be that selection on these other toxins isn't enough to remove them from the transcriptome entirely, making simple upregulation of major toxins the "easier" option for molecular venom modification. The large discrepancy in PLA_2 representation in *N. philippinensis* and *N. sumatrana* between the transcriptome and proteome levels may be due to the above discussed factors. In the case of *N. sumatrana*, there is the additional issue of RNA degradation due to long term cryostorage that may have resulted in a loss of capture for PLA_2 genes.

3.4.iii. Comparisons with the literature

Thus far, nine of the species of *Naja* that have been described in this chapter have had some degree of proteomic work previously performed in the literature. In the case of the African non-spitting species, the *Naja haje* and *N. subfulva* samples produced here from have a reduced number of toxin families recovered than the proteomes produced by Malih et al. (2014) and Lauridsen et al. (2017), with the literature samples containing a moderate amount of CRISPs, NGFs and SVMPs, as well as a large number of 'Other toxins' (Fig. 3.11B.). This is possibly due to differences in both locale and the number of individuals, especially in the case of *N. haje*, in which the Malih et al. (2014) sample contained 36 more individuals than those sampled in this study. In the case of *N. subfulva*, there is the additional difficulty of species

identification, with the venom used by Lauridsen et al. (2017) potentially being sourced from either *N. subfulva* (as in this study) or *N. melanoleuca*, as this species has since been split into multiple related 'forest cobra' species (Zancolli et al., 2019).

In the case of African spitting cobras, all proteomic data comes from a single comparative study by Petras et al. (2011) (see Fig. 3.11A.), which has the benefit of using the same bottom-up proteomics approach for all studied species. The results there are expected to be highly similar to those produced here as the animals of this species used for that study were also sourced from the same herpetarium as those used in this study (Table 3.2.). Indeed, the protein abundances are very similar, though the sample used here has notably higher abundances of 'other 3FTXs' and no SVMP. It is likely that different individuals may have been used to create the pooled venom sample used in Petras et al. (2011), different extraction times resulted in minor differences in venom protein composition, or differences in protein recovery methods. The proteomic abundances of the *N. mossambica* venom sample from this study is very similar to that of the earlier Petras study, although the previously published proteome has a higher abundance of PLA_2 and a small amount of SVMP not detected here. The *N. pallida* results are almost identical (see Fig. 3.11A.), which is perhaps surprising given that the locales of the individuals used are different. This hints at a low degree of venom variation across this species. Perhaps the most contrasting of these results are that of *N. nubiae*, in which the sample represented here has a lower diversity of toxins overall, a higher CTX abundance and lower PLA_2 and 'Other 3FTX' abundance. This is potentially due to the specimens used in this study being captive bred, as to the wild-caught African species used in the Petras et al. (2011) paper.

The Asian *Naja* in this study are somewhat more difficult to compare with that described in the literature, as all Asian specimen used in this study were captive bred due to the highly-restrictive export laws of India and other Asian countries. Many of the papers describing the venom proteomes of Asian non-spitting species used multiple locales for comparative analysis (see Fig. 3.12.). The *N. atra* from East and West Taiwan are near-identical in their venom protein composition (H. W. Huang et al., 2015), and very similar to the *N. atra* proteome described from China (Shan et

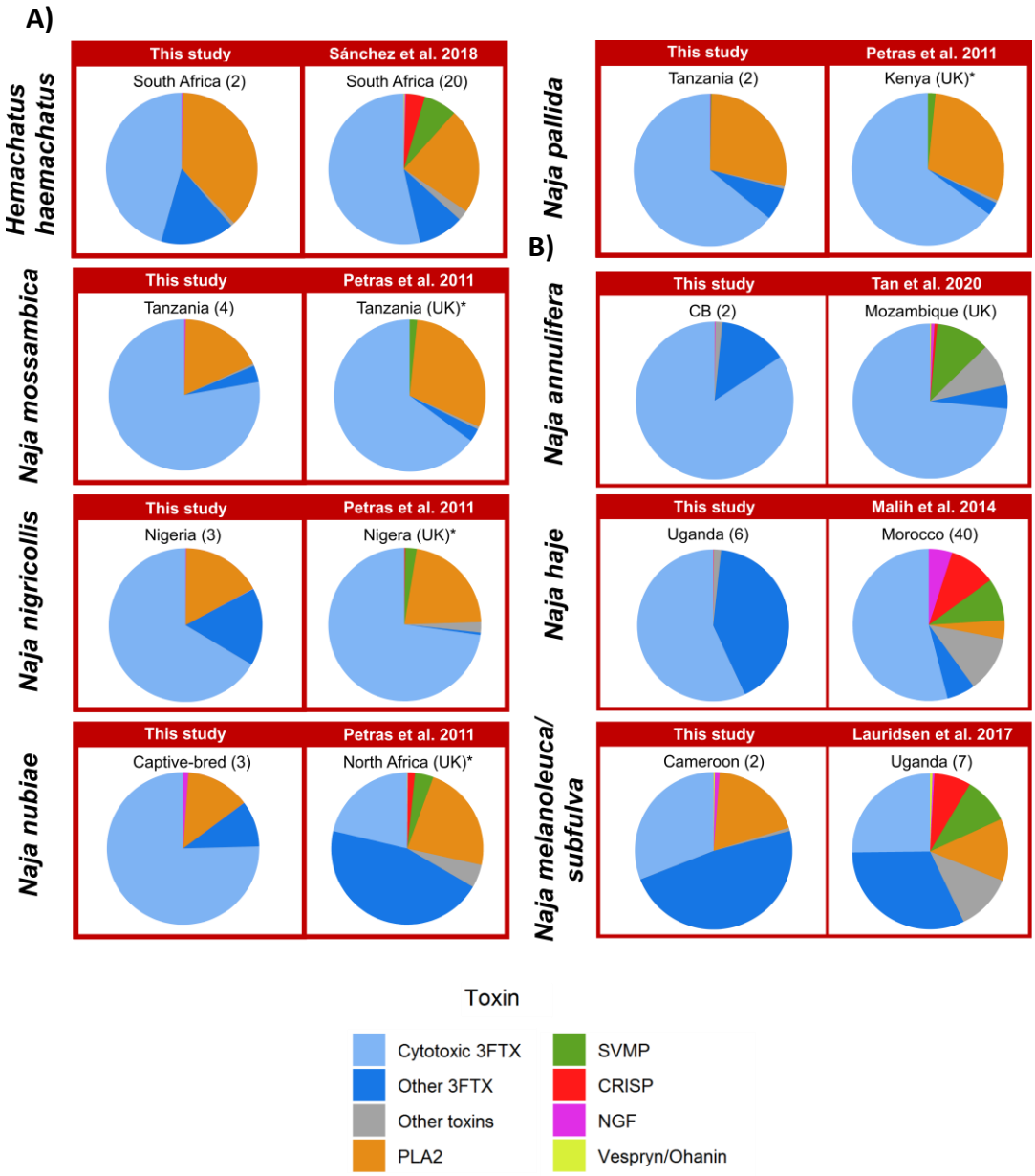


Figure 3.11. Comparison of the proteomes of African A) spitting cobras & *Hemachatus haemachatus*, B) non-spitting cobras from the literature with the proteomic data from this study. Text directly above each pie chart represents the region of origin of specimens used, with the number in parentheses representing the number of individuals used to make the pooled venom sample used in the respective studies. (UK) indicates that the number of individuals used was not specified/unknown. *The Petras et al. 2011 paper states that the cobras used for the study were also sourced from the Liverpool School of Tropical Medicine Herpetarium, so may potentially be the same individuals as those used for this study.

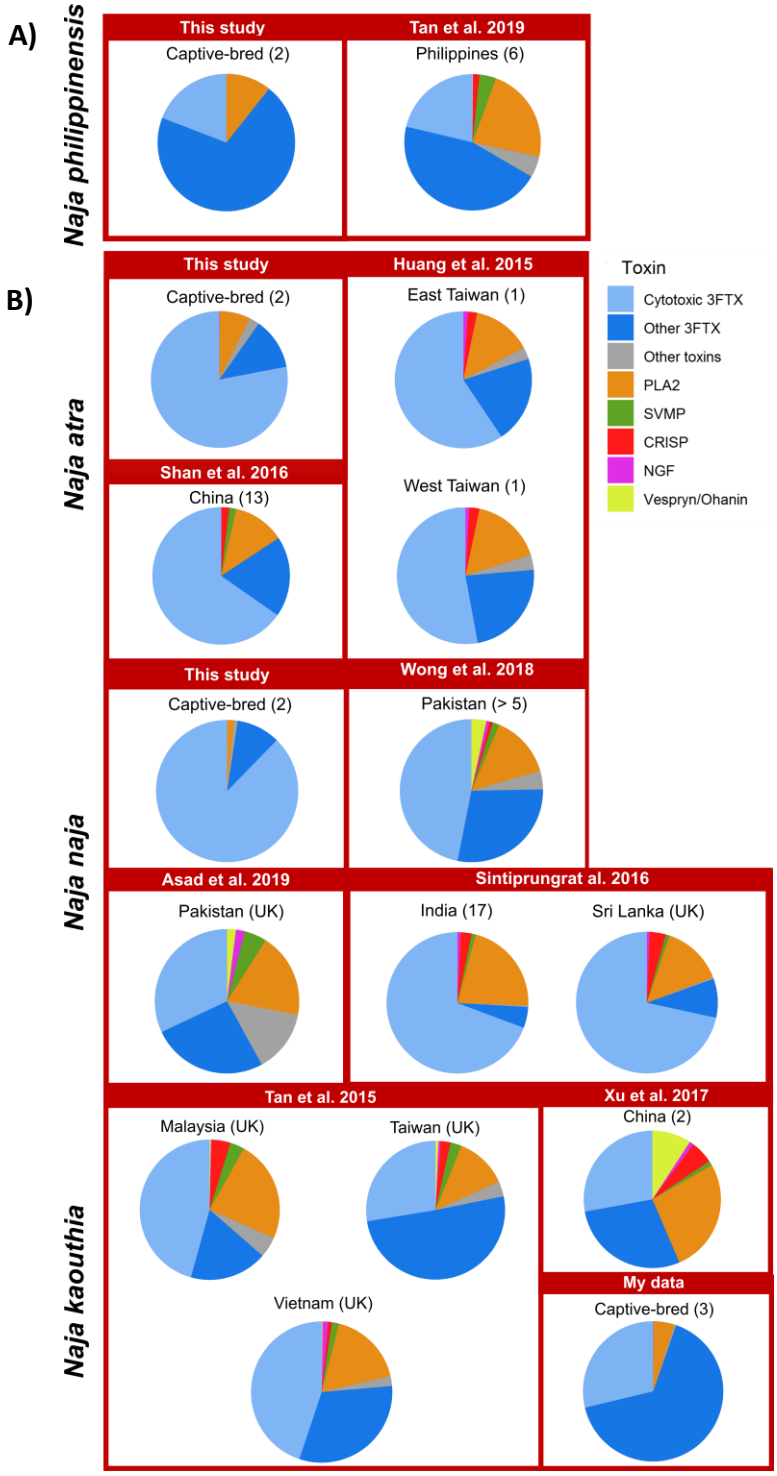


Figure 3.12. Comparison of the proteomes of Asian A) spitting cobras, B) non-spitting cobras from the literature with the proteomic data from this study. Text directly above each pie chart represents the region of origin of specimens used, with the number in parentheses representing the number of individuals used to make the pooled venom sample used in the respective studies. (UK) indicates that the number of individuals used was not specified/unknown.

al., 2016). These proteomes all have a moderate abundance of CRISP not detected in the *N. atra* sample of this study, along with a slightly higher abundance of 'other 3FTX' in comparison to CTXs. *N. kaouthia* shows perhaps the highest degree of venom variation in the cobras studied to date. The Malaysian and Taiwan samples described by Tan et al. (2015) are highly similar in their composition, though the Thai sample has a higher proportion of 'other 3FTXs' to CTXs. The Chinese *N. kaouthia* produced by Xu et al. (2017) has a much lower proportion of total 3FTX abundance and a higher PLA_2 abundance. In comparison, the *N. kaouthia* sample produced here has a larger proportion of 'other 3FTXs'. The Tan et al. (2015) and Xu et al. (2017) samples have a moderate amount of CRISP not seen in the Vietnam locale described by Tan et al. (2015) or the captive bred samples used in this study. The Chinese sample of *N. kaouthia* has the largest toxin family diversity, containing NGFs, CRISPS, SVMPs and a large amount of vespryn; a toxin not seen in this quantity in any other cobra species studied thus far. The two *N. naja* proteomes from the Sintiprungratet et al. (2016) are very similar in composition to one another but have a larger number of toxin families than the *N. naja* sample from this study, along with a much higher PLA_2 abundance. The only proteomic data described from an Asian spitting species is that of *N. philippinensis*, whose venom proteome was recently examined (Tan et al., 2019). This proteome has an increased number of toxin families recovered to the one produced by this study, with a larger abundance of SVMP and CRISP toxins.

Overall, the *Naja* proteomes in the literature tend to have recovered a larger number of toxin families than the samples here but there appear to be large discrepancies when it comes to PLA_2 abundance. The differences may stem from a combination of factors from different locales, to different numbers of individuals used for venoms, or to whether a top-down or bottom-up method was used to identify and quantify proteins in the venom. To account for these differences, for each species with previous proteomic literature (with the exception of *N. subfulva*, for which the previous study is uncertain of exact species), the average of the PLA_2 abundance from this study and all previous studies was taken. A two-way ANOVA was then rerun on this new literature-incorporated dataset and spitting cobras maintained a significantly higher PLA_2 abundance than non-spitting cobras (Two-way ANOVA: $F = 17.58$, $df = 1$, $p = 0.02$).

3.4.iv. Limitations

Venom variation can stem from a multitude of factors. As well as the biological factors stated previously, how the proteomes were generated may also have an effect on overall proteome composition. For instance, an increased number of individuals used for pooled venom samples is likely to lead to an increased number and diversity of toxin isoforms. While an average of 3 individuals from each species were used for the venom pools in this study, in the case of *N. sumatrana* only 1 individual was used, and in the case of *N. haje*, 6 individuals were used (Table 3.2.). Additionally, venom from *Aspidelaps scutatus* for proteomic work was from the subspecies *Aspidelaps scutatus scutatus* (Table 3.2.), which is a different subspecies to the individual used for trancriptome analysis (*Aspidelaps scutatus intermedius*, see Table 2.2.) as there was no supply of venom from that subspecies available for use. The differences in proteome composition may therefore be reflective of subspecies differences. In the future, it would be wise to only use extracted venom from an animal to be used for transcriptomic research for direct proteome-transcriptome analysis, preferably on the same day it is to be sacrificed to limit external factors that could influence fluctuations in expression, abundance or diversity in venom gland toxin expression or venom composition. Alternatively, multiple specimens of each species could be included but the proteomic data produced separately, so that individual variation in species can be observed and analysed. In addition, top-down proteomic methods can result in a decreased number of toxin families recovered due to mass sensitivity limitations, which may obscure the presence of some minor toxins.

Due to snake venoms being highly variable based on a number of both biological and methodological differences, I think there is good reason to call for standardisation in proteomic methods when studying snake venoms. In order for future literature to be comparable, they must use the same approach (top-down or bottom-up proteomics, or both), have a similar number of individuals used for pooled venom samples, and clearly state the locale of each species used to produce said samples. This way, methodological causes of venom protein variation can be minimised. Understandably, with the field containing researchers all around the

globe, this may be an idealistic goal but it would go a long way in making snake venom literature more comparative.

3.4.v. Concluding remarks

This chapter adds a wealth of new proteomic data to the field, including 7 previously unstudied *Naja* proteomes. Additionally, top-down proteomics allowed the comparison of full-length amino-acid sequences at a novel level via PCoA. The data here reveal that, while there are no distinct differences in the molecular composition of spitting and non-spitting cobras at the transcriptome level (Chapter 2), that spitting and non-spitting cobras have clearly diverged in terms of the proteomic composition of their venom. PCoA analysis on the toxin amino acid matrix generated here reveals that the three independent venom-spitting lineages have diverged from all non-spitting cobras and from one another. Despite this divergence within spitting cobra venoms, we also find evidence that these species have collectively, and thus convergently, upregulated the abundance of PLA_2s in their venoms, suggesting that this toxin family may contribute functionally to the defensive use of venom spitting. In the next chapter, the molecular evolution of toxins recovered at the transcriptome and proteome level of cobras will be further explored, with a particular emphasis on the two most abundantly expressed and secreted venom toxin families, the 3FTXs and PLA_2s.

4. MOLECULAR EVOLUTION OF COBRA VENOM COMPONENTS

4.1. Introduction

The origin of venom toxin genes in squamate reptiles is a complex topic (see 1.1.). One hypothesis is that the origin of venom was a single event at the base of the Toxicofera clade, a hypothetical taxonomic group that covers all venomous reptiles; snakes (*Serpentes*), iguanomorph lizards (*Iguania*) and anguimorph lizards (*Anguimorpha*) (Burbrink et al., 2020, Fry et al., 2009). These original venom genes were recruited and neofunctionalized from non-toxic forms. Such non-toxin genes, which may be expressed in multiple body tissues, such as the salivary glands, are thought to have undergone duplication, with duplicate genes becoming restricted predominately to expression in venom gland tissues while the original gene retains multi-tissue expression (Castoe et al., 2013; Fry et al., 2012; Fry et al., 2009; Reyes-Velasco et al., 2015). These duplicate genes are then thought to have undergone neofunctionalization into a toxic form via adaptive evolution. The extensive process of duplication and modification of these toxin genes has resulted in highly diverse toxin families, helping to form the various protein cocktails seen in the venoms of modern reptiles (Fry et al., 2012; Fry et al., 2006) (see Fig. 4.1.). Arguments against this hypothesis include evidence of toxin gene families being expressed in body tissues of both Toxicoferan and non-Toxicoferan squamates (Hargreaves et al., 2014) and that the duplication events in eukaryote genomes tend to be very low, with the estimated average duplication event occurring at 0.01 per gene per million years, and duplicate genes experiencing low half-life and relaxed selection pressures (Jordan, Wolf, & Koonin, 2004; Lynch & Conery, 2003). The opposing hypothesis proposes that venom toxin genes have emerged multiple times in squamates, through the duplication of non-toxic genes in all body tissues. These duplicate genes are then either restricted or recruited to the venom gland (Hargreaves et al., 2015; Hargreaves et al., 2014; Reyes-Velasco et al., 2015) (see Fig. 4.2.).

As mentioned above, gene duplication is thought to be an important step to generating toxin diversity (Kordiš & Gubenšek, 2000; Wong & Belov, 2012). Though general eukaryotic gene duplication rate has been estimated as being rare, there is

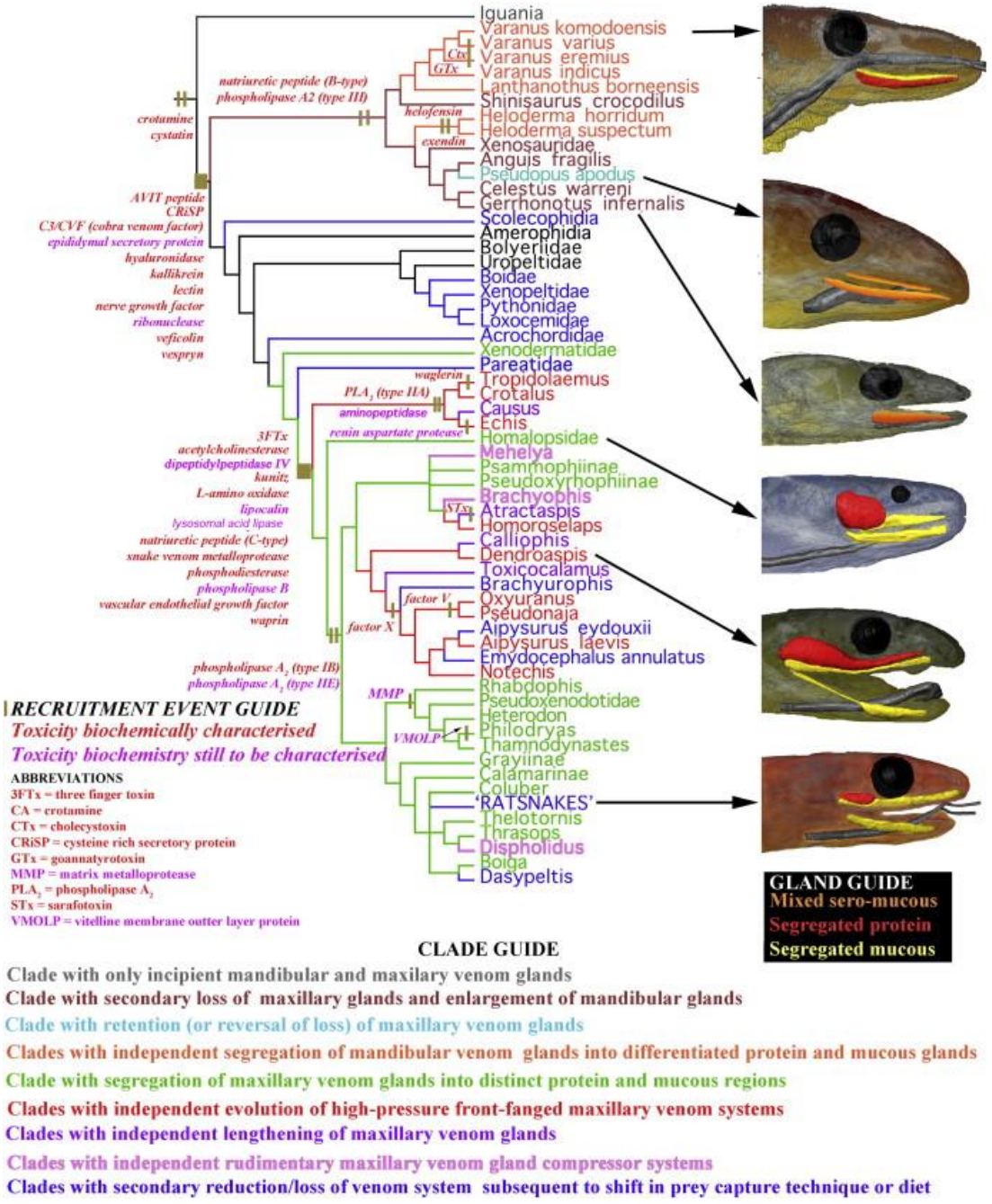


Fig. 4.1. Evolutionary tree showing toxin-recruitment events and evolution of the venom delivery systems according to the Toxicoferan hypothesis. Taken from Fry et al. 2012.

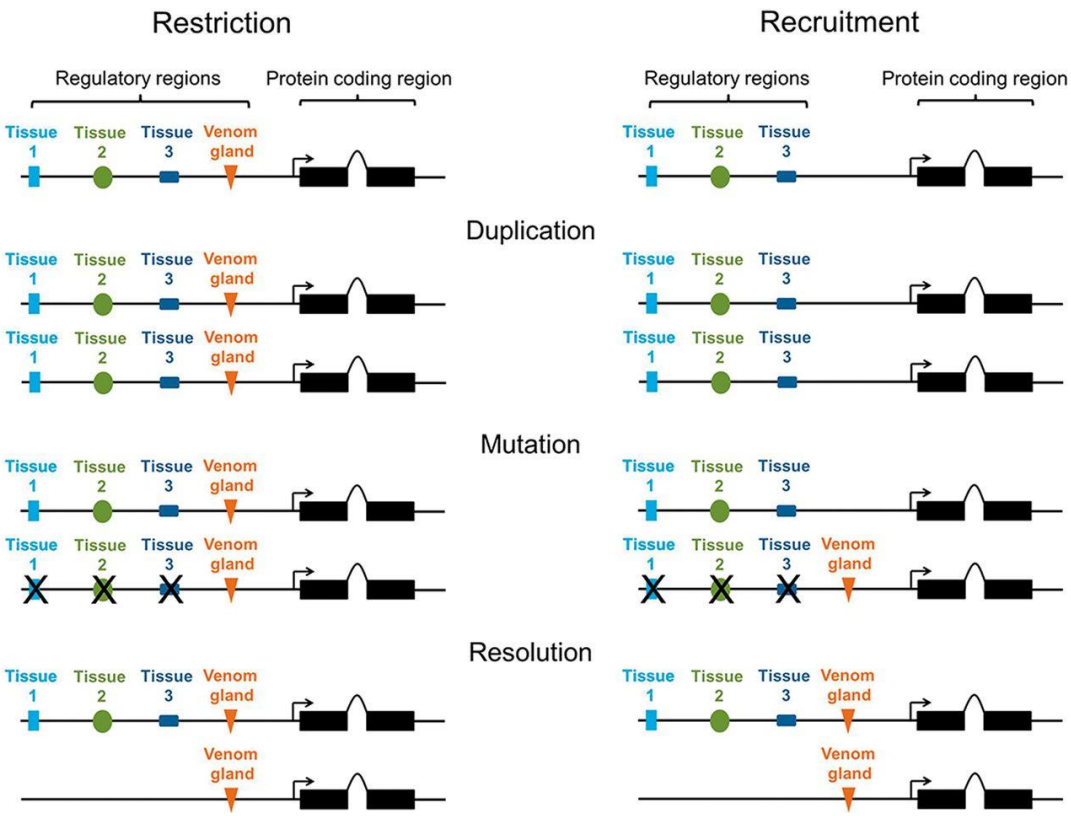


Fig. 4.2. Diagram illustrating the mechanisms of repeated incorporation of toxin genes into the squamate venom system. Taken from Hargreaves et al. 2014.

currently no large-scale study as of yet describing the duplication rates of specialised toxin genes, making it difficult to identify diversity stemming from this base level. However, toxin "superfamilies", such as three-finger toxins (3FTXs) and Snake Venom Metalloproteinases (SVMPs) contain several subfamilies and thousands of genes, suggesting they are either prone to duplication, have had several recruitment events of non-toxin genes or both. Venom gland transcriptomes reveal further evidence of duplication events in the three-finger toxin (3FTX), cysteine-rich secretory protein (CRISP), phospholipase A_2 (PLA_2), nerve growth factor (NGF) and SVMP families (Fry et al., 2003; Margres et al., 2013; Moura-da-Silva et al., 1996; Vonk et al., 2013; Wong & Belov, 2012), suggesting that these families are more prone to duplication events while other have limited susceptibility to such events.

In gene families containing large numbers of duplications, accelerated evolution is key for generating diversity in gene isoforms and thus shaping diversity in venom phenotype (Mebs, 2001). Indeed, venom genes can be subject to

extraordinary high levels of selection, as has been observed in cone snails (Chang & Duda, 2012). Similar phenomena have been observed in snakes, with evidence for accelerated evolution occurring in the 3FTX (Chang, 2007; Fujimi et al., 2003), SVMP (Brust et al., 2013; Shibata et al., 2018), PLA_2 (Chuman et al., 2000; Dowell et al., 2016; Gibbs & Rossiter, 2008; Kini & Chan, 1999; Lynch, 2007; Nakashima et al., 1995), Serine protease (SP) (Deshimaru et al., 1996; Shibata et al., 2018), C-type lectin (CTL) (Ogawa et al., 2018), CRISP (Shibata et al., 2018) and kunitz-type serine protease inhibitor (kunitz) (Župunski et al., 2003) toxin families at multiple taxonomic levels.

The ensuing diversity of snake toxins can be curtailed by physical gene loss, as is the case with lineage-specific losses of PLA_2 genes in rattlesnakes (Dowell et al., 2016; Gibbs & Rossiter, 2008), or by functional loss through reverse-recruitment into non-toxic forms (Casewell, 2016; Casewell et al., 2013; Casewell, Huttley, & Wüster, 2012). Evidence for reverse-recruitment in vertebrate taxa is sparse due to toxin gene sequences being prioritised in venom gland transcriptome analysis, though there is some evidence for reverse-recruitment of lectins in the king cobra (Vonk et al., 2013). In addition, processes such as alternate splicing (Keren, Lev-Maor, & Ast, 2010; Vaiyapuri et al., 2011), domain loss (Casewell et al., 2011), post-translational modification (Casewell et al., 2014), gene fusion (Pahari et al., 2007), exon switching (Doley et al., 2008) and rapid accumulation of variation/mutation in exposed gene residues (Sunagar et al., 2013) have all been implicated in the extensive diversification seen in venom composition across all taxonomic levels of snakes.

Evidence of accelerated evolution acting on toxin genes points towards an adaptive driver underpinning venom variability. The most well-studied external factors thought to affect venom composition is diet. Evidence for the arms race between predator (to evolve a more effective venom) and prey (to evolve more resistance to said venom) can be found in rock squirrels (*Spermophilus variegatus*), which show reduced levels of metalloprotease-mediated hemolytic activity caused by crotalid rattlesnake venoms (Biardi & Coss, 2011), and eels, which show higher resistance to the venom of a specialist eel-eating snake (*Laticauda colubrina*) than a generalist snake predator (*Aipysurus laevis*) (Heatwole & Poran, 1995). The reverse is also applicable; snakes can undergo a loss of venom functionality when experiencing dietary shift to less mobile or inactive prey. A good example of this is in

the sea snake *Aipysurus eydouxii*. Following a shift in diet from fish to fish eggs, this species has experienced loss of diversity of PLA_2 genes and a dinucleotide deletion in its only 3FTX gene, leading to a frame shift that resulted in loss of neurotoxicity (Li, Fry, & Kini, 2005a, 2005b).

Prey optimisation of venom can further be seen in the saw-scaled vipers (*Echis*). Species known to be arthropod specialists, as well as those with a higher proportion of arthropods in their stomach contents, have venoms more lethal to arthropod prey (Barlow et al., 2009; Richards et al., 2012). Additionally, in a study of 100 venomous snake species, those that prey on only a single taxonomic group showed decreased venom potency on the mouse model the higher the phylogenetic distance between the model species and natural prey species was, compared to snakes that had multiple taxonomic groups represented in their diet, in which there was no such relationship (Lyons et al., 2020).Thus, snake venom composition has been shown to change with diet in order to optimise the efficacy of acquiring target prey and this can affect venom variation, as was originally recorded in the Malaysian pitviper (*Calloselasma rhodostoma*), in which venom variation was closely correlated with the proportion of amphibians, reptiles, birds and mammals in their diet (Daltry et al., 1996). This work is further backed up by a more recent study on 258 snake species. That found that, in general, prey diversity is correlated with the diversity of toxicological effects of snake venoms (Davies & Arbuckle, 2019). Such variation affects the underlying gene composition. For instance, the PLA_2 loss and gain exhibited in *Sistrurus* rattlesnakes (as mentioned previously) tracks the divergence in prey type, with PLA_2 genes undergoing diversifying selection (Gibbs & Rossiter, 2008). Venom changes with diet are not only linked with the type of prey animal but may also tie in with the preferred metabolic state of the prey; for instance, Australian elapids lacking in venom clotting factor feed on more inactive/dormant prey, while those with the clotting factors present feed on more active prey (Jackson et al., 2016).

Related to the role of diet in venom evolution in snakes is that of ontogeny. Ontogenetic shifts in venom composition have been observed in brown snakes (*Pseudonaja* spp.) (Cipriani et al., 2017), with neonates lacking the toxic factors fVa and fXa, which are potently procoagulant toxins that rapidly induce coagulopathies in envenomed prey. Consequently, neonate brown snakes lack the coagulopathic

venom potency observed in adult specimens. In the rattlesnake *Crotalus simus simus*, individuals show a reduction in the expression of SP and PLA_2 as individuals age (Durban et al., 2013). Similarly, the pitvipers of the genus *Bothriechis* go through ontogenetic venom change, with *B. lateralis* venom becoming more SVMP-rich with age, whereas *B. schlegelii* becomes more PLA_2-rich (Pla et al., 2017). It is likely that shifts in venom composition with may simply be a reflection of the differential in prey niche between hatchling and adult resulting in postgenomic changes. Alternatively, it may be that some genes have evolved to become active or latent at certain ages due to young snakes needing more powerful venom to subjugate prey due to their smaller size.

Environment can also generate unique expression profiles in some snakes. Coral snakes (*Micrurus*) are a particularly renowned example of this, having two main toxins in their venom; PLA_2s and 3FTXs. A large-scale analysis of *Micrurus* proteomes found that species of *Micrurus* have either PLA_2-dominant or 3FTX-dominant venoms depending on their distribution in the American continent, with species inhabiting Central America having more PLA_2-dominant venoms, while species inhabiting the northernmost and central regions of South America having more 3FTX-dominant venoms (Lomonte et al., 2016). However, there was also evidence of phylogenetic influence on the venom composition, with PLA_2-dominant species generally being part of the "long-tailed" clade and 3FTX-dominant species a part of the "short-tailed" clade (Lomonte et al., 2016). It is clear then that a combination of species history and geography can result in changes in venom composition at the proteomic level. Changes at the transcript level can also be dictated by location. Such changes have been observed in *Crotalus oreganus helleri*, where lectin toxin genes were absent from the venom gland transcriptomes of the Idyllwild population and nerve growth factor (NGF) genes were absent from the Phelan population, with toxin composition significantly differing between four populations from different regions (Sunagar et al., 2014). Lectin chains were further found to undergo different levels of positive or negative selection depending on the population. As suggested by the author, such differences may be an artefact of changing prey availability or differences in elevation resulting in changing metabolic costs. Indeed, there are a lack of studies that comparatively analyse the venom composition of evolutionarily distinct lineages that

occupy the same environment. A recent study on the sympatric viper species *Crotalus adamanteus*, *Sistrurus barbouri* and *Agkistrodon piscivorus* suggests that the ecological specialisation of each species within the same region contributes to differences in venom composition (Margres et al., 2019). Given that the three species have drastically different degrees of prey specialisation, it appears again that the main selective force in this case is prey type. However there is evidence that in some snakes, such as the Mojave rattlesnake (*Crotalus scutulatus*), toxin gene frequencies can be strongly associated with climatic factors rather than prey type (Zancolli et al., 2019). It may then be that, at least in this snake species, genes associated with aspects of climate-mediated survival are linked with genes responsible for the transcribing of certain toxins. Alternatively, prey availability, rather than type, may fluctuate with climate, resulting in snake populations with which the urgency to subdue prey (due to lack of availability) becomes more pronounced and genes are selected either for their ability to produce more potent, fast-acting venom or for reduced metabolic cost of total venom production. This raises some interesting questions on how the aspects of man-mediated climate change may affect the evolution of snake venom.

Defensive adaptations, often more associated with non-serpentine taxa, can also play an important role in the evolution of toxins, with many defensive toxins resulting in swift onset of pain or incapacitation. In invertebrates such as jellyfish, centipedes and spiders, and in venomous fish, defensive venoms are often composed of toxins that aggravate ion channels or cause intense cytolytic activity to inflict such pain (Chen et al., 2016; Church & Hodgson, 2002; Cuypers et al., 2006; Geron, Hazan, & Priel, 2017; Jami et al., 2018). Often such venoms are accompanied by visual cues or behaviours to accompany or warn of their toxic potential, such as aposematism in poisonous frogs and arthropods, defensive posturing in Helodermatid lizards and the lifting of front limbs to reveal fangs in some spiders (Cloudsley-Thompson, 1995). Examples of a behavioural or morphological phenotype influencing snake venom evolution are rare, potentially due to snakes lacking a complex or diverse body plan in comparison with their vertebrate relatives. Similarly, snakes are generally believed to exhibit fewer complex behaviours than other reptiles and thus behavioural links (particularly defensive behaviours) with venom composition are generally

overlooked. Furthermore, a recent survey suggests that most snakebite do not result in the immediate severe pain often associated with defensive toxins (Ward-Smith et al., 2020). However, like the many non-serpent taxa that have visual displays to warn of their toxic potential, snakes also give visual (aposematic colouration in *Micrurus*, neck flattening in *Boiga*, mouth gaping in *Dendroaspis*) or auditory (rattling and striating in rattlesnakes and *Echis* spp.; "puffing" in *Bitis* spp.) warnings to repel non-prey aggressors. Thus, behaviour-linked mediators of venom evolution cannot be ruled out. There are still questions that are yet to be answered on how many such other factors such as body shape, head size or even scale pattern may affect venom composition, if at all. Additionally, most studies on venom evolution fail to take account of the effect of phylogenetic structure on venom evolution (Arbuckle, 2018). For instance, are the cases of accelerated evolution of PLA_2 toxins observed in *Trimeresurus* species (Nakashima et al., 1993; Pla, 1996) and *Crotalus* rattlesnakes (Dowell et al., 2016; Gibbs & Rossiter, 2008) restricted only to these sampled lineages? Or is this an ancestral characteristic of all viper PLA_2s? Without introducing statistical methods that account for phylogenetic structuring, meaningful interpretations on the factors influencing venom variation in a sampled group of species are inherently limited.

Cobras (*Naja* spp.) are model organisms for exploring the potential influence of behaviour and morphological adaptations influencing the venom compositional phenotype. Within this genus, species exhibit distinct, characteristic defensive behaviours - hooding and spitting. While all cobras (and a number of non-*Naja* species) are capable of laterally expanding their neck skin and underlying musculature and ribs, in a defensive behaviour known as hooding, only certain cobra species (and the near relative *Hemachatus haemachatus*) are capable of defensive venom spitting. Previous work has suggested that defensive hooding in elapid snakes was found to be strongly linked with enhanced cytotoxic venom activity when taking phylogenetic structure into account (Panagides et al., 2017), suggesting a clear link between behavioural and morphological phenotypes and venom phenotype. However, this study found no association between venom effects and venom spitting, and did also not investigate how variable venom composition across cobra

species might have evolved in response to the evolutionary origins of these defensive traits.

The purpose of this chapter is to use the integrated power of transcriptomic, proteomic and phylogenetic data to establish patterns of cobra venom toxin evolution, with a focus on delineating whether the emergence of defensive venom spitting in cobras corresponds with differential patterns of toxin evolution. To addresses the general lack of phylogeny-informed analysis of transcriptomic and proteomic data from venomous snakes, a robust species tree was incorporated to aid comparative informatic analyses of venom toxin family evolution, resulting in new insight into the consequences of a distinct defensive phenotype (venom spitting) on the underlying genotype of these venomous organisms.

4.2. Methods

4.2.i. Toxin phylogenetics

To investigate relationships between toxin genes, phylogenetic trees were generated for the toxin family RNA sequences identified and curated from snake venom glands as described in 2.2. (3FTX, PLA_2, CRISPs, Kunitz, NGF, SVMPs, NP and CTL) using the Bayesian inference software MrBayes v3.2.7a (Ronquist et al., 2012) on the CIPRES Science Gateway (Miller et al., 2010). Non-toxic homologues were not included in the analyses. After the quality-control steps outlined in 3.2.iii., the final alignments represented 17 species (the ingroup, *Hemachatus* & *Naja* spp. And the outgroups *Walterinnesia aegyptia* and *Aspidelaps scutatus intermedius*), with an average number of 76 sequences and average max length of 786bp across all toxin families (Table 4.1.). The most appropriate model for sequence evolution for each toxin family alignment was determined by the software jModelTest (Darriba et al., 2012; Posada, 2008) for nucleotide datasets and ModelGenerator (Keane et al., 2006) for amino acid data, based on the Akaike Information Criterion (see Table 4.1.). The selected sequence evolution models were then incorporated into the MrBayes. For each toxin family, representative sequences previously described from *Bungarus multicinctus* (Jiang et al., 2011), *Micrurus fulvius* (Margres et al., 2013), *Ophiophagus hannah* (Vonk et al., 2013) and *Aspidelaps scutatus intermedius* (Whiteley *et al.*, 2019) were

Table 4.1. Selection model determination for toxin family nucleotide and amino acid (translated directly from the nucleotide sequences) files. PLA_2 – Phospholipase A_2, 3FTX – Three-finger Toxin, SVMP – Snake Venom Metalloproteinases, Kunitz – Kunitz-type Serine Protease Inhibitors, CRISP – Cysteine-rich Secretory Proteins, NP – Natriuretic Peptides, CTL – C-type Lectins.

Toxin	Number of partial sequences	Number of complete sequences	Max. Length of alignment (bp)	Nucleotide Model	Amino Acid Model
PLA_2	25	14	465	TIM3+G	WAG+G
3FTX	114	143	444	GTR+G	WAG+G
SVMP	148	35	1989	GTR+I+G	JTT+ G
Kunitz	10	7	306	TIM2ef+G	JTT+G
CRISP	16	28	720	TPM3uf+I+G	JTT+G
NP	6	17	1110	TIM2+G	JTT+G
NGF	14	9	732	HKY+G	JTT+G
CTL	6	17	525	SYM+G	JTT+G

incorporated, along with a single sequence from the non-venomous *Python* spp. and *Anolis* spp., the latter of which were used as the defined outgroup. For each Bayesian inference analysis, four simultaneous runs with four chains (three hot, one cold) were implemented for 10×10^6 generations, sampling every 500th cycle from the chain, and using default settings in regard to priors. Burn-in was conservatively set at 25%; trees generated prior to this point were discarded, and a consensus tree was constructed from the remaining 75%. Following assessment of the resulting tree, the 3FTX gene tree was subjected to additional analysis. Although distinct groups were identified, each was poorly resolved, thus each major group was inputted into a separate phylogenetic analysis, using the same criteria defined above. Selection models for each 3FTX group can be found in Table 4.2. In addition to the above analyses performed for transcriptome-level data, only 3FTX and PLA_2 protein trees were constructed from proteomic data due to the lack of both species and sequence coverage from other toxin families across species. These were run using the parameters above and the respective amino acid models generated in Table 4.1.

Table 4.2. Selection model determination for the three-finger toxin (3FTX) group nucleotide and amino acid (translated directly from the nucleotide sequences) files.

Group	Nucleotide Model	Amino Acid Model
Ci – Short Neurotoxins	TVM+I+G	JTT+G
Cii – Neurotoxin-like Proteins	JC+G	WAG+G
Ciii – Long Neurotoxins	TPM1+G	WAG+G
Civ - Unknowns	TPM3uf+G	JTT+G
Cv - Cytotoxins	TVM+G	WAG+G
Cvi – Muscarinic Toxins	TIM3ef+I	WAG+G
Cvii – Weak Neurotoxins	TPM1+G	JTT+G

4.2.ii. Visualising Toxin Diversity using Principal Coordinate Analysis (PCoA)

As many of the amino acid phylogenetic trees generated from Bayesian inference were poorly resolved due to high variable short length proteins, an additional approach was applied to visualise the toxin diversity of 3FTXs and PLA_2s across the various cobra species. To test for the clustering of spitting and non-spitting lineages based on the amino acid sequences of these toxin proteins, Principal Coordinate Analysis (PCoA) was employed. This analysis was performed through the generation of a Euclidean dissimilarity matrix from the pairwise distance matrix produced by MEGA v 7 (method = JTT, rates = Uniform rates) (Kumar et al., 2016) from the toxin family amino acid sequence alignments used for the phylogenetic analysis of the 3FTX and PLA_2 families. The matrix was produced using the daisy function of the R package "cluster" (Maechler et al., 2019) and was followed by applying classical multidimensional scaling to the matrix using the cmdscale function in R Studio before being visualised with the ggplot 2 package.

4.2.iii. Conservation Analysis and Generation of 3D PLA_2 models

As the PLA_2 toxin families represent the most highly-expressed and diverse of those detected in the venom glands and venom of cobras (see chapters 2 & 3), they were subjected to informatic conservation and structural analyses. To ensure only biologically-relevant proteins were used for the conservation analysis, the PLA_2 protein sequence alignment was trimmed to contain only sequences with an

abundance in the toxin proteome of 1% or higher. These sequences were then uploaded to Jalview v2.11.0 (Waterhouse et al., 2009) and amino acids with a conservation score of 5.0 or lower highlighted. To generate the 3D models, the most highly-abundant amino acid sequence was selected from the PLA_2 proteome data (sequence ID: N.mossambica_1210_1102_1125_1292, abundance 10.37%) and used to search for the highest-matching structural template (template name 2osh.1.A (Hu et al., 2008) upon which the 3D protein model would be created using the SWISS-MODEL server (Waterhouse et al., 2018), before being visualised in PyMOL v2.0 (Schrödinger, 2015). Conservation scores were projected onto the resulting model using conservation scores from the ConSurf server (Ashkenazy et al., 2010) using the same sequence alignment used to generate conservation scores over amino acid sequences in Jalview.

4.2.iv. Ancestral State Estimation

To confirm the independent origins of spitting in cobras (Panagides et al., 2017; Wüster et al., 2007), the discrete ancestral states of "spitting" and "non-spitting" were estimated over a species tree (pruned from a larger tree provided by Wolfgang Wüster, generated from DNA sequence alignments of two mitochondrial markers and the phased partial exon sequences of five nuclear genes; see Fig. 4.3.) using the rerooting method function of the R package "phytools" (Revell, 2012). Comparison of the: "all rates different" (ARD), "equal rates" (ER) and "symmetric rates" (SYM) models using Log-likelihood (Log-lik) analyses revealed that both the ER and SYM models had the same likelihood, and so both were used to plot discrete traits over the species tree, and both resulted in visually-identical plots. To investigate the evolutionary history of toxin abundance, ancestral relative toxin abundances were estimated for each toxin family via maximum likelihood on the pruned species tree using the contmap function of the phytools package. To test for associations between toxin abundances with the evolutionary origin of venom spitting, we used the phylogenetic Generalized Least Squares (PGLS) approach using the pgls function

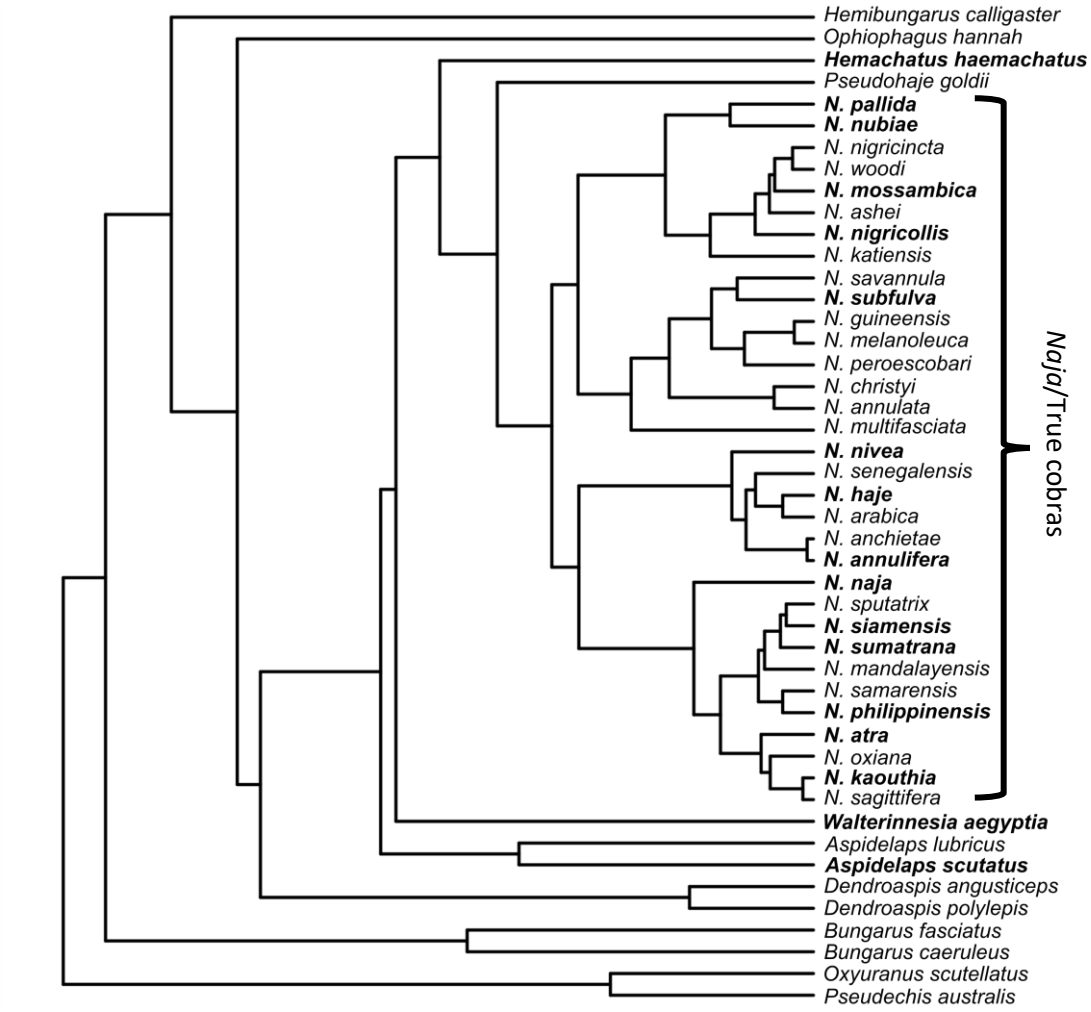


Fig. 4.3. Elapid phylogenetic species tree. The above tree was determined and provided to me by my colleague, Wolfgang Wüster, of Bangor University. Names in bold represent species used in this study.

(under the maximum likelihood approach) of the "caper" (Orme et al., 2018) package in RStudio (RStudio, 2016) on the pruned species tree.

4.3. Results

4.3.i. Three-finger toxins (3FTXs)

The resulting RNA 3FTX gene tree resolved into seven major groups based on functional descriptors determined via BLAST searches of the NCBI database (Fig. 4.4.). These seven groups contain 3FTXs exhibiting functional annotations consistent

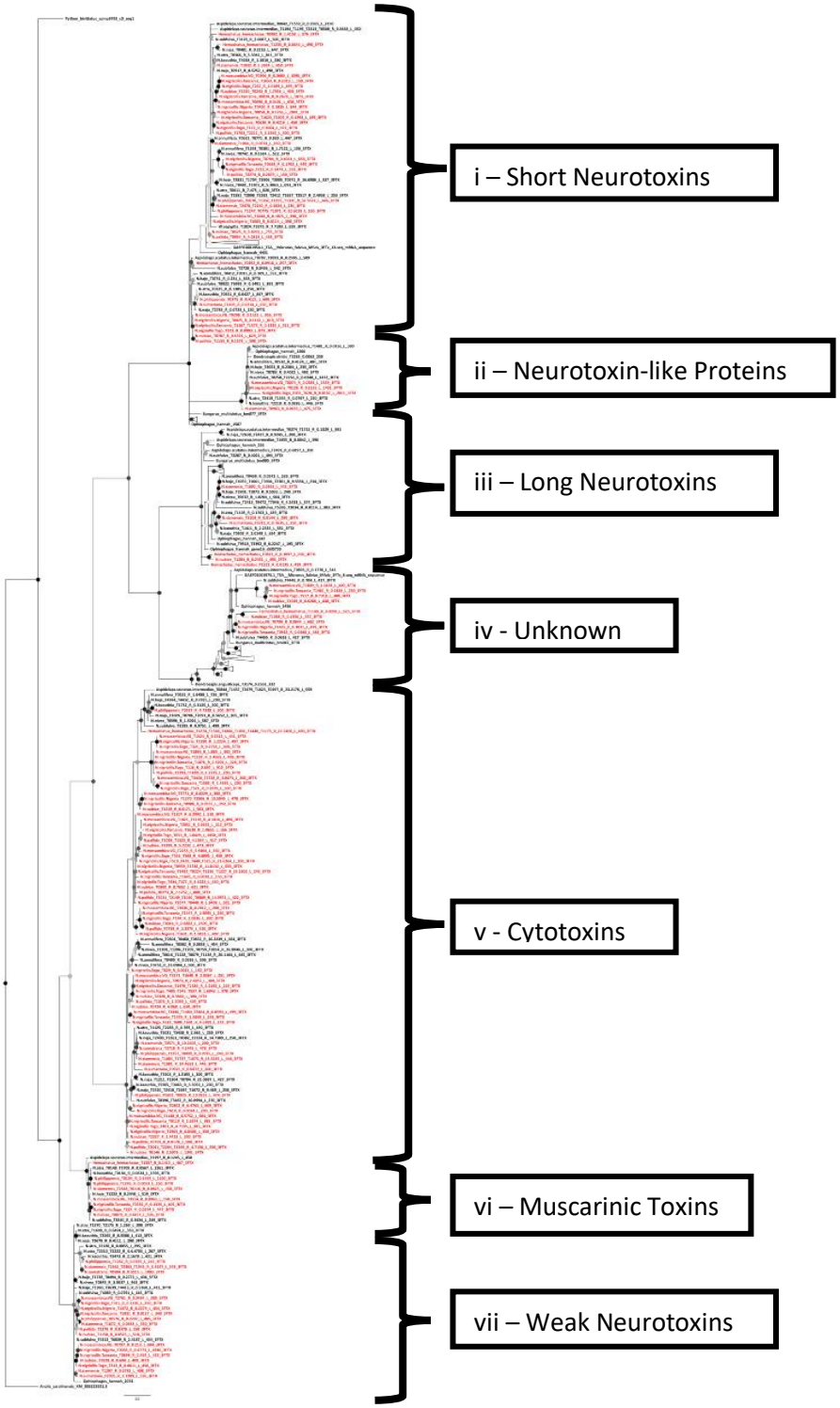


Fig. 4.4. Phylogenetic tree of cobra three-finger toxin (3FTX) genes, with clades representing 3FTX subfamilies (as determined by Nucleotide Blast) labelled. Spitting species are highlighted in red. Outgroup species nodes have been collapsed. Circles indicate node probabilities, with white circles representing 0.5, black circles 1.0 and grey circles shaded darker with increasing probability.

with: i) short neurotoxins (STXs), ii) neurotoxin-like proteins (NLPs), iii) long neurotoxins (LTXs), iv) unknown/unclassified 3FTXs (UNK), v) cytotoxins (CTXs), vi) muscarinic toxins (MTXs), vii) weak neurotoxins (WTXs). The node support values for these groups ranged from Bayesian posterior probabilities (bpp) of 0.75 for the WTX group to 0.99 for the MTX group. The 3FTX gene trees resulting from alignments containing amino acid translations of the RNA dataset, and the protein data derived from venom proteomics, exhibited a high proportion of polytomies, and thus are not discussed further due to a lack of meaningful resolution.

Cobras have two main subgroups of STX genes, labelled here STXa and STXb, similar to the related African outgroup species *Dendroaspis* and *Aspidelaps*, but distinct from *Walterinnesia*, which has retained only a single gene in STXa, and *Ophiophagus*, which has retained only STXb genes (Fig. 4.5.). There are no patterns of diversification in the STX tree that indicate an association with the ability to spit, with duplications in all spitting lineages observed in STXa and evidence for duplications in *N. subfulva* only in STXb (Fig. 4.5.). STXa has a higher number of sequences per species, ranging from 1-4, compared to 0-2 in STXb. This results in a higher transcriptome expression of STXa, with genes found in this subgroup ranging in transcriptomic expression levels from 0.01-32.56% of all toxins, compared with 0.03-0.54% in STXb. In the absence of a robust amino acid gene tree, principal coordinate analysis (PCoA) was used to explore the observed diversity of the STX group detected in cobras venoms. The most abundant STX proteins form 3-4 groups, and there appears to be no association between these groups or the ability to spit venom or cobra lineage (e.g. African spitting, African non-spitting, etc). However, a distinct and moderately to highly-abundant group of short neurotoxins unique to Asian cobras was detected (Fig. 4.6.).

The NLP group consists of far fewer toxins, with only one representative from each of nine of the 17 species sampled here, along with additional representation from the outgroup species (see Fig. 4.7.). Thus, there is no evidence that NLP 3FTXs have diversified (or indeed been lost) as the result of the evolutionary origin of spitting. Indeed, the phylogenetic patterns observed demonstrate that these genes do not separate into a group unique to spitting cobras and the toxins themselves are highly-related to the outgroup sequences, suggesting that cobra NLPs are largely

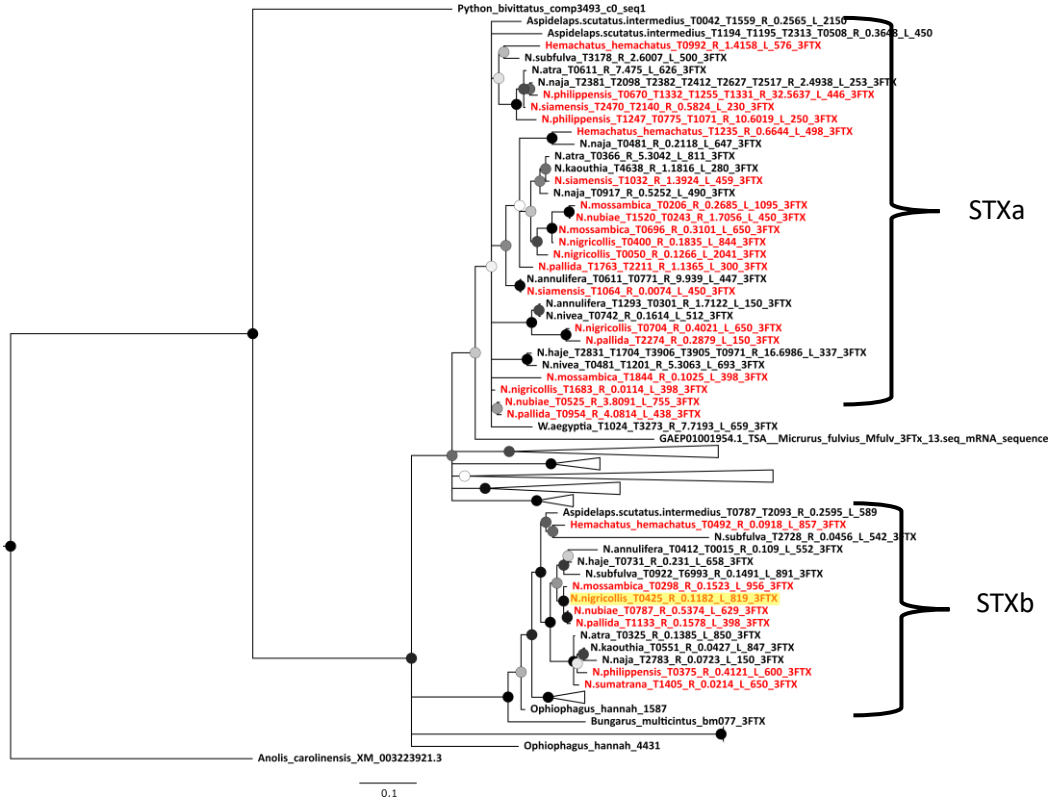


Fig. 4.5. Phylogenetic tree of the (i)/Short Neurotoxins (STXs) group of cobra three-finger toxins (3FTXs). Spitting species are indicated by red text and highlighted tip labels represent proteomically confirmed sequences. *Dendroaspis* genus nodes have been collapsed for clarity. Circles indicate node probabilities, with white circles representing 0.5, black circles 1.0 and grey circles shaded darker with increasing probability.

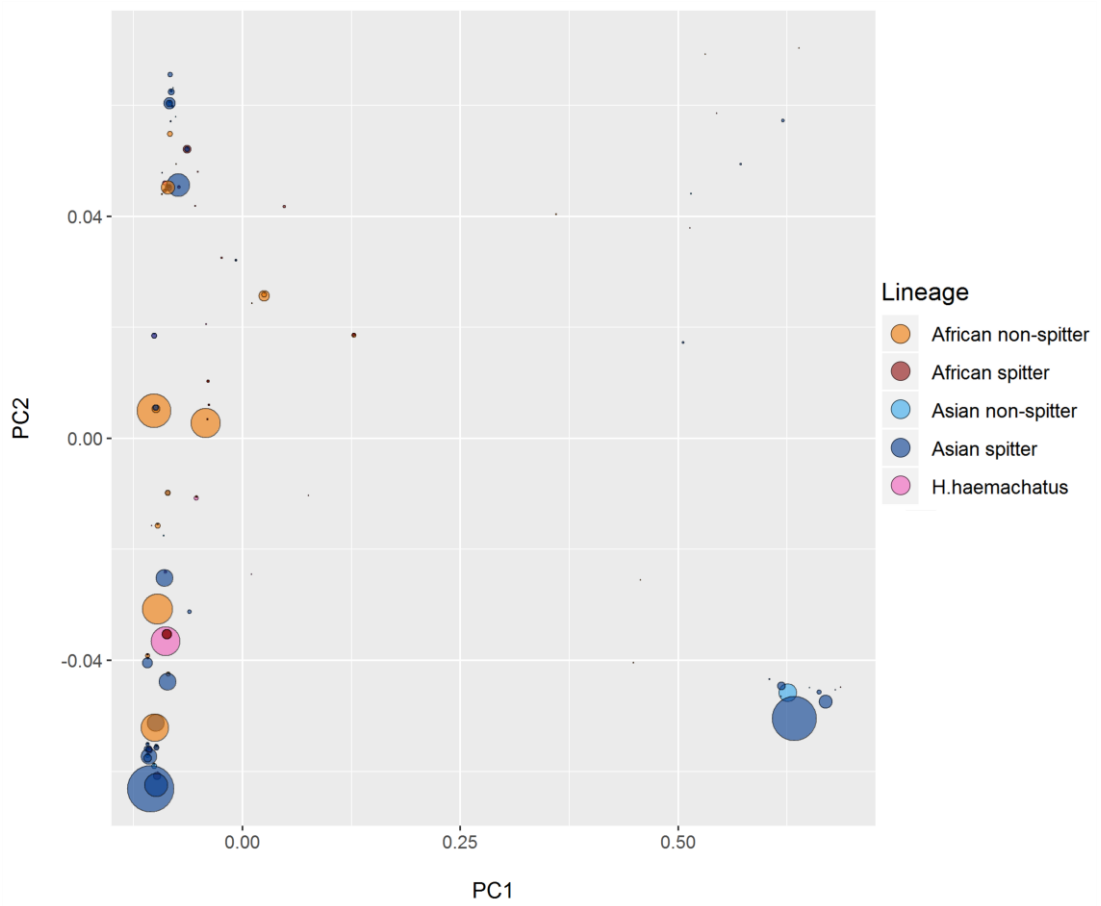


Fig. 4.6. Principal coordinate analysis (PCoA) of the amino acid composition of (i)/Short Neurotoxins (STXs) group proteins, sized by abundance in the proteome (%). The axes represent the distance in terms of amino acid differences between sequences, determined by pairwise analysis in MEGA v7.

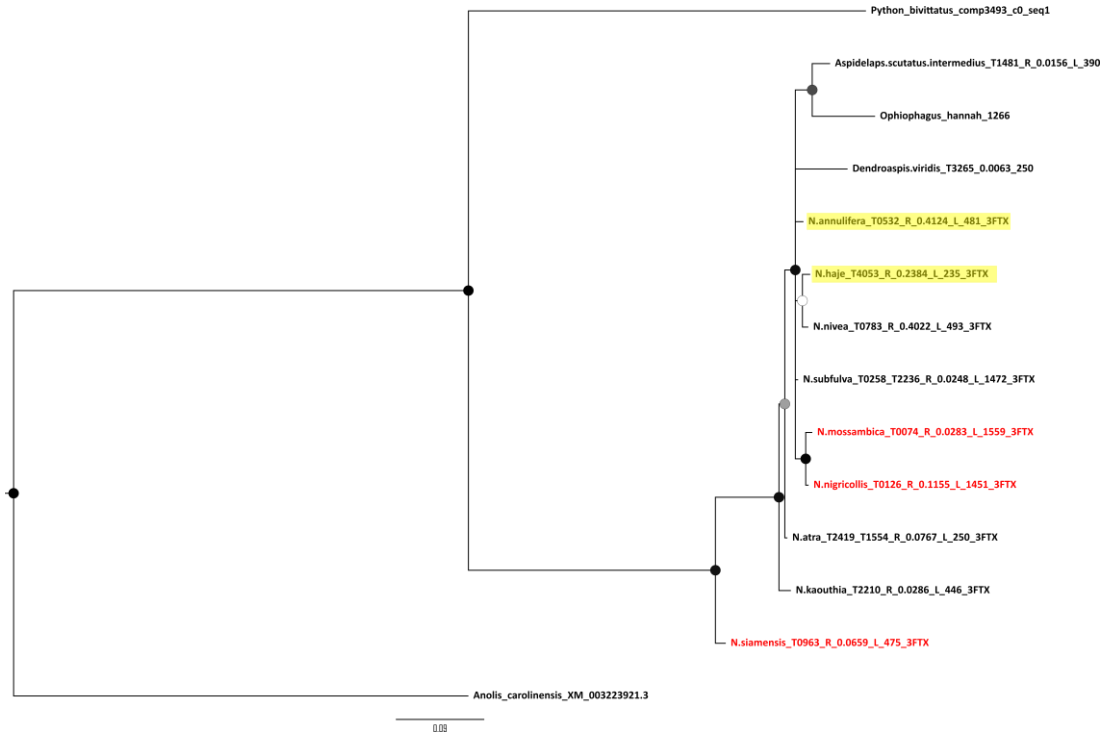


Fig. 4.7. Phylogenetic tree of the (ii)/Neurotoxin-like proteins (NLPs) group of cobra three-finger toxins (3FTXs). Spitting species are indicated by red text and highlighted tip labels represent proteomically confirmed sequences. Circles indicate node probabilities, with white circles representing 0.5, black circles 1.0 and grey circles shaded darker with increasing probability.

conserved with the ancestral form. In addition, the transcriptomic representation of these genes is low, with expression levels ranging from 0.01-0.24% of total toxins, which is much lower than many other subclasses of 3FTXs. As there were not at least two representative cii proteins for each species, PCoA was not performed.

The LTX is dominated by genes from non-spitting cobras, particularly the African non-spitting cobra *N. subfulva*, which has three gene representatives in this group, and the outgroup species (Fig 4.8.). Indeed, African non-spitting cobras have the highest transcriptome expression of genes found in this group, averaging expression levels of 3.13% of all toxins compared to 1.34% in Asian non-spitting cobras and 0.1% in all spitting cobras. PCoA analysis of the long neurotoxins detected in cobra venom proteomic data demonstrated that shows the majority of the highly-abundant toxins form a single group, although a highly-distinct protein from the venom of *N. haje* was also detected (Fig. 4.9.).

group iv of the 3FTXs represent an 'orphan' group of functionally unknown toxins (referred to here as unknowns) and are represented by the African spitting cobras, *H. haemachatus*, the African non-spitting cobra *N. subfulva* and the outgroups *Bungarus*, *Ophiophagus* and *Micrurus* (Fig. 4.10.). Only *N. subfulva*, *N. mossambica* and *N. nubiae* have more than one gene present in this group (all have two), with one of these isoforms closely resembling the ancestral form, given their relative position to sequences detected in the outgroup species *Micrurus* and *Ophiophagus*. However, the posterior probability of the key node in the relating to the group containing the cobra unknown toxins and the related non-*Dendroaspis* outgroups is fairly low (bpp = 0.60), making it difficult to determine whether *N. subfulva*, *N. mossambica* and *N. nubiae* underwent a duplication of these ancestral 'unknown' toxin genes or whether all species of cobras originally had two copies of group iv genes which were then lost in most species, with the two copies being retained in *N. subfulva*, *N. mossambica* and *N. nubiae*, and only a single copy retained in *H. haemachatus* and *N. nigricollis*. Given that the outgroups *Ophiophagus*, *Aspidelaps* and *Micrurus* only have one copy of this gene, it appears that the former hypothesis is more likely. In terms of transcriptome expression, the abundances of these toxin types are low, with the total expression ranging from 0.01% (in *H. haemachatus*) to 1.13% (in *N. mossambica*) of all toxins. As the majority of species

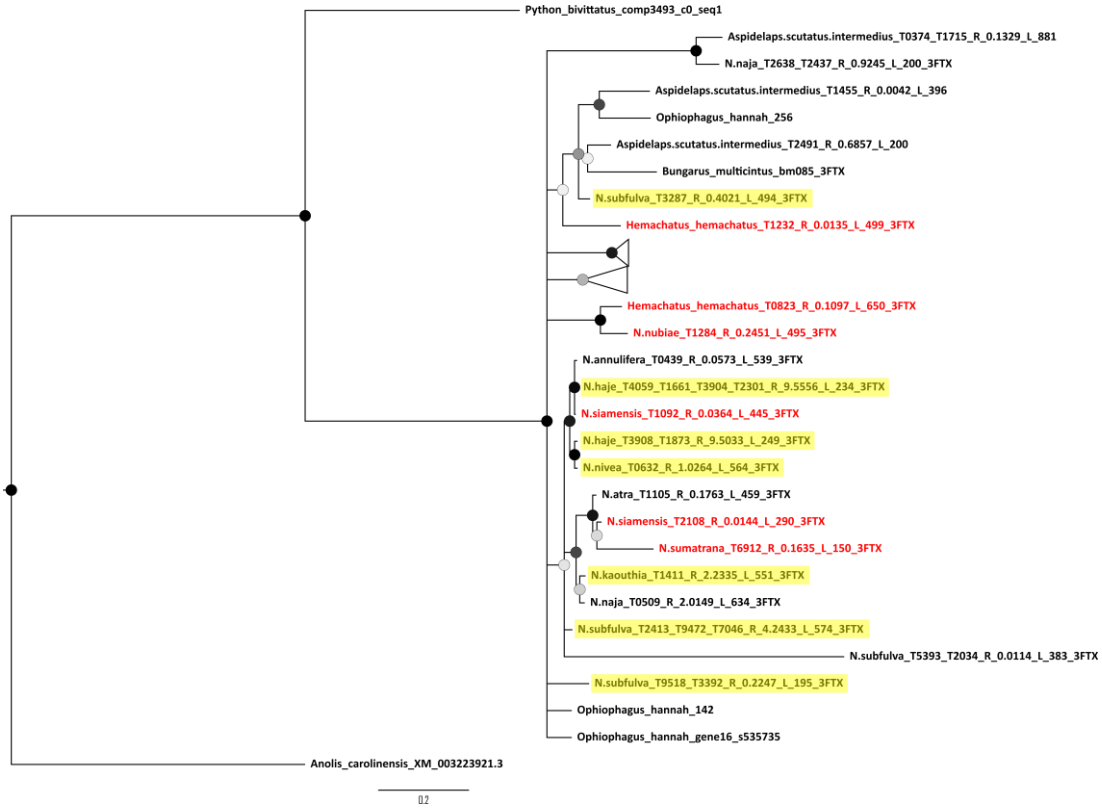


Fig. 4.8. Phylogenetic tree of the (iii)/ Long Neurotoxins (LTXs) group of cobra three-finger toxins (3FTXs). Spitting species are indicated by red text and highlighted tip labels represent proteomically confirmed sequences. *Dendroaspis* genus nodes have been collapsed for clarity. Circles indicate node probabilities, with white circles representing 0.5, black circles 1.0 and grey circles shaded darker with increasing probability.

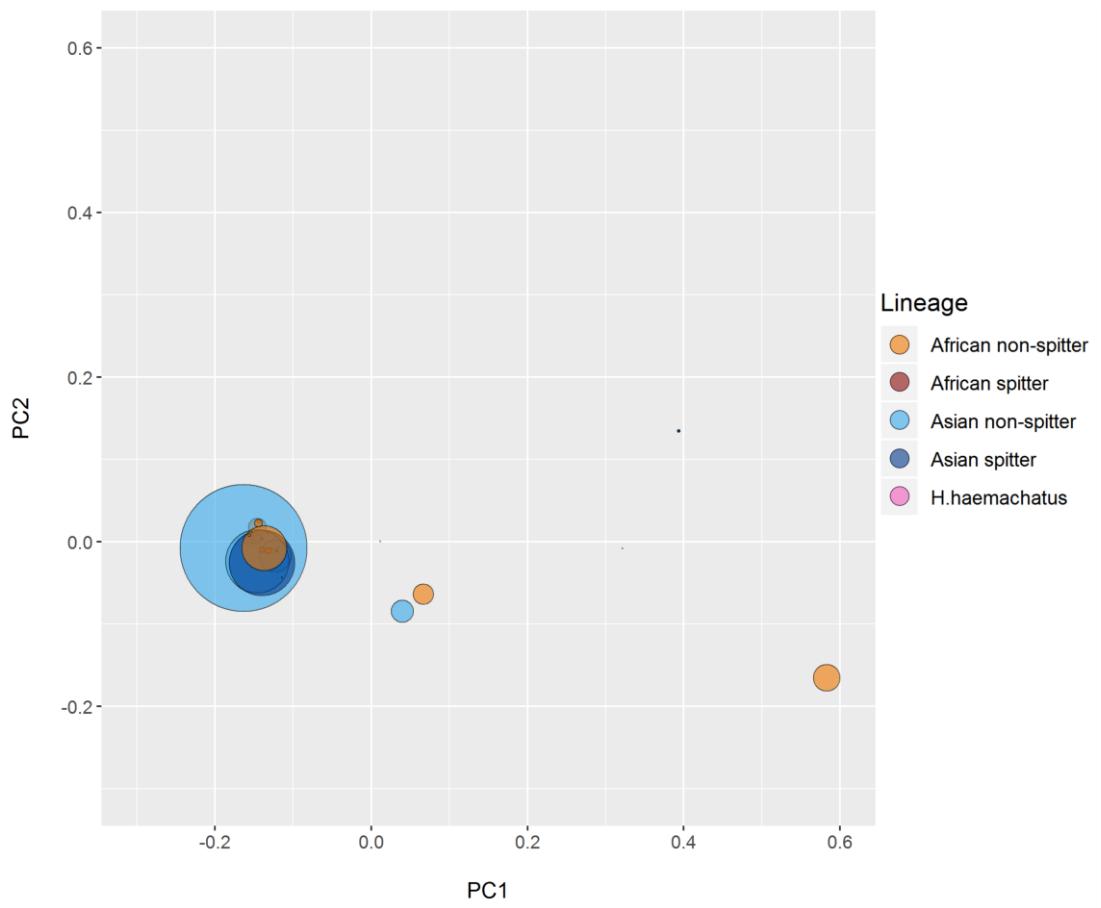


Fig. 4.9. Principal coordinate analysis (PCoA) of the amino acid composition of (iii)/ Long Neurotoxins (LTXs) group proteins, sized by abundance in the proteome (%). The axes represent the distance in terms of amino acid differences between sequences, determined by pairwise analysis in MEGA v7.

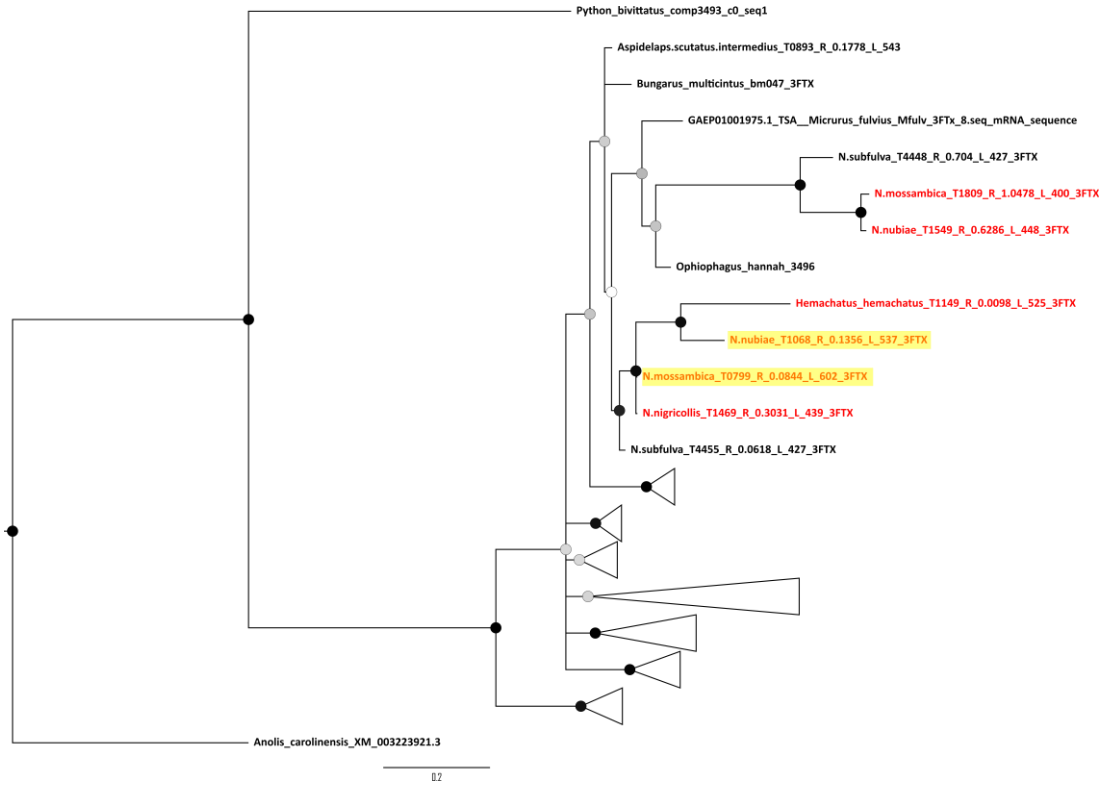


Fig. 4.10. Phylogenetic tree of the (iv)/Unknown (UNK) group of cobra three-finger toxins (3FTXs). Spitting species are indicated by red text and highlighted tip labels represent proteomically confirmed sequences. *Dendroaspis* genus nodes have been collapsed for clarity. Circles indicate node probabilities, with white circles representing 0.5, black circles 1.0 and grey circles shaded darker with increasing probability.

contained fewer than two representative group iv proteins, PCoA was not performed on this group of 3FTX genes.

The CTX group represents the most diverse of the 3FTX subclasses detected in cobras (Fig. 4.11.), with CTX diversity appearing to be much higher in spitting cobras, possessing an average of six genes, than non-spitting cobras, possessing an average of three. African spitting cobras in particular have undergone extensive duplication of CTXs, and contain an average of nine CTX genes, compared to only one from *H. haemachatus* and three from all other cobra lineages. This group also

contains the majority of 3FTX expression detected in the venom gland transcriptomes for most species, representing an average of 46.48% of toxin transcripts across *Naja* and *Hemachatus*, compared to 24.08% from all other 3FTX types combined. This group also has the largest range of expression, from 0.00% in *Walterinnesia* to 77.34% in *N. nivea*. PCoA analysis of CTXs proteins detected via venom proteomics reveals that highly-abundant CTXs cluster tightly together in terms of amino acid similarity (Fig. 4.12.), suggesting a relatively conserved structure and, perhaps, function of those isoforms most likely to be of pathogenic relevance CTXs.

The MTXs are another small group, containing single genes from nine cobra species of both spitting and non-spitting origin, and also *Aspidelaps*. Expression from these sequences range from 0.01% in *N. philippinensis* and *N. mossambica*, to 0.45% in *N. nubiae*, and represent an average expression level of 1.54% of all toxins detected in the venom gland transcriptomes (Fig 4.13.), making this a very lowly-expressed 3FTX group. The lack of diversity within this group, coupled with relatively low expression levels gives no clear link between these 3FTXs and the evolution of defensive venom spitting. As there were not at least two MTX representative proteins detected for each cobra species, PCoA was not performed on the data found within this group.

The last major 3FTX group represents the weak neurotoxins (WTXs) and this group resolves into 4 group: a well-supported, African spitter clade (bpp > 0.9), one moderately-supported clade containing Asian cobras (bpp = 0.73), one moderately-supported clade containing spitting cobras and *N. subfulva* (bpp = 0.72) and a poorly-supported clade containing Asian spitters and African non-spitters (bpp = 0.59) (Fig. 4.14.). Although the Asian cobra clade has the highest range of transcriptomic expression (0.01-4.47%), the clade containing sequences from the African non-spitters and Asian spitters *N. siamensis* and *N. sumatrana* has the highest average expression at 1.62%. The species with the highest single gene expression in the WTX clade are all non-spitters, and non-spitters have on average higher expression of genes in this clade than spitting cobras (1.39%, compared to 0.56%). PCoA analysis of the WTXs reveals a cluster composed of Asian WTXs and a few African spitter WTXs, one African non-spitter protein clustering with some lowly-expressed WTXs,

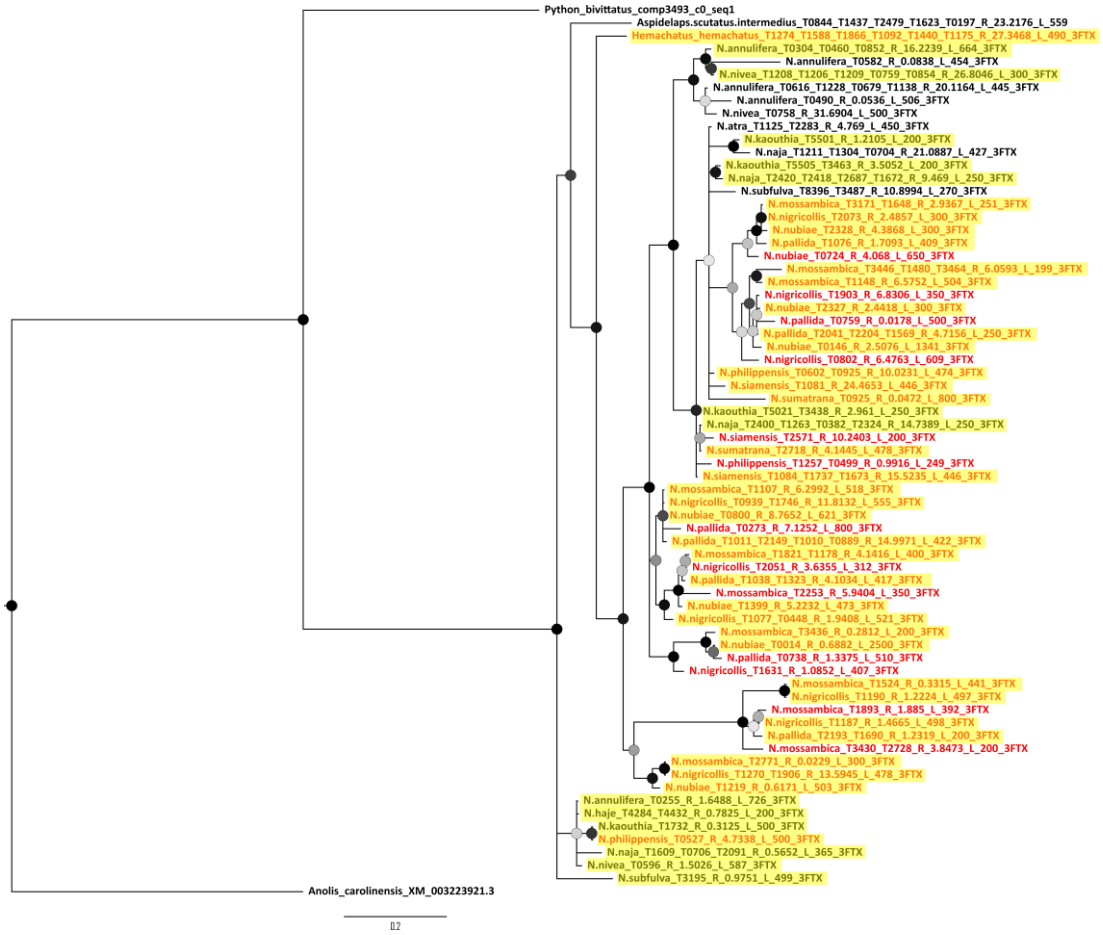


Fig. 4.11. Phylogenetic tree of the (v)/Cytotoxins (CTXs) group of cobra three-finger toxins (3FTXs). Spitting species are indicated by red text and highlighted tip labels represent proteomically confirmed sequences. Circles indicate node probabilities, with white circles representing 0.5, black circles 1.0 and grey circles shaded darker with increasing probability.

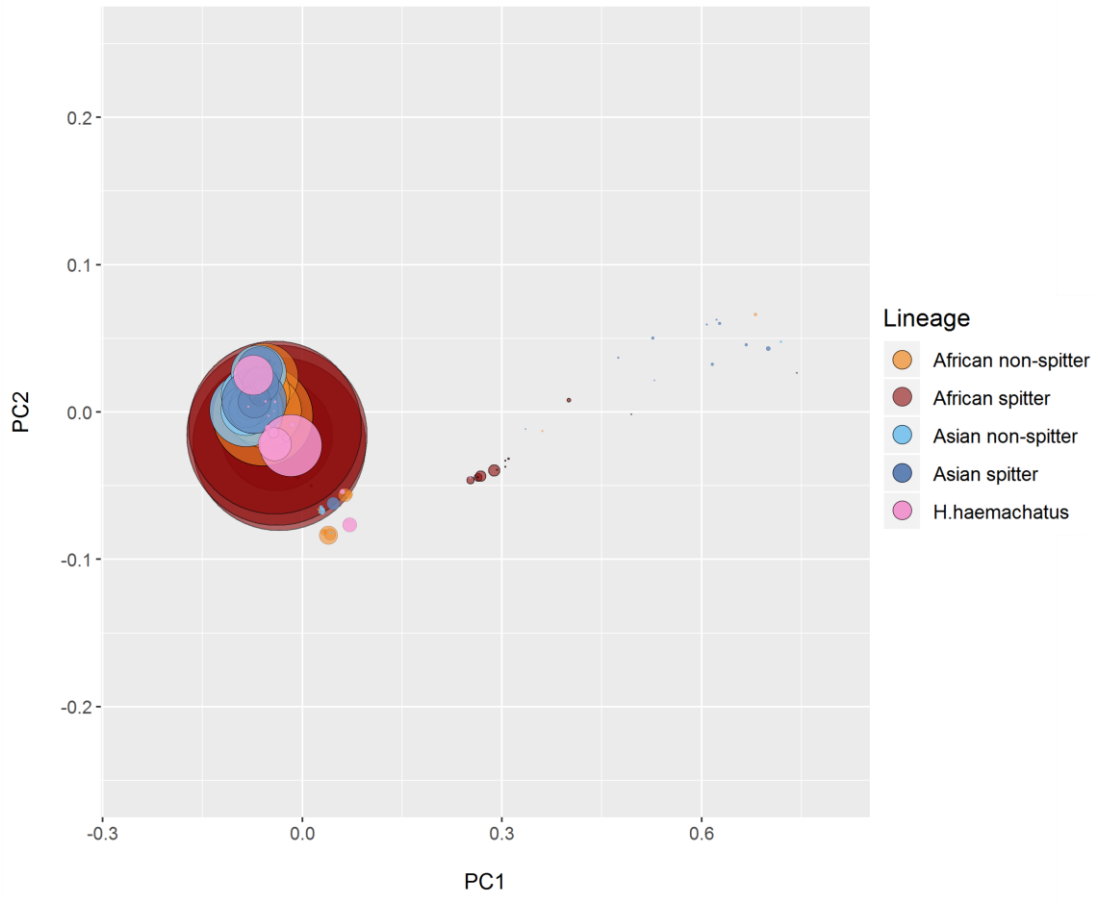


Fig. 4.12. Principal coordinate analysis (PCoA) of the amino acid composition of the (v)/Cytotoxins (CTXs) group of cobra three-finger toxins (3FTXs), sized by abundance in the proteome (%). The axes represent the distance in terms of amino acid differences between sequences, determined by pairwise analysis in MEGA v7.

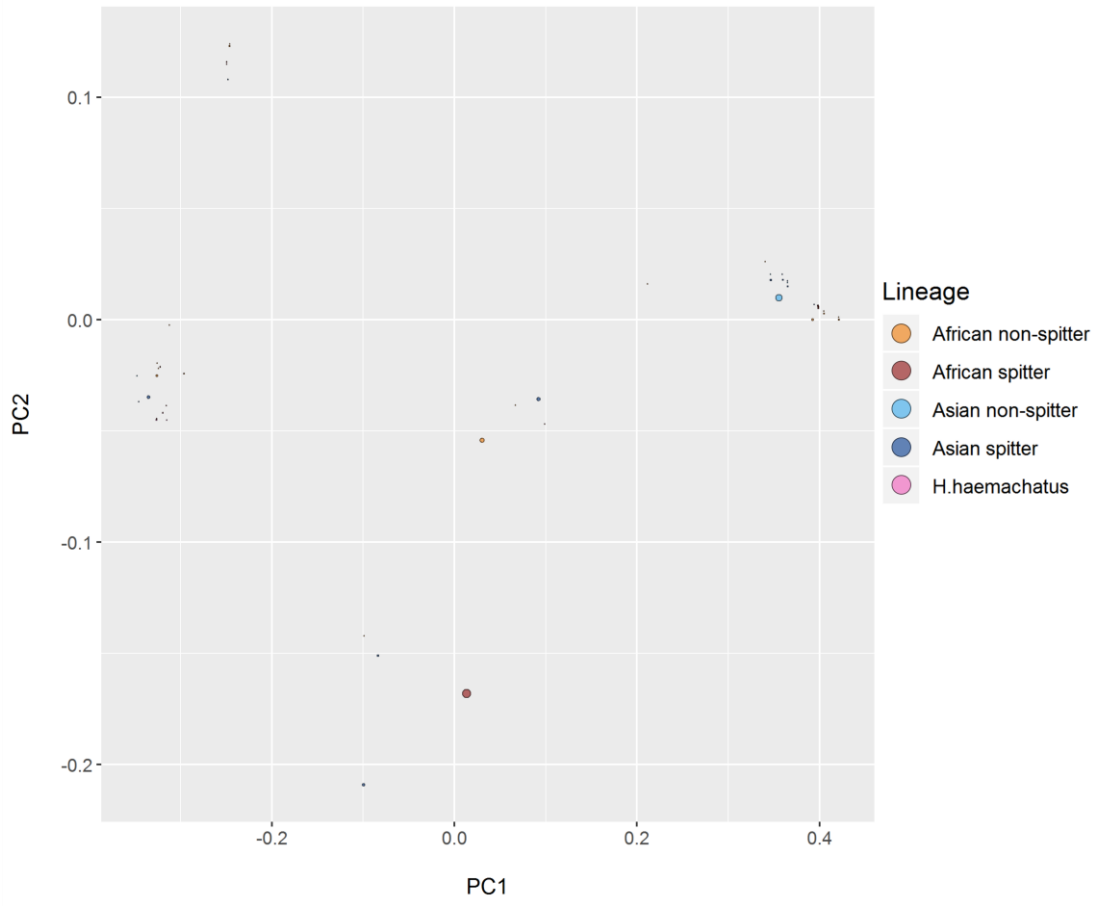


Fig. 4.13. Phylogenetic tree of the (vi)/Muscarinic toxins (MTXs) group of cobra three-finger toxins (3FTXs). Spitting species are indicated by red text and highlighted tip labels represent proteomically confirmed sequences. *Dendroaspis* genus nodes have been collapsed for clarity. Circles indicate node probabilities, with white circles representing 0.5, black circles 1.0 and grey circles shaded darker with increasing probability.

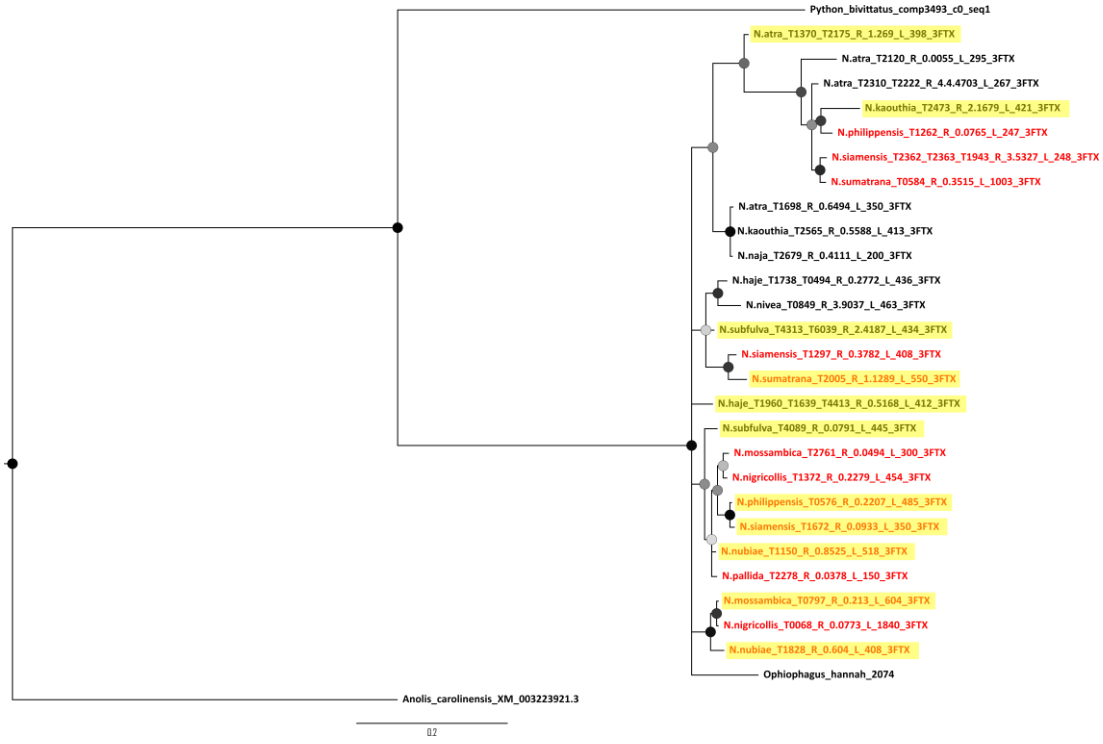


Fig. 4.14. Phylogenetic tree of the (vii)/Weak Neurotoxins (WTXs) group of cobra three-finger toxins (3FTXs). Spitting species are indicated by red text and highlighted tip labels represent proteomically confirmed sequences. Circles indicate node probabilities, with white circles representing 0.5, black circles 1.0 and grey circles shaded darker with increasing probability.

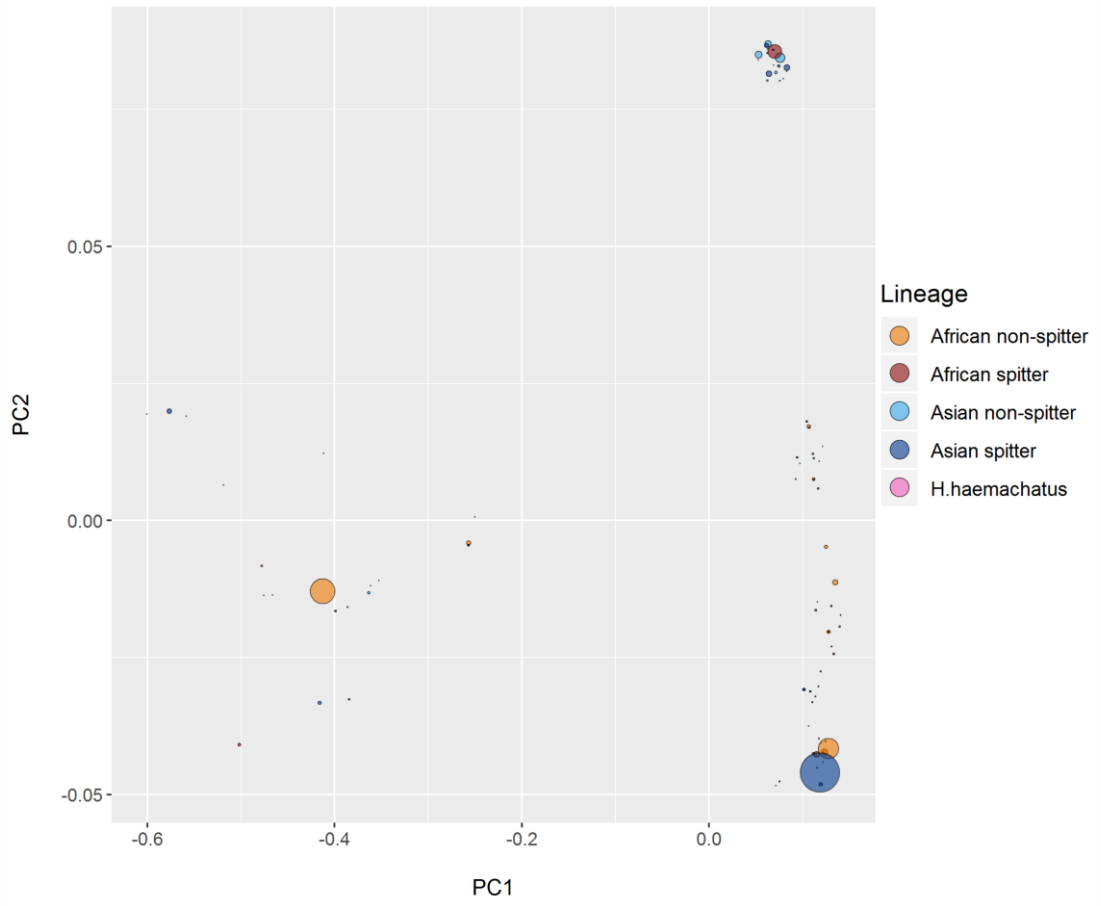


Fig. 4.15. Principal coordinate analysis (PCoA) of the amino acid composition of the (vii)/Weak Neurotoxins (WTXs) proteins, sized by abundance in the proteome (%). The axes represent the distance in terms of amino acid differences between sequences, determined by pairwise analysis in MEGA v7.

and a small group of Asian spitter and African non-spitter WTXs, with these three groups highly distanced from each other (Fig. 4.15.).

As with PCoA analyses, ancestral trait estimations were performed only for 3FTX groups that had sufficient species coverage. The resulting estimations are largely uninformative, with most groups having very low abundance throughout the cobra species tree (Fig 4.16.). Some interesting patterns of note are the increase of

STX abundance in *Walterinnesia*, *N. subfulva* and the Asian spitting cobras, and the upregulation of LTXs in *N. kaouthia*. Despite this, PGLS analysis reveals no association with any 3FTX clade with either spitting or geography (Table 4.3.). The most revealing ancestral trait map by far is that of the CTXs, which are shown to be universally high throughout the cobra species tree, only decreasing in abundance slightly in Asian *Naja* and dramatically in *Walterinnesia*. Phylogenetic Generalized Least Squares (PGLS) reveals that there is no significant link between CTX abundance and the emergence of spitting ($t = -0.38$, $t = 0.71$) (Table 4.3.) or geography ($t = -1.81$, $p = 0.09$) (Table 4.3.) implying that the reconstructed patterns are likely a result of phylogeny. This suggests that high CTX abundance is ancestral to the group containing the true cobras (*Naja*), *Hemachatus*, *Wallterinnesia* and *Aspidelaps*, further implying a general divergence away from the neurotoxicity generally associated with elapid venoms (Tasoulis & Isbister, 2017).

This interesting pattern of CTX abundance prompted further investigation into the origin of cytotoxicity. The origins of CTXs in elapids in the literature is currently uncertain, with some postulating that CTXs can be found in most elapids (Boffa et al., 1983) while others maintaining that they are restricted to the cobras (Dufton & Hider, 1988; Kini & Doley, 2010). To look into this, two methods were employed. Firstly, in order to reconstruct the evolutionary history of CTXs to determine their origin in elapid snakes, a refined elapid CTX tree was generated. To do this, the Uniprot database (Bateman et al., 2017) was searched using the terms “cytotoxin” and “cardiotoxin” (synonym) and elapids set as the taxonomic restriction. Complete sequences with a corresponding nucleotide sequence (see Table 4.4.) were added to the dataset containing all members of the CTX group from the 3FTX gene tree, and in addition to these sequences, representative 3FTXs from the other major six group of 3FTX were retained as outgroups. This reanalysis of the DNA 3FTX dataset with the addition of tentatively-annotated CTX sequences described from the literature (see Table 4.4.) revealed that all “cytotoxin” sequences previously described from *Bungarus* species were found nested within non-CTX groups, while CTXs described from *Ophiophagus* grouped with the CTX group (group v) of 3FTXs (Fig. 4.17.). As venom gland transcriptome data from *Dendroaspis* spp. revealed an absence of CTXs (Ainsworth et al., 2018), this

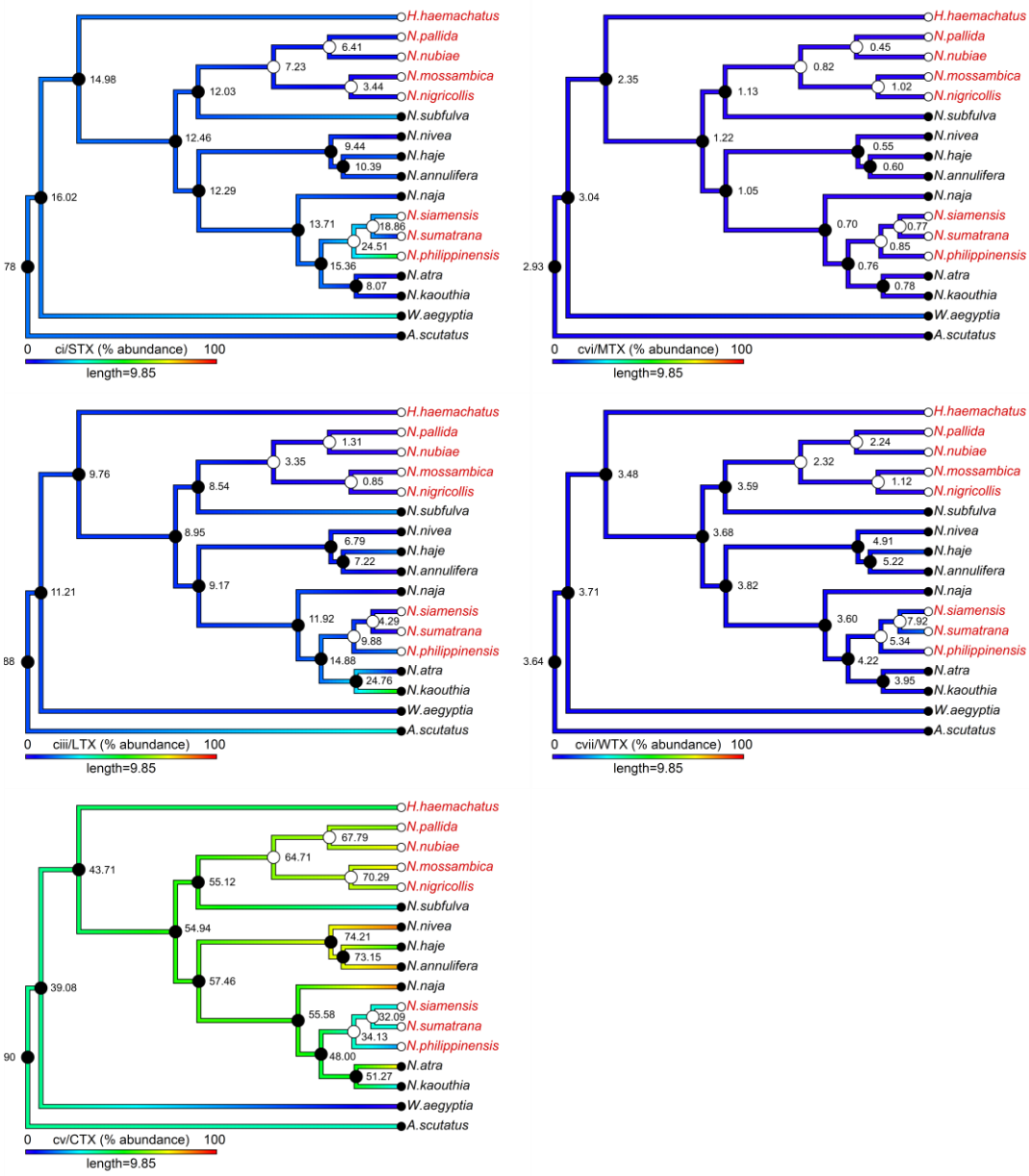


Fig. 4.16. Ancestral Trait Estimations of the three-finger toxin (3FTX) groups, mapped onto the cobra species tree. Ancestral trait estimations were calculated and mapped in RStudio using the "caper" and "phytools" packages. Species tree pruned from a larger elapid species tree provided by Wolfgang Wüster. groups that do not have proteomic abundance of above 1% in at least one species are not shown. Spitting species are indicated by red text. STX – short neurotoxin, MTX – muscarinic toxin, LTX – long neurotoxin, WTX – weak neurotoxin, CTX – cytotoxin/cardiotoxin.

Table 4.3. Phylogenetic Generalised Least Squares (PGLS) Analysis outputs. All analyses were performed in R Studio using the "phytools" package. Significant values are emboldened, and those that remain significant post Bonferroni-correction are represented by red text.

Test Variable	Factor	t	p
Ci/STX abundance	Group ("spitter or "non-spitter")	0.47	0.64
	Geography (Africa or Asia)	1.51	0.15
Ciii/LTX abundance	Group ("spitter or "non-spitter")	-1.97	0.08
	Geography (Africa or Asia)	0.65	0.53
Cv/CTX abundance	Group ("spitter or "non-spitter")	-0.38	0.71
	Geography (Africa or Asia)	-1.81	0.09
Cvi/MTX abundance	Group ("spitter or "non-spitter")	-0.25	0.81
	Geography (Africa or Asia)	1.76	0.10
Cvii/WTX abundance	Group ("spitter or "non-spitter")	-0.43	0.67
	Geography (Africa or Asia)	1.01	0.33
Non-CTX 3FTX abundance	**Group ("spitter or "non-spitter")**	**2.91**	**0.01**
	Geography (Africa or Asia)	-0.80	0.44
PLA_2 abundance	**Group ("spitter or "non-spitter")**	**4.27**	**0.0008**
	Geography (Africa or Asia)	0.92	0.37
% of PLA_2 abundance that is acidic	Group ("spitter or "non-spitter")	0.58	0.57
	Geography (Africa or Asia)	1.80	0.09
% of PLA_2 abundance that is basic	Group ("spitter or "non-spitter")	0.04	0.97
	Geography (Africa or Asia)	-1.65	0.12
SVMP abundance	Group ("spitter or "non-spitter")	-0.86	0.40
	Geography (Africa or Asia)	0.64	0.53
NGF abundance	Group ("spitter or "non-spitter")	-0.76	0.46
	Geography (Africa or Asia)	-1.37	0.19
Kunitz abundance	Group ("spitter or "non-spitter")	-0.68	0.51
	Geography (Africa or Asia)	1.86	0.08

Table 4.4. Full-length, non-cobra elapid three-finger toxins (3FTXs) nucleotide sequences characterised as "cytotoxins" or "cardiotoxins" in the Uniprot database.

Genbank ID	Species	Associated paper
GU190793.1	*Bungarus flaviceps*	Siang et al. 2010
GU190792.1	*Bungarus flaviceps*	Siang et al. 2010
GU190795.1	*Bungarus flaviceps*	Siang et al. 2010
Y17059.1	*Bungarus multicintus*	NA
DQ273578.1	*Ophiophagus hannah*	Li et al. 2006
DQ273580.1	*Ophiophagus hannah*	Li et al. 2006
DQ273581.1	*Ophiophagus hannah*	Li et al. 2006
DQ273577.1	*Ophiophagus hannah*	Li et al. 2006
DQ273579.1	*Ophiophagus hannah*	Li et al. 2006

subfamily of toxins must have either evolved once at the base of the clade containing *Ophiophagus*, *Dendroaspis*, *Walterinnesia*, *Aspidelaps*, *Hemachatus* and *Naja* before being lost independently in *Dendroaspis* and *Walterinnesia*, or evolved twice; once at the base of *Ophiophagus* and once at the base of the clade containing *Aspidelaps*, *Walterinnesia*, *Hemachatus* and the cobras, before being lost in the *Walterinnesia* genus. Next, a reconstruction of the ancestral state of the presence or absence of cytotoxins in elapids was attempted using a combination of a pruned elapid species tree (provided by Wüster) and the proteomic data on elapids in the literature (Table 3.1.), however this could not place the origin of cytotoxins and therefore failed to resolve the question of where precisely cytotoxins emerged in this snake family (Fig. 4.18.). Regardless, this analysis does clearly show that CTXs are not widely distributed across all elapid snakes, but are instead restricted to relatively few genera.

4.3.ii. Phospholipase A_2s (PLA_2s)

Phylogenetic analysis of the of the PLA_2 RNA sequences revealed the presence of two main clades of PLA_2s in cobras, both of which are well supported (bpp >0.9, Fig. 4.19). Moreover, each of these clades shows representation of at least one PLA_2 gene from representatives of each of the major spitting and non-spitting cobra groups, with the exception of the African non-spitting cobras, which appear to have lost (or genes were undetected due to extremely low expression levels) one of these forms. The first of these PLA_2 groups, named here PLA_2a, generally shows a high expression level in cobras (mean 4.30% of all toxins), but is highly variable across taxa (range of 0.01-14.8%). Spitting cobras in the *Hemachatus-Naja* ingroup have a higher expression of PLA_2a (mean 5.54%, range 0.006-14.80%) than non-spitting cobras (mean 4.47%, range 0.04-13.51%). Additionally, there is evidence of gene duplication events unique to the African spitting cobras occurring in this clade, each of which exhibits at least 2 of these PLA_2s, compared with 1 copy in all other cobras. Moreover, these PLA_2 paralogs are relatively highly expressed in African spitting cobras (mean expression 5.45%, range 1.69-8.27%) compared with other *Naja* (mean expression 4.57%, range 0.04-13.51%). PLA_2b genes more closely resemble ancestral forms, separating out

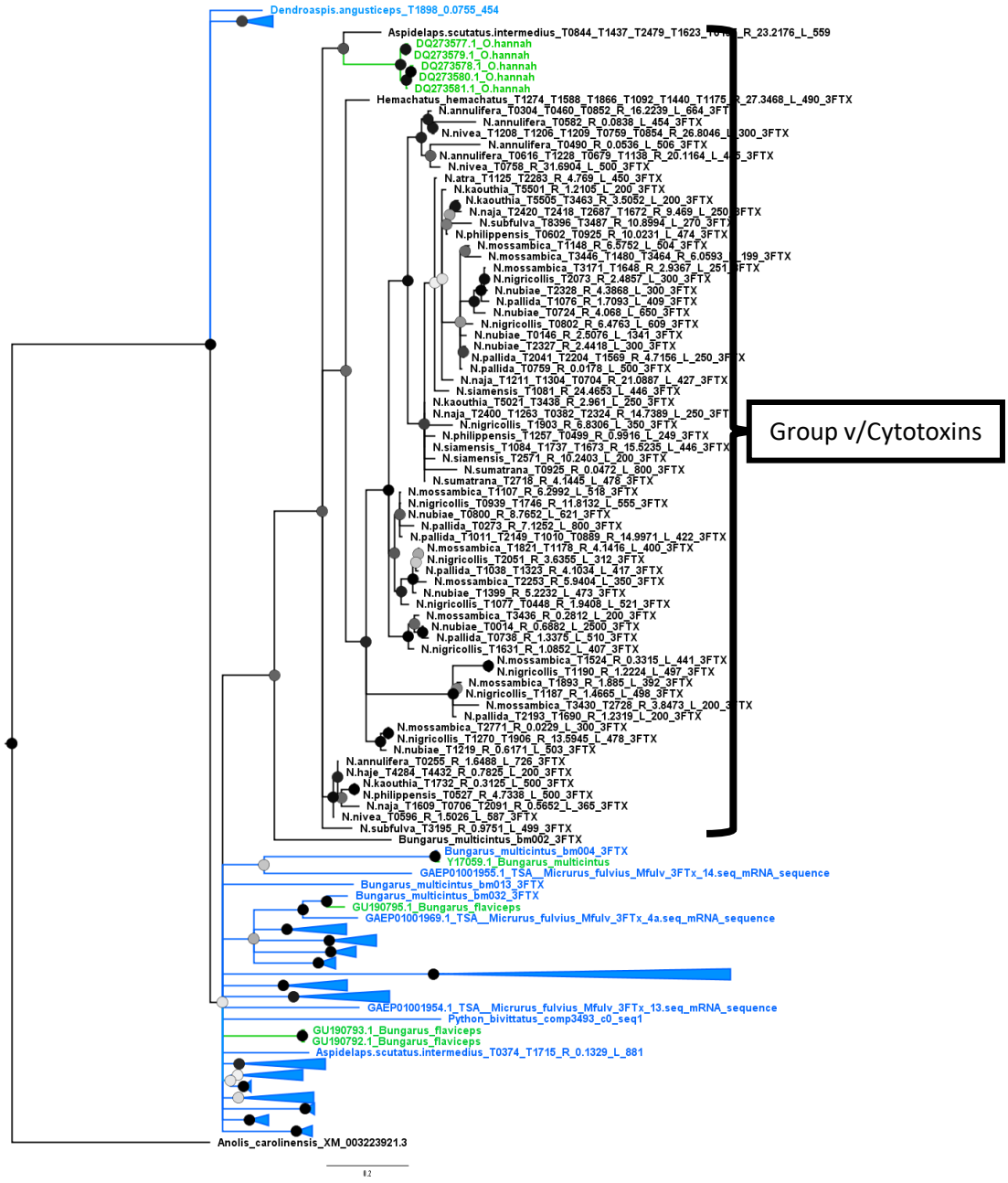


Fig. 4.17. Phylogenetic tree of full-length three-finger toxins (3FTXs) and putative cytotoxins (CTXs) from Genbank. Non-CTX sequences are indicated by blue text and collapsed blue nodes. The putative cytotoxins from the database (see Table 4.3.) are indicated by green text. Circles indicate node probabilities, with white circles representing 0.5, black circles 1.0 and grey circles shaded darker with increasing probability.

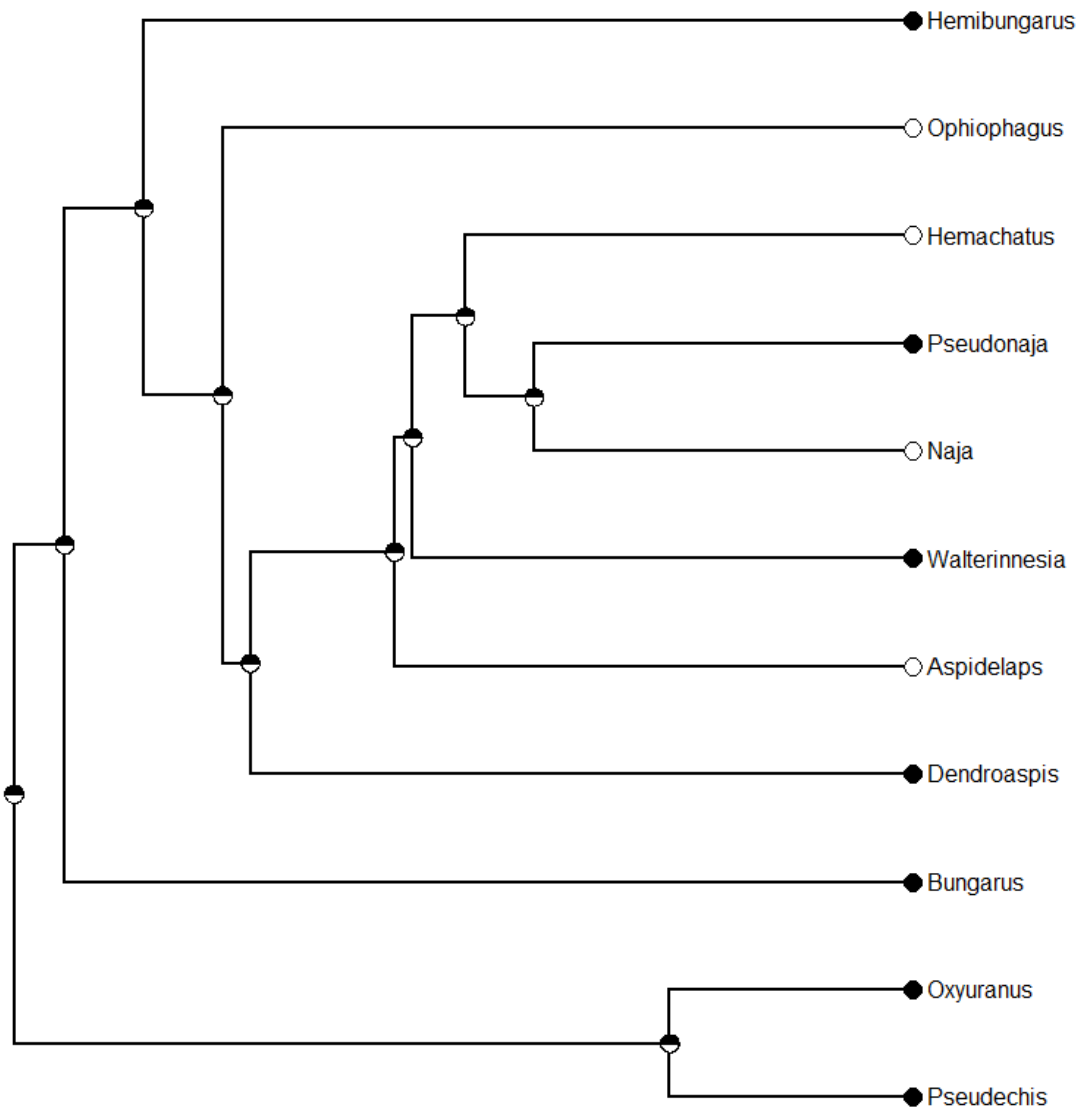


Fig. 4.18. Attempted ancestral state estimation of cytotoxicity in elapid snakes. Filled circles represent no cytotoxins in the transcriptome, while white/unfilled circles represent the presence of cytotoxins in the transcriptome. Both the "equal rates" and "symmetric rates" models were chosen (based on equal log likelihood values) for trait estimation.

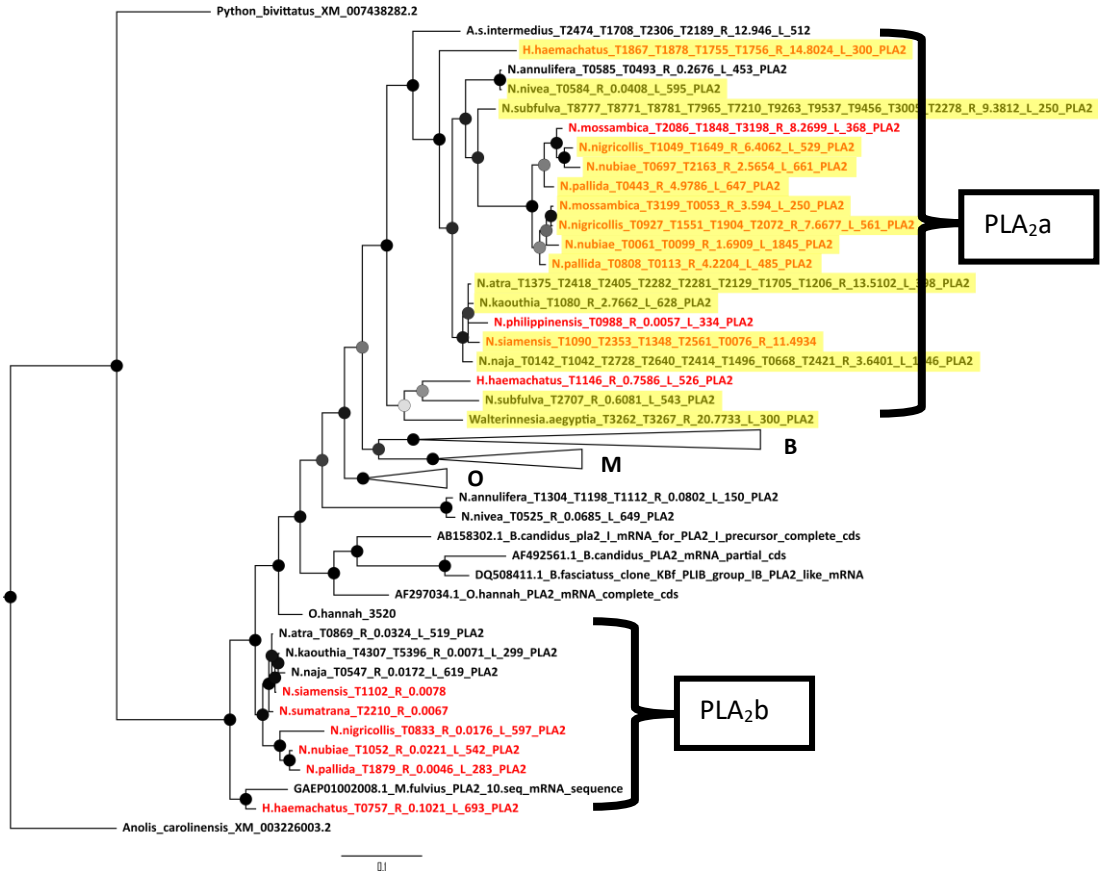


Figure 4.19. Phylogenetic tree of Phospholipase A_2 (PLA_2) toxin genes. A) Phylogenetic tree of PLA_2 genes with spitting species indicated by red text and highlighted text indicating proteomically confirmed sequences. Circles indicate node probabilities, with white circles representing 0.5, black circles 1.0 and grey circles shaded darker with increasing probability. Outgroup clades have been collapsed for ease of viewing; B – *Bungarus* gene radiation, M – *Micrurus* gene radiation, O – *Ophiophagus* gene radiation.

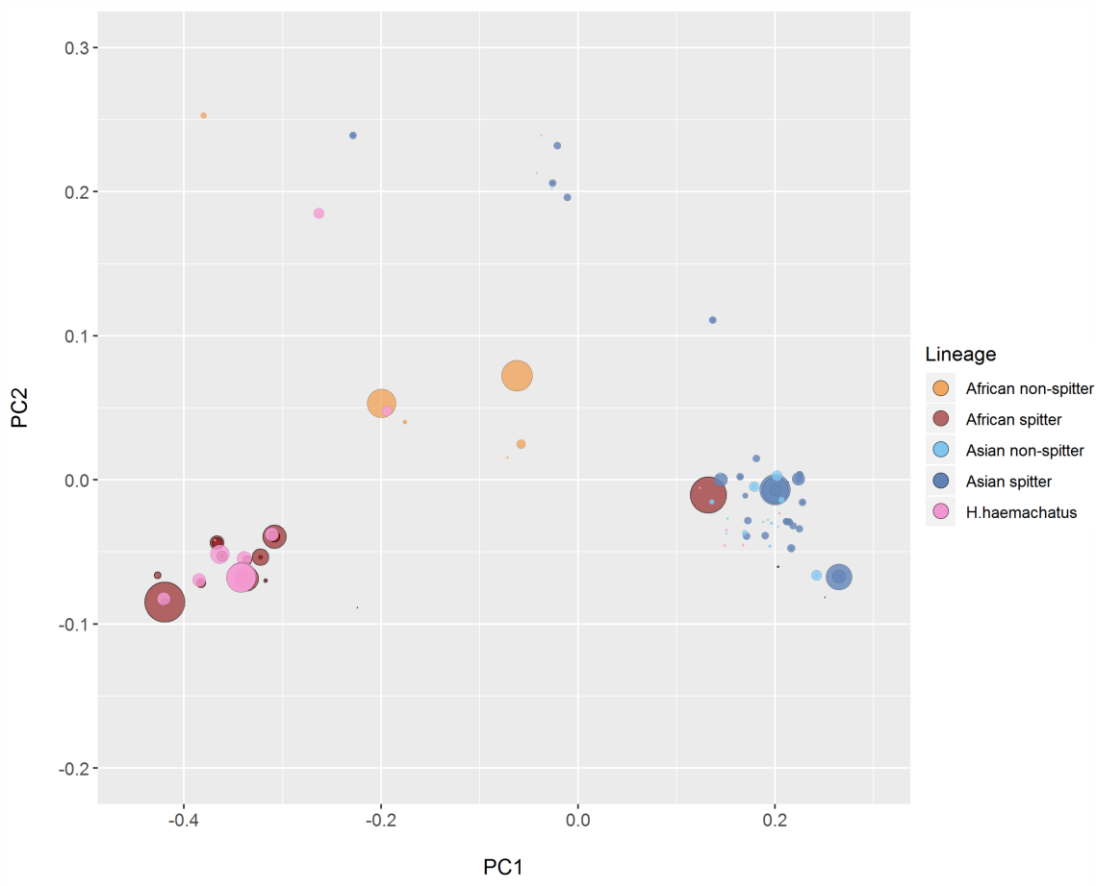


Figure 4.20. Principal coordinate analysis (PCoA) of the amino acid composition of Phospholipase A_2 (PLA_2) proteins, sized by abundance in the proteome (%). The axes represent the distance in terms of amino acid differences between sequences, determined by pairwise analysis in MEGA v7.

just before the *Bungarus*, *Micrurus* and *Ophiophagus* radiations (Fig. 4.19.). These genes are much lower in both number and expression than PLA_2as, containing 1 gene from each of only 8 representative species, representing Asian cobras and African spitting cobras, and having an average expression of 0.01%.

Ancestral state estimations of PLA_2 abundances in the venom proteomes suggest that the ancestor of the clade containing *Aspidelaps*, *Hemachatus*, *Walterinnesia* and *Naja* had moderate PLA_2 representation (14.85%) (Fig 4.21.). There is a slight decrease in PLA_2 abundance at the base of the *Naja* clade but a large upregulation of PLA_2 abundance at the base of the African spitting and Asian spitting cobra clades. Additionally, PGLS reveals a significant association of PLA_2 abundance in the proteome with the emergence of spitting in cobras ($t = 4.27$, $p = 0.0008$), showing that spitting cobras have convergently upregulated PLA_2 abundance. When breaking these down into acidic and basic PLA_2s based on isoelectric point, the patterns are very similar, though basic PLA_2s are notably absent in Asian cobras and *N. subfulva* (Fig. 4.21.). The percentage of total PLA_2 abundance made up of acidic or basic PLA_2s does not differ between spitting and non-spitting cobras, suggesting that there is no specific defensive use of PLA_2s of a high or low isoelectric point.

PCoA analysis on the proteins sequences themselves further revealed that cobra PLA_2s cluster together (in terms of amino acid composition) in to three main groups; those from African spitting cobras and *H. haemachatus*, those from Asian cobras, and those from African non-spitting cobras (Fig. 4.20.). This, combined with the results of ancestral trait estimation, suggest that PLA_2 proteins from African spitting species have converged upon a sequentially-similar form, while Asian cobras have converged upon a sequentially distinct form, with all spitters then upregulating the abundance of their respective PLA_2s in their venoms.

As PLA_2 proteins appear to exhibit considerable diversity, and elements of molecular convergence, based on these groupings, as well as displaying clear differences in proteomic abundances between spitting and non-spitting cobras, conservation and structural analyses of these proteins were pursued to identify any distinct differences in potentially functionally-relevant regions. Conservation analysis of the amino acid sequences show several regions of low conservation (Fig. 4.22.), including a deletion at position 58 in many of the PLA_2s in African spitting cobras and

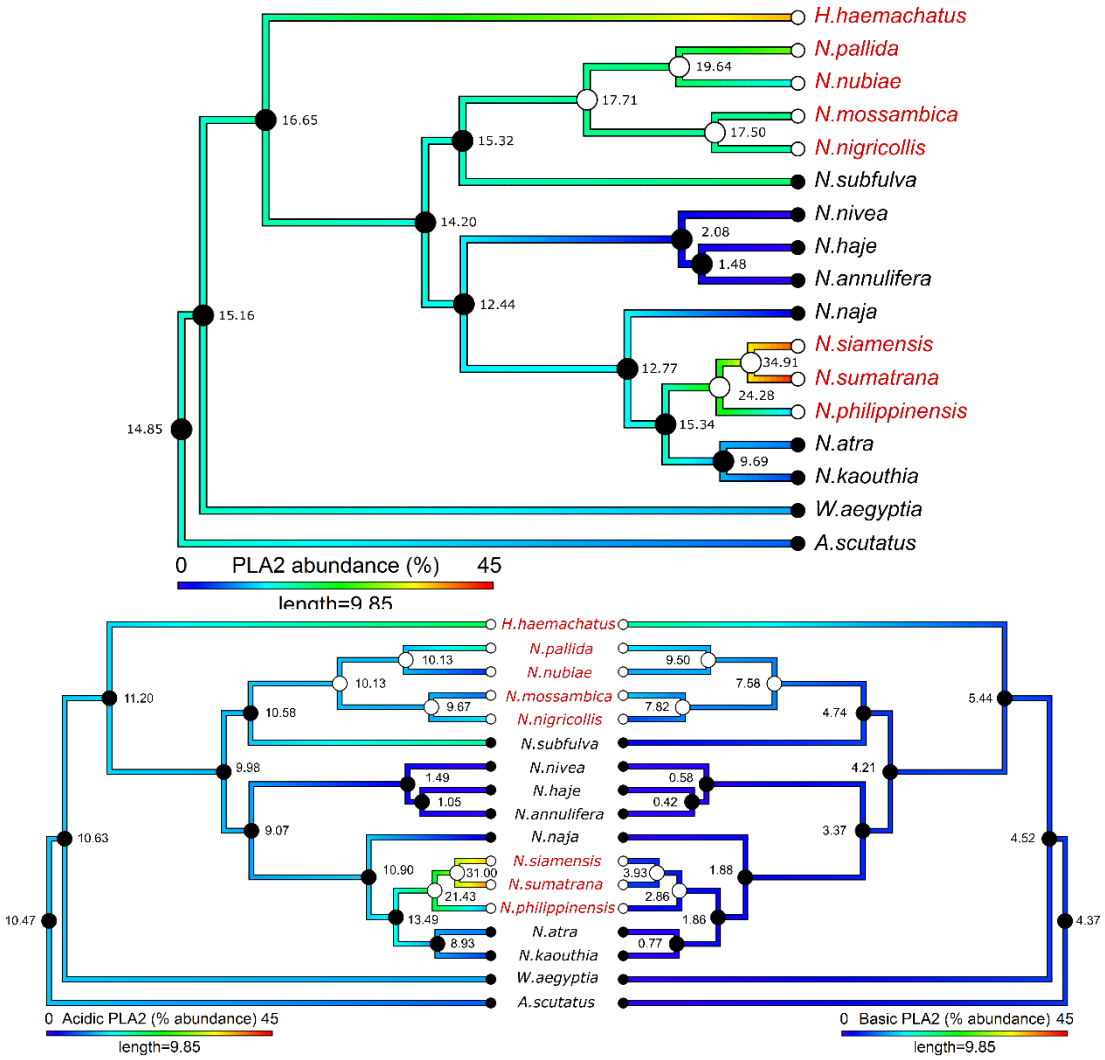


Fig. 4.21. Ancestral state estimations of Phospholipase A_2 (PLA_2) abundance. Black circles at nodes and tips represent a non-spitting lineage, while white circles represent spitting lineages. The species tree was made by pruning a larger tree received from Wolfgang Wüster.

H. haemachatus. This would cause a shift in the positioning of adjacent amino acids in one of the regions predicted to be an α-helix, potentially causing these amino acids to become more or less exposed on the protein molecule. Conversely, a protein from *N. subfulva* (see asterisk in Fig. 4.22.) appears to have undergone two insertions; an isoleucine at position 16 and a Glutamine followed by Tyrosine at positions 107-108. Again, this insertion may have led to a significant shift in positioning within the PLA_2 protein, which could impact its functional effects. Given its relatively low abundance

in the proteome (1.12%) compared to 3 of the 4 other *N. subfulva* PLA_2s (2.02%, 7.35%, 7.77%), it seems unlikely that any differences in functionality would be of large medical importance.

The predicted 3D protein structure of cobra PLA_2s is comprised of 3 central α-helices and several β-sheets (Fig. 4.23.). The helices appear to have several regions of hydrophobicity (Fig. 4.23A.) and positive charge (Fig 4.23B.), while the sheets mostly show regions of negative charge. When estimating conservation scores on the 3D model, a couple of areas estimated to have high variability appear to coincide with areas of negative charge, and there appears to be some overlap of highly variable areas with hydrophobic regions of one of the α-helices (Fig. 4.23C.).

4.3.iii. Snake Venom Metalloproteinases (SVMPs)

The SVMP gene tree did not resolve well (see Fig. 4.24.), making it difficult to analyse the evolutionary history of SVMPs in cobras. There were not enough SVMP protein sequences to run a protein tree. This lack of protein sequences in comparison to the large number of SVMP sequences suggests posttranslational suppression of SVMPs in cobra venoms. PGLS shows no association of SVMP expression in the transcriptome with the emergence of spitting ($t = -0.86$, $p = 0.40$).

4.3.iv. Other Toxins

Several other toxin gene trees were run successfully, though there were not enough representative protein sequences in which to run protein trees. Cobras appear to have one nerve growth factor (NGF) gene per species with the exception *of N. mossambica*, which has 3 (Fig. 4.25A.). Though the interrelationships of the cobra NGF genes do not have high support, they seem to be as expected from the species phylogeny, with the exception of the *H. haemachatus* gene, which segregates with genes from the Asian cobras. PGLS found no significant association between the emergence of spitting and NGF abundance in the proteome ($t = -0.76$, $p = 0.46$).

Cobra c-type lectins (CTLs) appear to have 2 distinct clades (Fig. 4.25B.) A single African non-spitter gene shares clade 1 with the outgroup species, initially

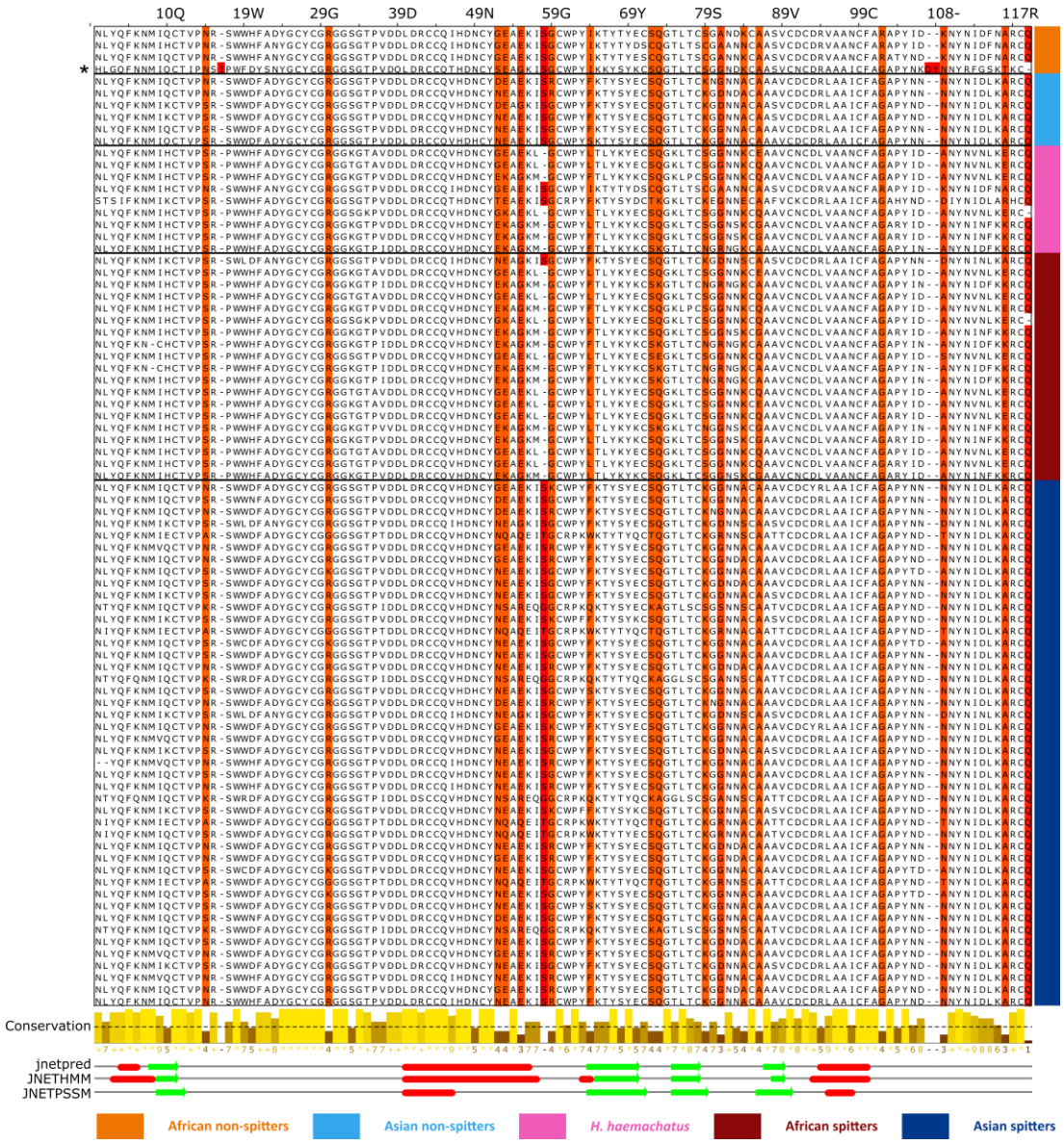


Fig. 4.22. Conservation analysis of phospholipase A_2 (PLA_2) proteins, highlighting regions of low conservation. Amino acids with conservation scores below 5.0 are highlighted, as estimated by Jalview (Waterhouse et al., 2009). Numbers above the sequences represent position and respective consensus amino acid. Estimates for tertiary structure are under the conservation scores and were calculated using jnetpred (Drozdetskiy et al., 2015). Sequence IDs have been removed for clarity. * This sequence from *Naja subfulva* is noted as being particularly unsual in comparison to other PLA_2 sequences.

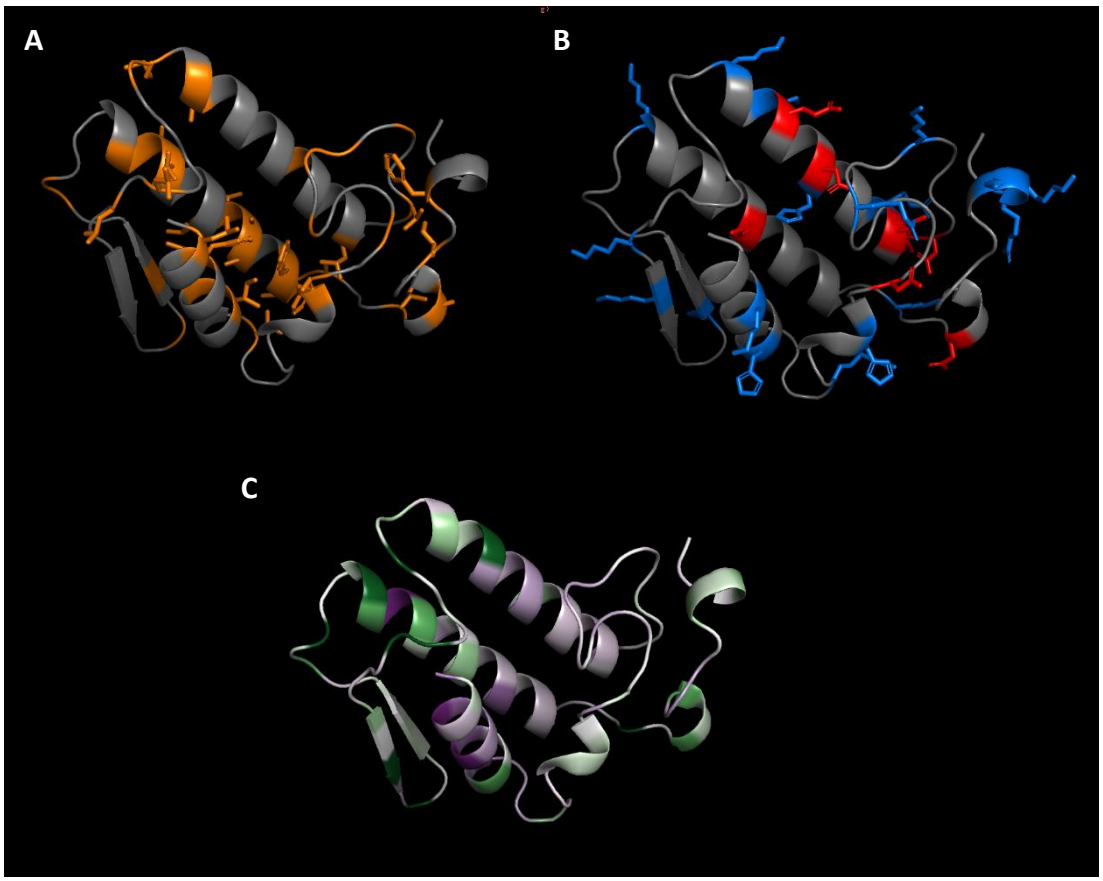


Figure 4.23. Predicted phospholipase A_2 (PLA_2) protein structure, based on a template generated from *Naja mossabica* PLA_2 amino acid sequence. Regions of **A)** Hydrophobicity (orange - hydrophobic) **B)** Charge (red – positive, blue – negative) and **C)** conservation (darker shades of purple indicate higher conservation, while darker shades of green indicate higher variability) have been highlighted. The template was generated and protein model built by SWISS-MODEL (Waterhouse et al., 2018), and regions of conservation calculated using the ConSurf server (Ashkenazy et al., 2010) on all PLA_2 protein sequences with abundances of 1% or higher.

Fig. 4.24. Phylogenetic tree of cobra snake venom metalloproteinase (SVMP) toxin genes. Spitting species are indicated by red text. Circles indicate node probabilities, with white circles representing 0.5, black circles 1.0 and grey circles shaded darker with increasing probability.

suggesting a loss of this gene in the African spitters and Asiatic cobras, however clade 1 itself does not have good node support, and the expression for this gene is extremely low (< 0.01). The second clade of CTLs splits into two further clades with gene representatives from each cobra group present in both of these clades, and expression levels for genes in this clade remains low (mean 0.16%, range 0.002-0.45%). There doesn't appear to be any patterns between spitting and non-spitting cobras. CTLs were absent from the proteome.

Similarly to CTLs, cysteine-rich Secretory Protein (CRISP) genes separate into 2 distinct clades, with many non-spitting species possessing 2 genes in the uppermost clade, as opposed to the single gene seen in other species (see Fig. 4.26A.). The lowermost lacks representatives from several spitting species and appears more closely related to the ancestral CTL form. CRISPs were absent from the proteome.

Kunitz genes appear to have an unusual pattern in that the genes from the Asiatic cobras appear to be more closely related to the *Dendroaspis* and *Ophiophagus* outgroups, though these genes exhibit very small expression (mean 0.39%) (see Fig. 4.26B.). The majority of African cobra genes cluster together in a highly-supported clade, and this clade presents the most highly-expressed of the kunitz genes in cobras (mean 0.74%). However, two African non-spitter genes segregate with counterparts from *Ophiophagus* and *Walterinnesia*. PGLS found no significant association between the emergence of spitting and Kunitz abundance in the proteome ($t = -0.68$, $p = 0.51$) (Table 4.3.).

Natriuretic peptides (NPs) genes are noticeably absent in African spitters, with only 1 representative each from *Naja nubiae* and *N. mossambica* (Fig. 4.27.). The gene tree can be split into 2 main clades, with the upper clade containing the majority of sequences. This is due to duplications seen in many of the Asian cobra NPs as well as the multiple *N. subfulva* sequences. This first clade also contains most of the transcriptome expression in the tree, with a mean of 0.19% and range of 0.005-0.6% expression in *Naja*, compared to 0.05% and 0.006-0.07%, respectively. NPs were absent from the proteome.

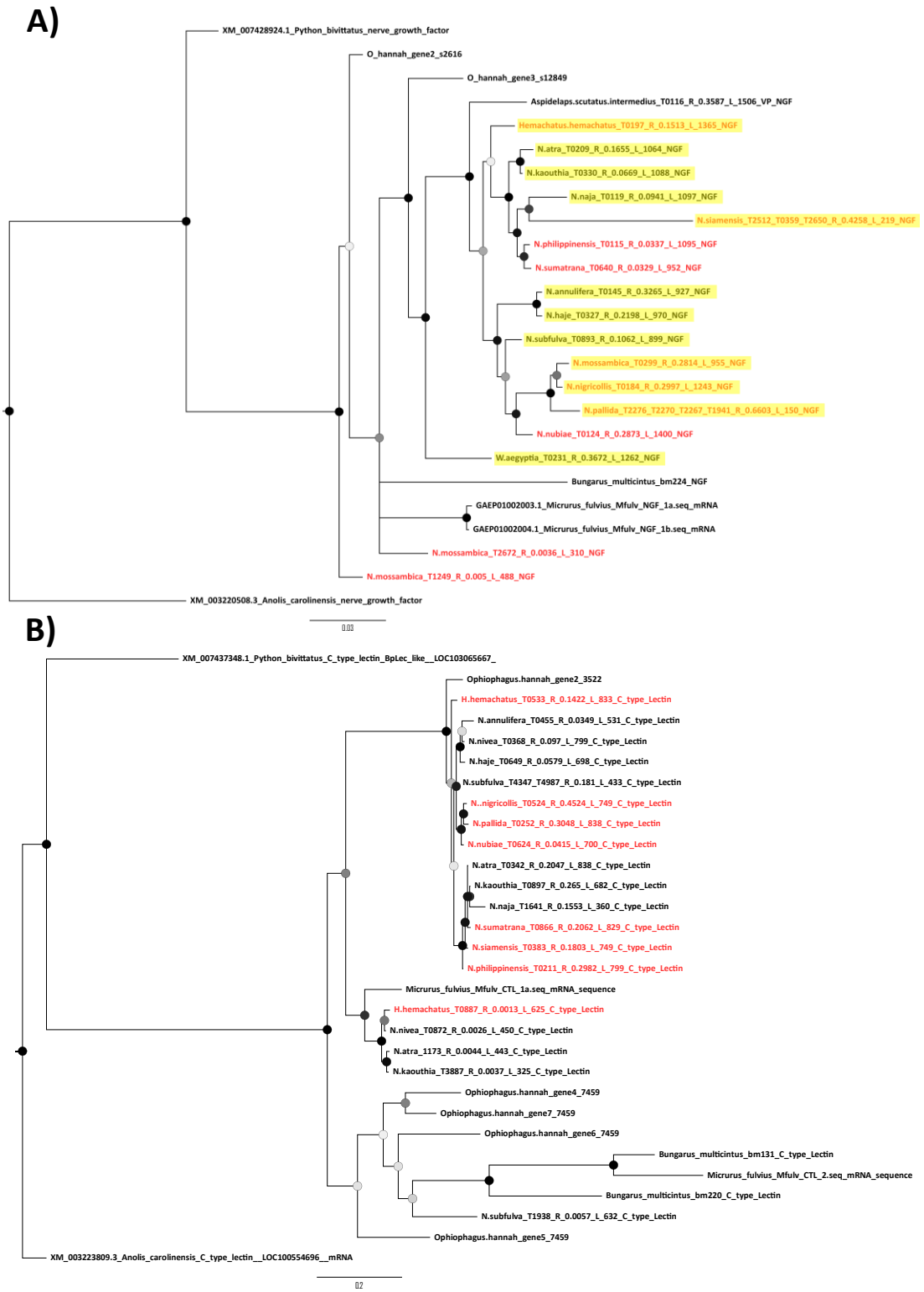


Fig. 4.25. Phylogenetic tree of cobra A) nerve growth factor (NGF) toxin genes B) c-type lectin (CTL) toxin genes. Spitting species indicated by red text and highlighted text indicating proteomically confirmed sequences. Circles indicate node probabilities, with white circles representing 0.5, black circles 1.0 and grey circles shaded darker with increasing probability.

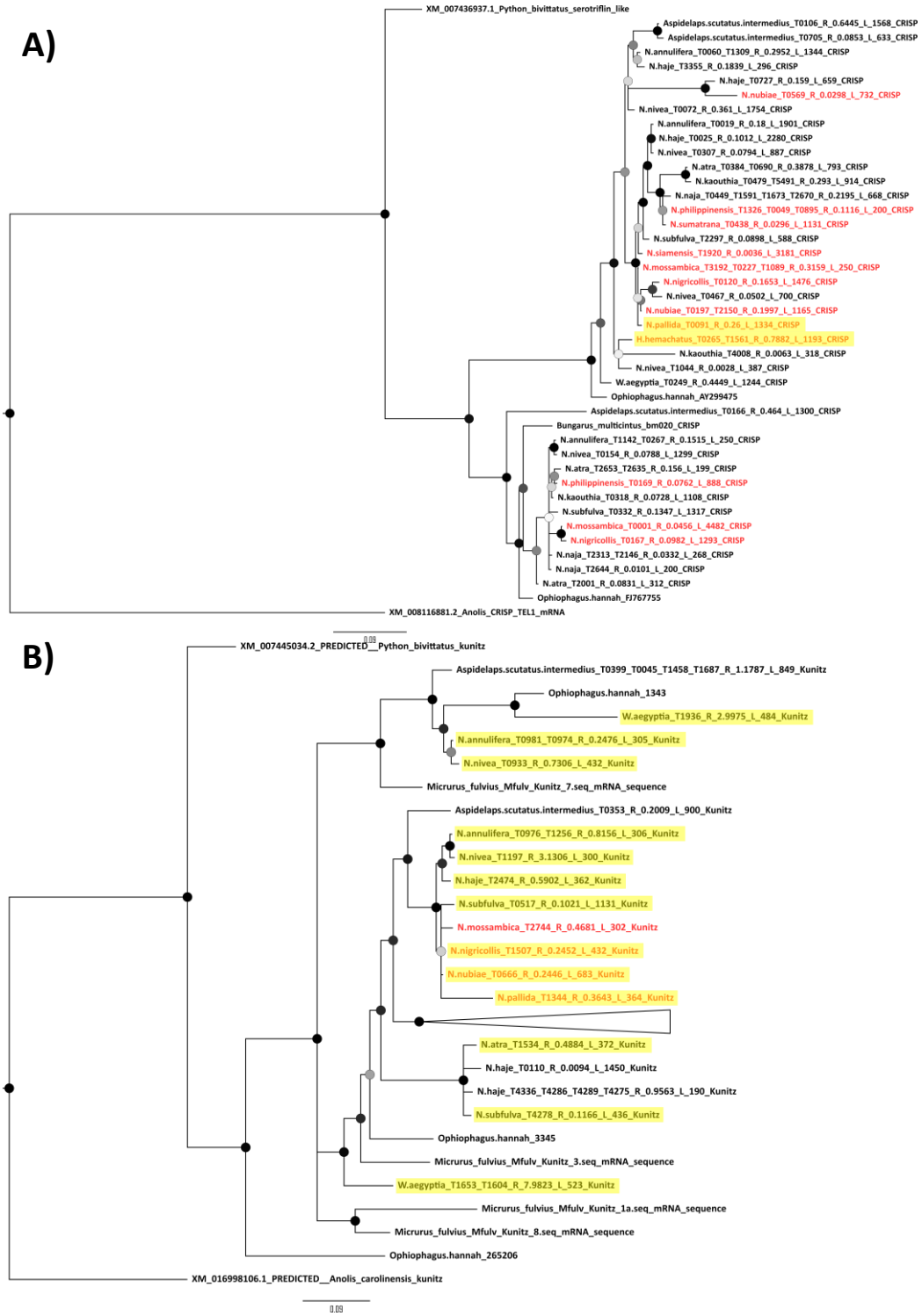


Fig. 4.26. Phylogenetic tree of cobra A) cysteine-rich secretory protein (CRISP) toxin genes B) kunitz-type serine protease inhibitors (kunitz). Spitting species indicated by red text and highlighted text indicating proteomically confirmed sequences. *Dendroaspis* genus nodes have been collapsed for clarity. Circles indicate node probabilities, with white circles representing 0.5, black circles 1. 0 and grey circles shaded darker with increasing probability.

Fig. 4.27. Phylogenetic tree of cobra Natriuretic Peptide (NP) toxin genes. Spitting species indicated by red text and highlighted text indicating proteomically confirmed sequences. Circles indicate node probabilities, with white circles representing 0.5, black circles 1.0 and grey circles shaded darker with increasing probability.

4.4. Discussion

4.4.i. Overview

The transcriptomic data presented in Chapter 2, proteomic data presented in Chapter 3, and the analyses presented herein, convincingly demonstrate that the major toxin families present in cobra venom are the three-finger toxins (3FTX) and Phospholipases A_2 (PLA_2). PLA_2s appear to be of particular importance to spitting cobras, with their higher abundances in the venom proteomes significantly correlated with the evolutionary origins spitting ($t = 4.27$, $p = 0.0008$), while evidence of gene duplication occurring in at least one spitting lineage was also detected. The majority of other toxin families were either very lowly represented in the venom proteomes or were absent entirely. Perhaps unsurprisingly, no association between the evolutionary histories or abundances of these minor toxin types and venom spitting was detected. Consequently, this chapter predominately focuses on

exploring the evolutionary association of 3FTXs and PLA_2s with cobras and the emergence of defensive venom spitting.

4.4.ii. Evolution of 3FTXs

The 3FTX gene family encodes an extensive and diverse array of related toxin isoforms that are capable of causing a variety of pathological effects, such as cytotoxicity, and neurotoxicity (Gasanov, Dagda, & Rael, 2014; Lomonte & Gutiérrez, 1989; Nirthanan & Gwee, 2004; Utkin, 2013). This diversity is extensively represented in cobras, with phylogenetic analyses reconstructing seven major groups, each of which are associated with different functional annotations, with varying differences in terms of transcriptomic expression and isoform diversity. These groups include toxins annotated as i) short neurotoxins (STXs), ii) neurotoxin-like proteins (NLPs), iii) long neurotoxins (LTXs), iv) unknown/unclassified 3FTXs (UNK), v) cytotoxins (CTXs), vi) muscarinic toxins (MTXs) and vii) weak neurotoxins (WTXs). The concept of multiple, functionally distinct, groups of 3FTXs has previously been described (Fry et al., 2003; Fry et al., 2008, 2013; Sunagar et al., 2013). This diverse evolutionary history of 3FTXs has been proposed to have been the result of the recruitment of a non-toxic 3FTX gene to the venom gland at the base of the Caenophidia clade, followed by extensive gene duplication and accelerated evolution of the protein coding regions (Fry et al., 2012; Sunagar et al., 2013), in turn leading to protein neofunctionalization. Work on α-neurotoxins suggests that diversification is predominately associated with the accumulation of mutations in exon II (Chang, 2007; Fujimi et al., 2003). The 3FTX gene reconstructed here (Fig. 4.4.) shows a number of similarities with the evolutionary relationships determined Fry et al. (2003) using amino acid sequences, although there are some notable differences. The functional classifications described here are not as extensive as those presented by Fry et al. (2003), with the latter labelling clades by SwissProt subfamily classifications as opposed to using BLAST annotation to the less thoroughly curated NCBI protein database. As a result, Fry et al. (2003) classified the 3FTX under 33 different subfamilies, compared to the seven major classifications described here. These 33 classifications included separating the long and short neurotoxins into Type I α-neurotoxins, Type II α- neurotoxins, Type III α- neurotoxins and κ-neurotoxins, the

muscarinic toxins into three further groups named A, B and C, and separating CTXs into type 1A and 1B. Of particular note were the description of numerous "Orphan" groups, classified as toxins lacking specific functional motifs but having 75% consensus sequence with each other. Despite these additional 'functional groups', it is worth noting that this prior analysis was generated by neighbour-joining and maximum likelihood analysis of amino acid sequences, compared with the Bayesian inference RNA approach undertaken here. Additionally, this study used over 400 sequences from 24 different elapid species, while Fry's work used 276 sequences from 38 species, giving their work a smaller coverage per species but larger coverage for Elapids overall. Although these analyses may therefore not be directly comparable, the resulting phylogenetic analyses are both poorly resolved, particularly within major clades, which is the direct result of extensive sequence mutations having occurred throughout the evolutionary history of these relatively small proteins (generally 80-100 amino acids in length). Despite this limitation, both trees clearly demonstrate that 3FTXs have extensively diversified as the result of gene duplication, although it is apparent that, when looking at the gene tree reconstructed here, CTXs are much more diverse in relation to related neurotoxin sequences (see clade v in Fig. 4.4., and Fig. 4.11.).

Of these clades, the CTXs are the most highly represented in both the transcriptome and proteome of most cobras. Despite a reasonable hypothesis being that CTXs would be more abundant in venom spitting species to facilitate defence, no significant difference in CTX abundance was observed between spitting and non-spitting cobras when phylogenetic structure (i.e. the species relationship of cobras) was taken into account (PGLS: $t = -0.38$, $p = 0.71$). However, despite the abundance of individual non-CTX clades not differing between spitting and non-spitting cobras, when combined as "other 3FTXs", which are predominately neurotoxins, non-spitting cobras have a significantly higher representation in the proteome (PGLS: $t = 2.91$, $p = 0.01$), which correlates with neurotoxicity observed from at least the African non-spitting cobras (Warrell, 2008c). Despite there being no association between their abundances in cobras and defensive venom spitting, the CTXs are the most abundant of the 3FTXs found in cobra venoms, and the evolutionary history of this unique subfamily of 3FTX in the context of elapids is largely unknown. Consequently, in this

chapter this was investigated in detail. The results of phylogenetic analyses show that there is no evidence of CTXs emerging in elapid snakes before the divergence of *Ophiophagus* from the ancestor of *Dendroaspis*, *Walterinnesia, Hemachatus, Aspidelaps* and *Naja* (Fig. 4.17.). Non-naja elapid snakes possessing CTXs are *Hemachatus haemachatus*, *Aspidelaps scutatus* and *Ophiophagus hannah*, which leads to three theories about the evolution of CTXs in this group: i) there was a single origin for CTXs at the base of the *Ophiophagus-Dendroaspis* split, followed by two losses; once in the *Dendroaspis* genus and once in *Walterinnesia*; ii) CTXs evolved twice, once in *Ophiophagus* and once in the clade containing *Aspidelaps*, *Naja*, *Hemachatus* and *Walterinnesia*, before being lost in *Walterinnesia*; iii) CTXs evolved repeatedly and independantly, once in *Ophiophagus*, once in *Aspidelaps*, and once in the clade containing *Hemachatus*, *Pseudonaja* and *Naja*. On the face of it, i) is the most parsimonious explanation, however with 3FTXs known to evolve rapidly the multiple-origin theories of CTXs cannot be ruled out without further data.

When performing ancestral state reconstruction on CTX abundances detected in the various venom proteomes, including *Dendroaspis* and *Ophiophagus* as additional outgroups (Fig 4.28.), the aforementioned lack of significance between the emergence of spitting in elapids and CTX abundance holds (PGLS: $t = -0.60$, $p = 0.56$). It is, however, apparent that high CTX abundance is an ancestral trait of the clade containing *Hemachatus* and *Naja*. This suggests that cobras (in general) have shifted from a neurotoxic 3FTX-rich venom, as observed in many elapid relatives (Barua et al., 2019; Tasoulis & Isbister, 2017), and instead exhibit CTX-rich venoms, presumably facilitated by the diversification of an ancestral CTX-like 3FTX genes. This is particularly evident in the African cobras. Consequently, ancestral reconstructions show that the abundance of other (likely neurotoxic) 3FTXs decrease in African cobras, but remain moderate in Asian species, and actually increase in *N. philippinensis* and *N. kaouthia*, which correlates with the strong neurotoxic activity observed in the venom of these species (Faiz et al., 2017; Watt et al., 1988).

4.4.iii. Evolution of PLA_2s

The PLA_2 toxin family is a major constituent of many snake venoms, and originates from multiple recruitment events in caenophidian snakes; once in viperids (group I),

once in Elapids (group II) and once in non-front fanged snakes (group IIE) (Fry & Wüster, 2004; Fry et al., 2009; Sunagar et al., 2015a, 2015b) (Fig. 4.2). The group I PLA_2s found in elapid venoms are derived from a non-toxic scaffold, which was recruited for use in venom prior to the diversification of elapid snakes. Subsequently, and in an analogous manner to the 3FTXs, surface residues of these molecules appear to have been modified via adaptive evolution (Kini & Chan, 1999), which in turn enabled the evolution of new functional activities, such as the myotoxicity observed in coral snake venoms (*Micrurus*) (Alape-Girón et al., 1999). Indeed, PLA_2s are well known for their multi-functional activities, which include myotoxic, cytotoxic, haemotoxic and neurotoxic effects (Ferraz et al., 2019), and there are various sources of evidence for their accelerated evolution in different clades of venomous snakes. This has been evidenced in the rapid PLA_2 evolution at both the genus (Dowell et al., 2016; Gibbs & Rossiter, 2008) and species level (Nakashima et al., 1993; Pla et al., 1996) in vipers, and even between different populations of *N. kaouthia* occupying distinct ecotypes (Chuman et al., 2000).

Here, phylogenetic analysis of the PLA_2 toxin family revealed that cobra PLA_2 genes predominately fall into two main clades; a major clade (PLA_2a) which contains representation from all sampled species and which comprises the vast majority of PLA_2 expression, and a minor clade (PLA_2b) which contains lowly expressed genes from the Asian cobras, African spitting cobras (excluding *N. mossambica*) and *Hemachatus haemachatus*. These findings confirm earlier suggestions that this toxin family has diversified into a multi-locus toxin family following initial recruitment (Lynch, 2007; Vonk et al., 2013). Moreover, the representation of PLA_2 genes from African spitting cobras detected demonstrates that they have undergone further gene duplications, which are not observed in any of the other major cobra lineages. Crucially, when taking into account phylogenetic structure, the varying abundances of PLA_2s detected in the venoms of cobras are significantly associated with the repeated evolutionary origins of defensive spitting (PGLS: $t = 4.27$, $p = 0.0008$). These findings also hold with the addition of further elapid outgroups *Dendroaspis* and *Ophiophagus*, mirroring the CTX analysis described earlier (PGLS: $t = 5.13$, $p < 0.0001$) (Fig. 4.28.). A major finding of these analyses is that increased abundances of PLA_2 toxins correlate well with the origin of defensive spitting, and thus may represent

defensive toxins. To address the issue of previously published cobra venom proteomes having varying PLA_2 abundances, the literature PLA_2 values were averaged together with the data from this study (see 3.4.iii.) and the PGLS analysis rerun. The resulting outcome found that spitting cobras maintained significantly higher PLA_2 abundance than non-spitting cobras (PGLS: t = 5.20, p = 0.0001).

Despite these similar patterns of upregulated toxin abundances detected in the African spitting cobras, Asian spitting cobras and *H. haemachatus*, the mechanisms underpinning these increases may differ. Indeed, as mentioned above, African spitting cobras appear to have duplicated their PLA_2 genes which may underpin a gene dosing increase in toxin levels. However, increased diversity of PLA_2s was not observed between Asian spitting and their non-spitting counterparts, while the spitting *H. haemachatus* only expressed one PLA_2 gene in its venom gland transcriptome. Similarly, Asian spitting cobras, with the exception of *N. siamensis*, exhibited very low levels of PLA_2 expression at the transcriptome level compared to

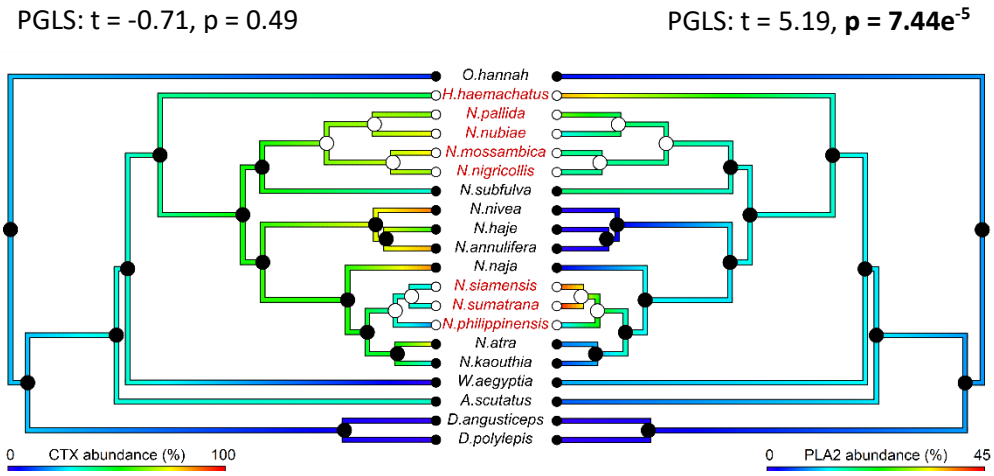


Fig. 4.28. Ancestral state estimations of the proteomic abundance of cytotoxic three-finger toxins (CTXs; left) and Phospholipase A_2s (PLA_2s; right) are mapped onto a species tree containing the cobras and representatives of the outgroup genera *Walterinnesia*, *Aspidelaps*, *Dendroaspis* and *Ophiophagus*. Black circles at nodes and tips represent a non-spitting lineage, while white circles represent spitting lineages. Phylogenetic Generalized Least Squares (PGLS) results are shown above each trait map. The species tree was made by pruning a larger tree received from Wolfgang Wüster.

African spitting cobras (see Fig. 2.4.) but all spitting cobras showed high representation of PLA_2 at the proteomic level (Fig. 3.2.), suggesting that African spitting cobras have a high representation of PLA_2s in their venoms due to direct underlying transcriptome levels, whereas Asian spitting cobras have undergone some form of post-transcriptional regulation that result in the increased representation of PLA_2 in the proteome when compared to the transcriptome.

The PCoA separated cobra PLA_2 proteins into 3 proteotypes, based on amino acid composition (Fig. 4.20.): African spitter/*Hemachatus* group (Group 1), The Asian cobra group (Group 2) and the African non-spitter group (Group 3). These proteotypes show varying levels of sequence conservation (Fig. 4.22.) but there are some more noticeable differences, such as the deletion in position 58 of many of the Group 1 PLA_2s. It is interesting to see that, despite there being no differences in representation in terms of PLA_2 abundance between African and Asian cobras, there is clearly a geographic difference in the amino acid sequences of these proteins, with the African and Asian sequences clustering closely together with the exception of the African non-spitters. It seems apparent then that, while upregulated PLA_2 abundance is a key feature of spitting cobras in general, the structure of spitting cobra PLA_2s is reflective of the differences between lineages likely resulting from phylogenetic distance of African and Asian cobras.

4.4.iv. PLA_2s as Defensive Toxins

As stated previously, PLA_2s are highly diverse in terms of function (Kini, 2003; Sunagar et al., 2015b; Zambelli et al., 2017). This makes it difficult to speculate as to why the upregulation of PLA_2 in spitting cobra venoms might incur some advantage. As the focus of this work is on the idea of defence-driven venom evolution, it can only be postulated that PLA_2s act in a way as to optimize defensive venom spitting in cobras. Some of the more effective ways of repelling an aggressor are to induce pain, and that is certainly one of the results of ocular venom affliction (Chu et al., 2010; Fung et al., 2009; Warrell & Ormerod, 1976). One of the many functions of venom PLA_2s is cytotoxicity (Debnath et al., 2010; Méndez et al., 2011; Panagides et al., 2017; Reali et al., 2003; Rudrammaji & Gowda, 1998; Suzuki-Matsubara et al., 2016), possibly resulting in pain through gradual destruction of ocular cells. Additionally, PLA_2s have

been shown to act synergistically with 3FTXs (especially CTXs) to increase the cytotoxicity of the venom (Chang et al., 1972; Harvey et al., 1983; Louw & Visser, 1978; Mukherjee, 2010). Alternatively, as with invertebrate defensive venoms, PLA_2s may cause pain through ion channel aggravation, or otherwise through the inducement of an inflammatory response or hyperalgesia, all of which have been shown to occur in rats injected with PLA_2 from the viper *Bothrops asper* (Chacur et al., 2003; Chacur et al., 2004; Zambelli et al., 2017). Further investigation into the functional aspects of isolated cobra PLA_2s would likely be informative in this area.

4.4.v. Limitations and Future Work

The limitations of this chapter are largely technical due to the large number of analyses and other computer-based processes performed. One issue concerns the failure to produce resolved protein trees. The failure of these trees may be due to the decrease in variability from nucleotide to amino acid due to nucleotide redundancy. It may have helped to include additional outgroups, though this may make any resulting protein trees less comparable to the already-produced gene trees unless they are re-generated using transcriptome sequences from the same outgroup species. Unfortunately, as protein sequences are shorter in length than untranslated RNA sequences, this can make attempting to construct trees from proteins more challenging in general. Additionally, the generated gene trees cannot account for pseudogenisation or reverse-recruitment, and the inclusion of such non-toxin isoforms can result in gaps in evolutionary history of toxin genes (Casewell, Huttley & Wüster, 2012).

In terms of future work, it would be exceedingly helpful to have a *Pseudohaje* transcriptome and proteome to look at the expression/abundance of CTXs and PLA_2s in the closest outgroup genus to the true cobras (*Naja*) and further test the single vs. multiple CTX origin theories. Similarly, it may have been helpful to have the transcriptome of *Aspidelaps lubricus* and more spitting species of *Naja* to further confirm whether PLA_2 abundance is undisputedly high amongst all spitting cobras. In the same vein it would be informative to perform tests for rates of evolution in PLA_2s in spitting cobras compared to the rates of evolution in a) the clade containing *Hemachatus*, *Pseudonaja* and *Naja* b) all elapids c) all snakes, to confirm whether

selection is acting uniquely on spitting cobra PLA_2s, implying further a defensive driver of PLA_2 evolution. Furthermore, a population-level proteomics approach looking at the representation and selection of PLA_2s in select spitting and non-spitting populations would help to confirm the importance of PLA_2 in defensive spitting.

4.4.vi. Concluding Remarks

The data represented in this chapter is one of few studies that have attempted to account for phylogenetic context when analysing snake venom toxin family evolution, and it also represents the most comprehensive evolutionary comparative analysis of toxins from cobras. Notable findings include the demonstration that cytotoxic 3FTXs are largely restricted to certain elapid species, and have been extensively upregulated in *Naja* species and their close relatives. Furthermore, evolutionary analyses demonstrated that a complex defensive behaviour in snakes can be linked to notable events in toxin evolution; in this case the convergent upregulation of PLA_2 abundance in cobras capable of spitting their venoms. In the following chapter, a number of functional activities of cobra venoms will be quantified and analysed in the context of the evolutionary history of these species.

5. FUNCTIONAL ANALYSIS OF COBRA VENOMS

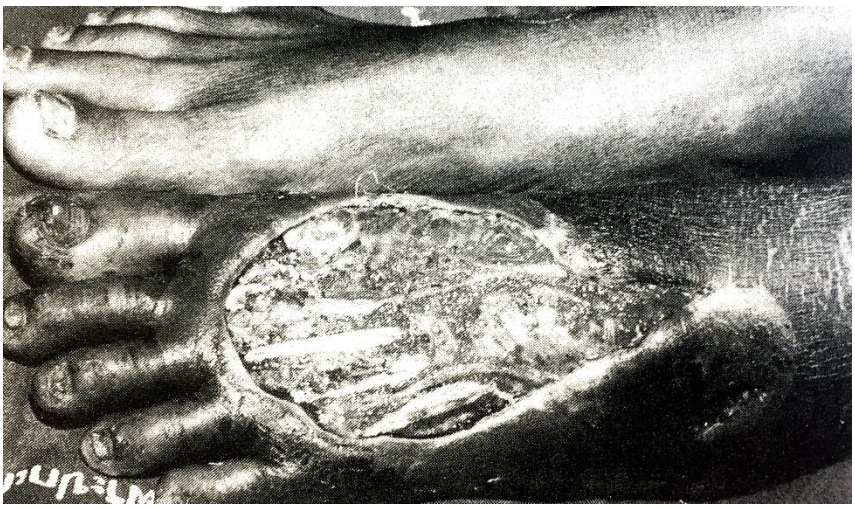
5.1. Introduction

5.1.i. Clinical symptoms of cobra envenomings

Globally, there are estimated to be to be 1,841,000 envenomings and 125,000 deaths a year from snakebite (Chippaux, 1998; Kasturiratne et al., 2008). Snake venom is a complex cocktail of toxins that has evolved with the purpose of subduing prey as quickly as possible to avoid prolonged confrontation. The extensive variation in symptoms of envenoming are fundamentally the result of differences in the toxin composition of these 'cocktails' between snake species, genera and families, which in turn is the result of variable evolutionary processes acting on snake venom toxins in different snake lineages. In general, bites from vipers cause extensive local swelling, haemorrhaging, coagulopathies and necrosis, while elapids tend to cause neurotoxic symptoms, and the medically-important colubrids (e.g. boomslang, twig snakes, keelbacks) cause disturbances in haemostasis (Gutiérrez et al., 2017).

Cobras are a type of elapid snake in which some species have evolved a defensive use for their venom, using muscular compression on the venom glands to eject venom through small holes at the front of each fang. This so called 'venom spitting' has evolved three times in cobras; twice in the genus *Naja* and once in the sister group *Hemachatus* (Wüster et al., 2007), a monospecific African genus. Cobra bites are frequent occurrences across much of their range in Africa and Asia, with most species coming under category 1 ("Highest medical importance") of the World Health Organisation (WHO) snakebite risk categories (see http://apps.who.int/bloodproducts/snakeantivenoms/database/default.htm). As snakebite occurs at higher frequency in impoverished areas, where access to medical facilities may be financially or physically limited (Chippaux, 1998), incidence and pathology data from cobra bites tends to be limited. Broadly speaking, bites by cobras are thought to result in neurotoxic symptoms and necrosis (Warrell, 2008c) (Fig. 5.1.). Clinical case studies relating to cobra envenomings are limited though (Table 5.1.), and those that exist in the literature predominately relate to bites by a small number of Asian cobra species. This may be due to the difficulty in

A)



B)

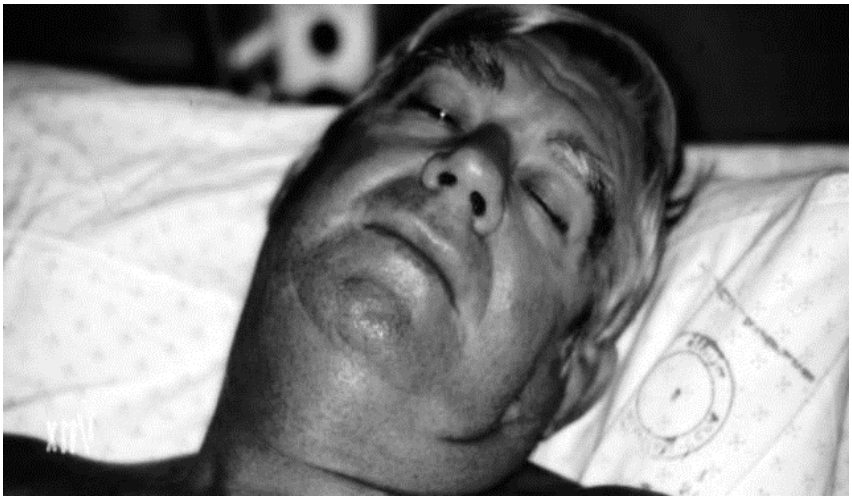


Figure 5.1. Symptoms of cobra bite. Featured are **A)** extensive necrosis of the foot 14 days following a bite from the Asian cobra *Naja kaouthia* (Warrell, 2008c) and **B)** ptosis (paralysis of the upper eyelid) following envenomation from the African cobra *Naja nivea* (Müller et al. 2012).

Table 5.1. A summary of clinical case studies from the literature involving cobra bite. Cases which contained only one patient, as well as those in which only specific bite symptoms were being looked for were excluded. Spitting species are highlighted in red.

				Number of patients exhibiting symptoms		
Citation	Species	No. of patients	Fatalities	Necrotic	Neurotoxic	Coagulopathic
(Bernheim *et al.*, 2001)	*Naja kaouthia*	3	0	2	2	0
(Blaylock, Lichtman and Potgieter, 1985)	*Naja nivea*	2	0	0	2	0
(Faiz *et al.*, 2017)	*Naja kaouthia*	70	2	19	58	0
(Khandelwal *et al.*, 2007)	*Naja kaouthia*	2	0	0	1	0
(Kularatne *et al.*, 2009)	*Naja naja*	25	2	8	9	3
(Muza *et al.*, 2004)	*Naja naja*	7	3	2	2	0
(Reid H. A., 1964)	*Naja sumatrana* & *Naja kaouthia*	47	2	20	4	0
(Strover, 1973)	*Naja mossambica*	2	0	2	0	0
(Tilbury, 1982)	*Naja mossambica*	17	0	13	0	1
(Trishnananda *et al.*, 1979)	*Naja kaouthia*	20	1	0	20	0
(Warrell *et al.*, 1976)	*Naja nigricollis*	14	2	10	0	14
(Warrell, Barnes and Piburn, 1976)	*Naja haje*	2	1	0	2	0
(Wang *et al.*, 2014)	*Naja atra*	292	0	292	0	0
(Watt *et al.*, 1988)	*Naja philippinensis*	39	2	3	38	0
(Wong *et al.*, 2010)	*Naja atra*	15	0	14	11	2
(Wongtongkam *et al.*, 2005)	*Naja kaouthia*	85	1	85	19	0
(Zouari and Choyakh, 1995)	*Naja haje*	2	0	0	2	0
	Total	545	15	385	150	6

attaining snakebite data from clinics in Sub-Saharan Africa, as many bites occur in rural areas where people may be hours or days away from a hospital, and many choose to seek traditional healers within the local community rather than seek medical aid (Warrell, 2010).

Nonetheless, from these case studies, cobra bites can be determined to result in a wide array of contrasting symptoms (Table 5.1.). This is perhaps best encapsulated by the description of necrosis as "the main clinical feature" of cobra envenoming from the Asian spitting cobra *Naja sumatrana* and non-spitting *N. kaouthia* (Reid, 1964). This same paper describes neurotoxic effects in human victims as 'rare', which conflicts with later observations by Strover (Strover, 1973), who noted that bites by African cobras typically result in neurotoxic pathology, which can be seen from the (admittedly limited) clinical manifestations of bites from *N. nivea* and *N. haje* (Blaylock, Lichtman, & Potgieter, 1985; Warrell, Barnes, & Piburn, 1976; Zouari & Choyakh, 1995). Cobra bites may additionally cause haemostatic disruptions, which may be seen in a small number of clinical cases from *N. nigricollis* (Warrell et al., 1976), *N. atra* (Wong et al., 2010) and *N. naja* (Kularatne et al., 2009), and in toxicological assays *in N. kaouthia* (Faiz et al., 2017). It seems then that the main function of cobra venoms in human victims has been in confusion for some time, and this is reflected by the large fluctuation in the proportion of patients that display neurotoxic or necrotic symptoms in the literature (Table 5.1.). Despite this, it is noted that symptoms of spitting cobra bites in Africa are generally necrotic, while non-spitting African cobras cause more neurotoxic symptoms such as ptosis and respiratory paralysis (Warrell, 2008c; WHO/Regional Office for Africa, 2010), whereas the symptoms of Asian cobras are often described as being both neurotoxic and necrotic, regardless of spitting ability (Warrell, 2008b; WHO/Regional Office for South-East Asia, 2016). The majority of cobra bite victims reported by the various studies outlined in Table 5.1 presented with substantial swelling and inflammation around the bite site, but as each paper usually details the symptoms of a single species, there is no cross-species comparison of function or analysis of differences between cobra species that can and cannot spit their venom.

In contrast to the pathologies that occur following envenoming caused by cobra bites, the symptoms observed following the introduction of venom into the eye of a victim, which is exclusively the results of encounters with spitting cobra species, are far more consistent. Such events initially cause a stinging or burning pain (Siraj & Joshi, 2012; Chu et al., 2010) and, in the majority of cases, progress into inflammation of different eye layers or a mild conjunctivitis (Siraj & Joshi, 2012; Goldman & Seefeld, 2010; Lanzetta et al., 2017; Warrell & Ormerod, 1976). In some cases, necrosis of the eye causes a temporary decrease in vision, which is usually recovered some time after treatment (Siraj & Joshi, 2012; Chu et al., 2010; Goldman & Seefeld, 2010). However, in rare cases, victims can become permanently blinded due to extensive corneal pathology (Warrell & Ormerod, 1976). There is no indication that the venom can be absorbed into the blood stream from ocular contact though, and as such systemic envenomings do not occur following venom spitting (Siraj & Joshi, 2012).

5.1.ii. Pre-clinical Analysis of Cobra Venoms

The issue with using clinical papers to determine the function of cobra venoms is that each study occurs at different hospitals or clinics. Subsequently, staff will have undergone different levels of training and each patient will be administered different doses of antivenom (if at all) at different times, making it difficult to determine definitive bite symptoms. Similarly, it is difficult to track the damage done by spitting cobra venom to the eye, as the pathological extent of damage depends on a variety of factors such as the volume of venom to hit the eye, whether both eyes were hit, the time taken to irrigate or otherwise treat the eye. Animal models offer a system to investigate the effects of snake venoms that is much easier to manipulate, as variables such as the site of injection, dose of venom, and time to pathology assessment can all be standardised. This comes with the necessary cost of the ethical implications of envenoming animals, potential species-specific effects in the animal model versus a human, and the low throughput nature of such studies.

Despite these inherent limitations, the necrotic effects observed in cases of human envenomings by cobras have been replicated in pre-clinical work. Both skeletal muscle necrosis and dermonecrosis have been shown to be caused by the actions of cytotoxic and cardiotoxic three finger toxins (3FTXs) found in *Naja*

nigricollis and *N. atra* venom, as well as PLA_2s to a partial extent (Ownby et al., 1993; Rivel et al., 2016). Cytotoxic 3FTXs from spitting cobras, as well as purified phospholipase A_2 (PLA_2) from *N. naja* venom, have been shown to cause severe dermonecrosis in murine models (Bhat & Gowda, 1989; Petras et al., 2011), as does whole *N. kaouthia* venom (Stringer et al., 1971). Furthermore, whole *N. nigricincta* venom has been shown to cause oedema and necrosis in rabbits (Kandiwa et al., 2018).

There have been several cases in which cobra venoms have been shown to affect coagulopathy in pre-clinical analysis, despite the lack of evidence of such activity in clinical case studies. Blood platelet counts and fibrinogen levels have been shown to drop with intramuscular injection of *Naja* venom (species unspecified) in rabbits (Rahman et al., 2006), and intravenous injection of a weak neurotoxin from *N. kaouthia* venom has been shown to cause drops in blood pressure in rats and mice (Ogay et al., 2005). Even oral ingestion of *N. naja* venom has been shown to cause a drop in blood cell and blood platelet count in rats (Malleswari et al., 2014).

Cobra venoms can also have extensive neurotoxic effects on animal models. Muscle paralysis resulting from cobra venoms have been observed in frogs (Rindani, 1952) and further respiratory paralysis shown in frogs, rabbits, cats, guniea pigs and dogs (Chou & Lee, 1969; Cushny & Yagi, 1916; Gode, Tandan, & Bhide, 1968). Other, less obvious neurotoxic effects have also been observed; mice injected with *N. kaouthia* venom are noted as becoming less mobile and more timid, with some of these effects remaining permanently (Mordvintsev et al., 2007). Additionally, *N. atra* venom injected into the infraorbital nerve of rats has been shown to cause learning and memory deficits (Zhang et al., 2017).

5.1.iii. In vitro Analysis of Cobra Venoms

Due to ethical concerns, lack of facilities or lack of animal technician training, animal models are not always suitable as a conduit for venom experimentation. *In vitro* analysis can allow venom or isolated venom components to be characterised without the need for an animal model, often greatly facilitating a higher throughput of data generation in a shorter amount of time.

Such studies have demonstrated that *Naja naja* venom possesses strong 5'-Nucleotidase and Phosphodiesterase activity and potent PLA_2 activity, and causes degeneration of fibrinogen in human plasma, resulting in prolonged thrombin and thromboplastin times (Wong et al., 2018). The venoms of *H. haemachatus*, *N. melanoleuca*, *N. nigricollis* and *N. mossambica* have also been shown to exhibit high PLA_2 activity (Lauridsen et al., 2017; Sánchez et al., 2018), and *N. melanoleuca* and *N. nigricollis* have additionally been shown to have anticoagulant effects through decreasing thrombin and thromboplastin production (Mackay et al., 1969). Furthermore, the venom of *N. nigricincta* has been shown to have procoagulant and thrombolytic activity on goat blood (Kandiwa et al., 2018). It has been suggested that cobra PLA_2s prevent the clotting of blood by binding to factor Va, factor Xa or both, providing a link between PLA_2 activity and haemolytic activity seen in cobra venom (Stefansson et al., 1990), and isolated proteins from *N. nigricollis* venom identified as PLA_2s were found to cause anticoagulant activity (Evans et al., 1980). Additionally, anticoagulant PLA_2 and 3FTX fractions have been found in *H. haemachatus* venom (Sánchez et al., 2018a), and the PLA_2 inhibitor varespladib has been shown to neutralise anticoagulant activity caused by spitting cobra venoms (Bittenbinder et al., 2018). However, PLA_2s from *N. kaouthia* venom have also been shown to cause neuromuscular blockade on rat and chick muscle preparations (Reali et al., 2003), once again suggesting multiple interactions and functions for PLA_2s in cobra venoms (Ferraz et al., 2019).

The venoms of *N. nigricollis*, *N. pallida*, *N. nubiae*, *N. kaouthia* and *N. mossambica* have all been shown to be highly cytotoxic to human leukemic U937 and K562 cell lines and myogenic cell line C2C12 (Debnath et al., 2010; Méndez et al., 2011). Additionally, the venoms of Asian cobras and African spitting cobra are highly cytotoxic against the human neonatal foreskin fibroblast and melanoma MM96L cell lines (Panagides et al., 2017). This extensive cytotoxicity, particularly in African spitting cobras, corresponds with the often-necrotic bite symptoms of these species (Warrell, 2008c) and is likely a result of the combination of high abundance of cytotoxic 3FTXs (CTXs) in the venom and moderate abundance of PLA_2s, known to possess some cytolytic functions (Dennis et al., 2011; Kini, 1997; Sunagar, Jackson, & Reeks, 2015), in the proteome (Fig. 3.2.).

Lastly, cobra venoms in general have been shown to have weak L-amino acid oxidase and SVMP activity (Modahl et al., 2016; Wong et al., 2018). The extent of the activity from other toxins is not-well document, probably due to being mostly-absent from the proteome (see chapter 3) (Tasoulis & Isbister, 2017).

5.1.iv. Chapter Overview

To date, there have been few comparative studies on the enzymatic or coagulopathic activities of cobra venoms (Bittenbinder et al., 2018; Panagides et al., 2017; Tan, Wong, Tan, et al., 2019). This chapter seeks to redress this literature deficiency by describing the comparative venom activities of cobra venoms in a series of distinct in vitro functional assays. The resulting data was examined to test whether any specific functional activities correlate with venom spitting, thereby suggesting that those functional activities might be associated with this defence specific adaptation.

In the previous chapters, it was determined that the vast majority of cobra venoms consist predominately of two main toxin families at the molecular level; three finger toxins (3FTXs) and phospholipase A_2s (PLA_2s). However, it is unlikely that the function of snake venoms can be predicted by molecular or proteomic data alone, as venom composition is dictated by the regulation of protein translation and post-translational modifications (Casewell et al., 2014; Durban et al., 2013), relating to often multifunctional venom toxins (Ferraz et al., 2019). To this end, I characterised the functional activity of the major enzymatic components found in 18 cobra snake venoms, namely PLA_2s, SVMPs and a lower abundance toxin family, the snake venom serine proteases (SVSPs), before characterising their coagulopathic activity in a small-scale plasma clotting assay.

The findings here demonstrate that: (i) all venoms exhibit anticoagulant activities, (ii) the potency of anticoagulant venom activity is likely influenced by the activity of PLA_2 toxins, and (iii) PLA_2 activity is positively correlated with venom spitting. This distinctiveness in PLA_2 function derived from spitting cobra venom could imply a defensive advantage that has been selected for, which would be the first recorded instance that venom composition in snakes has evolved as a result of defence rather than for prey acquisition.

5.2. Methods

5.2.i. Venoms Used

All venoms were extracted and pooled from snakes housed in the Herpetarium at the Centre for Snakebite Research & Interventions at the Liverpool School of Tropical Medicine. The species used to produce pooled venom samples are described in Table 3.2. Venoms were lyophilised and stored at 4°C until use. Approximately 10mg of each venom was reconstituted in 1mL PBS (pH 7.4) to yield 10mg/mL solutions, which were then stored at -80 °C until use.

5.2.ii. PLA_2 Activity

To quantify enzymatic PLA_2 activity, a recently-developed colorimetric absorbance-based assay was used (Neumann et al., 2020). The assay measures PLA_2 activity by quantifying colour change, mediated by the use of cresol red dye, caused by a pH change that results from the degradation of the phosphatidylcholine substrate into glycerophosphoric acids by PLA_2s. The change in the ability of the sample to absorb light as the substrate breaks down is then measured kinetically by a spectrophotometer at 572nm.

First, the PLA_2 reaction solution was prepared using 33.33µL of 49.5µM Cresol red dye, 25.75µL of 0.875mM Triton X-100 (made through diluting a 1.7M solution with deionised water) and 1mL of 5x salt mix, which was prepared using 0.29g of sodium chloride, 0.375g of potassium chloride and 0.55g of calcium chloride, and then made into a 10mL solution using 1mM Tris base, pH 8.5. The final reaction solution was made to 5mL using 1mM Tris base, pH 8.5. Each of the stock venoms were diluted to concentrations of 0.1µg/mL, 1µg/mL, 10µg/mL and 100µg/mL using 1mM Tris buffer (pH 8.5) and pipetted into wells of a Greiner Bio-One clear 384-well microplate at volumes of 10µL, and in replicates of 4 per concentration. Control wells contiained TRIS buffer only. Subsequently, 168.25µL of the substrate (L-α-phosphatidylcholine; stock concentration 26mM; SIGMA Life Sciences, PCode: 1002502139) was added to the 5mL reaction solution. The pH of this mixture was adjusted to 8.5 using either sodium hydroxide or hydrochloric acid, and 40µL was then added to each well of the plate using an Ergo One multidrop pipette, supplied and calibrated by StarLab. Immediately after addition, the plate was read on an FLUOstar® Omega plate reader.

The plate reader was set to a temperature of 25°C and reading kinetically at a wavelength of 572nm. The number of cycles was set to 42 and the cycle time was 46-47 seconds. After the plate was read, the data was extracted using Multivariate Adaptive Regression Splines® (MARS) software and exported to Microsoft Excel. PLA_2 activity was measured as the mean area under the concentration curve (mAUCC). Concentration curves were generated for each venom by plotting the mean control AUC of absorbance at 572nm, minus the mean area under the curve (AUC) for readings at four different concentrations of venom (0.1µg/mL, 1µg/mL, 10µg/mL and 100µg/mL) in replicates of four, using the run period from 0-10 minutes (when the substrate in at least one sample showed evidence of complete depletion).

5.2.iii. SVMP Activity

Snake venom metalloproteinase (SVMP) activity was measured via changes in fluorescence resulting from the cleavage of the target substrate (Mca-K-P-L-G-L-Dpa-A-R-NH_2 Fluorogenic Peptide Substrate IX; R&D Systems, catalog number ES010) by SVMPs. The substrate was initially reconstituted into a 6.2mM solution via dilution with dimethyl sulfoxide (DMSO). Subsequently, 10µM working concentrations were prepared by diluting 9µL of 6.2 mM substrate in 5mL assay buffer, which was in turn prepared from a 50mL stock of 150mM sodium chloride solution containing 50mM Tris-HCl (pH 7.5-8). Due to the light-sensitivity of the substrate solution, it was stored in the dark at 4°C when not in use. Immediately prior to each experiment, 10µL of the substrate was added to the 5mL reaction buffer. Subsequently, 10µL of 1mg/mL venom samples were pipetted in triplicate into the wells of a Greiner Bio-One clear 384-well microplate, followed by 90µL of the reaction buffer containing the substrate. Control wells were prepared in the same manner except containing 10µL of Phosphate Buffered Saline (PBS) in the place of venom. An Omega FluoSTAR spectrophotometer was used to measure the experimental reaction kinetically at 25°C, with an excitation wavelength of 320nm and emission at 405nm. The gain adjustment was set to 5% of a well expected to contain SVMP activity. The assay was run for 160 cycles at 22 seconds per cycle and the data was recorded on the MARS software and exported to Excel. SVMP activity was measured as the mean control

AUC for fluorescence intensity, minus the mean area under the curve (AUC) for readings at 100µg/mL venom in triplicate, multiplied to the power of -1.

5.2.iv. Serine Protease Activity

Serine protease (SP) activity was measured via changes in fluorescence following the cleavage of a specific substrate (H-D-Ile-Pro-Arg-pNA•2HCl, Chromogenix, S-2288) by serine proteases. To prepare the assay, the substrate was made into a 6mM solution using deionised water. Due to its sensitivity to light, the resulting stock was coated with foil and stored between 2-8°C until use. Next, 15µL of 1mg/mL venom was pipetted into a Greiner Bio-One clear 384-well microplate in quadruplicate. As the assay is run at 37°C, the venoms were incubated at 37°C for three minutes inside the FLUOstar plate reader to avoid a large temperature gradient upon performing the assay. Subsequently, 15µL of the reaction buffer, consisting of 100mM Tris pH 8.5 and 100mM NaCl, was pipetted into the same wells and the plate underwent another three-minute incubation period at 37°C. The substrate was then pipetted at 15µL into the wells using a 10-100µL Ergo One multidrop pipette and the plate was read by an FLUOstar Omega spectrophotometer at 405 nm for 70 cycles at 30 seconds per cycle. Serine Protease (SP) activity was measured as the gradient of mean fluorescence intensity over time of 1mg/mL venom samples in replicates of four, as produced by the cleaving of the substrate.

5.2.v. Effects on Plasma Clotting

To quantify the coagulopathic effect of the various cobra venoms, plasma clotting assays were performed using the recently described protocol (Neumann et al., 2020). Citrated bovine plasma (Equitech-Bio, SBPUC35-0100), which was stored at -80°C, was first defrosted at room temperature and, once thawed, centrifuged at 2000 x g for 4 min to remove precipitates. The supernatant was then transferred into a 15 mL Falcon tube. 10µL of each venom samples were pipetted in quadruplicate onto a Greiner Bio-One clear 384-well microplate at concentrations of 1µg/mL, 10µg/mL and 100µg/m in triplicate; 10µL PBS was pipetted into control wells. A 50mL solution of 20mM calcium chloride was freshly made for each experiment using deionised water and pipetted onto the plate using a Thermo Scientific™ Multidrop™ 384

Labsystems multidrop pipette. The multidrop was then flushed with water before being used to overlay the plate with 20µL of plasma into each well. The plate was then read kinetically at a wavelength of 595nm and a temperature of 25°C using a FLUOstar Omega plate reader. The assay was run for 54 cycles at a cycle time of 141 seconds.

For venom inhibition experiments, venom samples were mixed with toxin family-specific inhibitors, and the reactions consisted of 1µL of venom, 3.75µL of either 6mM of the PLA_2 inhibitor Varespladib (Sigma-Aldrich, SML1100-5MG) or 6mM of the SVMP inhibitor marimastat (Sigma-Aldrich, M2699-5MG) and 5.25µL PBS. Venom controls consisted of 1µL venom and 9µL PBS, and as the inhibitors were reconstituted in Dimethylsulfoxide (DMSO), both an inhibitor control and DMSO control were set up, consisting of 3.75µL Varespladib/Marimastat or DMSO and 6.25µL PBS. All reaction mixtures were then incubated at 37°C for 30 minutes before the addition of calcium chloride and plasma, and measurements collected as outlined above.

General coagulant activity was measured as the mean area under the concentration curve (mAUCC), standardised by dividing against the mAUCC of the results from *Dendroaspis jamesoni jamesoni* run on the same plate, which was used as a standard as not all of the study species could be fit onto a single plate, and inter-plate result variability was high. Concentration curves were generated for each venom by plotting the mean control AUC minus the mean area under the curve (AUC) for readings at three different concentrations of venom (1µg/mL, 10µg/mL and 100µg/mL) in replicates of four, using the run period from 0-100 minutes. For measuring the effect of inhibitors on clotting ability of cobra venoms, results were plotted as the mean AUC of each control type (PBS control for crude venom samples, 6mM marimastat, and 6mM varespladib) minus the AUC of each sample (venom concentration 100µg/mL). The effect of isolated toxins on bovine plasma were measured as the mAUCC for venoms at concentrations of 10µg/mL, 100µg/mL and 1000µg/mL.

5.2.vi. Statistical Analyses

To determine whether spitting cobra venoms exhibited significantly different functional activity to non-spitting cobra venoms, assess the potential influence of geography at the functional level, and give phylogenetic context to the above analyses (as per Chapter 4), Phylogenetic Generalized Least Squares (pGLS) was performed using the 'pgls()' function of the R package 'caper' in R Studio version 1.1.423 (Orme et al., 2018), setting the lambda value to "maximum likelihood" for all factors. Additionally, discrete and continuous characters were reconstructed on to an unpublished species tree, generated based on a combined analysis of mitochondrial and nuclear markers and provided by Dr. Wolfgang Wüster (Bangor University, UK), using the Equal Rates model in the rerootingMethod() and contmap() functions of the R package "phytools" (Revell, 2012).

5.3. Results

5.3.i. SVMP Activity

In general, cobra venoms exhibited no detectable SVMP activity (Fig. 5.2), with the few exceptions being the venoms of *N. subfulva, N annulifera, H. haemachatus* and *N. kaouthia*. However, when the SVMP activities of these species were compared with that of a viper (*Calloselasma rhodostoma*), known generally to have high levels of SVMP expression and activity than their elapid counterparts (Tasoulis & Isbister, 2017), these cobra SVMP activities are found to very low (Fig. 5.2). While non-spitting cobras (three of the four species with activity) were found to have a higher mean SVMP activity than spitting cobras (155615.57 and -126784.17, respectively), when comparative statistics were run to test for differences in SVMP activity between spitting and non-spitting cobras, unsurprisingly, no significance was detected (PGLS, $t = -1.98$, $p = 0.07$) (Fig. 5.3.), nor were there significant differences between African and Asian cobras ($t = -0.99$, $p = 0.34$)(Table 5.2.).

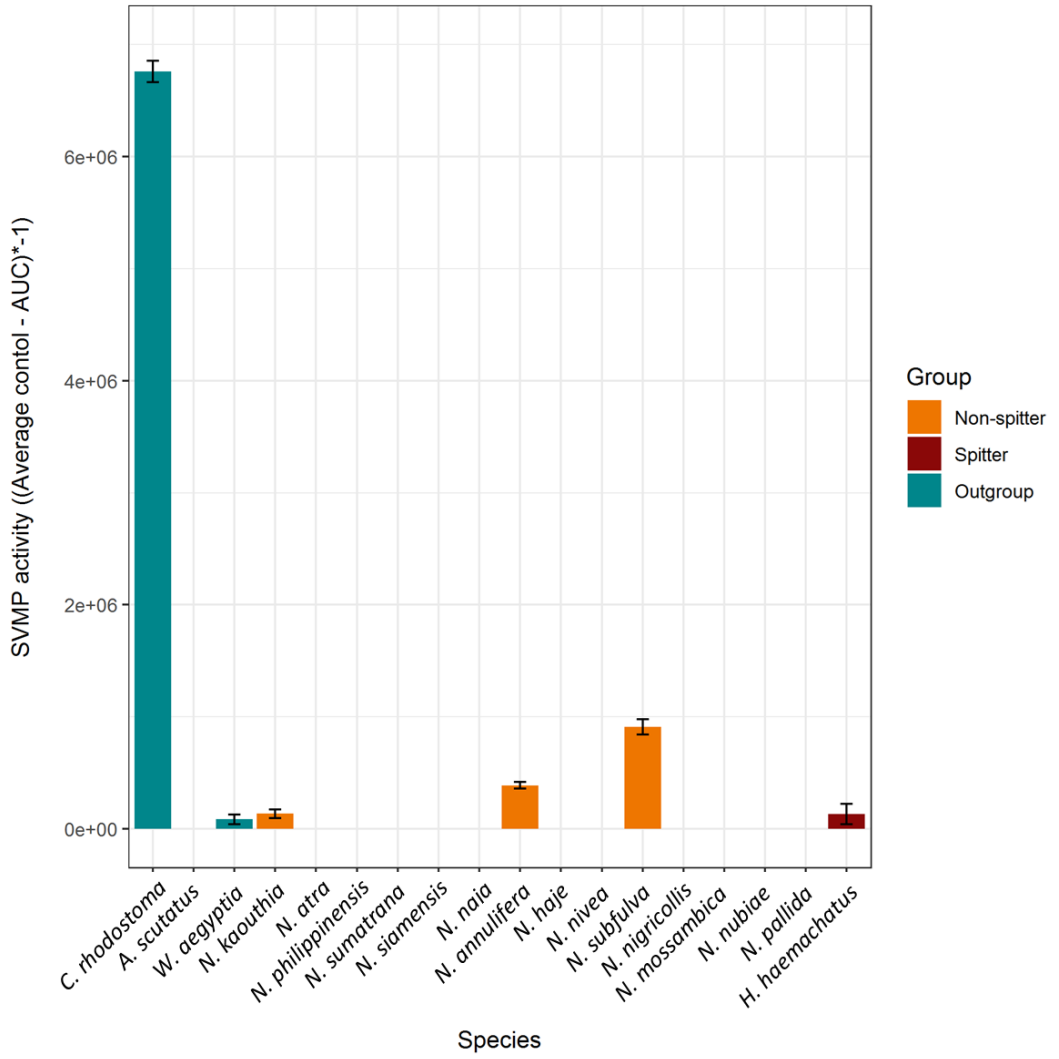


Figure 5.2. Bar chart of the snake venom metalloproteinase (SVMP) activity of cobra venoms in comparison with that of the viper *Calloselasma rhodostoma*. SVMP activity is measured as the mean of the control area under the curve (AUC) minus individual AUC of fluorescence intensity, multiplied by -1. Each sample was measured in triplicates of 10µL and error bars represent standard errors of the mean.

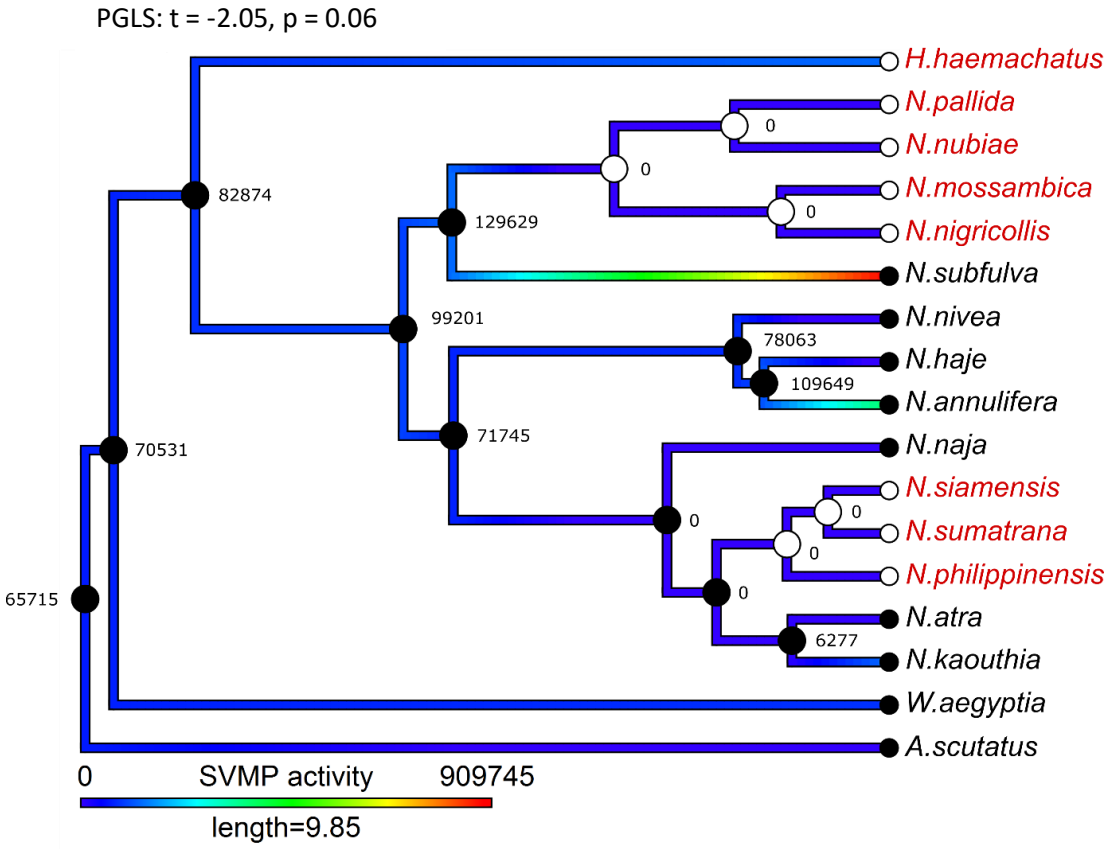


Figure 5.3. Ancestral trait estimation of Snake Venom Metalloprotease (SVMP) activity against the phylogeny of cobras. The Phylogenetic generalized least squares (PGLS) output of spitting vs. SVMP activity is labelled above. Spitting species are highlighted in red. The species tree was made by pruning a larger tree received from Wolfgang Wüster.

Table 5.2. PGLS output Table to determine the influence of spitting, geography, and the combination of both factors on the functional properties of the venoms from *Naja* and *Haemachatus* in the context of species phylogeny.

Test Variable	Factor	t value	p
SVMP activity	Group ("spitter or "non-spitter")	-2.05	0.06
	Geography (Africa or Asia)	-1.65	0.12
SP activity	Group ("spitter or "non-spitter")	-0.62	0.54
	Geography (Africa or Asia)	0.35	0.73
PLA_2 activity	Group ("spitter or "non-spitter")	0.69	0.50
	Geography (Africa or Asia)	1.00	0.33
Anticoagulant activity	Group ("spitter or "non-spitter")	1.47	0.16
	Geography (Africa or Asia)	1.46	0.17

5.3.ii. Serine Protease Activity

The results of the SP assay revealed that all cobra venom exhibit serine protease activity, with the venom of *H. haemachatus* being the most potent (Fig. 5.4.). However, similarly to SVMP activity, when the SP activity of the cobra venoms was contextualised via comparison with that of *Calloselasma rhodostoma*, the resulting activities were all found to be low (Fig. 5.4.). The mean SP activity of spitting and non-spitting cobras is equal (0.001), indicating there is likely no association between SP activity and emergence of venom spitting. Reconstructing the evolutionary history of this trait across the cobra species tree reveals an moderate increase in SP activity at the base of the Asian cobra radiation (Fig. 5.5.), however PGLS analysis detected no significant association between SP activity with the difference geographical locales of cobras ($t = 1.61$, $p = 0.13$), in addition to the origin of venom spitting ($t = -0.71$, $p = 0.49$) (Table 5.2.).

5.3.iii. PLA_2 Activity

All of the cobra venoms tested exhibited some degree of enzymatic PLA_2 activity, however this was substantial in spitting cobras and the elapid outgroup *Aspidelaps scutatus* (Fig. 5.6A.). These species had results comparable to the viper outgroups, *Bothrops asper* and *Daboia russelli*, which are generally known to have moderate-

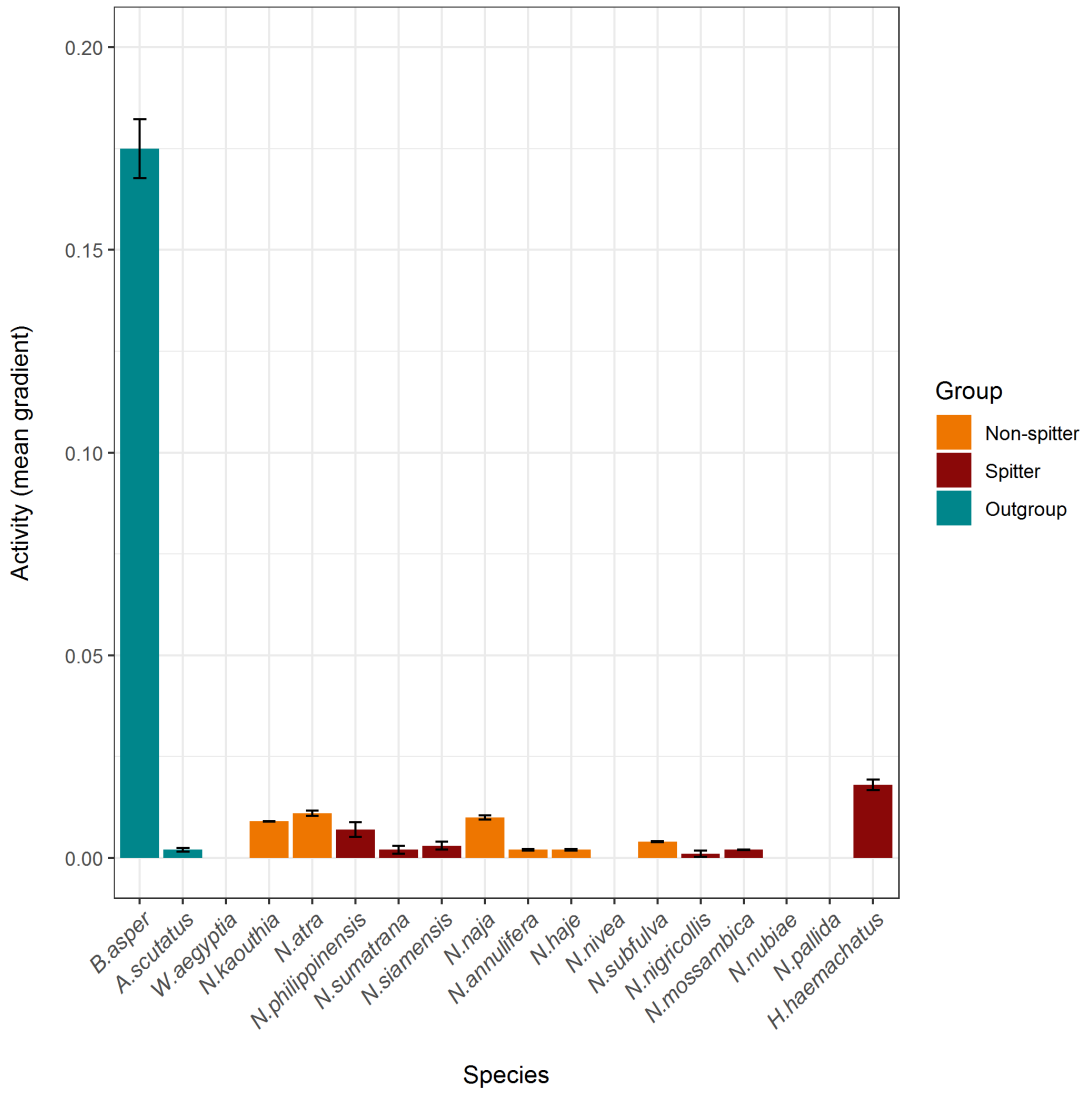


Figure 5.4. Bar chart of the enzymatic serine protease (SP) activity of cobra venoms as compared to that of the viper *Bothrops asper*. SP activity is represented by the gradient of the line produced by fluorescence intensity over time. Each sample was measured in 4 replicates of 1mg/ml.

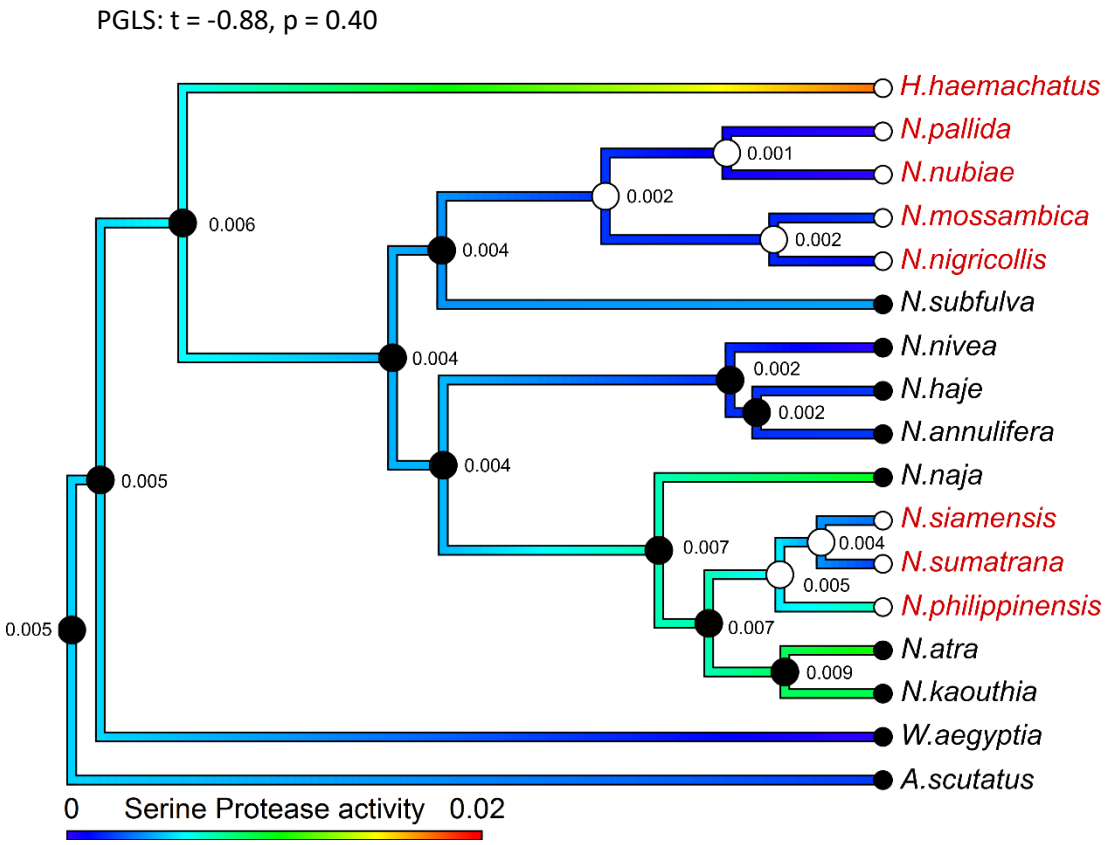


Figure 5.5. Character mapping and ancestral trait estimation of serine protease (SP) activity against the phylogeny of cobras. The Phylogenetic generalized least squares (PGLS) output of spitting vs. SP activity is labelled above. Spitting species are highlighted in red. The species tree was made by pruning a larger tree received from Wolfgang Wüster.

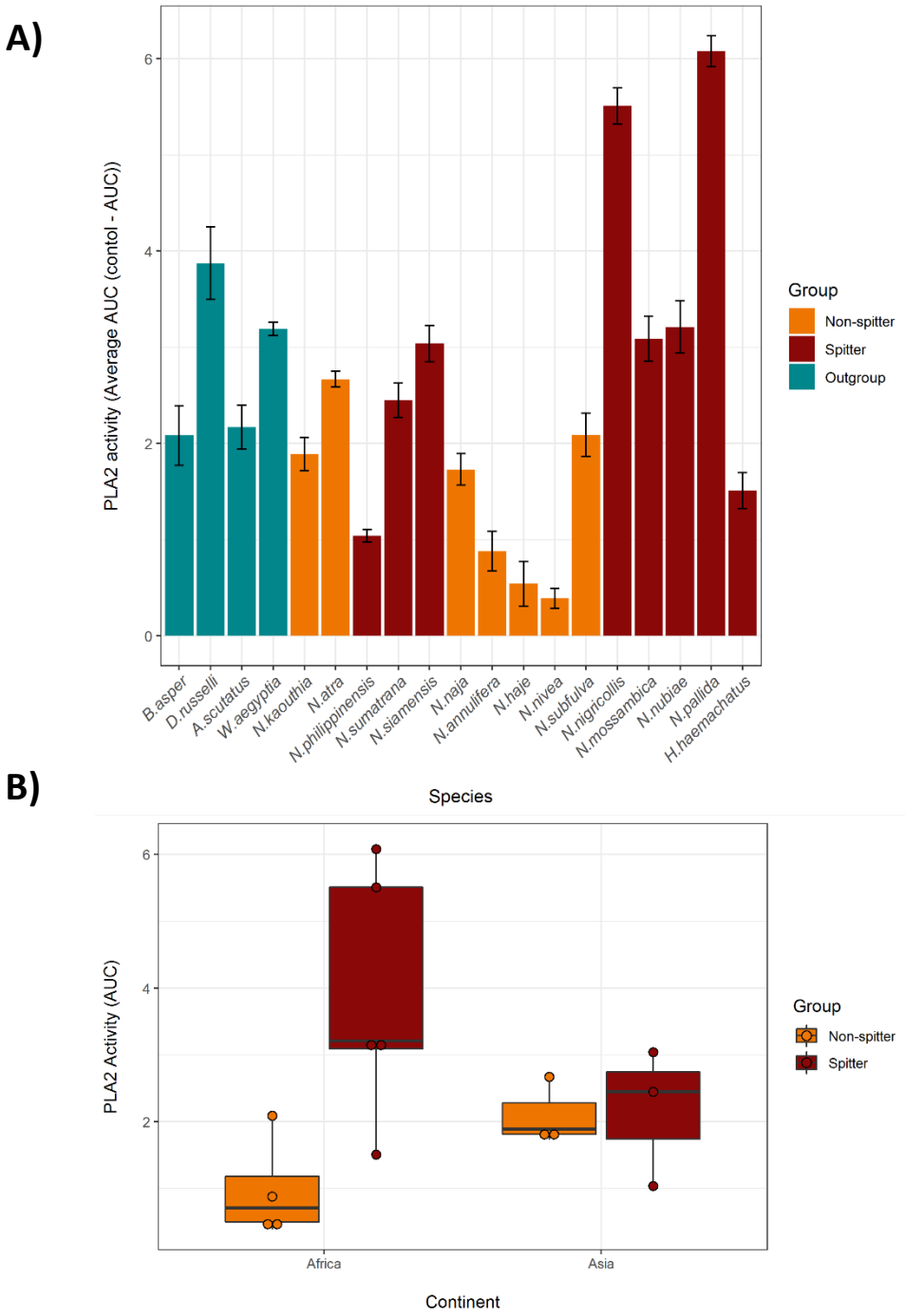


Figure 5.6. The phospholipase A_2 (PLA_2) Activity of cobra venoms, compared to the viper outgroups *Bothrops asper* and *Daboia russelli*. Represented by **A)** bar graph showing species-level comparisons **B)** box-and-whisker plot showing group-level comparisons and separated by continent. PLA_2 activity was measured as the mean area under the concentration curve (mAUCC). All venoms were pipetted as replicates of 4, in 10µL samples. Boxes show the interquartile range of each dataset. The emboldened horizontal line represents the median value. Horizontal lines protruding from the boxes cover data points that fall within 1.5x the interquartile range.

high abundance of PLA_2 in their venoms (Tasoulis & Isbister, 2017). These species were chosen as viper outgroups as opposed to *C. rhodostoma* due to their venoms displaying PLA_2 activity more typical to what would be expected from viper venoms in assays previously run by my colleague, Laura-Oana Albescu, while *C. rhodostoma* showed unusually low activity for the viper grouping and thus would not make for a realistic comparison. Spitting is not significantly associated with higher enzymatic PLA_2 activity ($t = 0.69$, $p = 0.5$) (Table 5.2.), though this may be due to the mean activity from African spitters being nearly double that of Asian non-spitting cobras (mAUCC 3.88, compared to 2. 10) and over triple that of African non-spitters (mAUCC 3.88, compared to 0.98), while the mean activity of Asian spitters was much closer to their non-spitting counterparts (mAUCC 2.18, compared to 2.10) (Fig. 5.6B.). PLA_2 activity was also the most variable for African spitting cobras, ranging from a mAUCC of 1.51 to 6.08, compared to 1.04-3.04 in Asian spitters, 0.39-2.09 in African non-spitters and 1.73-2.67 in Asian non-spitters (Fig. 5.6B.).

Reconstructing the evolutionary history of this trait across the cobra species tree reveals moderate to high PLA_2 activity as the ancestral state for this group, with losses of activity observed occurring independently in both *N. philippinensis* and the clade of African non-spitting species (Fig. 5.7.). PLA_2 potency was also observed to increase moderately at the base of the African spitting clade, with further increases occurring independently in *N. nigricollis* and *N. pallida* (Fig. 5.7). There is no significant association of enzymatic PLA_2 activity with geography ($t = -0.19$, $p = 0.85$) (Table 5.2.).

5.3.iv. Coagulopathic venom activity

Under normal conditions, when stimulated with calcium, citrated plasma will clot, resulting in an increase in absorbance over time following recalcification. All cobra venoms tested, including those of the outgroups not considered 'true cobras' (*Walterinnesia aegyptia* and *Aspidelaps scutatus*), caused an anticoagulant effect on the clotting ability of bovine plasma, resulting in delays to increases in absorbance ranging from a mAUCC of 5.27(in *N. annulifiera*) to 15.78 (in *N. sumatrana*) (Fig. 5.8.). Spitting cobras venoms cause a greater anticoagulant effect that than non-spitting cobras (mean of 9.90 and 7.72, respectively), although spitting itself is not

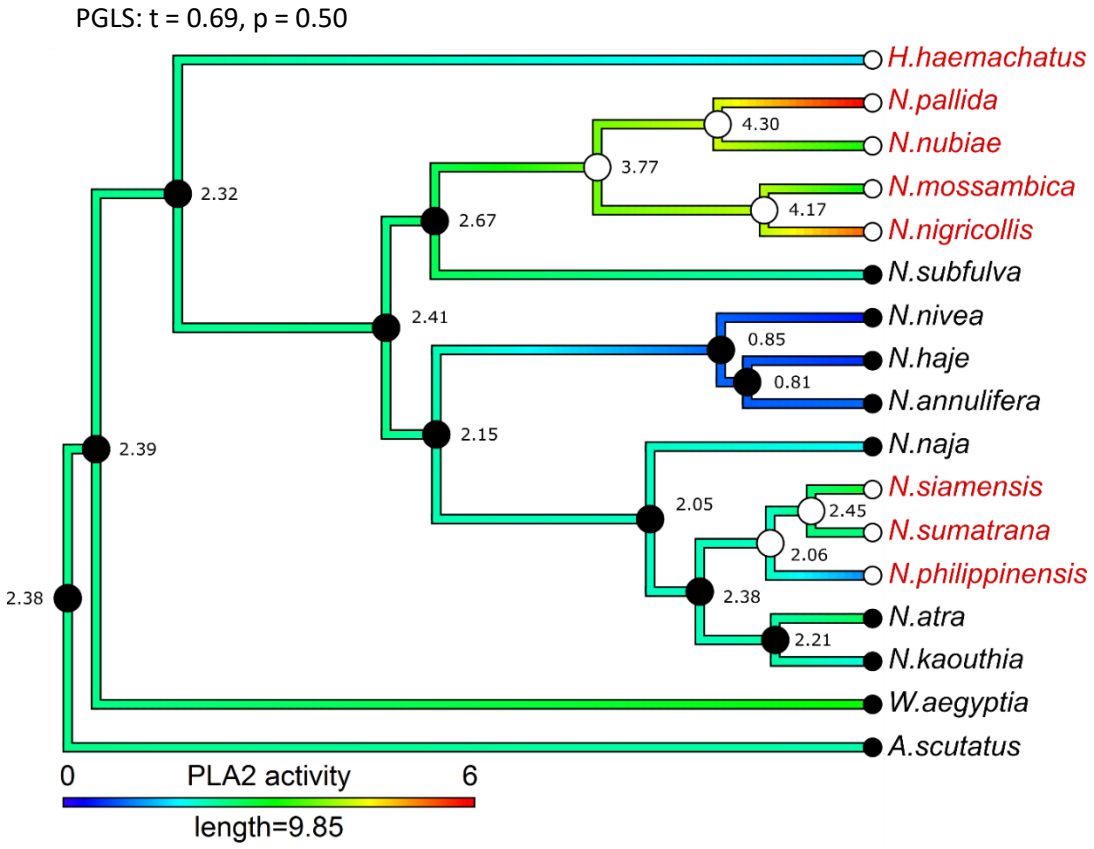


Figure 5.7. Character mapping and ancestral trait estimation of Phospholipase A_2 (PLA_2) activity against the phylogeny of cobras. The Phylogenetic generalized least squares (PGLS) output of spitting vs. PLA_2 activity is labelled above. Spitting species are highlighted in red. The species tree was made by pruning a larger tree received from Wolfgang Wüster.

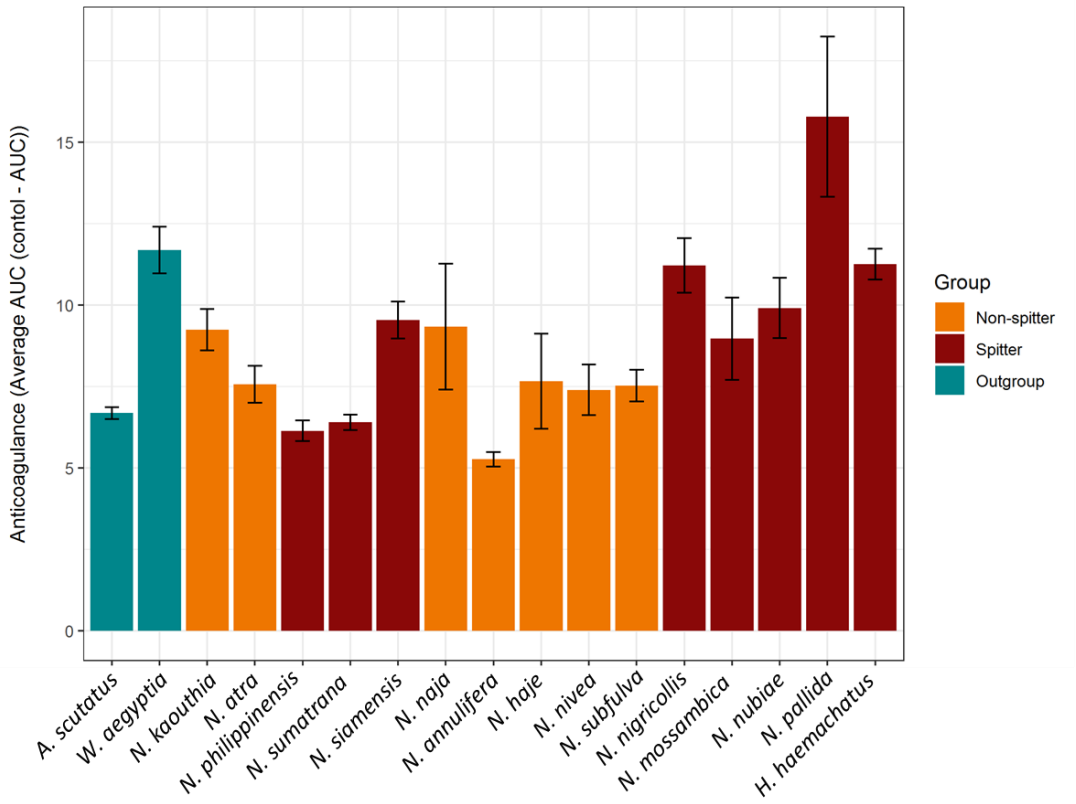


Figure 5.8. Bar chart of the effect of cobra venom on bovine plasma. The data presented is displayed as the was measured as the mean area under the concentration curve (mAUCC), taken from absorbance data collected at venom concentrations of 1µg/ml, 10µg/ml and 100µg/ml. This, the control measurements of normal clotting in the presence of no venom is represented by 0. All measurements were collected in quadruplicate, with means displayed and standard errors of the means represented by error bars.

significantly associated with anticoagulant activity (PGLS, t = 1.54, p = 0.14) (Table 5.2.). Character correlation analysis (Fig. 5.9.), reveals that anticoagulant venom activity appears to be an ancestral trait in this group of elapid snakes, but that anticoagulant potency has been reduced in the monophyletic group consisting of the Asian cobras and their closest African non-spitting cobra relatives, as well as in *N. subfulva* and *A. scutatus*. Contrastingly, anticoagulant venom potency appears to have slightly increased in the African spitting cobra clade, and is further enhanced in *N. pallida* (Fig. 5.9.). There is no association of anticoagulant activity with geography (t = -1.04, p = 0.32).

5.3.v. Identification of anticoagulant toxins

To investigate the toxin proteins responsible for causing the anticoagulant activity described above, I first co-incubated cobra venoms independently with two different inhibitory molecules; the phospholipase A_2 inhibitor varespladib, and the SVMP inhibitor marimastat. The addition of 6mM varespladib (Lewin et al., 2016) to cobra venoms resulted in dramatic reductions in anticoagulant activity in all species, with many venoms being neutralised to baseline levels, although the venoms of *N. nigricollis* and *N. nivea* were least effected (40.82% and 36.97% reduction in activity, respectively) (Fig. 5.10B.). Overall, treatment with varespladib resulted in significant decreases in anticoagulant activity (Analysis of Variance of Aligned Rank Transformed Data, F = 96.33, p < 0.001). The addition of the SVMP inhibitor Marimastat (A. H. Drummond, 1995) had no inhibitory effect on the coagulopathic venom activities observed, with the exception of a fairly substantial 37.82 reduction in activity in *N. annulifera* venom (Fig. 5.10A.). Indeed, marimastat actually caused an average of 10.56% increased anticoagulant activity in all other samples, and these were statistically significant (Analysis of Variance of Aligned Rank Transformed Data, F = 17.14, p < 0.001).

To test the hypothesis that PLA_2 toxins are the major venom components responsible for anticoagulant venom activities, next fractions of venom toxins separated from *N. nigricollis* venom were used in the plasma clotting assay. Major venom constituents of *N . nigricollis* venom (3FTXs and PLA_2s) were fractionated by Dr. Mark Wilkinson using electron gel filtration chromatography. This resulted in

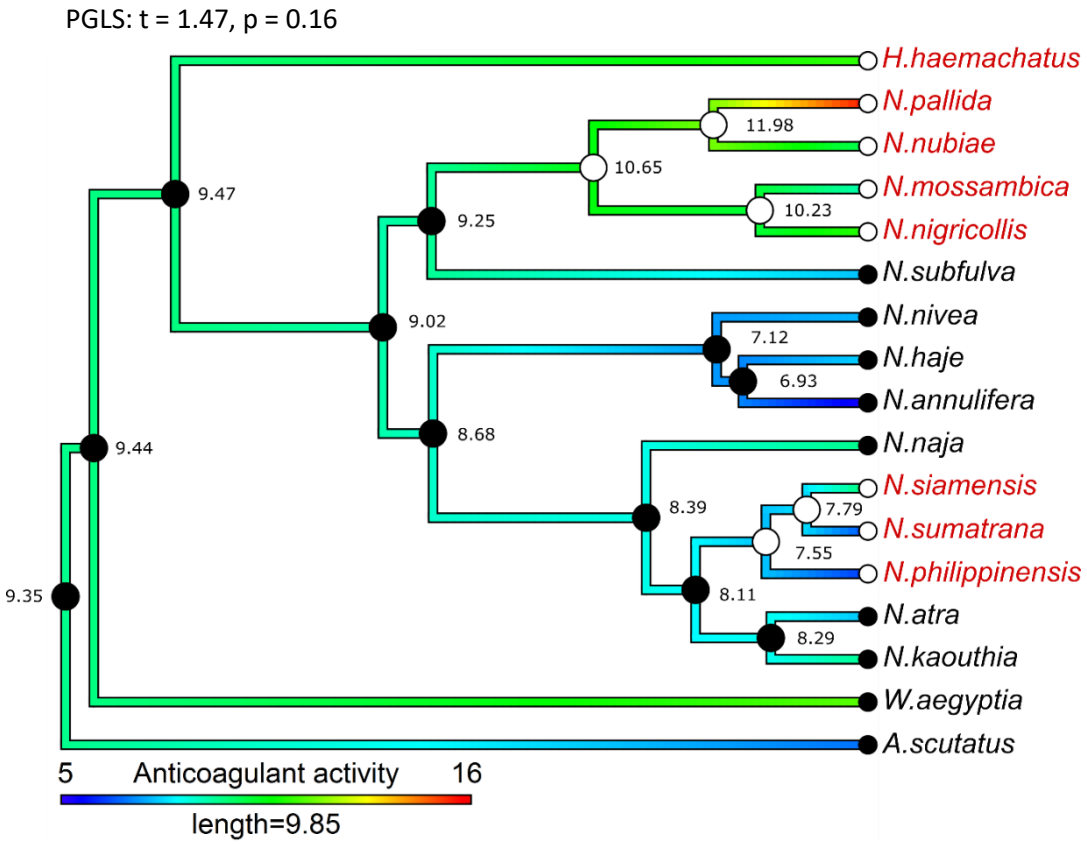


Figure 5.9. Character mapping and ancestral trait estimation of anticoagulant activity against the phylogeny of cobras. The anticoagulant activity scale represents area under the curve values, and node numbers reflect ancestrally reconstructed venom activity of hypothetical ancestors. The Phylogenetic generalized least squares (PGLS) output of spitting vs. anticoagulant activity is labelled above (p = 0.14). Spitting species are highlighted in red. The species tree was made by pruning a larger tree received from Wolfgang Wüster.

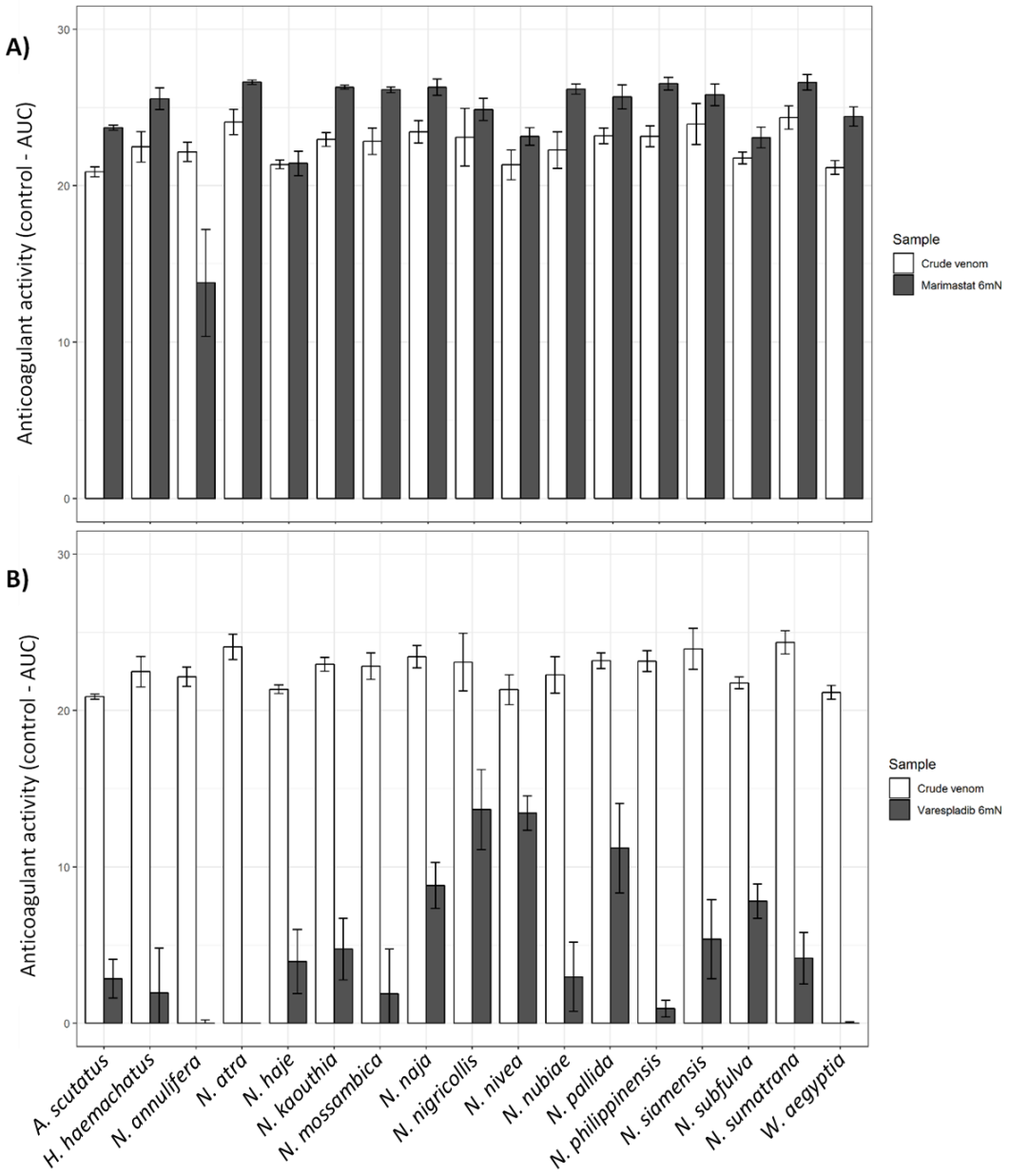


Figure 5.10. Anticoagulant activity of venoms with the addition of the A) the SVMP inhibitor Marimastat (Mars) or B) the PLA_2 inhibitor Varespladib (Vars). The data represents the average of the average area under the curve of each positive control (PBS for crude venoms, Marimastat and Varespladib), minus the area under the curve for each respective sample (venom concentration 100µg/ml). Error bars represent the standard error of the mean.

fractions, identified as PLA_2 and 3FTX proteins by subsequent gels. These fractions were then tested for anticoagulant venom activity in the bovine plasma assay at concentrations of 1mg/mL, 0.1mg/mL and 0.01mg/mL. Although neither the PLA_2 nor 3FTX fraction resulted in anticoagulant activities comparable with the crude venom, the PLA_2 fraction was found to exhibit significantly greater anticoagulant activity than the 3FTX fragment (Tukey post-hoc test, diff = 24.56, df = 3, p = 0.00001) (Fig. 5.11.) – findings in line with those described using the PLA_2 inhibitor varespladib. However, the 3FTX fraction did exhibit some anticoagulant effects, suggesting that both groups of these toxins may contribute towards this venom activity somewhat, but PLA_2s to a greater extent than 3FTXs. To our surprise, the simultaneous addition of a 1:1 ratio

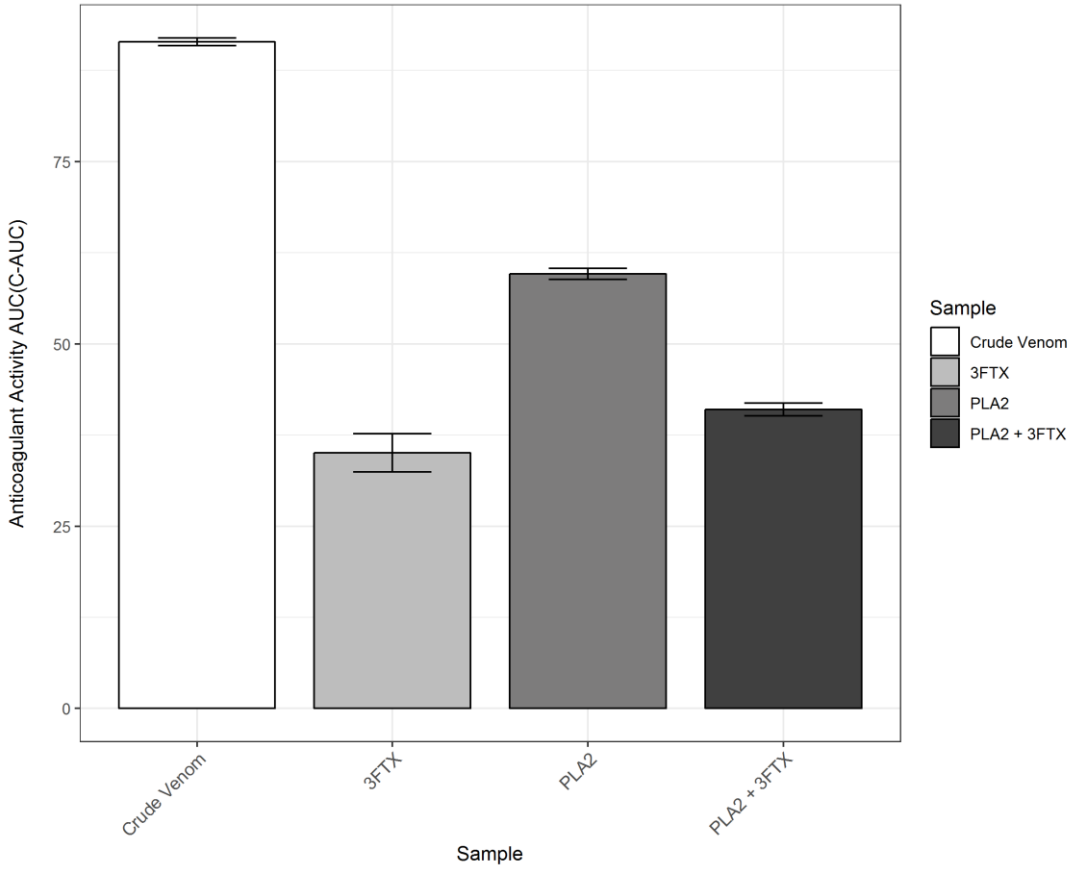


Figure 5.11. Bar chart of the anticoagulant activity of PLA_2 and 3FTX fragments extracted from Naja nigricollis venom. Bars represent the combined outcome of standardised concentrations of 1mg/ml, 0.1mg/ml and 0.01mg/ml. Anticoagulant activity was measured as the average control AUC minus the AUC from absorbance data of samples. Error bars represent standard error of the mean (SEM).

mixture of PLA_2 and 3FTX fractions to plasma did not result in enhanced activity over each of the fractions alone (Fig. 5.11.). While the combination of these findings suggest that PLA_2s contribute extensively to the anticoagulant effects caused by cobra venoms, I conclude that other toxin constituents, including 3FTXs, may also contribute to this venom activity.

5.4. Discussion

5.4.i. Overview

This chapter provides a detailed comparison of the function of venoms from cobras and their near relatives in the context of their enzymatic and coagulopathic activity. The findings described herein demonstrate that, although cobra venoms exert low SVMP and SP activity, many exhibit potent anticoagulant effects on bovine plasma and African spitting cobra venoms show high enzymatic PLA_2 activity. In regard to the toxin-specific venom activities described (e.g. SP, SVMP and PLA_2 activities), these results are perhaps not surprising; elapid snakes are known to have much lower abundances of SP and SVMP toxins than their viperid counterparts while PLA_2 toxins can make a large constituent of elapid toxins (Table 3.1.)(Tasoulis & Isbister, 2017). Somewhat surprisingly, there is no difference in PLA_2 activity between spitting and non-spitting cobras, despite spitting cobras being shown to have significantly higher PLA_2 abundances in previous chapters (see Table 3.3. and Table 4.3.), even when incorporating the data from previously published literature (see).

5.4.ii. PLA_2 Activity

Spitting cobras not having a significantly higher enzymatic PLA_2 activity is somewhat in conflict with evidence from previous chapters that suggests the importance of PLA_2s in their venoms. Gene evolution data presented in Chapter 4 demonstrated evidence of at least one PLA_2 gene duplication event restricted to the African spitting cobra lineage (see Fig. 4.19.) and proteomic quantification data presented in Chapter 3, which found significantly higher levels of PLA_2 toxin abundances in the venoms of

spitting species (see Table 3.3.). The issue may lie in the method of quantification and analysis of PLA_2 activity.

The assay used here to characterise the biological activity of PLA_2s only measure the enzymatic activity of PLA_2s through the cleavage of phosphatidylcholine, however there has long been suggested the idea that PLA_2s have a pharmacological activity separate from their enzymatic activity (Kini, 2003; R. Kini & Evans, 1989). This works through a more generalised "pharmacological site", which binds non-specifically as opposed to the substrate-specific enzymatic site (Kini & Evans, 1989), which may explain the huge multifunctionality of this toxin family (Gutiérrez & Lomonte, 2013). These pharmacological activities include haemorrhage, neurotoxicity (Rouault et al., 2006) and myotoxicity (Montecucco et al., 2008), and non-enzymatic PLA_2 activity has also been linked to lethality in *Naja atra* and *Naja mossambica* (Condrea et al., 1981). Unfortunately, there has yet to be developed a single, standalone assay that can measure solely pharmacological activity of PLA_2s, making it difficult to distinguish some of the biologically-relevant functional differences between spitting and non-spitting cobra venoms. In order to investigated further, the PLA_2s would need to be isolated, then their catalytic site disrupted before being run on a variety of functional assays.

A second issue lies in the method of analysis. The Phylogenetic Generalised Least Squares (PGLS) method employed here and in chapter 4 relies on the pgls() function of the phytools package to be able to estimate phylogenetic signal (Revell, 2012), measured as Pagel's lambda (Freckleton et al., 2002; Münkemüller et al., 2012), through a maximum likelihood approach. This worked well for all factors except PLA_2 activity, which threw up an error as a result of the estimation for lambda being out of bounds (>1 or <0.000001). The default value for lambda is 1 or strong phylogenetic signal, and under strong phylogenetic signal, PLA_2 activity is not significant. However, further testing found that a lambda value of 0.07 or lower (i.e. very low phylogenetic signal) does produce a significant difference in PLA_2 activity. Thus we can interpret that enzymatic PLA_2 activity is most likely not significant between spitting and non-spitting cobras unless phylogenetic signal is very weak.

5.4.iii. The Anticoagulant Effects of Cobra Venoms

The data presented in this chapter demonstrates that all cobra venoms exert anticoagulant effects on blood plasma. Statistical tests determine that there are no significant differences between the potency of spitting and non-spitting cobras in their anticoagulant activity (Table 5.2.). Coagulopathy is perhaps an unusual functional activity for elapid snake venoms, which are generally considered to be predominately neurotoxic (Postma, 2009). However, there are prior studies that show cobra venoms or venom components exhibiting anticoagulant activities, even when taken orally rather than through injection (Evans et al., 1980; Mackay et al., 1969; Malleswari et al., 2014; Ogay et al., 2005; Rahman et al., 2006; Stefansson et al., 1990). In one such study, Rahman et al. (2006) found prolonged thrombin times and a fall in blood platelet count when cobra venom (species unspecified) was injected intramuscularly into rabbits over a period of a few weeks. Though there are clear limitations with this study and its clinical relevance (e.g. the cobra species was not described, and multiple injections were delivered over a prolonged period of time, rather than mimicking a single-bite exposure), these findings do suggest that cobra venoms have the potential to exert haemotoxic effects in snakebite victims.

With cobra venoms being shown to have general anticoagulant properties, two major questions remain: (i) which toxins are responsible for causing such an effect? and (ii) what is their mechanism of action? The data presented in this chapter sought to answer the first of these questions. My findings suggest that PLA_2s appear to be the main toxin family responsible for causing anticoagulant effects, as co-incubation of various cobra venoms with the PLA_2 inhibitor varespladib significantly reduced anticoagulant venom activity across cobra species. Moreover, linear regression finds a positive association between anticoagulant venom activity and the abundance of PLA_2 in the proteomes of cobras (Fig. 5.12A.) and between anticoagulant activity and PLA_2 activity (Fig. 5.12B.), though this correlation is weakened when removing the outlier data point, *N. pallida* ($y = 0.03x + 7.87$, $R_2 = 0.08$; $y = 0.79x + 6.70$, $R_2 = 0.34$, respectively).

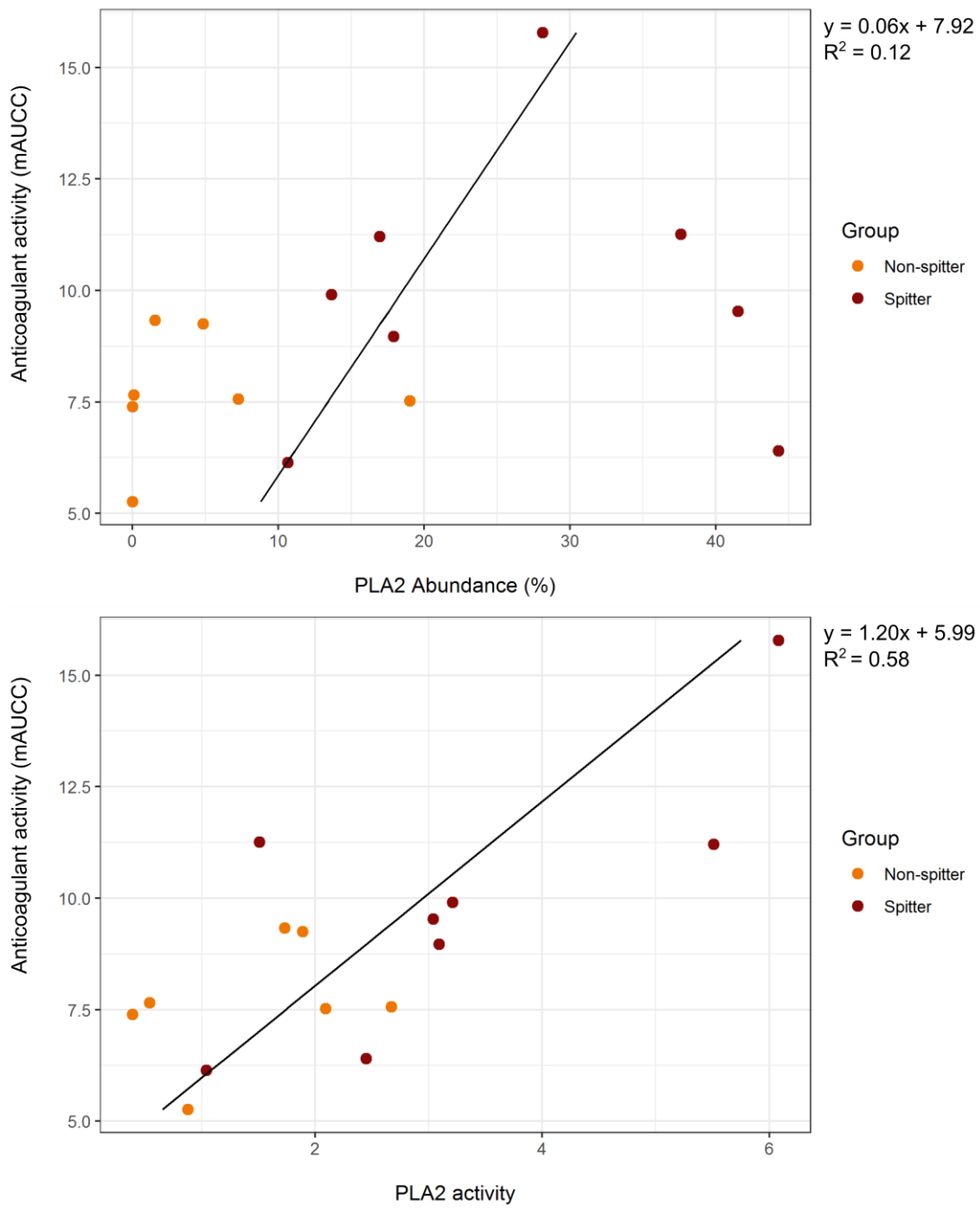


Figure 5.12. Scatter graph plotting the linear regression of anticoagulant activity against A) PLA_2 abundance B) PLA_2 activity. Data points and linear regression were made using data from the ingroup species (*Naja* & *Hemachatus* spp.).

These findings correlate with limited prior reports from the literature, including evidence of anticoagulant PLA_2s previously isolated from both *N. kaouthia* (Doley & Mukherjee, 2003) and *N. nigricollis* venom (Kerns et al., 1999), as well as the reduction in anticoagulant effects from cobra venoms with the addition of varespladib (Bittenbinder et al., 2018). In addition, *H. haemachatus* PLA_2s showed anticoagulant activity, though the authors also noted that fractions containing 3FTXs also contributed to the anticoagulant effect of the venom (Sánchez et al., 2018a). These findings conflict partially with my results which showed that the anticoagulant activity of *H. haemachatus* venom is almost completely abolished by the inhibition of venom PLA_2s with varespladib (Fig. 5.10B). However, it remains possible that 3FTXs are responsible for causing the remaining, low level, anticoagulant activity observed.

Assessing the contributory effect of 3FTXs to anticoagulant venom activity is challenging due to the absence of an appropriate inhibitory agent. To address this issue, *N. nigricollis* venom was fractionated into PLA_2 and 3FTX fragments and these tested both independently and as a mixture, alongside crude venom, on plasma clotting ability. Both 3FTX fraction and PLA_2 fraction have anticoagulant activity (fig. 5.11.), which ties with previous work by Sánchez et al. (2018), though the PLA_2 fraction shows substantially higher activity then the 3FTX fraction. Unexpectedly, the combination of both fractions had less activity than that of the independent PLA_2 fraction. Perhaps this is a direct result of reduced PLA_2 representation in the mixture compared to the independent fraction, or perhaps the 3FTXs themselves are somehow obstructing the anticoagulant mechanism of the PLA_2s. These findings further emphasise that anticoagulant activity in cobra venoms is directly associated with PLA_2 toxins. While there is a positive association between enzymatic PLA_2 activity and anticoagulant activity, this association is fairly weak ($R^2 = 0.12$). This may be the result of an independent, non-enzymatic anti-clotting mechanism or mechanisms of PLA_2s compared to their enzymatic activity of cell lysis. PLA_2 purified from *N. naja* venom has been shown to prevent clotting through the inhibition of thrombin-induced aggregation of plasma (Dutta et al., 2015). Similarly, PLA_2 from *N. nigricollis* binds to factor Xa to prevent the binding of factor Va, preventing the formation of prothrominase and subsequently preventing production of the thrombin, a crucial mediator of coagulation (Kerns et al., 1999; Kini, 2005).

As mentioned earlier, the PLA_2 assay only detects the enzymatic activity of these toxins. In addition, or perhaps alternatively, other venom toxins seem likely to also be contributing to the anticoagulant venom effects observed. As also mentioned, Sánchez et al. (2018) suggested that some *H. haemachatus* 3FTXs are anticoagulant, while Suzuki-Matsubara et al. (2016) proposed that cytotoxic 3FTXs might be responsible for the anticoagulant activity of *N. naja* venom. Indeed, my findings with *N. nigricollis* venom support these results by also demonstrating that some 3FTXs have an anticoagulant effect (Fig. 5.11.). Unfortunately, due to the lack of prior functional characterisation of 3FTXs in the context of coagulation, the specific toxin isoforms responsible remain unidentified, preventing correlation analyses across all cobras. It is possible that other toxin families may also be contributing to perturb coagulation. For example, SVMPs and SP are well known to disrupt normal coagulation by cleaving or activating various members of the blood clotting cascade, particularly in the context of viperid snake venoms (Slagboom et al., 2017). However, the results of the SVMP and SP assays present herein suggest that these toxin types are unlikely to be prominent anticoagulant toxins in cobras as their activities are low (Figs. 5.2. and 5.4.), while coincubation of cobra venoms with the specific metalloproteinase inhibitor marimastat did not result in any reduction in anticoagulant venom activity (Fig. 5.10A.).

Ultimately, additional work is required to clarify whether toxin families other than PLA_2s also contribute to the anticoagulant effects observed here, and whether such additional toxins vary among cobra species, or are consistently found across the genus. However, what is clear, from both the inhibitor and toxin fraction assays presented in this chapter (Figs. 5.10. and 5.11.), and the correlation between PLA_2 abundance and anticoagulant activity (Fig. 5.12.), is the crucial role PLA_2s play in the anticoagulant venom activities of cobras. Irrespective of whether they are solely responsible for this activity, their inhibition results in the near complete restoration of baseline coagulation irrespective of the venom tested. Finally, it is worth noting that these observations extend outside of the genera *Naja* and *Hemachatus*, with comparable anticoagulant activities also observed by the venoms of the outgroup species, *Aspidelaps scutatus* and *Walterinnesia aegyptia* (Fig. 5.8.). Furthermore,

these activities were also inhibited by the PLA_2 inhibitor varespladib (86.33% and 100% inhibited, respectively) (Fig. 5.10.). These findings suggest a generality about anticoagulant venom activities across elapid snakes, despite little mention of such pathologies in the clinical literature. This may simply reflect clinical observations being predominately directed towards the life-threatening pathologies observed following bites by these species (e.g. neurotoxicity), for the few case reports that exist. This being said, there are still some clinical reports of coagulopathic symptoms following bites from *N. nigricollis*, *N. naja*, *N. mossambica* and *N. atra* (Kularatne et al., 2009; Tilbury, 1982; D A Warrell, Greenwood, et al., 1976; O. F. Wong et al., 2010). It seems likely that preventing normal coagulation following bites by these species may facilitate the distribution of potent neurotoxins systemically, thus potentially increasing the rapidity with which neuromuscular paralysis can occur. Supportive evidence for this is shown by the absence of neurotoxic symptoms in mice envenomed by the Papuan taipan (*Oxyuranus scutellatus*) after oral dosing with the PLA_2 inhibitor varespladib (Lewin et al., 2018), and reduced lethality in pigs envenomed by the Eastern coral snake (*Micrurus fulvius*) after oral dosing or intravenous injection of varespladib (Lewin et al., 2018). However, as PLA_2s are also known to act as neurotoxins themselves (Kini, 2003), it is possible that the directly neurotoxic toxins are being inhibited rather than anticoagulant PLA_2s that might mediate 3FTX-based neurotoxicity. Future research is therefore required to robustly test this hypothesis.

5.4.iv. Concluding remarks

In summary, although here I have demonstrated an associated between a specific venom activity (PLA_2 activity) and venom spitting, my findings relating to SVMP, SVSP and anticoagulant effects show no strong association with the evolution of this adaptation. These findings are perhaps not surprising, as their functional activities, perhaps with the exception of the SVMPs, would likely not provide an adaptive advantage in the context of the use of venom spitting for defence. However, the lack of an identified association could be confounded by different lineages of spitting cobras evolving different solutions for the evolution of defensive toxins (i.e. in contrast to the convergence hypothesis tested by statistical analyses), and thus

undermining the comparative analyses performed here across all three spitting snake lineages. Despite seemingly occurring independently of venom spitting, the anticoagulant activities of cobra venoms were largely consistent among species. A major outstanding question is whether these *in vitro* findings can be validly extrapolated as a representation of *in vivo* pathology, particularly given the lack of literature (e.g. clinical case studies) describing anticoagulant effects following human envenomings by cobras (Warrell et al., 1976). However, it might be that such signs are masked by the more acute and/or life-threatening signs of cobra bites, such as descending neuromuscular paralysis or extensive local tissue damage, that rightfully garner the primary attention of an attending clinician. This, coupled with the typically under-resourced healthcare environment in the rural regions of sub-Saharan Africa and south Asia where most cobra bites occur, may be partially responsible for standard blood parameters rarely being described following such envenomings. The findings outlined here therefore strongly advocate that haemostatic characteristics should be monitored during the clinical management of patients suffering from cobra envenomings due to the potential for such individuals to suffer from clinically relevant coagulopathy. In addition, this work opens the door to further work on the role of PLA_2s (if any) as a defensive toxin; namely the investigation into PLA_2-mediated or exacerbated pain-inducing mechanisms.

6. COMPARATIVE COMPOSITIONAL ANALYSIS OF VENOMS COLLECTED FROM THE BITES AND DEFENSIVE SPITS OF COBRAS

6.1. Introduction

Animal toxins can be defined as offensive (used to incapacitate and/or kill a prey item for consumption) or defensive (used to incapacitate an aggressor to increase likelihood of escape). Offensive toxins tend to be those that can cause decreased movement through cell destruction, as is seen in the venom composition of vipers, or paralysis, as more commonly reflected by the large number of neurotoxic components in elapid venom (Tasoulis & Isbister, 2017).

Defensive toxins are primarily used to temporarily distract or deter an aggressor long enough to allow adequate time to escape and survive another day. One way of doing this is to cause an unpleasant sensation or some form of incapacitation. Such unpleasantness can be in the form of foul-tasting chemicals that make an animal difficult or undesirous to eat and this is a tactic employed by many insects (particularly Lepidoptera), amphibians and several birds through sequestering unpalatable chemicals from the food that they eat, such as cardenolides, grayanoids and alkaloids (Bartram & Boland, 2001; Daly et al., 1978; Nishida, 2002; Savitzky et al., 2012). Some defensive toxins offer more moderate forms of incapacitation, such as the diarrhetic symptoms caused by dinophysistoxins and pectenotoxins in some dinoflagellate-feeding shellfish (Yasumoto et al., 1985) and hypotension caused by neuropeptide Y in the venom of blenny fishes (Casewell et al., 2017). More severe forms of deterrence involve complete incapacitation, such as cardiac arrest induced by bufadienolides stored in the nuchal glands of the snake *Rhapdophis tigrinus* from its toad prey (Hutchinson et al., 2007) or batrachotoxins secreted by the skin of *Phyllobates* dart frogs (Daly et al., 1978). Some defensive toxins can even cause paralysis, with toxins that bind to ion channels like saxitoxin (blocks calcium and potassium channels) and tetrodotoxin (binds to sodium channels) found in some shellfish and pufferfish venoms (Chau et al., 2011; Costa et al., 2009; Llewellyn, 2006; Sato et al., 1997).

Perhaps the most effective deterrent method is to inflict pain. Predators can become averse to prey which produce painful stimuli (Linhart et al., 1976; Noboa & Gillette, 2013), sometimes to the extent of transmitting the learned aversion to conspecifics (Thorogood et al., 2018), which in turns reinforces the defensive signal of such toxins further. A common pain-inflicting strategy employed by poisonous and venomous animals is the use of compounds that activate nociceptors via targeting of ion channels (Jami et al., 2018). This appears to be a common strategy in venomous invertebrates such as jellyfish, spiders and centipedes, whose toxins aggravate transient receptor potential cation channel subfamily V member 1 (TRPV1) channels (Cuypers et al., 2006; Geron et al., 2017) resulting in pain through the release of inflammatory molecules. Similarly, bee venoms contain melittin, which can also directly activate TRPV1 receptors via PLA_2 cascade pathways or indirectly activate ion channels through the formation of pores in tissue and mast cells (Chen et al., 2016). Helodermatid lizards also have PLA_2s in their venoms, which have been shown to have more similar sequence similarity to bee PLA_2s than those from venomous snakes (Gomez et al., 1989), perhaps suggesting a similar role in this notoriously painful venom. An alternative strategy to inducing pain via ion channels is to cause extensive cell death or necrosis via cytotoxic venom effects. This appears to be more common in vertebrate defensive venoms, such as in the toadfish *Thalassophryne nattereri* (Lima et al., 2003), which combines cytotoxicity with inducing inflammatory responses to inflict pain, and some sea anemones, which use cytotoxic PLA_2s to both incapacitate prey and repel predators (Maček, 1992). Some animals, such as scorpions, appear to have a venom composition that utilises both of the above methods, targeting potassium and sodium channels as well as containing multiple cytotoxic components (Sunagar et al., 2013).

Defensive toxin composition can vary for a variety of reasons, including diet, environment and ontogeny (Speed et al., 2012). Fascinatingly, some animals, including representatives of the scorpions, cone snails and assassin beetles, exhibit bipartite venoms, whereby venom composition exhibits a degree of plasticity and differs with its intended function (Dutertre et al., 2014; Gangur et al., 2017; Walker et al., 2018). For example, the assassin bug *Pristhesancus plagipennis* possesses a physical delineation between venom used for predation and defence, with each

being produced in a separate and distinct gland (Walker et al., 2018). The fish-eating cone snails *Conus geographus* and *Conus marmoreus* also utilise an entirely different suite of toxins for predatory and defensive purposes, with predatory-venom containing more fish-specific sodium channel inhibitors and non-paralytic toxins, while the defensive-venom contains mostly paralysis-orientated toxins that effectively target mammalian receptors (Dutertre et al., 2014). Unlike the assassin bug, the differences in defensive and offensive venom composition were found to originate from separate parts of the same gland, with defensive toxins produced by the proximal duct and offensive toxins by the distal regions of the gland. Scorpions, another invertebrate shown to exhibit differences in defensive and offensive venom (Gangur et al., 2017; Inceoglu et al., 2003) do not exhibit physically separate glands or spatially-separated glandular regions. Instead, their venoms contain more defensive toxins with increased exposure to predation threat, with the scorpion *Liocheles waigiensis* producing venoms that were more toxic to mammalian cells after repeated exposure (Gangur et al., 2017) and *Parabuthus transvaalicus* producing first a "prevenom" containing mostly k^+ salts and pain-inducing peptides before switching to a more potent and proteinaceous venom composition (Inceoglu et al., 2003). These examples suggest that defensive venom use, even in animals whereby the primary function of the venom gland is to produce secretions to facilitate prey capture, can act as a selective force on the defence-specific composition of venom, and can also result in temporal compositional and/or functional or long-term, physical delineation of venom producing tissue.

Spitting cobras also have a bipartite venom, using it both for predation (through biting) and defence (through spitting). Currently there is no literature on the potential partitioning of the snake venom gland, leaving open the possibility that functionally different toxins could be produced by different regions. Additionally, there is the possibility that the accessory gland, which is directly linked to the fangs through the secondary venom duct (Fig. 1.2.), produces a distinct toxin profile that could be used for defensive venom. Previous work has suggested that snakes can meter their venom expenditure (Young et al., 2002) and that venom gland contraction differs between spitting and biting actions (Hayes et al., 2008). Additionally, the spitting cobra Naja pallida has been shown to produce venom that

initially contains a high concentration of cytotoxins (CTXs), which decrease significantly following the 20th spit (Cascardi et al., 1999), suggesting that spat venom initially contains an abundance of potentially eye-damaging and thus painful toxins. It is therefore possible that selection may act on cobras to exert control on the composition of venom that is used defensively through spitting as opposed to that used for predation through biting; an idea that has not yet been addressed by the field at large.

The delivery mechanism of defensive toxins varies from the simple ingestion of sequestered or self-synthesised toxins (poisons), such as in Monarch butterflies (Malcolm & Brower, 1989), frogs (Santos et al., 2016) and some crustaceans and bivalves (Asakawa et al., 2010; Biessy et al., 2019), to the specialised spines, barbs or teeth of venomous animals, such as in cnidarians (Jouiaei et al., 2015), fish (Baumann et al., 2014; Smith & Wheeler, 2006), some frogs (Jared et al., 2015), and Helodermatid lizards (Russell & Bogert, 1981). The ability to project venom as a spray ("spitting") in cobras is a unique defensive adaptation in snakes, however some non-serpentine taxa are capable of projecting their toxins in a similar manner, such as the "bombardier beetles" *Brachynus ballistarius* (Eisner, 1958) and *Paussus favieri* (Muzzi & Di Giulio, 2019), which spray toxic chemicals from the abdomen, the scorpion *Parabuthus transvaalicus* (Nisani & Hayes, 2015), which can eject venom from its stinger, and *Anisomorpha* stick insects (Brutlag et al., 2011), which sprays chemicals from glands on its thorax. While there is diversity in both the type of animal and delivery mechanism of these defensive toxins, the function of these projections is to protect the producing animal from predators and/or aggressors.

In chapters 3-4 of this thesis, spitting cobra venoms were demonstrated to have significantly higher PLA_2 abundance than venoms from non-spitting cobra. PLA_2s have been convergently recruited into the venoms of multiple invertebrate orders, as well as several times in advanced snakes (Fry et al., 2009). Indeed, these toxins almost certainly contribute to the defensive activity of bee and Helodermatid lizard venoms (Chen et al., 2016; Gomez et al., 1989; Russell & Bogert, 1981), and this toxin family has an extremely broad functional diversity, including inducing cytotoxicity (Kini, 1997). Thus, PLA_2 toxins seem likely to play at least some defensive role in spitting cobra venoms. Cytotoxins (CTXs), which cause cell death and necrosis

in cobra venoms (Panagides et al., 2017; Rivel et al., 2016), may also be a defensive component in spitting cobras, although no significant differences in the proteomic abundances of CTXs were detected between spitting and non-spitting cobras in this study (chapter 4). One remaining outstanding question relating to defensive venom spitting is whether the composition of venom sourced from spits and bites differ, in a manner analogous to the compositionally dichotomous venoms of certain cone snails, scorpions and assassin bugs. One might predict that venom spits might contain higher abundances of defensive venom toxins compared with venom, particularly since spits contain less venom than biting (Cascardi et al., 1999), and are inherently less accurate (e.g. less venom will actually hit/enter the target). However, such a finding would raise major questions relating to whether the production and storage of venom in the venom gland is homogenous.

This chapter seeks to address the question of whether venom from spitting cobras that is ejected via spitting differs to that of venom delivered through biting. Using four species of spitting cobra from two of the three spitting lineages (*Hemachatus haemachatus* and African spitting cobras), here I compared the protein composition of spat and 'bite' venom samples using SDS-PAGE gel electrophoresis and reverse-phase high performance liquid chromatography (RP-HPLC), and assessed *in vitro* the functional activities of these samples using snake venom metalloproteinase (SVMP), serine protease (SP), phospholipase A_2 (PLA_2) and coagulation biochemical assays.

6.2. Methods

6.2.i. Collecting venom from spitting cobras

Venom 'spits' were collected successfully from the following species: *Hemachatus haemachatus*, *Naja pallida*, *Naja mossambica* and *Naja nigricollis* (Table 6.1.). To collect venom spits, a makeshift 'face' was created using the image of a large black circle, 17cm in diameter. To make the 'eyes', 2 smaller circles, roughly 3cm in

Table 6.1. The species, IDs and localities of the spitting cobras used for the research presented in this chapter.

ID	Species	Locale
HemHae.CB001	*Hemachatus haemachatus*	Captive bred
NajMosTZA001	*Naja mossambica*	Tanzania
NajNigTZA003	*Naja nigricollis*	Tanzania
NajPalKen001	*Naja pallida*	Kenya

diameter and white (to make them as contrasting as possible) were placed on the upper section of the 'face', spaced approximately 5.5cm apart (see Fig. 6.1.). This 'face' was adhered to the inside of a visor mask using clear tape, and the visor was suspended from the hole at the top using a snake hook (see Fig. 6.2.). When interacting with the cobras, all participants in the room were wearing scrubs, visors and suitable footwear, and were equipped with snake hooks. The 'face' was presented close to the head of each cobra and moved erratically to encourage defensive venom spitting. The snake was safely returned once it was determined that sufficient spat venom had been collected on the visor, or if it was determined that the individual was unlikely to spit after several failed attempts. Before each individual spitting session, the visor was used to tare a set of digital laboratory scales (OHAUS Pioneer PA214C) and wet venom on the visor was weighed immediately after spitting. The venom on the visor was then allowed to dry over a period of <5 mins, before being scraped off into a petri dish using either the rounded edges of a blank white plastic card or the edge of a razor blade. In some cases (when the venom would not dry effectively), the wet venom was pipetted into the dish. The spat venom was then weighed again before the petri dish was covered with parafilm and stored at -20°C overnight, prior to lyophilisation. Extracted venom samples (i.e. from biting), referred to as "post-spitting extraction" (PSE) samples, were collected from each individual after their respective spitting session by restraining the snake and allowing it to bite a parafilm covered petri dish. Venom was weighed, stored and lyophilised as per spat venom.

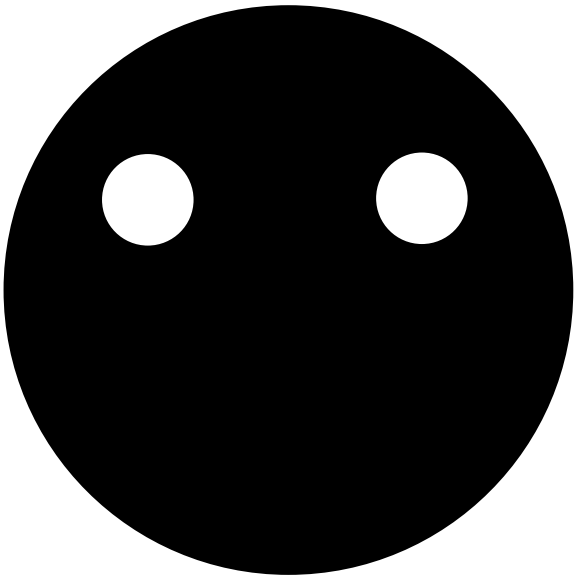


Fig. 6.1. The “face” design used to stimulate spitting from cobras.

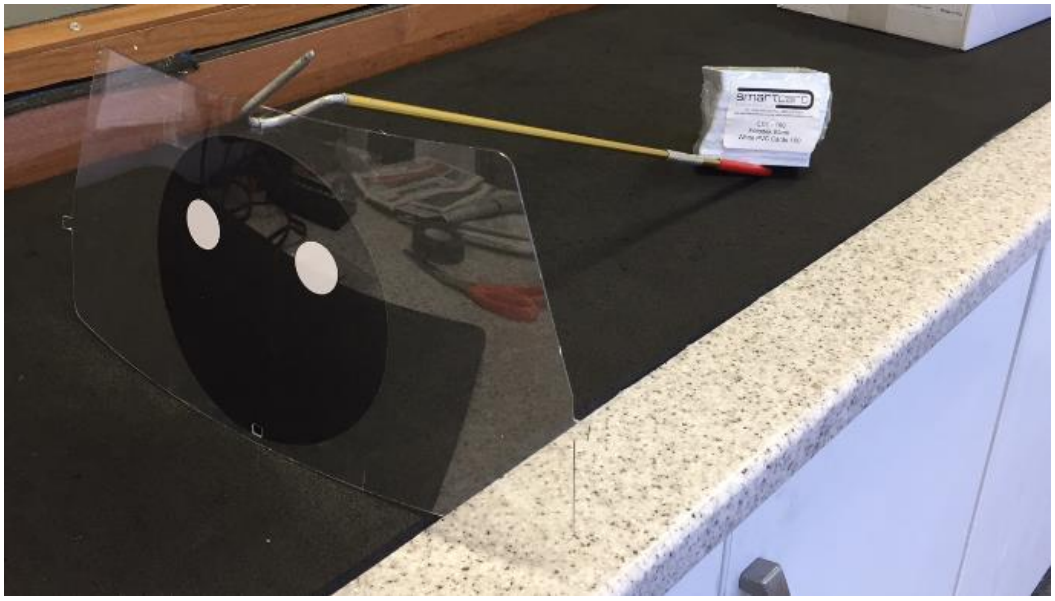


Fig. 6.2. The setup for collecting spat venom. This utilises an isolated mask visor, snake hook and printed paper bearing the “face” design depicted in Fig. 6.1.

6.2.ii. SDS-PAGE gel electrophoresis

Hand-cast Bis-acrylamide SDS-PAGE gels were made using a 15% resolving gel containing a mixture of 3.75 mL deionised H_20, 2.5 mL 1M tris(hydroxymethyl)aminomethane (TRIS) (pH 8.8), 3.75 mL 40% Bis-acrylamide, 100 µL 10% sodium dodecyl sulfate (SDS), 60 µL 10% APS and 7 µL Tetramethylethylenediamine (TEMED). This resolving gel was allowed to set for 20-30 minutes before adding the stacking gel, which was made using 2.5 mL deionised H_20, 1 mL 1M TRIS (pH 6.8), 350 µL 40% Bis-acrylamide, 30 µL 10% APS and 5 µL (TEMED). A 1mm plate and comb was used to create wells in the stacking gel, which was allowed to set for 30 minutes. All venom samples were reconstituted to 1mg/mL with the addition of PBS, pH 6.2. Samples were prepared by adding 12 µl of 1 mg/mL to 12 µl loading dye (diluted from a 2x mixture of 3.55 mL H_20, 1.25 mL 0.5M TRIS [pH6.8], 2.5mL glycerol, 2.0 mL 10% sodium dodecyl sulfate [SDS] and 1.5 mL saturated bromophenol blue). To samples intended to make reduced gels, 150 µl of of B-mercaptoethanol was added to the 2x loading dye before dilution, and the venom-dye mixture was allowed to denature for 10 minutes in a heating block at 95 degrees. A ThermoFisher Mini Gel Tank (catalogue number A25977) was filled to the loading point with SDS page buffer, diluted from a 5x stock solution (made with 151g Tris-base, 720g glycine, 50g SDS and deionised H_20 to make a 10 L solution) using deionised water. Into the first well, 5 µL of Promega Broad Range Protein Molecular Marker (10–225kDa; reference number: V849A) was pipetted, followed by 20 µL of each sample into their respective wells (see individual chapter figures). Gels were run at 110V, 44mA for around 25 minutes followed by 200V for around 15 minutes. Resulting gels were stained with bromethyl blue dye overnight and destained the following morning with bromethyl blue destaining solution.

6.2.iii. Venom Profiling – Buffer Exchange Step

Venom samples were diluted in PBS as above to 1mg/mL, which can be damaging to chromatography columns. In order to replace the buffers from the samples and identify any non-peptide small molecules with potential pharmacological effects, a 5 mL Sephadex G25 desalting column (GE Healthcare) was set up on an AKTA Pure M chromatography system and pre-equilibrated with 0.2µm filtered buffer (25mM

sodium phosphate). 500µL of venom samples were then injected and the column was run with 50mM sodium phosphate, pH 6.0 at a flow rate of 0.5 mL/min. Elution was monitored at 280nm and 0.5mL fractions were collected sequentially.

6.2.iv. Venom Profiling – Cation Exchange

To identify if spat and PSE venoms differ by protein charge, 300µL of venom from the buffer exchange step described above were loaded onto a 1mL Resource S cation exchange column (GE Healthcare) set up on an AKTA Pure M chromatography system and equilibrated in 50mM sodium phosphate, pH 6.0. The proteins were then separated at 0.3 mL/min with a 15mL gradient of 0-0.75 M NaCl in 50 mM sodium phosphate, pH 6.0. Elution was monitored at 214 nm.

6.2.v. Venom Profiling – Reverse-phase HPLC

To identify if spat and PSE venoms differ by the hydrophobicity of their proteins, RP-HPLC of venom proteins was carried out on a Dionex ICS3000 HPLC system. From the buffer exchange step, 85µL of venoms were made up to 0.05% in trifluoroacetic acid (TFA) and centrifuged at 10,000 x g for 5 minutes. These were then injected onto a Phenomenex Jupiter C4 RP-HPLC column (250 x 4.6mm) equilibrated in 0.1% TFA. The column was run at 0.7 mL/min and the proteins were separated over 60 minutes with a 2 - 72% gradient of acetonitrile in 0.1% TFA. Elution was monitored at 214 nm.

6.2.vi. Functional Assays

Spat and PSE venom were compared to identify whether there are functional differences between spat venom and venoms extracted at potentially different venom gland depletion levels. For functional analyses, each sample was analysed using the Snake venom metalloproteinase (SVMP), Serine Protease (SP), Phospholipase A_2 (PLA_2) and plasma assays outlined in 5.2.

6.2.vii. Statistical Analyses

To determine if the functional activities of spat venom differed to that extracted post spitting (PSE), a one-way ANOVA or Kruskal-Wallis test was performed in R, with the results from each functional assay treated as a separate dataset. A residuals versus

fits plot and QQ plots were used to determine if these datasets conformed to the assumptions required to perform a one-way ANOVA. If these assumptions were violated, a Kruskal-Wallis rank sum test was performed instead.

6.3. Results

6.3.i. Spat Venom Yield

All cobras were successfully encouraged to spit at the "face" visor at least once. There was substantial variation in the wet and dry weights of the spat venom collected from spitting cobras (Table 6.2.), ranging from 13.2mg to 41.7mg wet weight and 9.6mg to 24.4mg dry weight, although these data ranges are clearly influence by the variable numbers of spits collected for each species. Across the species tested, dry venom weight collected from spits equated to 48.29-72.73% of the collected wet weight. Interesting, venom yield appears to have an inverse relationship with the number of spits collected (Fig. 6.3.); *Hemachatus haemachatus*, which spat the most before becoming uninterested (n = 16), had the lowest average venom yield per spit (1.8mg wet, 0.9mg dry), while *Naja nigricollis*, had the highest average venom yield per spit (9.8mg wet, 6.1mg dry, n = 2). In terms of venom collected via biting after the spitting experiments (PSE), wet venom weight varied between 529.4mg to 958.2mg, with dry venom equating to 17.04-32.33% of wet weight (Table 6.3.). Spat venom wet weight represents 2.49-4.35% of PSE wet venom weight, showing how small a proportion of venom reaches the face when spitting.

Table 6.2. The basic characteristics of spat venom collected from spitting cobras.

Species	Wet weight (mg)	Dry Weight (mg)
N. pallida	529.40	126.10
H. haemachatus	694.10	118.30
N. mossambica	958.20	309.80
N. nigricollis	643.30	117.80

Table 6.3. The basic characteristics of post-spitting extraction (PSE) venom collected from the animals.

Species	Number of venom spits collected	Wet weight (mg)	Dry Weight (mg)	Average Spit Wet weight (mg)	Average Spit Dry Weight (mg)
N. pallida	5	13.20	9.60	2.64	1.92
H. haemachatus	16	29.20	14.10	1.83	0.88
N. mossambica	5	41.70	24.40	8.34	4.88
N. nigricollis	2	19.50	12.20	9.75	6.10

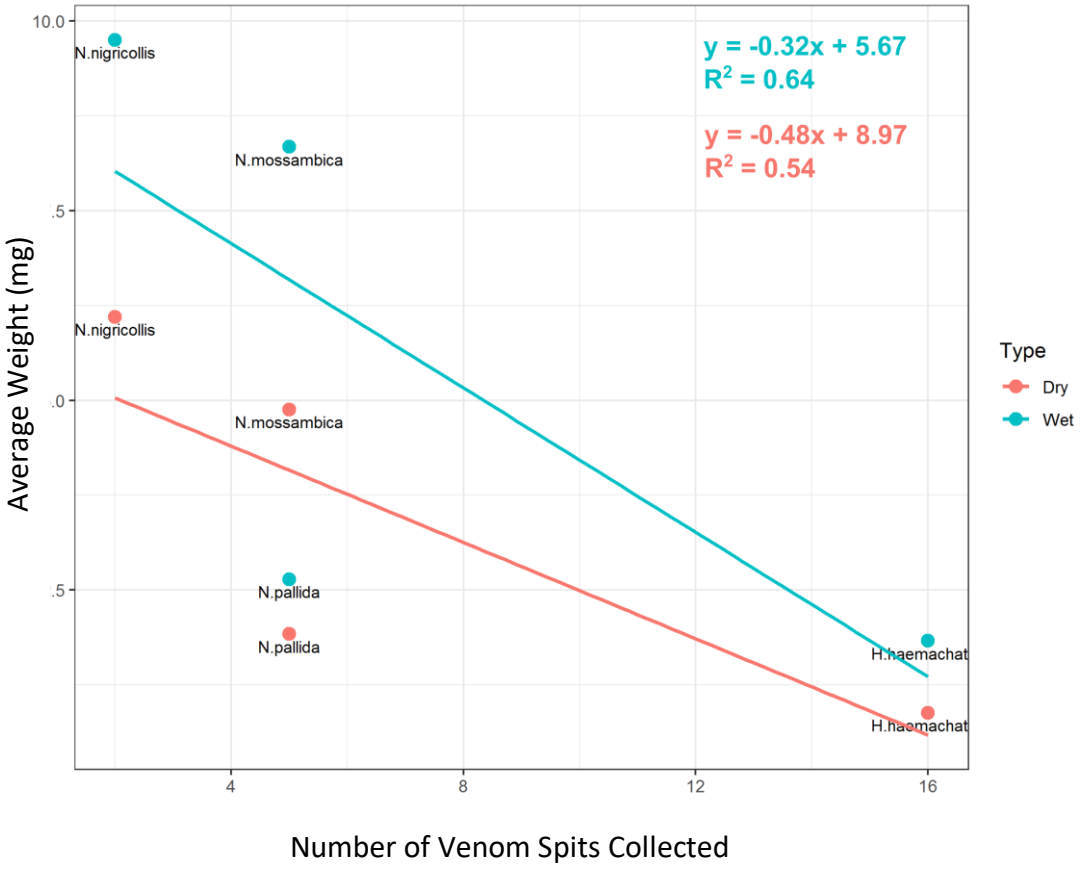


Fig. 6.3. The relationship between number of times spat by spitting cobra and the average venom yield in mg. The lines represent linear regression for the mean of each venom type.

6.3.ii. Venom protein profiles by SDS-PAGE

In both reduced and non-reduced gels, all venoms exhibit protein profiles that are dominated by toxins that are <25 kDa in size (Fig. 6.4). All venoms show greatest abundance of proteins at the ~12 and ~13-14 kDa size under reducing conditions, which is consistent with the venom proteomes of these species being dominated by three-finger toxins (3FTXs) and PLA_2s (Fig. 3.2.). *Hemachatus haemachatus* has a slightly fainter 15kDa band in both the spat and PSE samples, suggesting a lower proportion of the higher-weight 3FTX than other spitting cobras. Similarly, both gels show a band at the 25kDa weight, which is likely to be Phospholipase A_2 (PLA_2). This band varies quite strongly across species in the standard gel (see Fig. 6.4A.), being the most pronounced in *H. haemachatus*, suggesting the highest PLA_2 content among the spitting species sampled. This corresponds with the proportional abundance of PLA_2 in these species seen in the proteomic data (see Fig. 3.2.). In both gels, there are faint bands between 50-225kDa, which may be a combination of other toxins, including the heavy Snake venom metalloproteinases (SVMPs) likely to be near the 225kDa end, non-toxic elements, and protein complexes. In the 15-25kDa region of the standard gel there are some unusually strong bands, particularly in *Naja pallida* (Fig. 6.4A.). It is possible that these are low-weight 3FTX that have formed complexes, as these bands disappear in the reduced gel (see Fig.6.4B.). There does not appear to be any pronounced differences in spat and PSE venom at this level.

6.3.iii. Venom protein profiles by cation exchange

Annotation of the cation exchange profiles of spitting cobras is based on previous work on *Naja mossambica* venom done by M. Wilkinson (personal communications). All spitting cobra venoms displayed at least one peak around the 6mL mark, signifying neutral PLA2 ($nPLA_2$), with all except *N. nigricollis* having two $nPLA_2$S. There are no major differences in profiles between spat and extracted/PSE venoms in terms of protein diversity, though in H. haemachatus there is a mild peak at the 12mL mark of the spat sample that does not appear in the extracted sample. Additionally, *H. haemachatus* and *N. nigricollis* appear to have a higher concentration of PLA_2s and 3FTXs in the extracted samples than the spat samples.

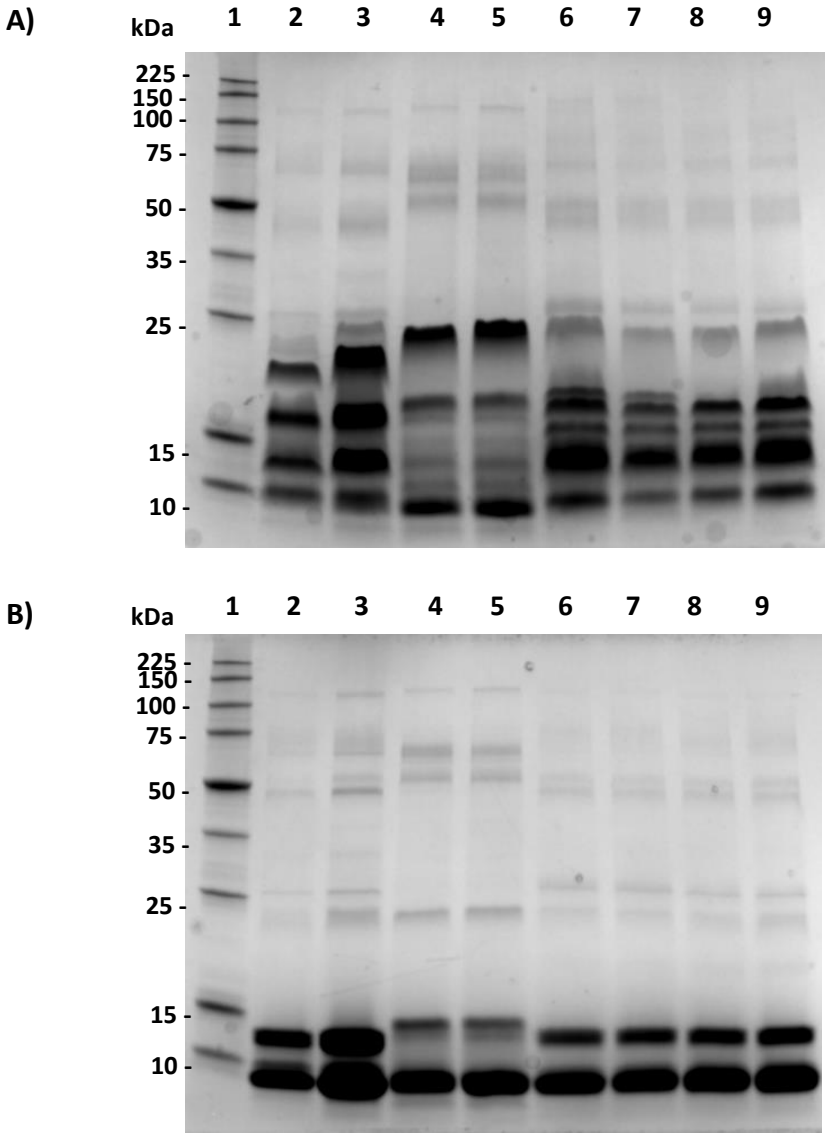


Fig. 6.4. SDS-PAGE gel (15%) electrophoresis profiles of spat and extracted spitting cobra venoms run under non-reducing (A) and reducing (B) conditions. 1 - Protein Marker, 2 - Naja pallida (spat), 3 - Naja pallida (post-spitting extracted/PSE), 4 - Hemachatus haemachatus (spat), 5 - Hemachatus haemachatus (PSE), 6 – Naja mossambica (spat), 7 – Naja mossambica (PSE), 8- Naja nigricollis (spat), 9 – Naja nigricollis (PSE).

6.3.iv. Venom protein profiles by RP-HPLC

The RP-HPLC profiles of the four spitting cobra venoms are similar, with three major peaks observed in each species. For *N. nigricollis*, *N. mossambica* and *H. haemachatus* these peaks elute between 25-35 mins, whereas in *N. pallida* the two most major peaks elute after 40 mins (Fig. 6.5.-6.8.). In addition, the venom of *H. haemachatus* exhibits two additional peaks in the 25-25 min time frame not observed in the other species. Most noticeable, and broadly consistent with the findings from the gel electrophoresis and cation exchange experiments, is the observation that spat and PSE venom profiles were near-identical, suggesting that there are no major compositional differences between defensive venom spits and predatory bites.

6.3.vi. SVMP Activity

Compared to the activity of the viper *Calloselasma rhodostoma*, the SVMP activity of both spat and PSE venoms are low (Fig. 6.9.). *H. haemachatus* spat venom appears to have more SVMP activity than PSE venom, while, contrarily, PSE venom from *N. pallida* has substantially higher activity than spat venom. Values for SVMP activity, measured as the mean of the control area under the curve minus individual AUC of fluorescence intensity, multiplied by -1 $(mAUC)^{-1}$, ranged from 396581.9 to X in spat venoms and X to X in PSE venoms. The mean SVMP activity $(mAUC)^{-1}$ for spat venoms is 1089502.92, compared to 1221390.50 from PSE venoms, and this difference was not significant (Kruskal-Wallis rank sum test, $X^2 = 0.85$, $p = 0.36$) (Table 6.4.).

6.3.vii. SP Activity

All venoms exhibited little to no visible SP activity, particularly when compared to the activity of venom from the viper *Bothrops asper* (Fig. 6.10.). Mean activity, measured as the averaged gradient, ranged from 0.00 to 0.002 in both the spat and PSE samples, with mean values of 0.000264 and 0.000181 respectively. Unsurprisingly, there was no significant difference in SP activity between spat and PSE samples (Kruskal-Wallis rank sum test, $X^2 = 0.48$, $p = 0.49$) (Table 6.4.).

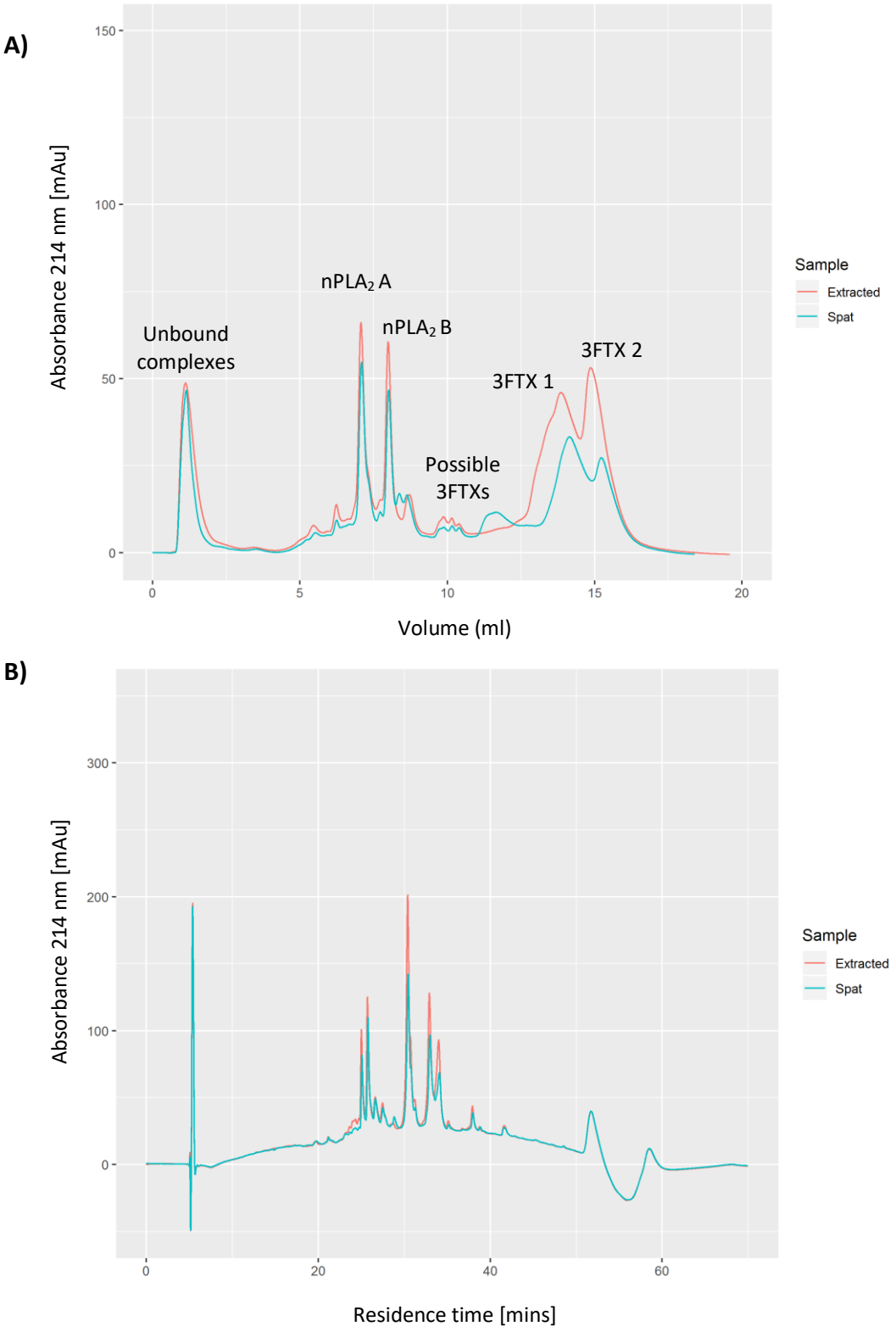


Fig. 6.5. Chromatography profiles of *Hemachatus haemachatus* spat and extracted (PSE) venom. A) Cation exchange **B)** RP-HPLC. nPLA$_2$ – Neutral PLA$_2$.

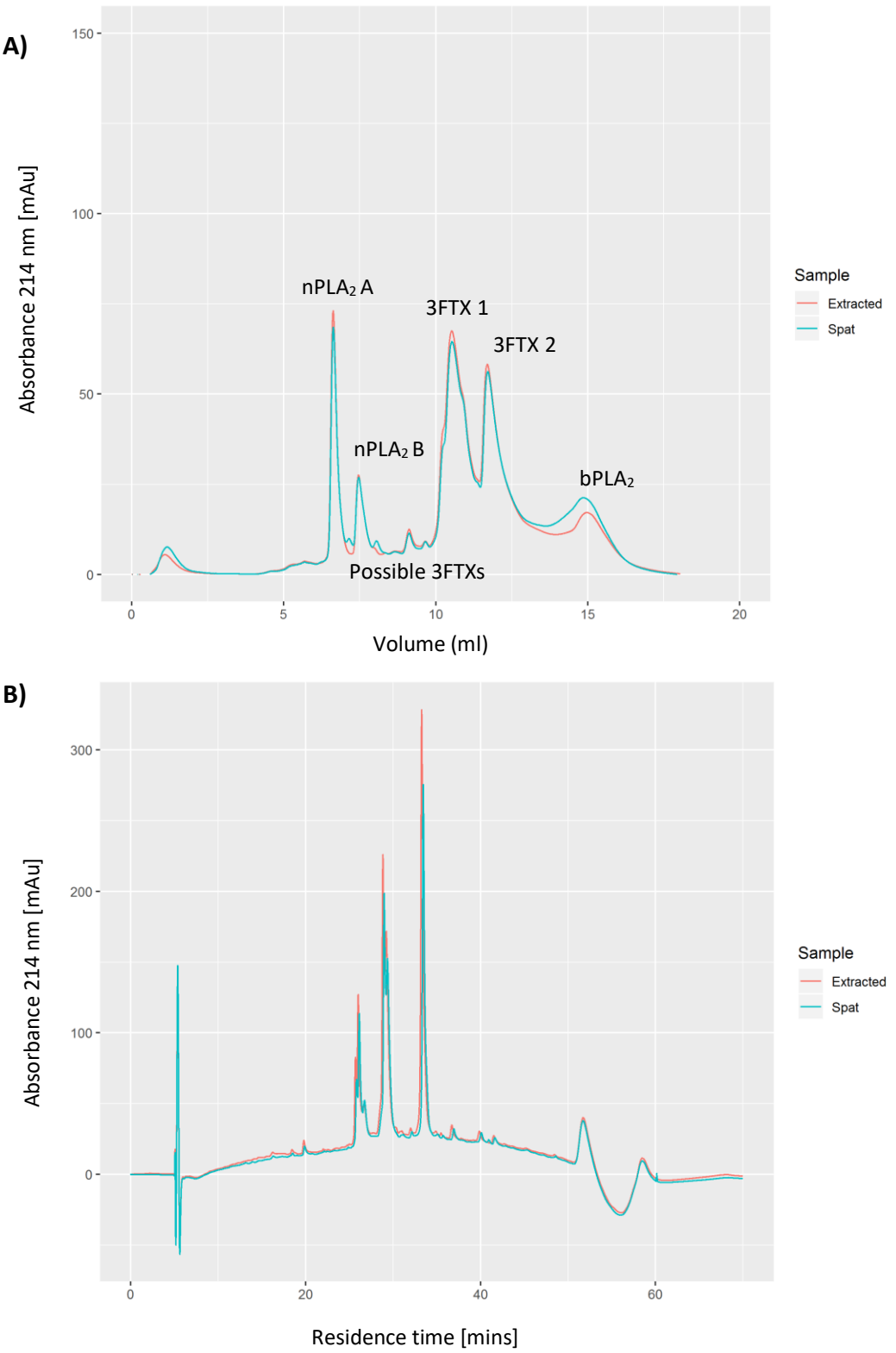


Fig. 6.6. Chromatography profiles of *Naja mossambica* spat and extracted (PSE) venom. **A)** Cation exchange **B)** RP-HPLC. $nPLA_2$ – Neutral PLA_2; $bPLA_2$ – Basic PLA_2.

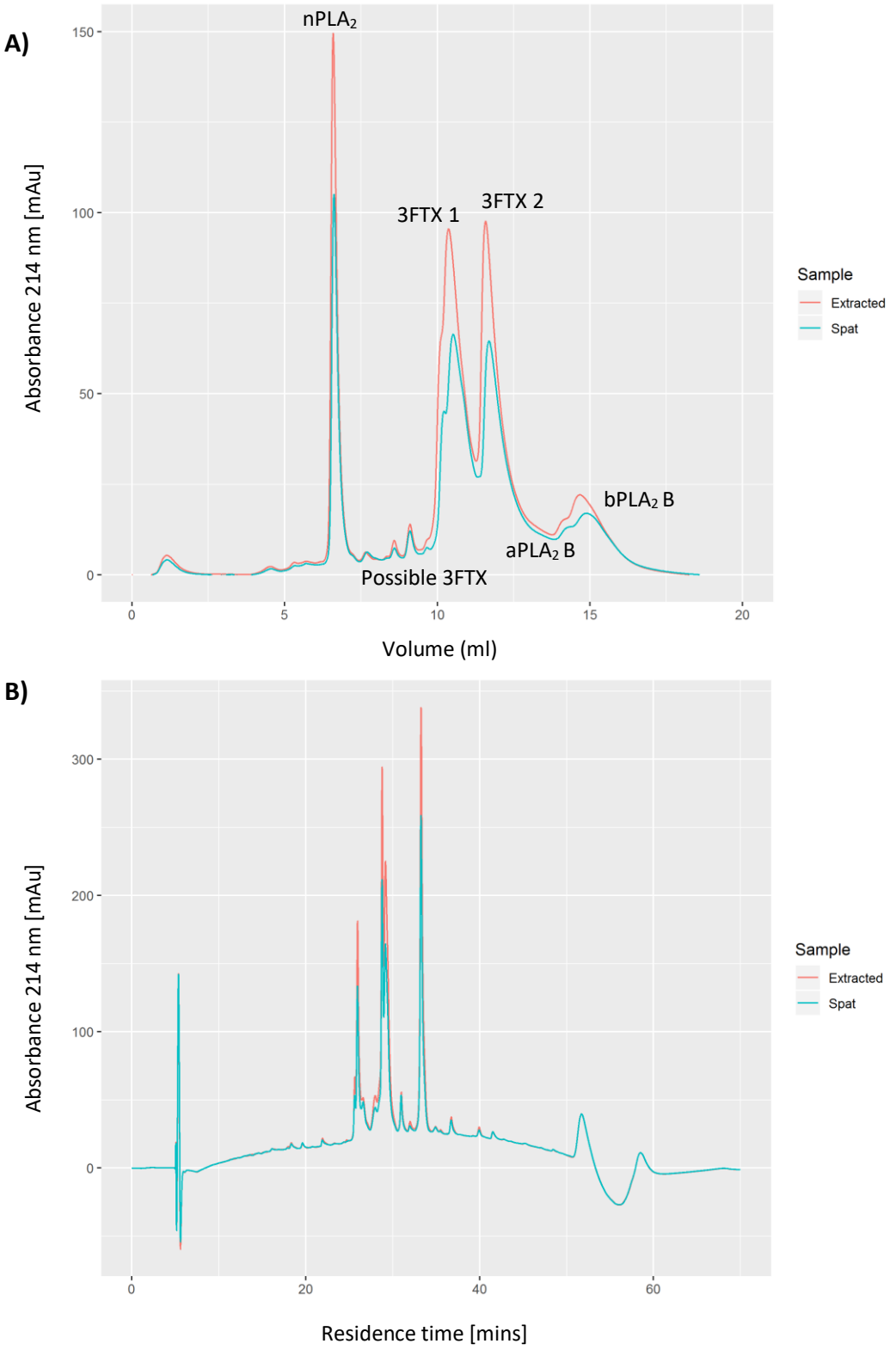


Fig. 6.7. Chromatography profiles of *Naja nigricollis* spat and extracted (PSE) venom. A) Cation exchange **B)** RP-HPLC. $nPLA_2$ – Neutral PLA_2; $bPLA_2$ – Basic PLA_2.

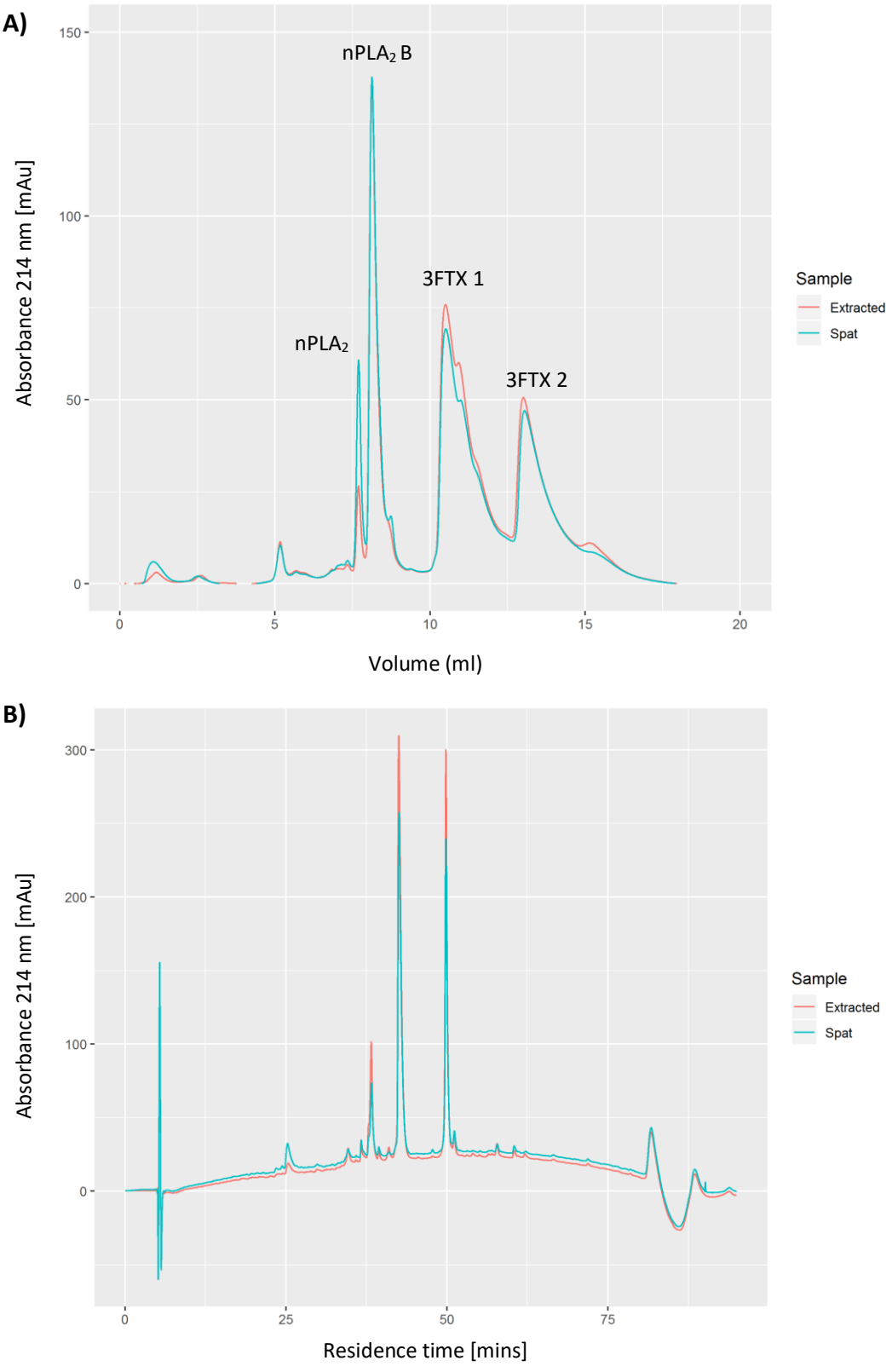


Fig. 6.8. Chromatography profiles of *Naja pallida* spat and extracted (PSE) venom. **A)** Cation exchange **B)** RP-HPLC. $nPLA_2$ – Neutral PLA_2.

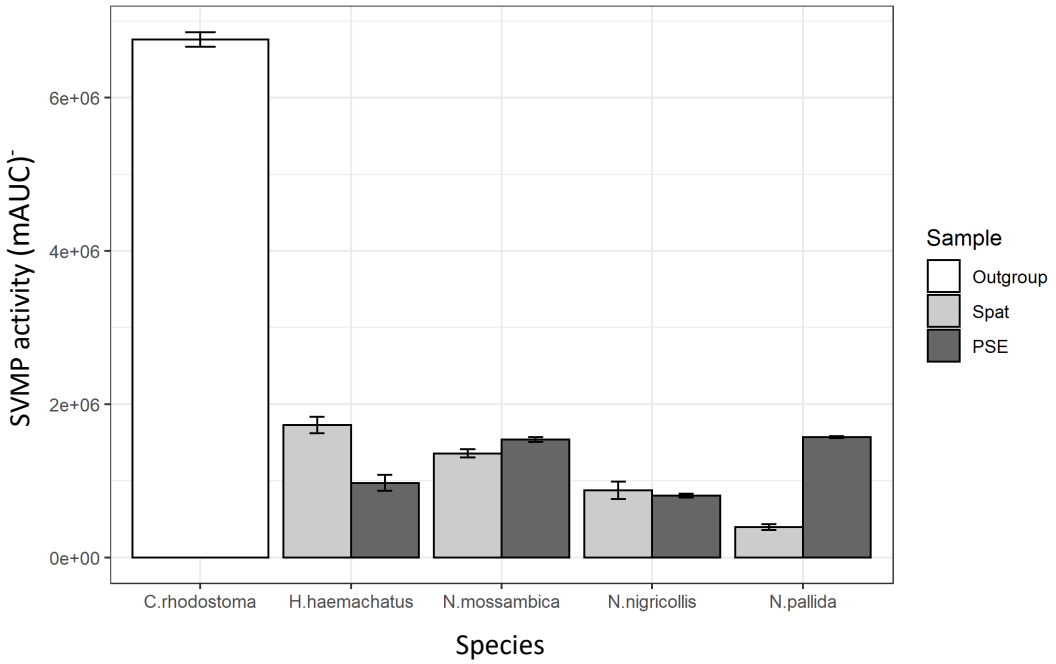


Fig. 6.9. Snake venom metalloproteinase (SVMP) assay of spat and post-spitting extracted (PSE) venoms from spitting cobras, compared to the activity from the viper *Calloselasma rhodostoma*. SVMP activity is measured as the mean of the control area under the curve (mAUC) minus individual AUC of fluorescence intensity, multiplied by -1 $(mAUC)^{-1}$. Each sample was measured in triplicates of 10µL and error bars represent standard errors of the mean.

6.3.viii. PLA_2 Activity

All venom samples showed moderate PLA_2 activity (Fig. 6.11.), though this was substantially less than the results from the pooled venoms used in the previous chapter (Fig. 5.6A.) when compared with the same viper outgroups, *Bothrops asper* and *Daboia russelli.* PLA_2 activity, measured as the mean of the control AUC minus the AUC from absorbance data at venom concentrations of 100 µg/mL (mAUC), ranged from 4.04 to 7.13 in spat samples, with a mean of 5.24 and from 3.85 to 9.29 with a mean of 6.47 in PSE samples. There was no significant difference in PLA_2 activity between spat and PSE samples (Kruskal-Wallis rank sum test, $X^2 = 1.76$, $p = 0.18$) (Table 6.4.).

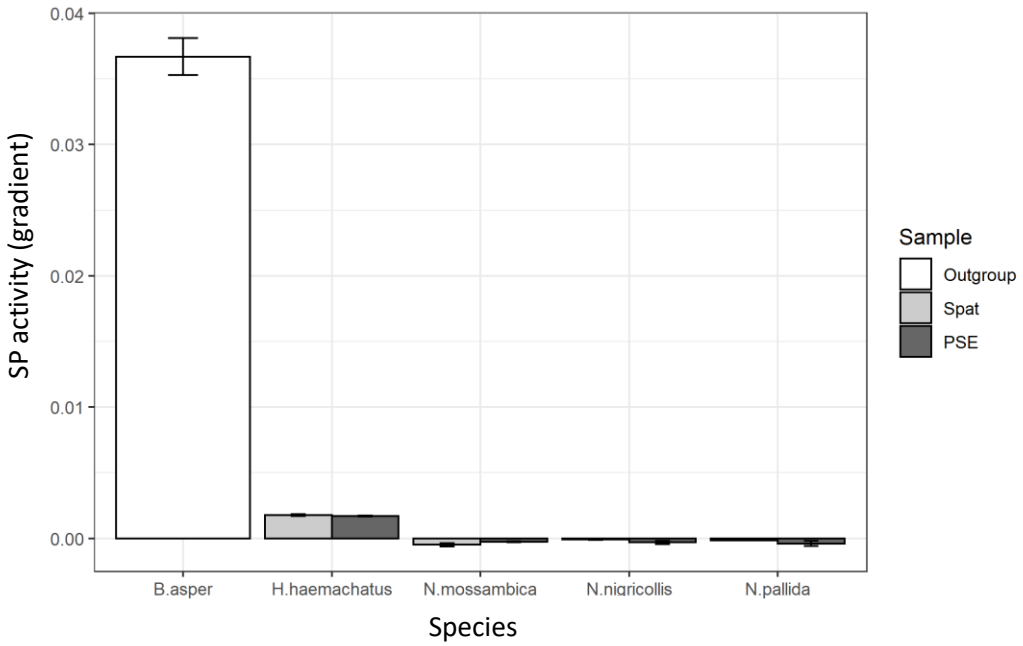


Fig. 6.10. Serine protease (SP) assay of spat and post-spitting extracted (PSE) venoms from spitting cobras, compared with that of the viper *Bothrops asper*. SP activity is represented by the averaged gradient of the line produced by fluorescence intensity over time. Each sample was measured in 4 replicates of 10µL.

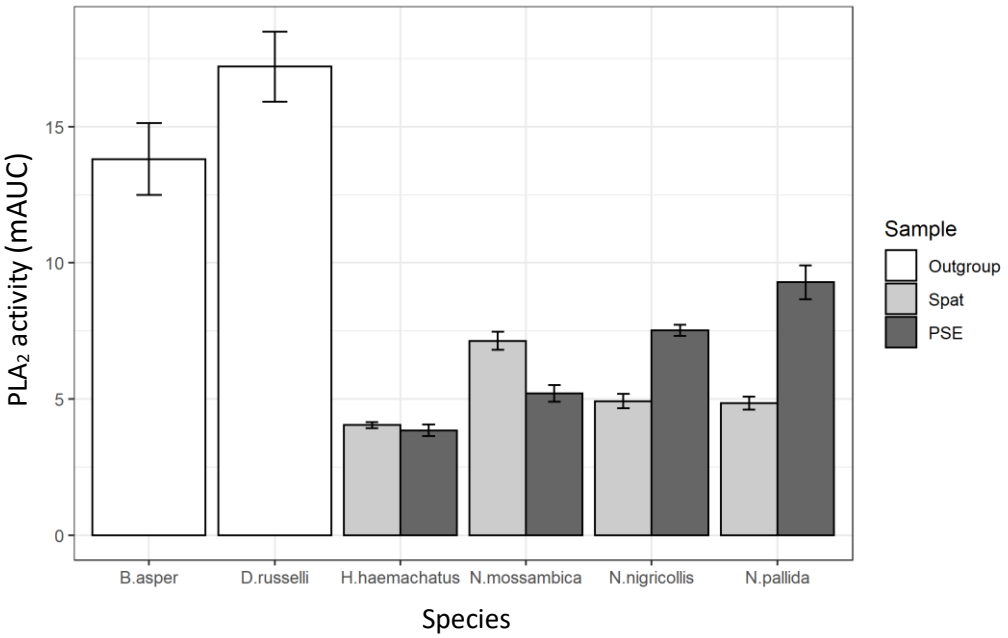


Fig. 6.11. Phospholipase A_2 (PLA_2) assay of spat and post-spitting extracted (PSE) venoms from spitting cobras, compared with the activity of the vipers *Bothrops asper* and *Daboia russelli*. PLA_2 activity is measured as the mean of the control AUC minus the AUC from absorbance data at venom concentrations of 100 µg/mL (mAUC). All venoms were pipetted as replicates of 4, in 10 µL samples.

Table 6.4. Test statistics from ANOVA or unparametric analyses assessing the effect of whether venom is spat or extracted (PSE) on functional activities. SVMP – snake venom metalloproteinase, SP – serine protease, PLA_2 – phospholipase A_2.

Factor	Test type	Test statistic	p
SVMP	Kruskal-Wallis rank sum test	$X^2 = 0.85$	0.36
SP	Kruskal-Wallis rank sum test	$X^2 = 0.48$	0.49
PLA_2	Kruskal-Wallis rank sum test	$X^2 = 1.76$	0.18
Plasma	Kruskal-Wallis rank sum test	$X^2 = 0.00$	1.00

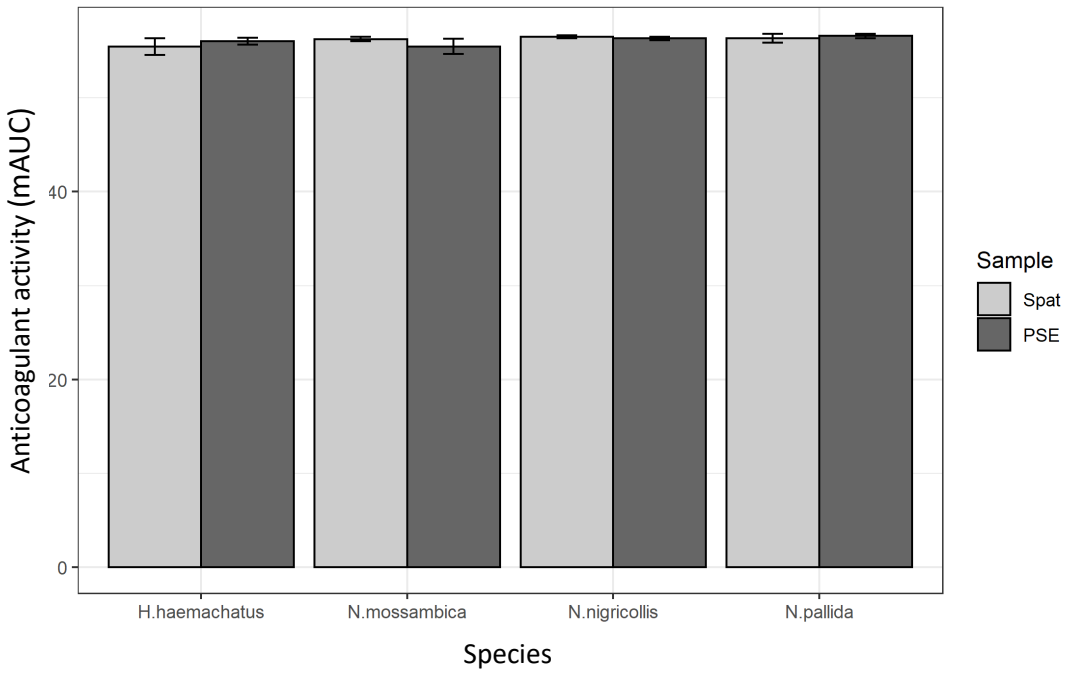


Fig. 6.12. Plasma assay of extracted (standard), spat and post-spitting extracted (PSE) venoms from spitting cobras. Anticoagulant activity is measured as the mean control AUC minus the AUC from absorbance data of venom at concentrations of 100µg/mL (mAUC).

6.3.ix. Activity Against Bovine Plasma

All venoms displayed anticoagulant activity (Fig. 6.12.), consistent with results from the previous chapter (Fig. 5.8.). Anticoagulant activity, measured as the mean control AUC minus the AUC from absorbance data of venom at concentrations of 100µg/mL (mAUC), showed very little variation across samples, ranging from 55.47 to 56.51 in spat samples and from 55.48 to 56.60 in PSE samples, with corresponding means of 56.15 and 56.12 respectively. There was no significant difference in anticoagulant activity between spat and PSE samples (Kruskal-Wallis rank sum test, $X^2 = 0.00$, $p = 1.00$) (Table 6.4.).

6.4. Discussion

Previous studies have shown that bipartite venoms are used by some animals for the separate purposes of predation and defence, and that the composition of these venoms often differs with its intended purpose (Dutertre et al., 2014; Gangur et al., 2017; Inceoglu et al., 2003; Walker et al., 2018). This chapter explores whether this is the case in spitting cobras, which also have bipartite venom use, through investigating whether the proteomic and functional activities differ between spat venom and venom injected through biting (simulated by venom extraction).

This compositional and functional profiling of cobra venoms collected following spitting or biting revealed that these different secretions are highly similar to one another. For example, the three profiling approaches applied here revealed no proteins that were unique to either spat or extracted venom in the four spitting cobras tested here. Functional analyses also revealed no significant differences between spat and post-spitting extracted (PSE) venom in any of the functional assays (Table 6.4.). Crucially, the lack of functional differences in PLA_2s, which have been previously shown to have a significant difference in abundance and function between spitting and non-spitting cobra species (See chapters 4 and 6), suggests that these defensive toxin components are not differentially ejected depending on the use of the secreted venom.

The SDS-PAGE, cation exchange and RP-HPLC profiles clearly demonstrate that venom collected via biting immediately after spitting does not differ

substantially, if at all, from venom ejected during defensive spitting (see Fig. 6.4.-6.8.). These findings clearly suggest that the defensive venoms of spitting cobras do not contain different protein compositions to the venom ejected during biting for predominately predatory purposes. These observations contrast with the few limited examples of comparative analysis of defensive and predatory venoms seen in other venomous species, such as cone snails (Dutertre et al., 2014), scorpions (Gangur et al., 2017) and assassin beetles (Walker et al., 2018), where toxin compositional and/or functional differences were observed. There are likely many reasons for these distinctions, not least the independent origins of the venom systems of these animals. However, the cone snails *Conus geographus* and *Conus marmoreus*, and scorpion *Parabuthus transvaalicus* are able to identify predation threat and exert immediate control over their venom composition as a result, with *Parabuthus transvaalicus* first producing a defensive "prevenom", that contains more K^+ ions and channel-aggravating peptides to induce pain (Inceoglu et al., 2003) and cone snails producing paralytic toxins that target mammalian receptors (Dutertre et al., 2014). Similarly, the scorpion *Liocheles waigiensis* has been shown to identify and respond to predation threat when exposed over a longer period of 42 days, changing the proportion of proteins in its venom (Gangur et al., 2017).

The only potential predation threat experienced by spitting cobras at the Herpetarium of the Liverpool School of Tropical Medicine are the times when they are handled for venom extractions, which is less than once a month. Venom composition is therefore not reflective of repeated predator exposure, though this would be interesting to investigate further. Previous studies have shown that individual snakes can change venom composition over time (Gibbs et al., 2011) but this tends to be over a period of years, where hatchling snakes go through a number of ontogenetic changes and dietary shifts that may induce differences in venom composition so it is difficult to factor in the role of predation threat during this time without thorough long term study that controls for external factors like temperature and prey availability. However, the spitting cobra *Naja pallida* has been shown to exhibit compositional differences in spat venom after the 20^{th} spit (Cascardi et al., 1999) so it is possible that long-term or persistent exposure to a predation threat may also act as a selective factor for changes in venom composition for an individual.

Further testing of venom composition over spits, as above, across a larger number of spitting species would help to answer this question.

Unlike the assassin beetle, cobras do not have a separate gland for defensive venom. Venom is produced in the venom glands, which are singular entities located at the rear of the head, connected to an accessory gland, positioned anteriorly, via ducts (Rosenberg, 1967) (Fig. 1.2.). It is possible that these accessory glands produce entirely different toxins to the main venom gland, which has been seen in transcripts from the king cobra (*Ophiophagus hannah*) (Vonk et al., 2013) but not in the jararaca viper (*Bothrops jararaca*) (Valente et al., 2018). It may therefore be difficult to exert immediate control over venom composition at the molecular level. However, cone snails also only have a single tissue source for venom production, the venom bulb, yet are able to switch the type of venom evoked from predatory to defensive instantly after only one exposure to a predator as separate sections of the same gland produce different toxins (Dutertre et al., 2014). There is no evidence of this form of glandular separation in snakes as of yet, though the MALDI imaging used in the Dutertre study could be used to identify any such regions in snake venom glands in future studies. It may be then that there is a limitation on venom plasticity in reptiles or even vertebrates in general. A couple of factors may make it easier for selection to act on active separation of defensive and offensive venom components in invertebrates than the large vertebrates that comprise venomous snakes: i) Their smaller size may make them more frequent targets of predation and thus the selective pressure on defence may be higher ii) invertebrates often mature quickly and reproduce more rapidly than vertebrates, meaning that turnover time is considerably longer. The lack of functional differences between spat venoms and those simulating a bite are therefore unsurprising.

The interpretations of this chapter come with some limitations. Firstly, in this pilot study sample sizes are small, with only one individual representing each spitting species, and each individual subjected to only one round of spitting to limit stress to snakes and exposure of staff to danger. Additionally, only two of the three spitting cobra lineages were tested here, limiting our comparisons between distinct spitting lineages. While captively held specimens of *N. siamensis* and *N. philippinensis* were

subjected to the venom collection strategy outlined in 6.2.*i*, neither could be encouraged to spit even after repeated attempts.

Overall, this chapter represents the first work to extensively compare spat and extracted venoms from spitting cobras to address the question of whether spitting cobras evoke compositionally distinct toxic secretions in response to the dual ecological role of their venom. The findings of this pilot study strongly suggest that spitting cobras do not have active or passive control of the molecular diversity of the venoms that they evoke for predatory or defensive purposes, although further research relating to increased breadth of individuals and species of spitting cobras, along with investigations of the spatial heterogeneity of toxin production and storage in the venom gland of these snakes would likely be revealing.

7. DISCUSSION

7.1. Overview of this thesis

This thesis has compiled the transcriptomic, proteomic and functional data from 14 species of true cobra (genus *Naja*), the spitting elapid known commonly as the rhinkals (*Hemachatus haemachatus*) and the non-spitting elapid outgroups *Walterinnesia aegyptia* and *Aspidelaps scutatus*. This data was then analysed in the context of the threat of snakebite envenomation and venom evolution, with a focus on patterns emerging with the convergent evolution of defensive spiting behaviour in snakes. This analysis has revealed that all cobras have venoms rich in three finger toxins (3FTX), with cytotoxins being the most abundant 3FTX subclass present. While no significant differences in 3FTX abundances were detected between spitting and non-spitting cobras, I found that spitting cobras have convergently upregulated the abundances of the second most abundant toxin family, the phospholipase A_{2s} (PLA_2s), in their venom proteomes. African spitting cobras and the rhinkals (*Hemachatus haemachatus*) have achieved this upregulation through increased transcriptomic expression, which directly translates to increased proteome abundance, while Asian spitting cobras may have increased the proteomic PLA_2 abundances through post-transcriptional mechanisms, as elevated gene expression levels were not detected. In addition, such increases in PLA_2 abundance may be relevant to anticoagulant and other pathological symptoms seen in the bites from spitting cobras, especially in African spitting cobras, where previous evidence suggests these snakes cause distinct symptoms compared with their non-spitting counterparts (David A. Warrell, 2008c).

The conclusions described above are the result of integrated data analyses from the transcriptome, proteome and functional levels of investigating cobra venoms. In Chapter 2, African spitting cobras and the related spitting species *H. haemachatus* were shown to have high PLA_2 expression levels in venom gland transcriptomes, while elevated PLA_2 expression is not detected in the Asian cobras, with the exception of *N. siamensis* (Fig. 2.4.), leading to a lack of significance between the PLA_2 expression of spitting and non-spitting cobras (Two-way ANOVA: $F = 0.08$ $df = 1$, $p = 0.79$; Table 2.4.). Phylogenetic reconstructions of the evolutionary history of

the PLA_2 toxin family, described in Chapter 4, suggests that the high expression levels of these toxins found in African spitting species appears to be the result of gene duplication events. These have resulting in a number of moderately-expressed PLA_2 genes, which when combined together, result in increased PLA_2 diversity and summed abundance compared with non-spitting counterparts. Contrastingly, a single, highly-expressed PLA_2 gene and one, very lowly-expressed gene were detected in *H. haemachatus*, evidencing less PLA_2 diversity that African spitting cobras, despite comparable expression levels (Fig. 4.19.). Thus, while these two different spitting cobra lineages appear to have convergently increased the expression levels of PLA_2s, they have done so via different molecular processes.

In Chapters 3 and 4, analysis of venom proteomic data revealed that PLA_2 abundances are significantly higher in spitting than non-spitting cobras (Two-way ANOVA: $F = 14.03$, $df = 1$, $p = 0.003$; Table 3.3.), and this holds up when reanalysed while taking into account phylogenetic signal (phylogenetic generalized least squares; $t = 4.27$, $df = 1$, $p < 0.001$; Table 4.3.). It was noted that there seemed to be some discrepancies in PLA_2 abundance between what has been recorded in this study and data from previous studies, especially concerning the Asian cobras, which may be a result of this study using a small number of captive bred specimens, as opposed to a larger or unknown number of wild-caught individuals (Asad et al., 2019; Huang et al., 2015; Tan, 2016; Shan et al., 2016; Sintiprungrat et al., 2016; Tan et al., 2019a; Wong et al., 2018b; Xu et al., 2017). To account for this, additional analyses were performed incorporating previous literature into the dataset, and the significantly higher PLA_2 abundance of spitting cobras was maintained both with (phylogenetic generalized least squares; $t = 5.20$, $df = 1$, $p = 0.0001$; Table 4.3) and without (Two-way ANOVA: $F = 17.58$, $df = 1$, $p = 0.002$) the context of phylogenetic signal. Moreover, venom proteome PLA_2 abundances are considerably increased in the Asian spitting cobras from that predicted by the venom gland transcriptomes, suggesting that the upregulation of PLA_2 abundance in the proteome may be due to post-transcriptional modifications in Asian spitting cobras, such as alternate splicing or RNA editing to produce multiple PLA_2 isozymes from a single gene. Such mechanisms have been shown to contribute to increased abundance of c-type lectins in *Echis* vipers (Casewell et al., 2014) and ontogenetic changes in the venom of the

rattlesnake Crotalus simus are thought to be a result of changes in the targets of regulatory miRNAs (Durban et al., 2013), though conflicting evidence suggests that post-transcriptional mechanisms by themselves contribute little to venom variation in snakes (Rokyta et al., 2015). Potentially then, post-translational modifications may have led to a large number of PLA_2 protein isoforms with a large combined abundance in the proteome. These type of mechanisms have been shown to result in and increased protein diversity of serine proteases, c-type lectins and l-amino acid oxidases in *Echis* (Casewell et al., 2014), and increased diversity of nerve growth factor toxins in Australian elapid venoms (Earl et al., 2006).

There is an issue in the interpretation of what the presence or absence of PLA_2 itself means in terms of both snakebite envenomation and snake venom evolution. PLA_2s are known to have a diverse array of functional activities that make interpretations problematic in the context of the detection of increased levels of PLA_2 abundance in spitting cobras without directly testing isolated fragments in a multitude of assays. Chapter 5 explored the functional activities of cobra venoms using assays relevant to PLA_2 toxins, including their enzymatic activity and anticoagulant potency (Fig. 5.6., 5.10., 5.11.). This chapter revealed an association between anticoagulant activity with PLA_2 abundance (see Fig. 5.6., 5.10., 5.11), which is possibly clinically relevant in the context of snakebite envenoming (Warrell et al., 1976). However, despite significant differences in PLA_2 abundance between spitting and non-spitting cobras, and divergence in the amino acid composition of PLA_2 isoforms detected between spitting cobra lineages (Fig. 4.21), no significant differences in enzymatic PLA_2 activity or anticoagulant activity were detected. We found no significant association between enzymatic PLA_2 activities (PGLS: $t = -0.69$, $p = 0.50$) and the evolution of venom spitting, suggesting that convergent increases in PLA_2s associated with defensive venom spitting may be linked to non-enzymatic activities. Indeed, previous studies have provided evidence that there is often little to no relationship between enzymatic PLA_2 activity and their pharmacological activity (Gutiérrez & Lomonte, 2013), with several papers suggesting that many PLA_2s may have an alternate mechanism of action to standard hydrolysis of phospholipids via the targeting of proteins within the phospholipid bilayer (Kini & Evans, 1989). Additionally, PLA_2s can interact with Nicotinic acetylcholine receptors (nAChRs) to

produce neurotoxic effects (Vulfius et al., 2013), and the products of enzymatic phospholipid hydrolysis themselves can also have neurotoxic effects (Rigoni et al., 2005).

Chapter 6 revealed that there is no major difference in the protein composition or functional activity of spat venom compared to venom that is extracted (venom injected through biting). This suggests that spitting cobras are not capable of controlling the differential release of different toxins based on the stimuli they are presented with, unlike several venomous invertebrates (Dutertre et al., 2014; Inceoglu et al., 2003; Nisani & Hayes, 2011). Consequently, any components relevant for defensive spitting are detected via the proteomic data described herein, and are therefore not influenced by the method of venom delivery or collection.

Overall, these data suggest that the significant differences in PLA_2 abundance observed are likely driving defence-specific activities via mechanisms that do not rely on anticoagulation or enzymatic activity. If indeed post-translational modifications have led to an increased diversity of PLA_2 proteins, as speculated for the Asian spitting cobras above, it is possible that this has led to an increased diversity of functions, many of them potentially non-enzymatic and undetectable by the assays used here.

7.2. PLA_2s as Defensive Toxins in Venom Spitting

The problem of these functionally hyper-diverse toxins (Kini, 2003; Sunagar et al., 2015a, 2015b; Zambelli et al., 2017) will continue to impede interpretive analysis of venom composition due to the difficulty in creating a standardised, relevant functional test. Due to this, it is difficult to ascertain with any certainty at this time why increased PLA_2 abundance may provide a selective advantage for spitting cobras. However, if cobra venom PLA_2s are responsible for causing cell destruction via their cytotoxic effects, as previously alluded to (Debnath et al., 2010; Méndez et al., 2011; Panagides et al., 2017; Reali et al., 2003; Rudrammaji & Gowda, 1998; Suzuki-Matsubara et al., 2016), then it is possible that spitting cobras have upregulated PLA_2 in their venoms to cause more potent cytotoxicity in the eyes of their aggressors when hit with a “spit”. Consistent destruction of cells within the eye would invoke gradual and intensifying pain through the liberation of internal cellular components

and may additionally inhibit the ability of an aggressor to see, potentially hindering it from targeting the cobra again after it recovers from the initial pain. However, general tests of cytotoxicity caused by cobra venoms on mammalian cell lines failed to detect general differences between spitting and non-spitting cobra species (Panagides et al., 2017) and tests of irritation using the vasculature of hen's eggs (Luepke, 1985) shows no difference in irritative or cytotoxic potential between spitting and non-spitting cobras (Appendix I).

Alternatively, PLA_2 toxins may induce more immediate pain through the activation of sensory neurons or aggravation of the immune system to cause inflammation. Venom PLA_2s have been associated with pain caused by bee stings (Sharma, 1993), and snake venom PLA_2s have been shown to cause hyperalgesia and inflammatory pain in rats or rat-derived rat trigeminal ganglia (Chacur et al., 2003; Chacur et al., 2004; Zambelli et al., 2017; Zhang et al., 2017) and which appear to be at least partially mediated by the immune system (Mamede et al., 2016). Furthermore, group I PLA_2s from the elapid *Micrurus tener tener* have been shown to activate sensory neurons when in complex with Kunitz toxins (Bohlen et al., 2011), and recent work on the effect of isolated venom components on mouse sensory neurons seems to suggest that cobra PLA_2s have a potentiating effect on the ability of cytotoxins to cause calcium influx into neuronal cells, implying an increase in pain stimulation by PLA_2s (Appendix I). Future work focused on testing the effect that crude venoms and isolated cobra PLA_2 toxins have on ocular tissues and pain-inducement could be particularly revealing in the study of defence-driven snake toxins.

7.3. The Uniqueness of Spitting in Cobras: why only these Afro-Asian Elapids?

If the upregulation of PLA_2s occurred to increase the efficacy of spitting, as postulated above, why is it that spitting is unique to a select number of African and Asian elapids we know as "cobras"? PLA_2 proteins are not unique to cobras; they can be found in large abundances in many Latin American and Australian elapids (Tasoulis & Isbister, 2017) (Table 3.1.). Similarly, cytotoxin abundance and cytotoxicity have not been shown to have any association with spitting (Table 4.3.) but instead with defensive hooding (Panagides et al., 2017). The answer may lie in the unique combination of

pre-existing behaviours with said cytotoxicity. Hooding is a long-distance visual defensive display. The elevated posture provided by this hooding, coupled with pre-emptive striking and accompanied by occasional premature releases of venom, may have provided a behavioural precursor for the evolution of more targeted venom spitting. Phylogenetic reconstructions performed here place the origin of the CTX subfamily of three-finger toxins (3FTXs) prior to the divergence of *Ophiophagus* from the ancestor of the clade containing *Dendroaspis*, *Walterinnesia, Aspidelaps*, *Hemachatus* and *Naja* (fig 4.3 & Fig. 4.13). The existence of cytotoxins, associated with hooding (Panagides et al., 2017) and largely absent from the other elapid venoms (Tasoulis & Isbister, 2017) (Table 3.1.) likely provided sufficient baseline ocular damage that made the retention of spitting advantageous (Santra & Wüster, 2017). Subsequent upregulation of PLA_2 toxins, speculated here to increase the pain-inflicting capacity of cytotoxins may be crucial for causing rapid and severe pain, enough to rapidly deter aggressors and allow for a swift exit.

The question of how spitting and the subsequent convergent upregulation of PLA_2s may have evolved has thus been addressed but the question of why may be more speculative in nature. Most discussions of defensive behaviour involve predation interactions, and snakes do have many mammalian and avian predators (Greene, 1988; Valkenburgh & Wayne, 2010). However, cobras are still successfully predated by these animals. Beyond predators, potential threats to snakes include inadvertent trampling and pre-emptive defensive killing. That spitting evolved to prevent snakes being trampled by herds of ungulates in African savannas (Barbour, 1922) does not explain the existence of primarily forest-dwelling Asian spitting cobras (Chu et al., 2010). Moreover, large ungulates typically have relatively lateral eyes and primarily use olfactory and auditory cues to detect danger, making them unlikely to be especially vulnerable to spitting.

One possible candidate for favouring the repeated evolution of spitting in the Afro-Asian cobras are the emergence of bipedal hominids in these regions. There are several reasons for this: (i) growing evidence suggests that snakes have profoundly influenced primate neurobiology and behaviour (Isbell, 2009), and that interactions between these two lineages have been important throughout the 75 million year history of primates (Greene, 2013; Headland & Greene, 2011). (ii) Compared to

carnivorous mammals, anthropoid primates are highly visually acute, cognitively complex, and have a profound sense of culture (Isbell, 2009). (iii) said anthropoids group up to attack snakes, with some able to distinguish between harmless and dangerous species, killing the latter as a pre-emptive defence (Greene, 2013; Greene, 2017; Headland & Greene, 2011). (iv) Vision and group mobbing are enhanced among bipedal, larger brained hominins (Greene, 2013; Isbell, 2009), making them a potentially important, long-distance threat to Afro-Asian snakes (Faurby et al., 2020). African spitting cobras may have diverged as recently as 6.7 million years ago (MYA) (Appendix I), and this follows closely after the divergence of hominins from bonobos and chimpanzees more than 7 MYA, coinciding with early evolution of bipedalism, enlarged brains, tool use, and occupation of savanna habitats by the former (Pozzi et al., 2014), with a spitting cobra fossil additionally being found in a known hominin site in Africa (Vilakazi et al., 2018). Similarly, the origin of the Asian spitting cobras around 2.5 MYA approximately coincides with the arrival of *Homo erectus* in Asia (Han et al., 2017; Prat, 2018). Additional fossils of individual spitting lineages, along with more finely-tuned dating of relevant cobra and primate divergences may help strengthen this currently rather romantic hypothesis.

7.4. Wider contributions to the field

More broadly, this work contributes 16 transcriptomic and proteomic datasets, of which 13 of the venom gland transcriptomes and 6 of the venom proteomes represent comparative data from never-before characterised snake species. Comparative proteomic analysis has previously been performed on the venoms of African spiting cobras (Petras et al., 2011), and the evolution of defensive hooding and spitting has been examined in the context of the cytotoxic capacity of cobra venoms (Panagides et al., 2017). However, this study integrates multi-level comparisons (gene, protein, function) of all three spitting lineages in comparison with closely and more distantly related non-spitting species, and additionally cross-examined these multi-level factors in the context of phylogenetic structuring, to investigate the influence of defensive spitting on the evolution of snake venom. Indeed, the inclusion of 14 species of true cobras (genus *Naja*) makes this work the second largest comparative venom analysis of any genus described in the literature

to date. More broadly, because venom spitting is a defensive trait unique to this group of snakes, this project has enabled a better understanding of how defensive drivers may underpin venom evolution in venomous snakes, and how the integrated evolution of defensive behaviours and morphological adaptations may influence the venom phenotype at the molecular level.

This study also provided some interesting findings relevant to the context of snakebite envenoming, which represents a life-threatening neglected tropical disease that affects millions of people annually (Gutiérrez et al., 2017). Cobras are medically important snakes that cause thousands of deaths each year in sub-Saharan Africa and Asia (Warrell, 2008b, 2008c). Though major differences in clinical symptoms have been observed following bites by spitting and non-spitting cobras (e.g. cytotoxicity vs neurotoxicity, respectively), particularly in African species (Warrell, 2008c), the root of such pathological differences are poorly understood. This study identifies a potential culprit for these pathologies in the form PLA_2 toxins. Spitting cobras, whose venoms have been shown here to contain a significantly higher abundance of PLA_2 toxins may cause more noticeable local cytotoxic pathologies due to the destructive action of cell membranes. Indeed, the combination of abundant PLA_2s in the presence of cytotoxic 3FTXs may substantially enhance cytotoxic venom potential in a manner comparable to that previously observed on red blood cells (Dutta et al., 2019) and mouse neuronal cells (Appendix I). This provides a clear target for future antivenom research and intervention for cobra bites. Particularly interesting is ongoing research into the potential use of small molecules such as the PLA_2 inhibitor Varespladib (LY315920) to prevent symptoms. Varespladib has already shown to reduce PLA_2-mediated haemorrhage, oedema, myonecrosis and neurotoxicity (Gutiérrez et al., 2020; Wang et al., 2018), making it a promising candidate for treating PLA_2-mediated anticoagulant activity and cytotoxicity from spitting cobra bites.

7.5. Study limitations

The limitations of this work can be divided into technical and interpretive limitations. Concerning the former, we must first be cautious regarding interpretation of the venom gland transcriptomic data. In the case of this study, transcriptome assembly

can only inform us about the venom gland gene composition of a single individual, taken at a single point in time. Thus, arguably, greater weight should be applied to the proteomic data described in this study, which represents for most species pools of venom collected from multiple individuals, with proteomic annotations informed by the generated venom gland transcriptomic data. Another limitation is that de novo snake venom gland transcriptome assembly is challenging, because in the absence of a reference dataset (e.g. genome, transcriptome) robustly assembling highly related toxin isoforms encoded by multilocus gene families is problematic, and can result in the generation of chimeric sequences or underclustering (Archer et al. 2014). To offset these risks, here we used a validated pipeline (Ainsworth et al., 2018; Giribaldi et al., 2020; Pla et al., 2018; Pla et al., 2017; Whiteley et al., 2019) consisting of MiSeq 2x250bp sequencing technology, applied to aid assembly, and the venom gland-dedicated assembly program VTBuilder (Archer et al., 2014). Furthermore, annotation of transcriptome sequences is reliant on the accuracy of the NCBI database and corresponding resources which are, along with assembly and annotation, naturally prone to some degree of human error. These issues may be lessened, in time, with the gradual addition of sequences to the database, and with the development of techniques that allow transcriptome sequencing of multiple individuals from the same species without loss of life. Similarly, the proteomic data is also beholden to the same errors associated with database-led annotation. A standardisation of transcriptome sequence assembly, snake venom pooling and proteome assembly would further make data more comparable across the literature.

In terms of interpretive limitations, the work described herein would of course benefit from the inclusion of additional ingroup species of the genus *Naja*. Currently, 14 of the 33 described species of *Naja* were analysed, but additional species would increase the power of the applied phylogeny-based statistical analyses, namely the phylogenetic generalized least squares (PGLS). Perhaps most relevant to this would be the inclusion of additional African cobra species closely related to *N. subfulva* (subgenus *Boulengerina*), such as *N. melanoleuca*, *N. annulata* or *N. multifasciata*, to increase phylogenetic coverage in this distinct cobra clade (Fig. 4.3.). In the same vein, acquiring additional venom gland transcriptomic and venom proteomic data from the immediate sister group to *Naja*, snakes of the genus

Pseudohaje (*P. goldii* and *P. nigra*) (see Fig. 4.3.), would provide further insight into the evolution of cytotoxic 3FTXs (CTXs) and PLA_2s. The sampling of additional, more distantly related, elapid groups (e.g. beyond *Aspidelaps* and *Walterinnesia*) could also provide wider context to the ancestral trait reconstructions described here. In particular, the sampling and inclusion of genera such as *Micrurus* and *Bungarus*, and many of the Australian elapids, which do not hood or spit but still have moderate to high PLA_2 representation in the proteome (Table 3.1.; see also (Tasoulis & Isbister, 2017) and evidence of PLA_2 activity in several genera (Pycroft et al., 2012) would be valuable.

A final limitation of this work is the classification throughout this thesis of *Naja atra* and *Naja kaouthia* as non-spitting cobras. However, there have been limited records of certain individuals from certain populations of these snake two Asian cobras displaying some degree of spitting ability (Paterna, 2019; Santra & Wüster, 2017). While this 'spitting' is relatively ineffective compared with the release of venom by 'true spitting' species, it is prudent to reassess the data presented here in light of these observations. To account for the potential of these species being spitting cobras, the most evolutionarily-relevant statistical analyses, PGLS, was performed in an identical manner except that *N. atra* and *N. kaouthia* were recoded as spitting species. The majority of recoded analyses resulted in little difference in terms of statistical significance, or lack thereof (Table 7.1.), however, recoding these two species does result in changes in the prior association between 3FTX abundance and venom spitting becoming non-significant ($p = 0.15$, previously $p = 0.01$). Most importantly, increased PLA_2 abundance in spitting species remained statistically significant ($p = 0.02$, previously $p < 0.001$), even when incorporating the PLA2 data from previous literature ($t = 3.58$, $p = 0.003$), showing the importance of PLA_2 abundance as a factor in spitting cobra venoms.

7.4. Future work

In terms of future work, the focus should be on characterising the primary purpose for increased PLA_2 abundance observed in spitting cobra venoms. Preliminary data from collaborators indeed suggests that PLA_2 toxins from spitting cobra species enhance the activation of sensory neurons when combined with cytotoxins

Table 7.1. Summary statistical Table with *Naja atra* and *Naja kaouthia* coded as either spitting or non-spitting cobras. Only the phylogenetic generalized least squares (PGLS) results are displayed as they take phylogenetic structuring into account and are thus the most evolutionarily relevant. Significant values are emboldened, and those that remain significant post Bonferroni-correction are represented by red text.

	Test Variable	*Naja atra* and *Naja kaouthia* coded as:	t	p
Transcriptome	PLA_2 expression	Non-spitters	-1.08	0.30
		Spitters	0.11	0.91
	3FTX expression	Non-spitters	-0.05	0.96
		Spitters	-0.77	0.46
	CTX expression	Non-spitters	0.93	0.37
		Spitters	0.49	0.63
	'Other 3FTX' expression	Non-spitters	-1.24	0.23
		Spitters	-0.89	0.39
Proteome	**PLA_2 abundance**	**Non-spitters**	**4.27**	**0.0008**
		Spitters	**2.72**	**0.02**
	3FTX abundance	**Non-spitters**	**-2.81**	**0.01**
		Spitters	-1.53	0.15
	CTX abundance	Non-spitters	-0.38	0.71
		Spitters	0.15	0.88
	'Other 3FTX' abundance	Non-spitters	-1.16	0.27
		Spitters	-1.22	0.24
Function	PLA_2 Activity	Non-spitters	0.69	0.50
		Spitters	1.00	0.33
	Plasma activity	Non-spitters	1.47	0.16
		Spitters	1.46	0.17
	SVMP activity	Non-spitters	-2.05	0.06
		Spitters	-1.65	0.12
	SP activity	Non-spitters	-0.62	0.54
		Spitters	0.35	0.73

(Appendix I). The development and application of more specific bioassays to investigate the specific mechanism of action of pain-inducing toxins would be particularly revealing. In addition, the use of an increased breadth of cobra species from which specific toxins, such as CTXs and PLA_2s could be isolated from for testing, would enable increased robustness of these findings determined from this exciting pilot work. Indeed, functional analyses of pain inducement enhanced by different

isolated PLA_2 isoforms from the same venom, would be particularly revealing in terms of understanding the specificity of diverse venom PLA_2s, and also the dichotomous relationship observed between PLA_2 diversity detected in African spitting cobra venoms (isoform-rich) versus that of *Hemachatus* (one PLA_2). An extension of this would be to examine differences in the functional activities of the basic and acidic isoforms of PLA_2s detected in a number of cobra venoms (Fig 2.11. & 3.9.), and the potential role that differential post-translational modifications, such as glycosylation, may play in the PLA_2 differences observed between spitting and non-spitting cobras. That latter point may be particularly pertinent given that many snake venom toxins are subjected to post-translational modifications (Andrade-Silva et al., 2016; Casewell et al., 2014; Earl et al., 2006), along with previous evidence demonstrating that the glycosylation of a cytotoxin isolated from *Naja kaouthia* venom exhibits restricted biological activity (Osipov et al., 2004), thus such mechanisms likely result in biologically-relevant effects.

7.5. Concluding remarks

The results from this thesis demonstrate that a unique defensive behaviour in snakes, that of venom spitting, has had substantial consequences on the venom composition that have evolved this adaptation. In addition to morphological changes to the venom delivering fangs that facilitate this unique behaviour, we detected here significant increases in the abundances of PLA_2 toxins in the venoms of spitting cobras. Crucially, these increases have occurred convergently with the three independent origins of venom spitting, although different spitting cobra lineages have utilised distinct strategies to upregulate these proteins in their venom. The convergent evolution of such defensive venom toxins observed here represents the first such instances detected in the venom snakes, and may enable spitting cobras to induce increased pain via ocular contact during defensive encounters.

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APPENDIX I

The following is a manuscript (submitted in April 2020), written from the combination of the data produced from this thesis and data from our collaborators across the globe.

Title: Convergent Evolution of Pain-Inducing Defensive Venom Components in Spitting Cobras

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Abstract: Convergent evolution provides unparalleled insights into the selective drivers underlying evolutionary change. While snakes use venom primarily for predation, and venom composition often reflects diet specificity, three lineages of spitting cobras have independently evolved the ability to use venom as a defensive projectile. Using gene, protein and functional analyses, we show that the three spitting lineages possess venom characterized by an upregulation of PLA_2 toxins, which potentiate the action of venom cytotoxins to activate mammalian sensory neurons and cause enhanced pain. These repeated independent changes provide a fascinating example of convergent evolution across multiple phenotypic levels driven by exaptations. Notably, the timing of their origins suggests that defensive venom spitting may have evolved in response to the emergence of bipedal hominids in Africa and Asia.

One Sentence Summary: Venom spitting by snakes coincides with the emergence of hominins and is underpinned by convergent increases in pain-enhancing toxins

Main Text:

Convergent evolution, the independent emergence of similar traits, provides unparalleled opportunities to understand the selective drivers and mechanisms of adaptation (Losos, 2017). Thanks to their discrete function and direct genotype-phenotype link, animal venoms represent a superlative model system for unravelling both the driving forces and the underlying genetic mechanisms of molecular adaptation. Snake venoms consist of variable mixtures of proteinaceous components that cause potent hemotoxic, neurotoxic and/or cytotoxic pathologies in both prey and potential adversaries, including humans (Gutiérrez, Calvete, et al., 2017). Previous evidence suggests that venom variation is largely driven by dietary variation (Daltry et al., 1996), but defensive drivers of snake venom evolution are rarely considered (although see (Ward-Smith et al., 2020)). The evolution of venom projection or 'spitting' in cobras offers an excellent model system for exploring the evolution of defensive toxins, as this behavior has no role in prey capture, targets specific sensory tissues, and is the only long-distance, injurious defensive adaptation among almost four thousand species of snakes. Remarkably, venom spitting evolved independently three times, all within a single clade of closely related elapid snakes (Panagides et al., 2017; Wolfgang Wüster et al., 2007): the African spitting cobras (*Naja*: subgenus *Afronaja*), Asian spitting cobras (*Naja*: subgenus *Naja*) and the rinkhals (monotypic *Hemachatus*). All use modified fangs with small, rounded, front-facing orifices (Bogert, 1943) to produce a spray of venom, which may reach distances of 2.5 m (Rasmussen et al., 1995), and targets the eyes of an aggressor (Westhoff, G., Boetig, M., Bleckmann, H., Young, 2010) (Fig. S1). This suite of behavioral, morphological, and biochemical traits results in intense ocular pain and inflammation, which can lead to the loss of eyesight (D. A. Warrell & Ormerod, 1976). The three independent origins of spitting, solely within a clade more generally characterized by

the visually defensive behavior of hooding (Panagides et al., 2017; Wolfgang Wüster et al., 2007), provides an ideal model system to test whether similar selective pressures have resulted in convergent changes in molecular venom composition and overlying morphological and behavioral levels.

We used a multi-disciplinary approach consisting of transcriptomic, proteomic, functional and phylogenetic comparisons of 17 widely distributed elapids: 14 *Naja* (true cobras), the rinkhals *Hemachatus haemachatus*, and two non-spitting immediate outgroup species, *Walterinnesia aegyptia* and *Aspidelaps scutatus*, to investigate the evolutionary consequences of venom spitting. First, we reconstructed the phylogeny of these snakes using a multilocus coalescent species tree approach based on sequences from two mitochondrial and five nuclear genes. Fossil-calibrated molecular dating suggests that spitting originated in African spitting cobras 6.7-10.7 million years ago (MYA), and likely around 4 million years later in the Asian spitting cobras (2.5-4.2 MYA) (Fig. 1A). The origin of spitting in *Hemachatus* could not be dated, other than determining that it occurred <17 MYA, following its divergence from true cobras (*Naja*) (Fig. 1A).

Next, we used a top-down proteomics approach underpinned by venom gland transcriptomic data (Ainsworth et al., 2018) to characterize the toxins found in the venom of each species. Those analyses demonstrated that all cobra venoms are dominated by three finger toxins (3FTX), while in many species phospholipases A_2 (PLA_2) are the second most abundant toxin family (Fig. 1A, Fig. S2). Principal coordinate analysis (PCoA, Bray-Curtis), performed on a proteomic data-derived venom composition matrix of the ingroup species (*Naja* & *Hemachatus* spp.), separated the spitting lineages into three clusters that are distinct from the homogeneous cluster of venoms from non-spitters (Fig. 1B). The sole exception to this was *N. philippinensis*, a species reported to have a purely neurotoxic venom despite being able to spit (Watt et al., 1988), and whose venom composition placed it alongside non-

spitting species (see Fig. 1B). Nonetheless, these findings demonstrate that each spitting cobra lineage exhibits distinct venom compositions that collectively differ from those of non-spitting cobras – a finding consistent with differences in venom-induced pathology observed following bites to humans (Meier & White, 2008).

3FTXs are major venom components in many elapid snakes (Tasoulis & Isbister, 2017) (Table S1). They are encoded by a multilocus gene family, resulting in numerous functionally distinct isoforms, including neurotoxins that interfere with various neuromuscular receptors, and cytotoxins that disrupt cell membranes to cause cytotoxicity (Ferraz et al., 2019). Proteomic data revealed that cytotoxic 3FTXs (CTXs) are typically the most abundant toxins found in *Naja* and *Hemachatus* venoms (mean 57.7% of all toxins), contrasting with the sister group species *W. aegyptia* and *A. scutatus* (Whiteley et al., 2019) and other elapids (Fig. 1A, Figs. S2, S3, Table S1). Despite ocular cytotoxicity presumably stimulating pain of value for enemy deterrence, we found no significant difference in the abundances of CTXs between spitting and non-spitting species (PGLS; $t = -0.83$, $df = 15$, $p = 0.42$) (Table S2), and ancestral state estimations suggest that the origin of CTX-rich venom preceded that of venom spitting (Fig. S4) (Panagides et al., 2017). Moreover, PCoA analysis of a CTX Euclidean distance matrix derived from venom proteomic data revealed that all highly abundant cobra CTXs cluster tightly together (Fig. 1C), regardless of spitting ability. These findings extended to functional activities: measures of irritation, stimulated by high doses (1 mg/mL) of cobra venoms applied topically to non-sentient chick embryos (Tables S3, S4), revealed no association between cytotoxicity (Figs. S5, S6) and venom spitting (PGLS; $t = 1.08$, $df = 15$, $p = 0.30$) – results consistent with prior reports of comparable cytotoxicity to mammalian cells across the venoms of cobra species (Panagides et al., 2017). Thus, the main toxins thought to be responsible for cytotoxic venom effects are similar across the different spitting and non-spitting cobra species.

Pain inflicted via the slow development of cytotoxicity may be less defensively relevant than that caused by a more immediate route, that of direct, algesic activity. To investigate venom-induced nociception, we assessed, via calcium imaging, the activation of trigeminal neurons – sensory neurons derived from the trigeminal ganglia that innervate the face and eyes (Fig. S7). Venoms from all cobra species activated trigeminal sensory neurons, though a lack of substantial activity was noTable in African non-spitting cobras and the Asian non-spitting cobra *Naja kaouthia* (Fig. S7). The mechanism of activation for all venoms was characterized by rapid non-cell-type-specific calcium influx followed by dye release from the cells into the extracellular space, indicative of non-specific disruption of cell membranes. We next determined potency values for each venom in sensory neuron-derived F11 cells. The resulting half maximal effective concentrations (EC_{50}) demonstrated that spitting cobras had more potent venoms (PGLS; t = -4.48, df = 15, p = 0.0007) (Fig. 2A, Fig. S8, Table S2). These data therefore strongly support the hypothesis that the emergence of venom spitting in cobras is associated with convergent elevations in venom-induced activation of sensory neurons, and that spitting cobra venoms are more effective in causing pain than their non-spitting counterparts.

To determine the toxins responsible for this effect, we repeated these experiments using fractionated venom from three representative spitting cobras (*N. nigricollis*, African; *N. siamensis*, Asian; *H. haemachatus*, rinkhals) (Fig. S9). For all species tested, only fractions corresponding to CTXs activated sensory neurons, while those corresponding to other toxins (e.g. neurotoxins, PLA_2s, etc) were inactive (Fig. S9). However, none of the CTX fractions by themselves completely recapitulated the effects of whole venom or those of re-pooled venom fractions, suggesting that multiple venom components are acting synergistically.

PLA_2s are nearly ubiquitous, typically enzymatic, multifunctional toxin components found in snake venoms (Ferraz et al., 2019; Tasoulis & Isbister, 2017). As the hemolytic

activity of CTXs was previously shown to be potentiated by PLA_2 toxins (E. Condrea, 1974), we hypothesized that, although venom PLA_2s are not independently capable of activating sensory neurons, they may potentiate the effects induced by CTXs. Consequently, for each of the three representative spitting cobra venoms, we quantified the activation of sensory neurons stimulated by CTX fractions in the presence or absence of a corresponding PLA_2 venom fraction. Consistent with our expectation, the proportion of viable sensory neurons activated by each CTX fraction was significantly increased when preceded by the addition of a PLA_2 fraction, and this result was consistent for each representative of the three spitting lineages (unpaired t-test; *N. nigricollis*, t = 18.77, df = 2, p = 0.003; *N. siamensis*, t = 5.75, df = 4, p = 0.005; *H. haemachatus*, t = 4.18, df = 4, p = 0.01) (Fig. 2B, Fig. S10). To confirm the pain-potentiating activity of PLA_2 toxins, we compared the potency of sensory neuron activation caused by whole venoms from our eight spitting species in the presence or absence of the PLA_2 inhibitor varespladib. Significant reductions in sensory neuron activation occurred in the presence of this inhibitor (unpaired t-test; t = 2.77, df = 14, p = 0.02) (Fig. 2C, Fig. S11), providing further compelling evidence that PLA_2s potentiate CTX effects on sensory neurons.

In line with the above findings, comparative analysis of (i) the proteomic abundance of PLA_2 toxins and (ii) the quantified enzymatic PLA_2 activity, determined via specific in vitro colorimetric assay, revealed that spitting cobra venoms have significantly higher PLA_2 abundance and activity than those of non-spitting species (PGLS; t = 4.24, df = 15, p = 0.0007 and t = 2.24, df = 15, p = 0.04, respectively) (Fig. 3A, 3B, Fig. S12, Table S2). Analysis of a PLA_2 Euclidean distance matrix derived from venom proteomic data revealed substantial distinctions between the different lineages of spitting and non-spitting cobras, particularly in the case of African species (Fig. 3C). Additionally, *H. haemachatus* PLA_2s clustered tightly with those from African spitting cobras (Fig. 3C), despite the divergence of *Hemachatus* ~17 MYA. These findings suggest that the PLA_2s of African spitting cobras and *H. haemachatus*

exhibit some evidence of molecular convergence, and divergence from the PLA_2s of Asian spitting cobras (Fig. 3C). The combination of these data, plus evidence from the sensory neuron assays, demonstrate that convergent evolution of spitting is tightly linked with convergent upregulation of PLA_2 toxins in those venoms.

To exclude the possibility that functional distinctions simply reflect general differences in venom potency, e.g., venom lethality for prey capture, rather than specifically defensive adaptations, we tested each of the venoms in a murine lethality assay (median lethal dose, LD_{50}). These experiments revealed highly variable venom potencies (2.36-33.75 µg/mouse across *Naja*) (Table S5) and no significant differences between spitting and non-spitting species (PGLS; t = 0.86, df = 15, p = 0.40) (Table S2). This strongly suggests that the enhanced pain caused by spitting cobras is explicitly associated with the defensive use of those venoms, rather than being an evolutionary spandrel from selection for prey subjugation.

While the Asian cobras *N. atra* and *N. kaouthia* have been considered as non-spitters throughout this study, recent reports suggest that certain individuals/populations of these two species show some spitting ability (Paterna, 2019; Santra & Wüster, 2017). However, reanalysis of our data with *N. atra* and *N. kaouthia* treated as spitting species revealed only minor differences in statistical significance for the various analyses (Table S6). Importantly, our key results concerning associations between venom spitting and (i) increased PLA_2 abundance and (ii) enhanced activation of sensory neurons remaining highly significant (PGLS: t = 2.86, df = 15, p = 0.01 and t = -4.59, df = 15, p = 0.0004, respectively; Table S6), suggesting that our choice of coding of these two species does not impact our conclusions.

Our results detail the genetic and functional consequences of the evolution of venom spitting and demonstrate that defense can be a major driver of snake venom composition. Spitting probably only evolved within one relatively small clade of Afro-Asian elapids due to the integrated exaptation of a unique combination of pre-existing behaviors and cytotoxic

venom activities. Early evolution of cytotoxic venom activity in cobras and near relatives (~26 MYA; Fig. S4) has previously been linked to defense, as cytotoxicity co-originates with 'hooding' (Panagides et al., 2017), a long-distance visual defensive display. This elevated posture, coupled with pre-emptive striking and accompanied by occasional premature releases of venom, may have provided a behavioral precursor for the evolution of more targeted venom spitting. Pre-existing CTXs, largely absent from the other elapid venoms (Table S1), likely provided the necessary baseline ocular toxicity to favor the inception and retention of spitting (Santra & Wüster, 2017). Subsequent independent increases in PLA_2 toxins, which act in synergy with pre-existing CTXs, resulted in increased venom-induced activation of nociceptors (Fig. 2). This potentiating effect of PLA_2s may be crucial for causing immediate pain with sufficient intensity to rapidly deter aggressors, allowing the snake to escape. Consequently, our results from spitting cobras highlight how convergence at molecular, morphological, behavioral, and functional levels, in combination with behavioral and molecular exaptations, can drive complex adaptations in ecologically important traits.

Rare but repeatedly evolved adaptations likely result from similar but unexpected ecological relationships. Most discussions of defensive behavior involve potential predators, and certain mammals and birds commonly eat snakes (H. W. Greene, 1988; Valkenburgh & Wayne, 2010). However, predation events involving spitting cobras still occur often, therefore these interactions appear unremarkable in terms of evolutionary history and biogeography. Beyond predators, potential threats to snakes include inadvertent trampling and pre-emptive defensive killing. That spitting evolved to prevent snakes being trampled by herds of ungulates in African savannas (Barbour, 1922) does not explain the existence of primarily forest-dwelling Asian spitting cobras (Chu et al., 2010). Moreover, large ungulates typically have laterally-located eyes and primarily use olfaction and hearing to detect danger, making them unlikely to be especially vulnerable to spitting.

Several considerations make ancient hominins a more compelling candidate for favoring repeated evolution of spitting in the Afro-Asian cobras: (i) growing evidence suggests that snakes have profoundly influenced primate neurobiology and behavior (Lynne A. Isbell, 2009), and that interactions between these two lineages have been important, sometimes reciprocally, throughout the 75 million year history of primates (H. W Greene, 2013; Headland & Greene, 2011). (ii) Compared to obligately carnivorous mammals, anthropoid primates are more visually acute, cognitively complex, and culturally sophisticated (Lynne A. Isbell, 2009). (iii) Diverse anthropoids mob snakes, and some distinguish between harmless and dangerous species, eating the former and killing the latter with clubs or projectiles (H. W Greene, 2013; Harry W Greene, 2017; Headland & Greene, 2011). (iv) Characteristics (ii) and (iii) are enhanced among bipedal, larger brained hominins (H. W Greene, 2013; Lynne A. Isbell, 2009), which could have posed a singularly important, long-distance threat to dangerous Afro-Asian snakes (Faurby et al., 2020). The initial divergence of Africa spitting cobras as recently as 6.7 MYA (Fig. 1) occurred soon after the divergence of hominins from *Pan* (bonobos and chimpanzees) ~7 MYA, thus coinciding with the early evolution of bipedalism, enlarged brains, tool use, and occupation of savanna habitats by the former (Pozzi et al., 2014). Likewise, the origin of the Asian spitter clade ~2.5 MYA is approximately contemporaneous with the arrival in Asia of *Homo erectus* (Han et al., 2017; Prat, 2018). Additional fossils and more finely-tuned dating of relevant cobra and primate divergences will enable further testing of this hypothesis. Nonetheless, the repeated evolution of spitting cobras might well amount to an expansion of 'Lucy's Legacy' (Jolly, 1999), alluding to the famous *Australopithecus afarensis* fossil that initially shed so much light on bipedalism and big brains in our own lineage.

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Supplementary Materials:

Materials and Methods

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Tables S1-S9

Data S1-S3

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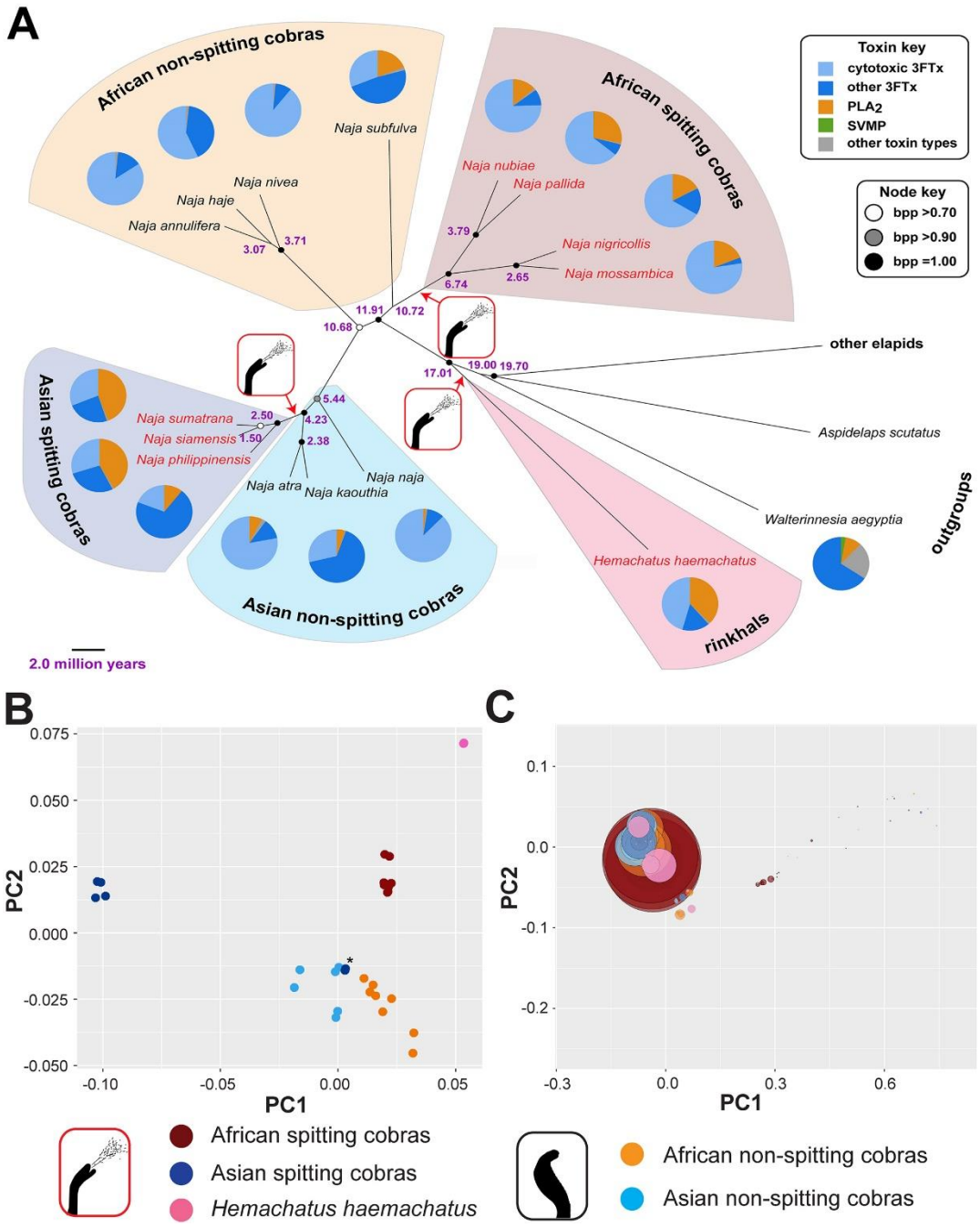

Fig. 1. Reconstruction of the evolutionary origin of venom spitting and comparative analysis of venom composition. (A) The species tree is derived from five nuclear and two mitochondrial markers reconstructed using a multispecies coalescent model in *BEAST and pruned to display the taxa whose venoms were analyzed in this study. Support for the various nodes is indicated by colored circles, representing the following Bayesian posterior probabilities (bpp): black = 1.00, grey >0.90, white >0.70. Purple node labels indicate calculated divergence times from the *BEAST analysis (see Fig. S13 for calculated divergence time ranges). Spitting species are highlighted by red tip labels, and the three independent origins of venom spitting are

indicated by the red-boxed spitting images and arrows. Pie charts adjacent to the tip labels represent the proteomic toxin composition of each of the sampled species as a percentage of total toxin proteins: light blue, cytotoxic 3FTx; dark blue, other 3FTx; orange, PLA_2; green, SVMP; grey, all other toxin types. (B) Principal Coordinate Analysis (PCoA) of cobra (*Naja* spp.) and rinkhals (*H. haemachatus*) venom toxins reveal major distinctions between the spitting (red, Africa; dark blue, Asia; pink, *H. haemachatus*) and non-spitting (light blue, Asia; orange, Africa) lineages. The asterisk highlights Asian spitting species *N. philippinensis*, which exhibits greater similarity to non-spitting Asian and African species than to its nearest relatives (other Asian spitting cobras). Note each species is represented by two, typically overlapping, data points, which represent technical proteomic duplicates. (C) PCoA of cobra (*Naja* spp.) and rinkhals (*H. haemachatus*) CTXs reveals that the most abundant CTXs detected in venom exhibit little sequence diversity among spitting and non-spitting lineages. PCoA analysis was performed on a Euclidean distance matrix containing CTX amino acid data derived from top-down venom proteomics. Circle sizes reflect relative abundances of CTXs detected in the venom proteomes. The different lineages are colored as in B.

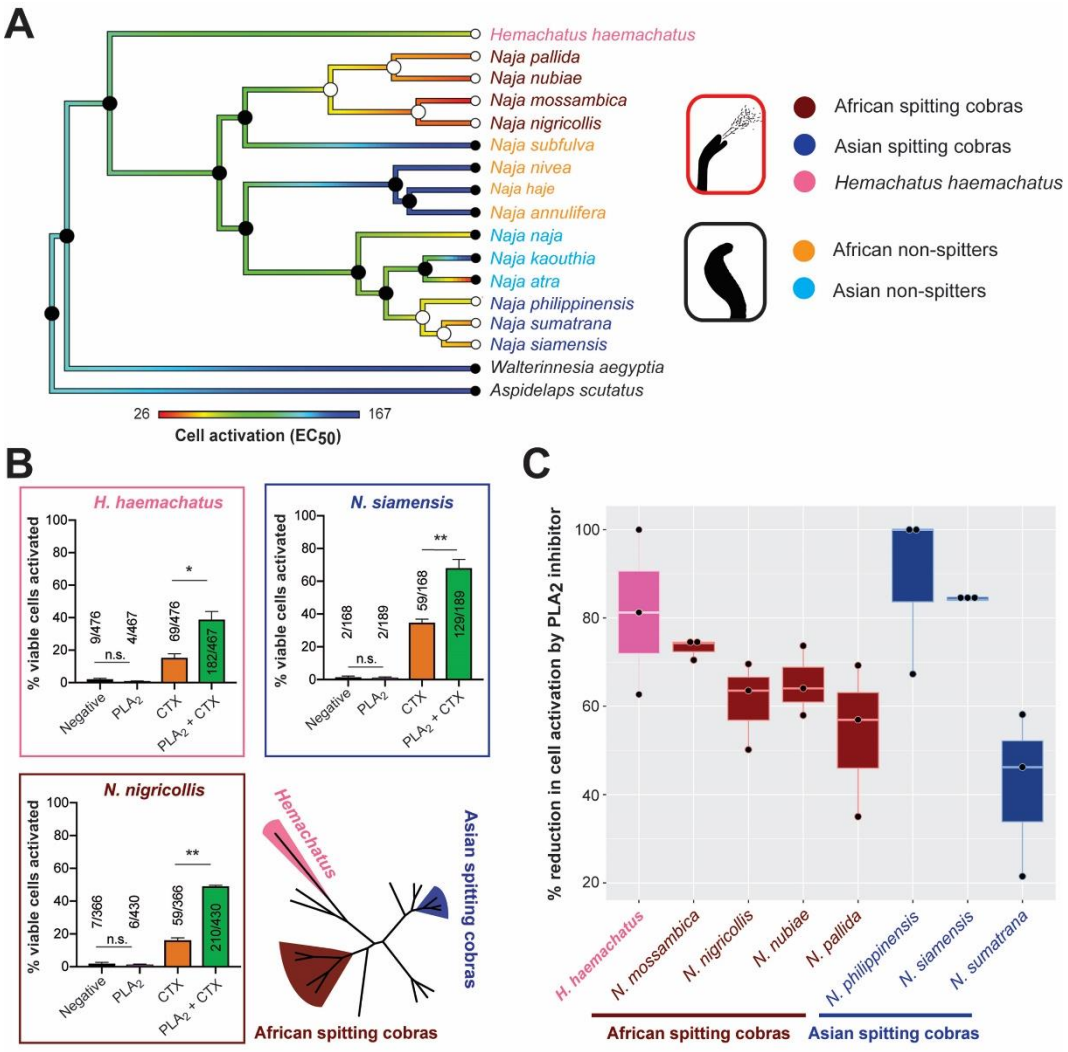

Fig. 2. Spitting cobra venoms cause significantly greater activation of sensory neurons than non-spitting cobras, mediated via potentiation by PLA_2 toxins. (A) Ancestral state estimation of the half maximal effective concentrations (EC_{50}) of venom-induced activation of neuronal cells shows a significant association between venom potency and venom spitting (PGLS, t = -4.48, p = 0.0004). EC_{50} values were derived from concentration-response curves fitted to the data using a four-parameter Hill equation with variable Hill slope, and are expressed as the mean of triplicate measurements. Colored branches are scaled according to levels of cell activation (red, low EC_{50} and thus high venom potency; blue, high EC_{50} and thus lower venom potency). At each node and tip of the tree, filled or empty circles represent estimated ancestral states of non-spitting or spitting, respectively, and colored tip labels correspond to different lineages detailed to the right: spitting (red, Africa; dark blue, Asia; pink, *H. haemachatus*) and non-spitting (light blue, Asia; orange, Africa). (B) PLA_2 toxins in spitting cobra venoms potentiate the activating effect of CTXs on sensory neurons. A CTX fraction from each venom was added to dissociated mouse DRG neurons in the presence or absence of a corresponding PLA_2 fraction (added 1 min prior). Neuronal activation (i.e. a rapid increase in $[Ca^{2+}]_i$) was monitored following the first (1 min window) and second (2 min window) additions and presented as a proportion of viable cells. Data are expressed as mean ± SEM and are representative of 2-3 experiments. Statistical comparisons were made using unpaired parametric *t*-tests in Graphpad Prism (version 8.02). *, $p < 0.05$; **, $p < 0.01$. (C) The PLA_2 inhibitor varespladib reduces neuronal activation stimulated by spitting cobra venoms. Calcium influx in F11 cells was measured on a FLIPR instrument incubated in the

presence of venom from spitting species (2.4 µg or 4.8 µg [in the case of *H. haemachatus* and *N. philippinensis*] venom) and in the presence or absence of varespladib (13 µM). The data displayed represents the percentage of venom only cell activation stimulated by treatment with venom and varespladib. Values are max-min (n = 3). The error bars represent standard error of the mean (SEM) with the box mid-lines representing the median value.

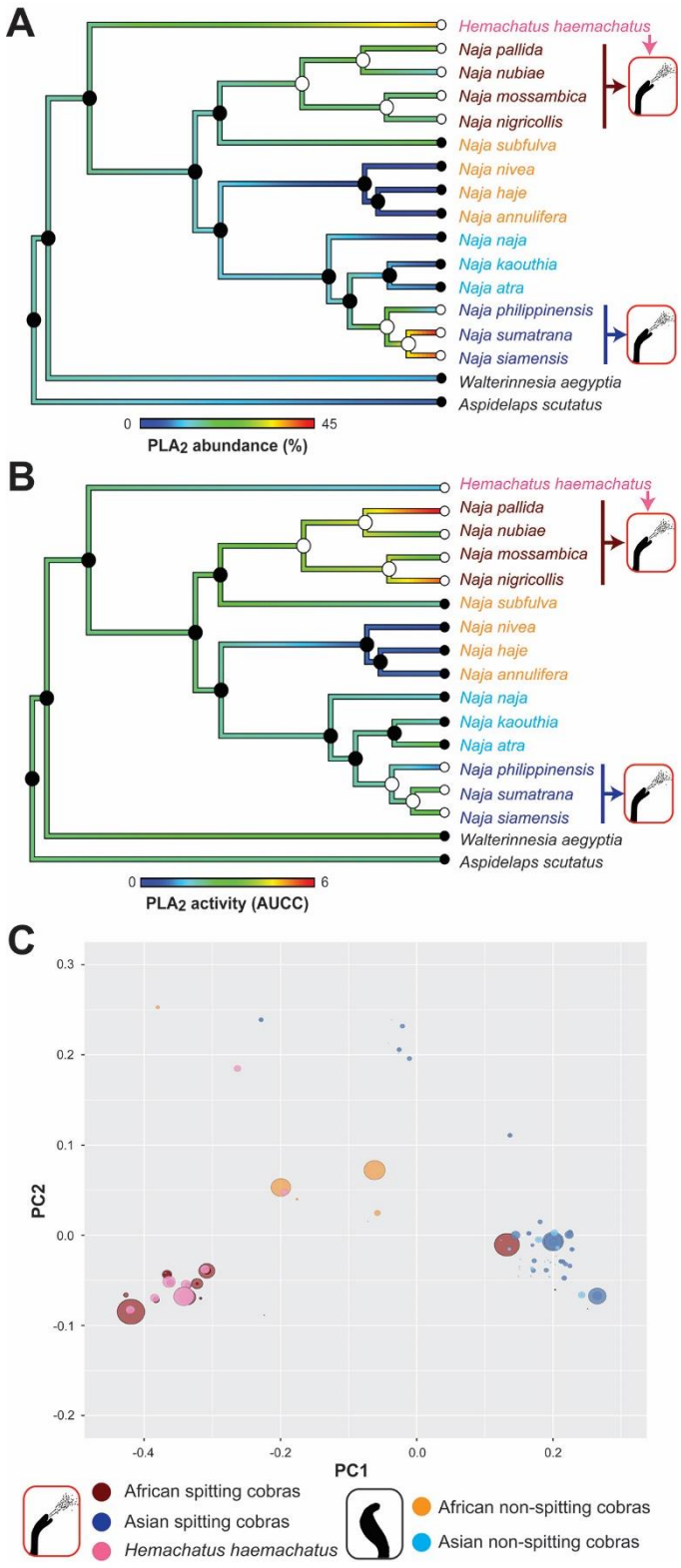

Fig. 3. The abundance, enzymatic activity and diversity of phospholipase A_2 (PLA_2) toxins is associated with convergent evolution of venom spitting. (A) Ancestral state estimation of proteomic abundance of PLA_2s, represented as the percentage of PLA_2 toxins in the venom proteomes, revealed a significant association with venom spitting (PGLS, t = 4.24, p = 0.0007). Colored branches are scaled according to PLA_2 abundance (blue, low abundance; red, high

abundance). At each node and tip of the tree, filled or empty circles represent the estimated ancestral state of non-spitting or spitting, respectively, and colored tip labels correspond to the different lineages detailed in C. (B) Ancestral state estimation of enzymatic PLA_2 activities, determined via area under the curve of concentration curves (AUCC) analysis of a kinetic *in vitro* colorimetric assay, revealed a significant association with venom spitting (PGLS, $t = 2.24$, $p = 0.04$). Colored branches are scaled according to PLA_2 activity (blue, low activity; red, high activity). Labels as in (A), and see Fig. S12 for PLA_2 activity concentration curves. (C) Principal Coordinate Analysis (PCoA) of cobra (*Naja* spp.) and rinkhals (*H. haemachatus*) PLA_2 toxins reveals major variation between African spitting and non-spitting lineages, but little variation between Asian spitting and non-spitting lineages. Note also the convergent placement of *Hemachatus* PLA_2 toxins with those of African spitting cobras. PCoA analysis was performed on a Euclidean distance matrix containing PLA_2 amino acid data derived from top-down venom proteomics. Circle sizes reflect relative abundances of PLA_2s detected in the venom proteomes. Spitting lineages are colored red (African), dark blue (Asian) and pink (*H. haemachatus*) and non-spitting lineages are colored light orange (African) and light blue (Asian).

Supplementary Materials for

Convergent Evolution of Defensive Venom Components in Spitting Cobras

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Materials and Methods

Venoms and venom glands

Venoms from cobras and related species were pooled from wild-caught or captive bred (CB) specimens maintained in the Liverpool School of Tropical Medicine Herpetarium (Table S7). Crude venoms were lyophilized post-extraction and stored at 4°C until analysis. For venom gland transcriptomics, single specimens from each species were euthanized three days after

venom extraction, and dissected venom glands were immediately flash frozen in liquid nitrogen and stored cryogenically until use. Details of the specimens used in this study are displayed in Table S7.

Species tree reconstruction

We reconstructed the phylogenetic relationships and divergence times of 46 species of cobra and related elapid taxa, representing almost all species of *Naja* and cobra-clade elapids under a multispecies coalescent model. This analysis was based on DNA sequence alignments of six genetic loci: mitochondrial DNA (partial CytB and ND4 gene sequences; Table S8); and the phased partial exon sequences of the nuclear genes CMOS, NT3, PRLR, UBN1 and RAG1. Briefly, we obtained blood samples, ventral scale clips, shed skins or other tissue in the field or from captive collections. Whole genomic DNA was extracted using a Qiagen DNeasy™ Tissue Kit (catalogue no. 69506 – www.qiagen.com), following the manufacturer's instructions, except for blood samples, where PBS buffer was not used and 200 µL of blood in Tris-EDTA buffer was added to 20 µL proteinase K. Where shed skins were used, the samples were left to lyse overnight. PCR was carried out in 15 µL volumes, using ThermoScientific 2X DreamTaq buffer. Primer sequences and thermocycling parameters are given in Table S8. Sanger dideoxy sequencing was carried out by Macrogen (dna.macrogen.com), using the forward primer for mitochondrial genes and forward and reverse primers for nuclear loci.

Sequences were checked, trimmed, translated into amino acid sequences and checked for unexpected indels or non-sense codons in CodonCode Aligner version 3.7.1 (www.codoncode.com). In nuclear locus sequences, heterozygous positions were replaced with the IUPAC uncertainty codes. The alleles of length heterozygotes were reconstructed using the online tool Indelligent 1.2 (http://dmitriev.speciesfile.org/indel.asp) (Dmitriev &

Rakitov, 2008). Individual allele sequences (haplotypes) were then estimated from diploid nuclear loci using the software PHASE v. 2.1.1 (Stephens et al., 2001; Stephens & Scheet, 2005) over 5000 iterations with a burn-in of 500 and a thinning interval of 10, after preparation of the sequence data using SEQPHASE (Flot, 2010). PHASE was run three times to confirm burn-in and convergence across multiple runs. PHASE was run separately by genus, or, in the case of *Naja* by subgenus.

Details of the final DNA sequence alignments were as follows: mitochondrial DNA, 1,316 bp, 281 sequences; CMOS, 628 bp, 206 sequences; NT3, 657 bp, 340 sequences; PRLR, 551 bp, 456 sequences; UBN1, 504 bp, 414 sequences; RAG1, 879 bp, 281 sequences. Sample details and accession numbers are shown in Table S8. For phylogenetic reconstruction, we used a multispecies coalescent model employed in *BEAST in BEAST v.1.8.2 (A. J. Drummond et al., 2012). Our approach was to initially generate a species tree for the entire set of 46 taxa, including virtually all cobras and cobra-like elapids, and then to extract the tree of the focal 17 species from that tree. The multispecies coalescent model implemented in *BEAST coestimates individual gene (locus) trees embedded within the overall species tree while accounting for incomplete lineage sorting. It requires prior assignment of sequences to species, and that each of these taxa be represented by at least one sequence of each locus. We lacked CMOS and RAG 1 sequences for certain taxa (*N. christyi* and *N. sputatrix*), so a single "dummy" sequence consisting of Ns was inserted for each of these taxa into the respective alignments in order to initiate the analysis.

Preliminary *BEAST runs using node calibrations based on the fossil record to estimate divergence times on an unconstrained species tree failed to converge, indicating overparameterisation. To overcome this, we adopted a two-step approach. First, an uncalibrated analysis was carried out estimating the topology of the species tree with the substitution rate of the mitochondrial alignment fixed at an arbitrary value and the substitution rate of the other gene alignments estimated relative to it. Then, a calibrated

analysis was carried out to estimate divergence times by applying internal node calibrations based on the fossil record, with the monophyly of all species tree nodes that were well supported in the uncalibrated analysis (posterior probability > 0.99) constrained.

For the uncalibrated analysis, the precise model specification was determined by trialing more complex models initially, and then incrementally reducing model complexity (in terms of the number of free parameters) in order to achieve convergence. A Yule process was specified for the species tree with a piecewise constant population size model. A single GTR+G nucleotide substitution model with empirical base frequencies was specified for nuclear DNA, by linking the substitution model partitions of the respective five nuclear gene alignments. A separate GTR+G nucleotide substitution model with estimated base frequencies was specified for the mitochondrial alignment. A separate, unlinked strict molecular clock model was specified for each locus. The substitution rate of the mitochondrial locus was arbitrarily fixed to 1, and the substitution rates of the five nuclear loci estimated relative to it by assigning uninformative, uniform priors between 0 and 1. It was necessary to run the MCMC chain for hundreds of millions of generations, sampling every 1,000 generations, to achieve burn in and adequate sampling of parameters. This was run until the physical limits of the computer's hard drive were reached, at which time all parameters had burned in and achieved effective sample sizes > 200, except for two parameters which were > 170, assessed using the program Tracer. Trees sampled during burn in were removed, and the posterior sample of trees downsampled to approximately 50,000 trees using the program LogCombiner. The maximum clade credibility tree was then selected from these ~50,000 trees, with node heights centered on the median of the subsample, using TreeAnnotator.

For the calibrated analysis, the model specification was identical except the monophyly of species tree nodes that were well supported in the uncalibrated analysis were

enforced, and node calibrations based on the fossil record were used to estimate divergence timings. These were: an exponential prior with a 0.01 MY mean and 17.0 MY offset on the MRCA of *Naja*, *Hemachatus* and *Pseudohaje*, which effectively fixed the age of this node at 17 MYA (Head et al., 2016); since fossil calibrations constrain the minimum but not the maximum age of a node, this represents the youngest plausible age of this node. We also applied a uniform prior between 0 and 10 MY on the MRCA of the Australian genera *Oxyuranus* and *Pseudechis*, effectively placing a maximum divergence time on these genera of 10 MYA (Head et al., 2016). The substitution rate of the mitochondrial locus was estimated based on these calibrations within an uninformative, uniform prior between 0 and 2% per million years. The substitution rates of the nuclear loci were estimated using uninformative uniform priors between 0 and 1.5% per million years. The MCMC chain was run for sufficient length to achieve burn in and effective sample sizes > 200 for all parameters. The maximum clade credibility tree was obtained as described above. We then pruned taxa not included in venom analyses from the tree while retaining the original node ages, and used this tree for all subsequent analyses.

Venom gland transcriptomics

Dissected venom glands were homogenized using a pestle and mortar, before RNA was extracted using the TRIzol Plus RNA Purification kit. RNA samples were then DNAse treated and mRNA selected for using the Dynabeads mRNA DIRECT purification kit protocol (Life Technologies). The RNA-Seq libraries were generated from 50 ng of each enriched sample using the TruSeq Stranded mRNA HT Sample Prep Kit (Illumina). As per the Illumina cBot User guide protocol, samples were then denatured and loaded at a concentration of 10 pM, and sequencing was carried out on multiple lanes (six samples multiplexed per lane) of an Illumina MiSeq with 2 × 250 bp paired-end sequencing (Centre for Genomic Research,

University of Liverpool). The resulting reads were quality processed using CGR's standard protocols (Ainsworth et al., 2018; D. Pla, Sanz, Whiteley, et al., 2017; Whiteley et al., 2019), and then assembled into contigs using the transcriptome assembly program VTBbuilder (Archer et al., 2014). The following parameters were used for assembly: minimum transcript length 150 bp, minimum read length 150 bp, minimum isoform similarity 96%. As part of this process, the VTBuilder algorithm performs normalized read mapping approach on the assembled contigs to generate relative transcript expression data, which is expressed for each contig as a percentage of the total transcriptome expression (Archer et al., 2014). The resulting assembled contigs were then converted into six frame translations, to provide amino acid databases for later protein spectrum matching. Assembled DNA contigs were also annotated with BLAST2GO Pro v3 (Conesa et al., 2005) using a BLASTx algorithm and a threshold of 1e-3 against the NCBI non-redundant protein database (GenBank release 219). Manual curation of BLAST2GO annotations were undertaken using the BLASTx annotations on all contigs with expression levels of 0.1% of the transcriptome or higher. Based on these annotations, contigs were classified into toxins (those showing homology with known snake venom toxins previously identified in the literature), non-toxins or 'unknown' (if no BLAST annotation or only hypothetical matches were detected). Toxins were further classified by toxin family and the contigs of all toxin families with >1% expression in at least one cobra species were subjected to downstream analysis in MEGA v7 (Kumar et al., 2016). As a quality control step, contigs were removed from these datasets if any of the following conditions were not met: i) sequence length was less than 100 bp, ii) 50% or more of the sequence did not match the target toxin in a BLASTx search, iii) the first 50% of the sequence was interrupted by a stop codon, indicating the presence of a pseudogene or misassembly, or iv) the sequence was made up of two exonic regions interspersed by an intron, indicating genomic DNA contamination. For some sequences, it was apparent that 'underclustering' had occurred during the assembly process; a common by product of assembling isoform rich

gene data (Archer et al., 2014), but more desirable than the alternative scenario of frequently producing chimeric contigs. To resolve this issue, contigs were merged if a ≥50 bp overlap resulted in ≥98% similarity. Sequences were then trimmed to the open reading frame and aligned using the MUSCLE algorithm (Edgar, 2004) in MEGA's amino acid space, before the alignments were visually inspected for errors, and edited manually if required.

Top-down venom proteomics

Denaturing top-down proteomic experiments were performed as described in previous papers (Petras et al., 2011; D. Pla et al., 2018). In short, venom samples were dissolved in ultrapure water to a final concentration of 10 mg/mL, and centrifuged at 12,000 x g for 5 min. For reduction of disulfide bonds, 10 µL of dissolved venom was mixed with 10 µL of 0.5 M tris(2-carboxyethyl) phosphine (TCEP), and 30 µL of 0.1 M citrate buffer (pH 3). After 30 min incubation at 65 °C, samples were mixed with 50 µL of acetonitrile/formic acid/H_2O (10:1:89, v/v/v) and centrifuged at 12,000 x g for 5 min. After centrifugation, 5 µL of supernatant of reduced samples were injected for LC-MS/MS analyses. LC-MS/MS experiments of two technical replicates were carried out on a Vanquish ultra-high-performance liquid chromatography (UHPLC) system coupled to a Q-Exactive hybrid quadrupole orbital ion trap (Thermo Fisher Scientific, Bremen, Germany). LC separation was performed on a Supelco Discovery Biowide C18 column (300Å pore size, 2 x 150 mm column size, 3 µm particle size) at a temperature of 30 °C. A flow rate of 0.5 mL/min was used and the samples were eluted with a gradient of water with 0.1% formic acid (FA) (solution A) and 0.1% FA in acetonitrile (ACN) (solution B). Gradient elution started with an isocratic phase with 5% B for 0.5 min, followed by a linear increase to 40% B for 50 min, and 40-70% B for 60 min. Finally, the column was washed with 70% B for 5 min and re-equilibrated at 5% B for 5 min. ESI settings of the mass spectrometer were adjusted to 53 L/min sheath gas, 18 L/min

auxiliary gas, spray voltage 3.5 kV, capillary voltage 63 V, S lens RF level 90 V, and capillary temperature 350 °C. MS/MS spectra were obtained in data dependent acquisition (DDA) mode. Mass spectra were acquired with 1 micro scan and 1000 ms maximal C-trap fill time. AGC targets were set to 1E6 for MS1 full scans and to 3E5 for MS/MS scans. Both, the survey scan and data dependent MS/MS scans were performed with a mass resolution (R) of 140,000 (at m/z 200). For MS/MS, the three most abundant ions of the survey scan with known charge were selected for Higher-energy C-trap dissociation (HCD) at the apex of a peak within 2 to 15 s from their first occurrence. Normalized collision energy was stepwise increased from 25% to 30% to 35%. The default charge state was set to z = 6, and the activation time to 30 ms. The mass window for precursor ion selection was set to 3 m/z. A window of 3 m/z was set for dynamic exclusion within 30 s. Ion species with unassigned charge states as well as isotope peaks were excluded from MS/MS experiments.

For data analysis, LC-MS/MS .raw files were converted to .mzXML file format using MSconvert of the ProteoWizard package (version 3.065.85). Extracted ion chromatograms (XICs) of intact proteins were generated of deconvoluted of multiple charged spectra with XTRACT of the Xcalibur Qual Browser version 2.2 (Thermo, Bremen, Germany). XICs of mono-isotopic deconvoluted LC-MS runs were performed with MZmine 2 (version 2.2). A 1.0E4 signal intensity threshold was used. The mass alignment for the creation of XICs was performed with a minimum peak width of 30 s, and 3.0E4 peak height. Mass tolerance was set to 10 ppm. For chromatographic deconvolution, the baseline cutoff algorithm with 1.0E4 signal threshold was used. Maximum peak width was set to 10 min. Feature alignment was performed with 10 ppm mass accuracy and 0.5 min retention time tolerance. For protein spectrum matching, multiple charged MS/MS spectra were then deconvoluted using MS-Deconv (version 0.8.0.7370). The maximum charge was set to 30, maximum mass was set to 50,000, signal-to-noise threshold was set to 2, and m/z tolerance was set to 10 ppm. Protein spectrum matching was performed using TopPIC

(http://proteomics.informatics.iupui.edu/software/toppic/) (Kou et al., 2016) (version 1.1.0) against the corresponding venom gland transcriptomic derived protein sequence database as well as all protein sequences from members of the genus *Naja* obtained from the NCBI protein database (Nov 2017), containing a total of 1,764 sequences. The search database also contained 116 protein sequences of known typical contaminants from the common Repository of Adventitious Proteins, (cRAP). TopPIC mass error tolerance was set to 10 ppm and a 1% false discovery rate (FDR, target-decoy) cut-off was used. The maximal allowed unexpected PTMs were set to one. Pairing of MS/MS derived protein ID from TopPic with MS1 XICs was performed by mass matching with an in-house R script available as a jupyter notebook at https://github.com/DorresteinLaboratory/match_tables_by_exact_mass. For relative quantification, area under the curve of XICs was normalized to total ion current (TIC) (see Data S1 and Fig. S2). Amino acid sequences of proteins were converted to FASTA format and imported into MEGA v7 (Kumar et al., 2016) for alignment. Proteins were merged if found to be identical using MEGA's pairwise distance measurement.

Data analyses

Ancestral states for spitting (vs non-spitting) were estimated over the species tree using the rerooting method function of the R package "phytools" (Revell, 2012). After Log-likelihood (Log-lik) comparison of the all rates different (ARD), equal rates (ER) and symmetric rates (SYM) models, both ER and SYM (which had equal Log-lik values) were used to plot these discrete traits over the tree, with both resulting in visually-identical plots. Ancestral states for all continuous characters were also estimated via maximum likelihood on a pruned version of this species tree, using the contmap function of the phytools package under default settings. To test for statistical support for differences between spitting and non-spitting cobras and the influences of geography at the proteomic and functional levels, we

used the phylogenetic Generalized Least Squares (PGLS) approach using the pgls() function of the "caper" (Orme et al., 2018) package in RStudio (RStudio, 2016), with the formula set as ([toxin characteristic, e.g. PLA_2 abundance] ~ grouping [spitter or non-spitter]) and lambda set to "Maximum Likelihood". Analyses were performed on the species tree pruned to those species whose venom was analysed in this study. To test for the clustering of spitting and non-spitting lineages based on the amino acid sequences of toxin proteins, we used Principal Coordinate Analysis (PCoA). To do this, a pairwise distance matrix was firstly conducted on the toxin amino acid sequences using the JTT matrix-based model (Jones et al., 1992) in MEGA v.7. The rate variation among sites was modelled with a gamma distribution (shape parameter = 0.8657) for PLA_2s and uniform rates for CTXs. A Euclidean dissimilarity matrix was generated from each of these matrices using the Daisy() function from the R package "Cluster" (Maechler et al., 2019) and was followed by applying classical multidimensional scaling to the matrix using the cmdscale() function in R Studio.

Phylogenetic analyses

Phylogenetic analysis of three-finger toxin (3FTX) gene family was conducted using Bayesian inference on the aligned sequences derived from the venom gland transcriptomes. Construction of an initial 3FTX tree also used representative sequences from *Aspidelaps scutatus intermedius* (Whiteley et al., 2019), *Bungarus flaviceps* (Siang et al., 2010), *Bungarus multicinctus* (52), *Dendroaspis* spp. (Ainsworth et al., 2018), *Micrurus fulvius* (Margres et al., 2013) and *Ophiophagus hannah* (53, 54) for phylogenetic context, and a non-toxic *Python regius* 3FTX sequence (A. D. Hargreaves et al., 2014) was used as the outgroup for rooting the tree. The final DNA alignment consisted of 56,781 bp with 285 sequences from 28 taxa. The selected model for nucleotide sequence evolution, "GTR+G", was determined by jModelTest v2.1.6 based on the Akaike Information Criterion (Darriba et al., 2012; Posada,

2008). Subsequently, Bayesian inference phylogenetic analysis was performed using MrBayes v3.2.6 (Ronquist et al., 2012) on the CIPRES Science Gateway (Miller et al., 2010). The analysis used four simultaneous runs with four chains (three hot, one cold) for 10×10^6 generations, sampling every 500th cycle from the chain and using default settings for priors. Burn-in was set at 25%; trees generated prior to this point were discarded, and a consensus tree constructed from the remaining 75%. From the resulting initial 3FTX tree, a strongly supported CTX clade was identified via BLASTx searches on individual sequences and a separate pared-down CTX-specific analysis was also performed to explore the evolutionary history of CTXs sequences in elapids. In addition to the CTXs identified in the original analysis, this second dataset included CTX sequences from *Ophiophagus hannah* (53, 54) and used the non-CTX 3FTX from *Bungarus flaviceps* (GU190795.1) (Siang et al., 2010) as the outgroup for rooting. The resulting DNA alignment consisted of 18,098 bp with 80 sequences from 17 taxa, phylogenetic analyses were performed as described above, except using the selected "TVM+G" model of nucleotide substitution, selected using the same methods described above in jModelTest. The two sequence alignments are displayed in Data S2 and S3, and the resulting phylogeny of the CTX-specific analysis is displayed in Fig. S3.

Venom cytotoxicity via hen's egg test-chorioallantoic membrane (HET-CAM) assay

Lyophilized venom (Table S9) was dissolved in sterile, deionized water to make a stock solution. The protein concentration was determined using a NanoDrop 1000 UV/Vis Spectrophotometer (Thermo Fisher Scientific, Bleiswijk, the Netherlands) at an absorbance of 280 nm. The stock solution was snap-frozen using liquid nitrogen and stored at −80°C till further use. Just before the experiment the venom was prepared in Hanks' balanced salt solution (HBSS: Cat. H9394, Sigma Aldrich, Zwijndrecht, the Netherlands) to a final total protein concentration of 1 mg/mL. Unincubated hen's eggs were obtained from a

commercial supplier. The eggs were incubated in a horizontal position at 38°C on stationary shelves in a humidified incubator for 10 days. Following incubation, eggs were candled and placed vertically with the blunt end upwards. A hole in the eggshell overlying the air sac was made with No. 3 watchmakers' forceps. The shell membrane was moistened with 200 µL HBSS, and then punctured and carefully peeled away using No. 5 watchmakers' forceps to reveal the chorioallantoic membrane (CAM) below. Excess HBSS was pipetted away. Venom or control solution (100 µL) was then pipetted onto the CAM and a timer started. Any hyperaemia, haemorrhage and/or coagulation was documented over a 5 min period. Photographs were taken at 15 s intervals for semi-quantitative analysis. Each venom and control sample was tested in triplicate (using three different eggs) in a single-blinded and randomized manner to avoid bias. The positive controls were 1% sodium dodecyl sulphate (Carl Roth, Karlsruhe, Germany) and 1 mg/mL of capsaicin (Sigma Aldrich, Zwijndrecht, the Netherlands), both known eye-irritants; and 10 mg/mL of histamine (Sigma Aldrich, Zwijndrecht, the Netherlands), a potent vasodilator. The negative control was HBSS. A semi-quantitative analysis was performed using the resulting photographs, where the severity of any hyperaemia, haemorrhage and/ or coagulation was manually scored at 0.5, 2.0 and 5.0 min using the method developed by Luepke (Luepke, 1985) (Tables S3, S4). The average score of three replicates was taken and the cumulative score was matched with the corresponding 'irritation potential' previously developed (Luepke, 1985) (Table S4).

Calcium imaging on mouse sensory neurons

Trigeminal ganglia (TG) or dorsal root ganglia (DRG) from 4-6 week old male C57BL/6 mice were dissociated and plated in DMEM (Gibco, MD, USA) containing 10% fetal bovine serum (FBS) (Assaymatrix, VIC, Australia) and penicillin/streptomycin (Gibco, MD, USA) on a 96-well poly-D-lysine-coated culture plate (Corning, ME, USA) and maintained overnight. Cells were

loaded with Fluo-4 AM calcium indicator, according to the manufacturer's instructions (ThermoFisher Scientific, MA, USA). After loading (1 h), the dye-containing solution was replaced with assay solution (1x Hanks' balanced salt solution, 20 mM HEPES). Images were acquired at 20x objective at 1 frame per second (excitation 485 nm, emission 521 nm). Fluorescence corresponding to $[Ca^{2+}]_i$ of 100–150 cells per experiment was monitored in parallel using an Nikon Ti-E Deconvolution inverted microscope, equipped with a Lumencor Spectra LED Lightsource. Baseline fluorescence was monitored for 30 s. At 30 s, assay solution was replaced with venom (100 ng/µL in assay solution) or venom fractions (estimated equivalent of amount for 100 ng/µL whole venom, in assay solution) and monitored for an additional 2 min. Experiments involving the use of mouse tissue were approved by The University of Queensland animal ethics committee.

FLIPR assays of F11 cells

F11 (Neuroblastoma × dorsal root ganglion (DRG) neuron hybrid) cells were cultured as previously described (Vetter & Lewis, 2010). Briefly, cells were maintained on Ham's F12 media supplemented with 10 % fetal bovine serum, 100 µM hypoxanthine, 0.4 µM aminopterin, and 16 µM thymidine (Hybri-MaxTM, Sigma Aldrich). A 384-well imaging plate (Corning, Lowell, MA, USA) was seeded 48 h prior to calcium imaging resulting in 90 – 95% confluence at imaging. Cells were incubated for 30 min with Calcium 4 assay component A, according to manufacturer's instructions (Molecular Devices, Sunnyvale, CA) in physiological salt solution (PSS; composition in mM: 140 NaCl, 11.5 D-glucose, 5.9 KCl, 1.4 $MgCl_2$, 1.2 NaH_2PO_4, 5 $NaHCO_3$, 1.8 $CaCl_2$, 10 HEPES) at 37 °C. Ca^{2+} responses were measured using a FLIPRTETRA fluorescent plate reader equipped with a CCD camera (Ex: 470-490 nm, Em: 515-575 nM) (Molecular Devices, Sunnyvale, CA). Signals were read every second for 10 s before, and 300 s after the addition of crude venoms (final concentration range: 1.3 – 333 ng/µL) in

PSS supplemented with 0.1% bovine serum albumin. All data represent the mean ± standard error of the mean (SEM) of a representative assay in triplicate unless otherwise stated. The mean fluorescence changes during venom addition was compared to a control solution addition, (0.1% BSA in PSS). The resulting maximum-minimum florescence in the 300 s period after venom was added was recorded as the response. A four-parameter Hill equation (Variable slope, two-site) was fitted using Graphpad Prism8.

Venom fractionation and bottom-up sequencing

To identify the venom constituents responsible for sensory neuron activation, 500 µg of venoms from *N. siamensis*, *N. nigricollis* and *H. haemachatus* were each separated on a Phenomenex Gemini NX-C18 column (250 x 4.6 mm, 3 µm particle size, 110 Å pore size) using a gradient of 15–45% solvent B (90% ACN, 0.05% TFA) over 30 min at a flow rate of 1 mL min^{-1}. Fractions were collected on the basis of absorbance at 214 nm. Fractions were dried by vacuum concentration and each resuspended in 50 µL pure water from which 1 µL aliquots were used for calcium imaging experiments (diluted in 100 µL assay solution). Bottom-up proteomics was used to confirm the identity of the major component/s of each fraction. 20 µL of each resuspended fraction was dried by vacuum centrifugation. Each sample was reduced and alkylated with reagents in gas phase according to the protocol described by Hale et al. (Hale et al., 2004). Reduction/alkylation reagent (50% 0.1 M ammonium carbonate, 48.75% ACN, 0.25% triethylphosphine, 1% 2-iodoethanol (by volume)) was placed in the cap of each inverted tube and incubated at 37 °C for 60 min. Reduced and alkylated fractions were then digested by incubating with 20 ng/µL trypsin overnight at 37 °C according to the manufacturer's instructions (Sigma-Aldrich, MO, USA). Trypsin digestion was quenched by addition of FA to a final concentration of 0.5%. Each sample was then separated on a Shimadzu (Japan) Nexera uHPLC with an Agilent Zorbax stable-bond C18 column (2.1 mm ×

100 mm, 1.8 µm particle size, 300 Å pore size), using a flow rate of 180 µL/min and a gradient of 1–40% solvent B (90 % ACN, 0.1% FA) in 0.1% FA over 30 min and analyzed on an AB Sciex 5600 TripleTOF mass spectrometer. MS survey scans were acquired at 300–1800 m/z over 250 ms, and the 20 most intense ions with a charge of +2 to +5 and an intensity of at least 120 counts were selected for MS/MS. The unit mass precursor ion inclusion window mass ± 0.7 Da, and isotopes within ±2 Da were excluded from MS/MS, with scans acquired at 80–1400 m/z over 100 ms and optimized for high resolution. Using ProteinPilot version 4.0 (ABSciex), MS/MS spectra were searched against a database of the toxin sequences (translated open-reading frames) derived from the corresponding venom gland transcriptomes.

Phospholipase A_2 assay

We characterized the enzymatic PLA_2 activity of each venom using a recently described colorimetric assay (Neumann et al., 2020). A PLA_2 reaction solution was made using 49.5 µM Cresol red dye, 0.875 mM Triton X-1007 M, and 1 mL of 5x Salt Mix (1mM Tris base pH 8.5, 500 mM sodium chloride, 500 mM potassium chloride and 50 mM calcium chloride). The pH of the reaction solution was checked using a pH strip and corrected (if necessary) with 1 M sodium hydroxide. To this solution 168.25 µL of the substrate (L-α-phosphatidylcholine; stock concentration 26 mM, SIGMA Life Sciences) was added. Venom samples were prepared in tenfold dilutions (0.1-100 µg/mL) of Tris buffer (pH 8.5), with 10 µL pipetted into the wells of a Greiner Bio-One clear 384-well microplate at volumes in quadruplicate using a 230V Multidrop 384 (Labsystems), with control wells containing Tris buffer only. The plate was then overlaid with 40 µL of reaction solution in each well and read kinetically on a FLUOstar Omega (BMG LABTECH) spectrophotometer at 572 nm over 42 cycles (~46 s/cycle) at 25 °C. Concentration curves were generated for each venom by plotting the mean control AUC of

absorbance at 572 nm, minus the mean area under the curve (AUC) for readings at each venom concentration, using the values from the run period of 0-10 min (Fig. S12). The resulting mean area under the concentration curves (AUCC) were used for comparisons.

Venom median lethal doses (LD_{50})

We calculated the lethal dose 50 (LD_{50}) of each of the venoms using a previously established murine model of venom lethality (Theakston & Reid, 1983). Groups of five mixed sex CD-1 mice (18-20 g) received intravenous (i.v.) injections, in the caudal vein, of various doses of each venom (Table S5), dissolved in 0.1 mL of 0.12 M NaCl, 0.04 M phosphates, pH 7.2 (PBS). Deaths were recorded at 24 h and the LD_{50}, as well as the 95% confidence limits, were estimated by probits. The experimental protocols for these experiments were approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUA) of the University of Costa Rica (approval number CICUA 82-08).

CT imaging of snake fangs

Fangs were scanned in pairs on a Nikon XTH225ST CT scanner at the University of Bristol. All scans were performed on a rotating target with a tube voltage of 70 kV and beam current of 101 µA with a 1 s exposure per projection with a total of 3141 projections in 360° at a resolution of between 3.9 to 4.5 microns. The CT scan data were aligned, reconstructed and cropped to generate individual tiff image stacks for each fang using VG Studio MAX (Volume Graphics GmbH, Heidelberg, Germany). The tiff stacks were subsequently segmented to generate a 3D model of each fang in Avizo 9 (FEI, Hillsboro, Oregon, USA).

Supplementary Figures

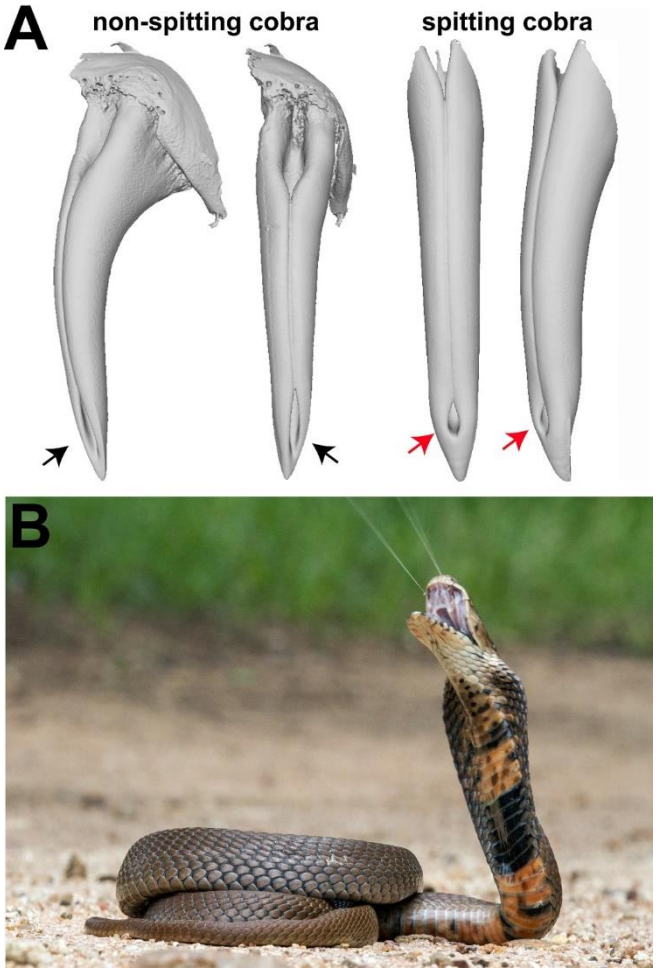


Fig. S1. The morphological adaptations associated with venom spitting. (A) MicroCT visualizations of fangs from representative non-spitting (*Naja nivea*) and spitting (*Naja nubiae*) cobra species. Fangs are visualized from the anterior perspective (central images) and rotated 45° (outer images). Black and red arrows highlight the distinct ejection orifices of non-spitting and spitting cobras, respectively. (B) Venom spitting displayed by a *Naja mossambica* from Limpopo Province, South Africa. Photograph by Wolfgang Wüster.

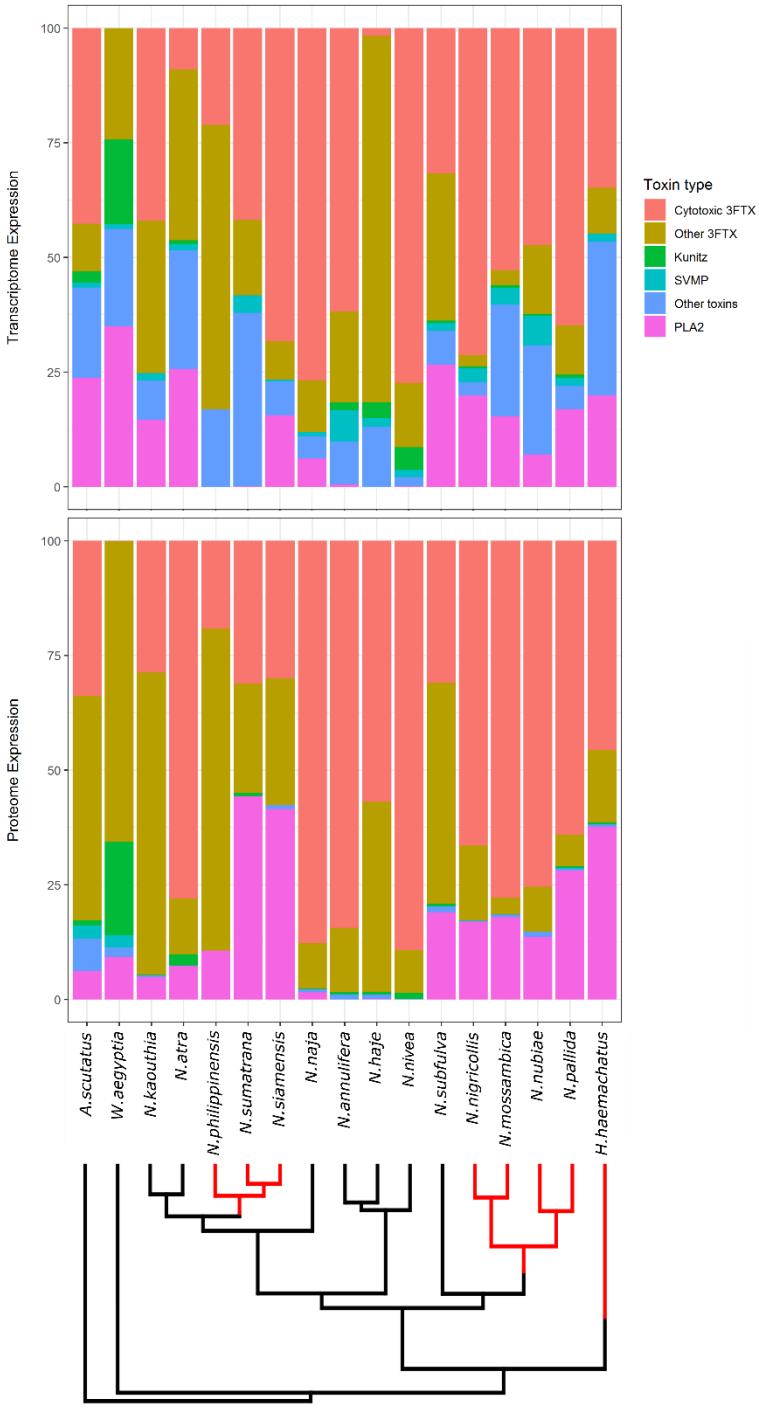


Fig. S2. Stacked bar charts representing the relative abundances of toxin families detected in the venom gland transcriptomes (top) and venom proteomes (bottom) of cobras and related species. The transcriptome data presented represents the percentage of all venom toxin-encoding gene expression detected in each venom gland transcriptome based on normalized read mapping to assembled contigs. The proteome data represents the percentage of all venom toxins detected in each venom proteome based on top-down proteomic analyses and quantification via area under the curve of extracted ion chromatograms (XICs) normalized to total ion current (TIC). The bars are plotted over the phylogenetic tree of the species used in this study. Branches of the tree highlighted in red represent spitting lineages. Note that the transcriptomic and proteomic abundances for *A.*

scutatus are taken from (Whiteley et al., 2019), and while the transcriptomic data is directly comparable, the proteomic analyses used a different measure of quantification than that applied for the remainder of the species investigated in this study.

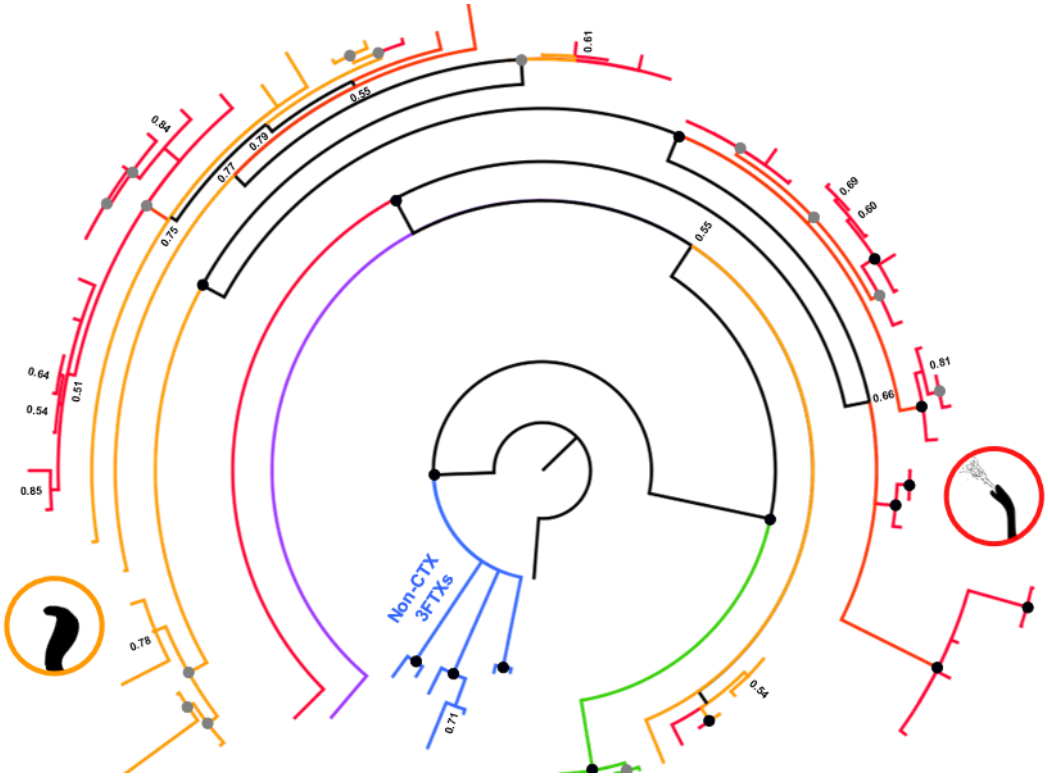


Fig. S3. Bayesian inference-derived DNA phylogeny of elapid three finger toxin cytotoxin (CTX) sequences demonstrates that the vast majority are from cobra species (genus *Naja*). Black-filled circles at each node represent Bayesian posterior probabilities of 1 and grey-filled circles represent values between 0.90 and 0.99. Tips labels have been removed to aid visualization. Colored branches represent non-CTX 3FTX outgroups (blue) and CTXs found in: the king cobra *Ophiophagus hannah* (green), *Aspidelaps scutatus* (purple), non-spitting cobras (orange), and spitting cobras (including *H. haemachatus*) (red). No bona fide CTX sequences were detected in the venom gland transcriptome of *Walterinnesia aegyptia*.

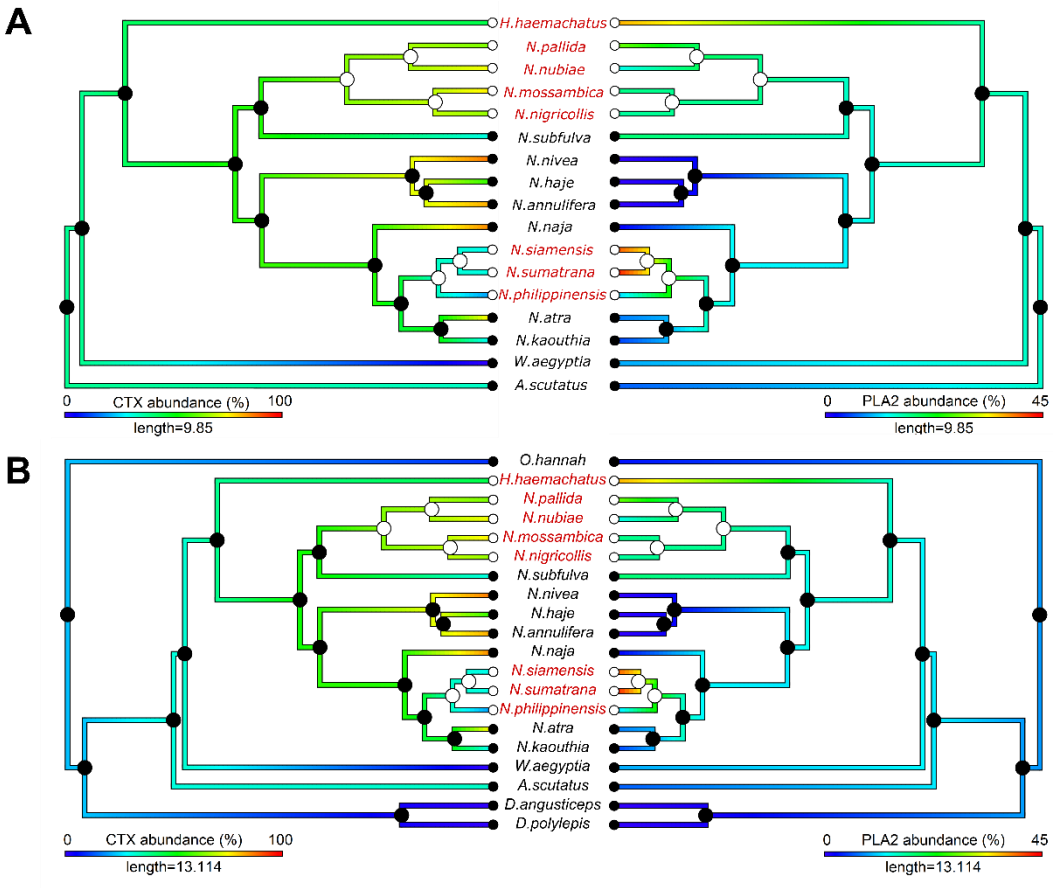
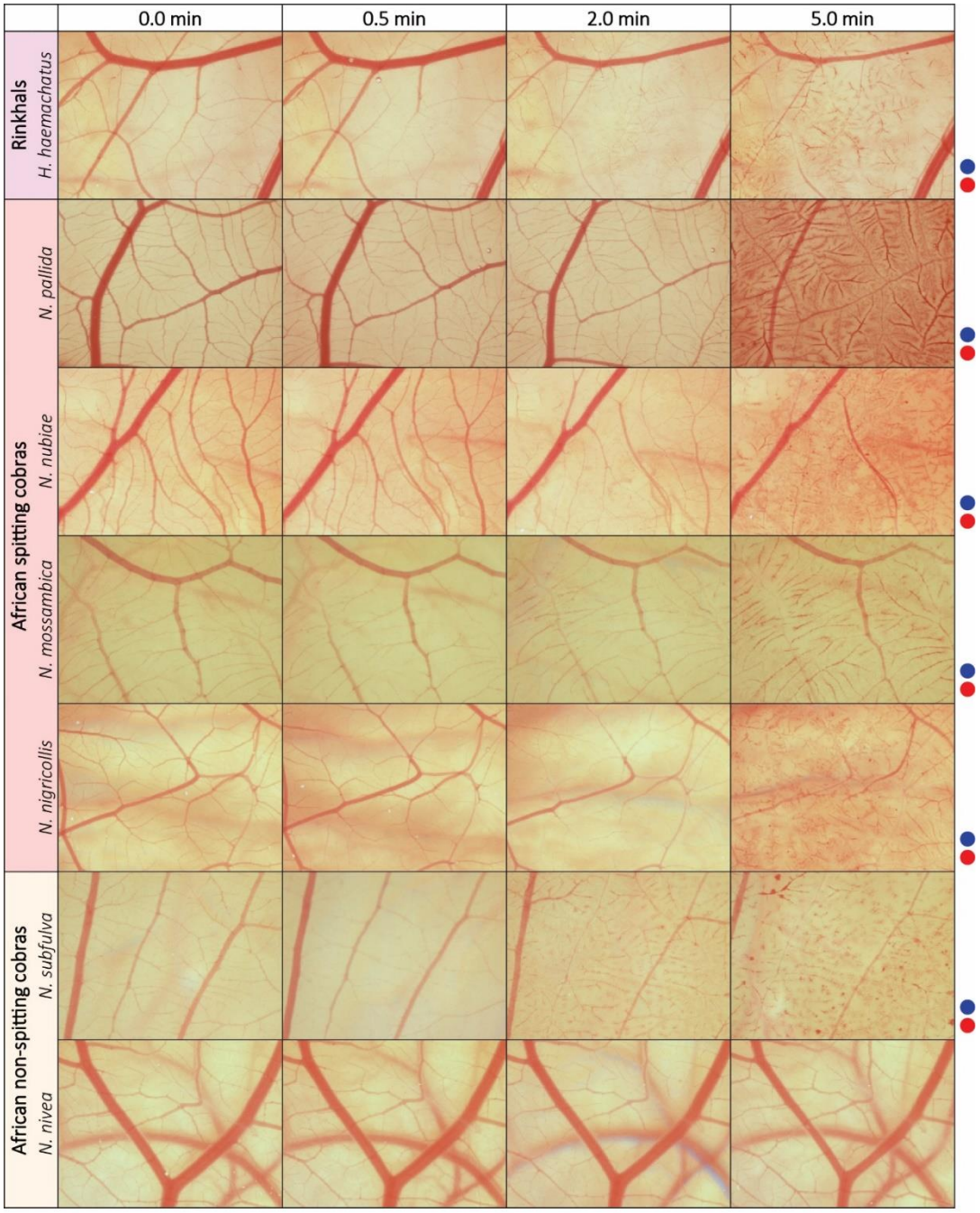
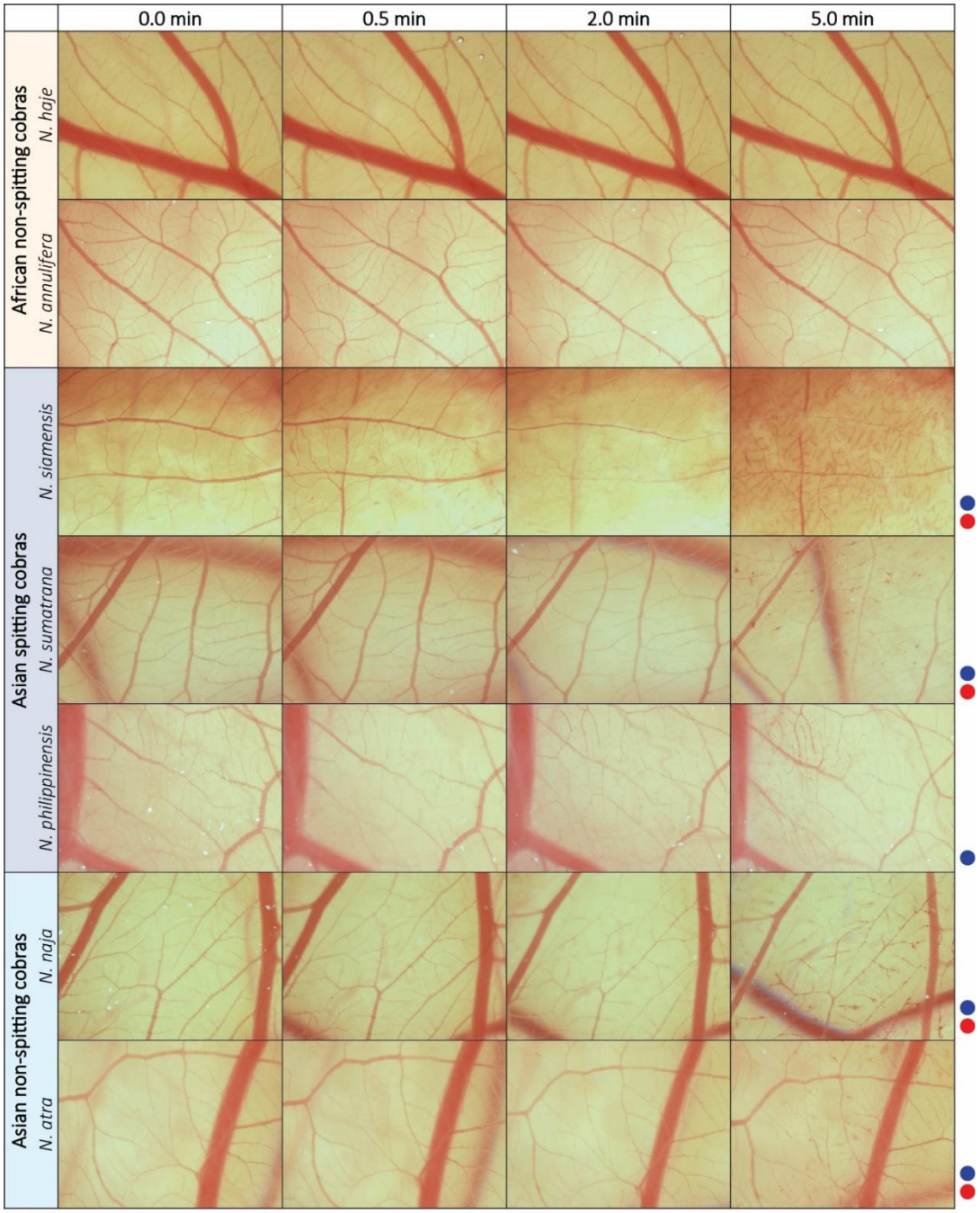


Fig. S4. Proteomic abundances of phospholipase A_2 (PLA_2) but not cytotoxic 3FTXs (CTXs) are significantly associated with the emergence of venom spitting. (A) Ancestral state estimations of the proteomic abundance (percentage of all toxins) of CTXs (left) and PLA_2s (right) mapped onto a species tree containing the cobras and representatives of the outgroup genera *Walterinnesia* and *Aspidelaps*. (B) Ancestral state estimations of the proteomic abundance of CTXs (left) and PLA_2s (right) mapped onto a species tree containing those taxa displayed in A, as well as the additional more distantly related elapid outgroups *Dendroaspis* (*D. angusticeps* and *D. polylepis*) and *Ophiophagus hannah*. Additional outgroups were incorporated due to the high reconstructed ancestral abundance of CTXs observed in A. For both (A) and (B) filled or empty circles at tips and nodes represent the observed or estimated ancestral state of non-spitting and spitting, respectively. Red tip labels are also used to highlight the spitting lineages. The Phylogenetic Generalized Least Squares (PGLS) statistics for each trait are as follows: CTX abundance with *Aspidelaps* as outgroup (A, left); t = -0.83, df = 15, p = 0.42, and with *Ophiophagus* as outgroup (B, left); t = -0.72, df = 18, p = 0.48. PLA_2 abundance with *Aspidelaps* as outgroup (A, right); t = 4.27, df = 15, p = 0.0007, and with *Ophiophagus* as outgroup (B, right); t = 5.12, df = 18, p = 0.00007. Proteomic abundances for *Aspidelaps scutatus*, *Dendroaspis* spp. and *Ophiophagus hannah* were taken from (Ainsworth et al., 2018; Petras et al., 2015; Whiteley et al., 2019).


0.0 min
0.5 min
2.0 min
5.0 min
Rinkhals
H. haemachatus
African spitting cobras
N. pallida
N. nubiae
N. mossambica
N. nigricollis
African non-spitting cobras
N. subfulva
N. nivea


0.0 min
0.5 min
2.0 min
5.0 min
African non-spitting cobras
N. haje
N. annulifera
Asian spitting cobras
N. siamensis
N. sumatrana
N. philippinensis
Asian non-spitting cobras
N. naja
N. atra

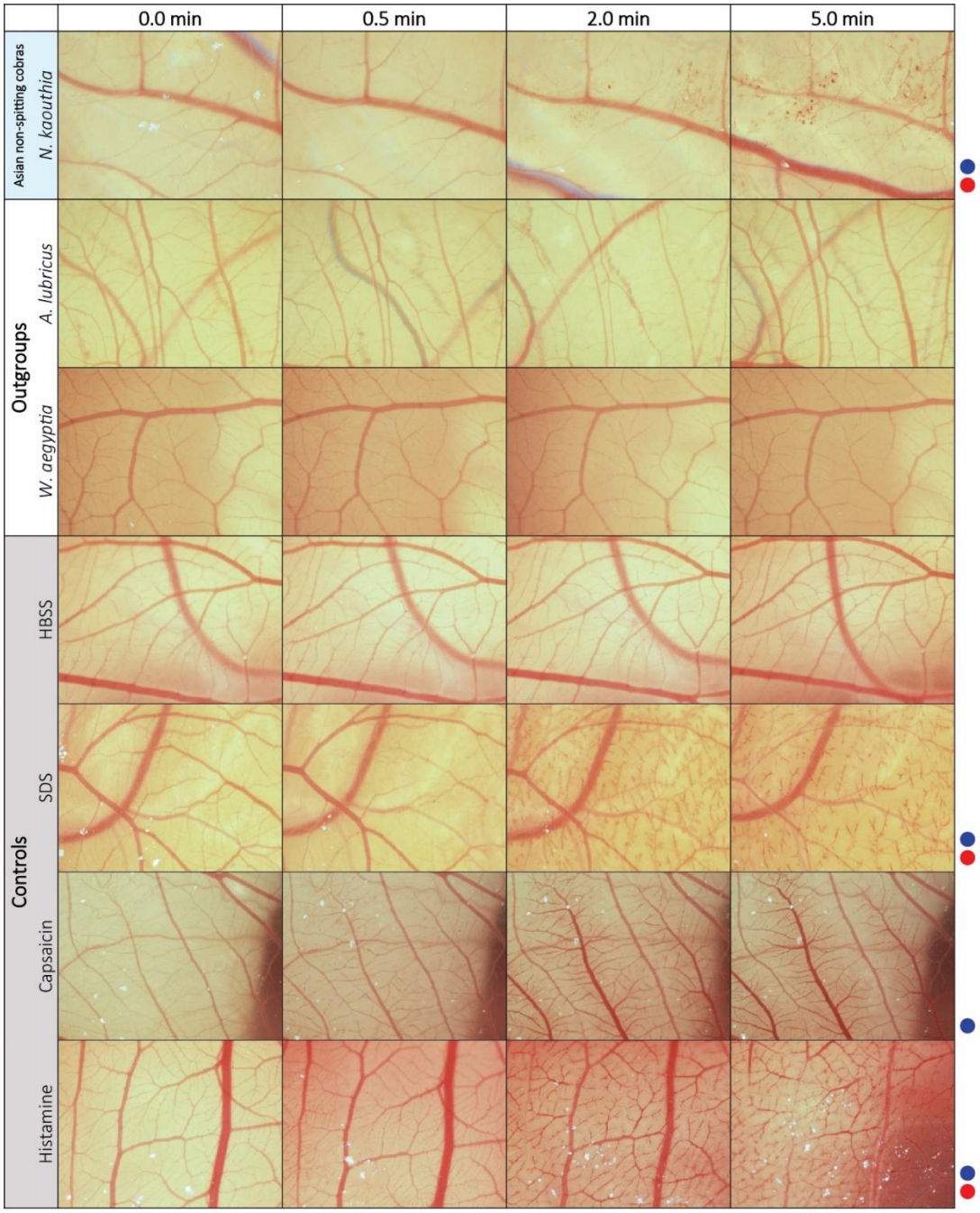


Fig. S5. The vascular pathologies induced by spitting cobra venoms. The eye-irritation potential of snake venoms and controls were assessed using the *in vivo* hen's egg test-chorioallantoic membrane (HET-CAM) assay (Luepke, 1985). The vascular effects observed are indicated on the right of each series if present: hyperaemia (●) and coagulation (●). All series were also assessed for evidence of haemorrhage, but this pathology was not observed in any sample. Key: HBSS, Hanks' balanced salt solution; SDS, sodium dodecyl sulphate.

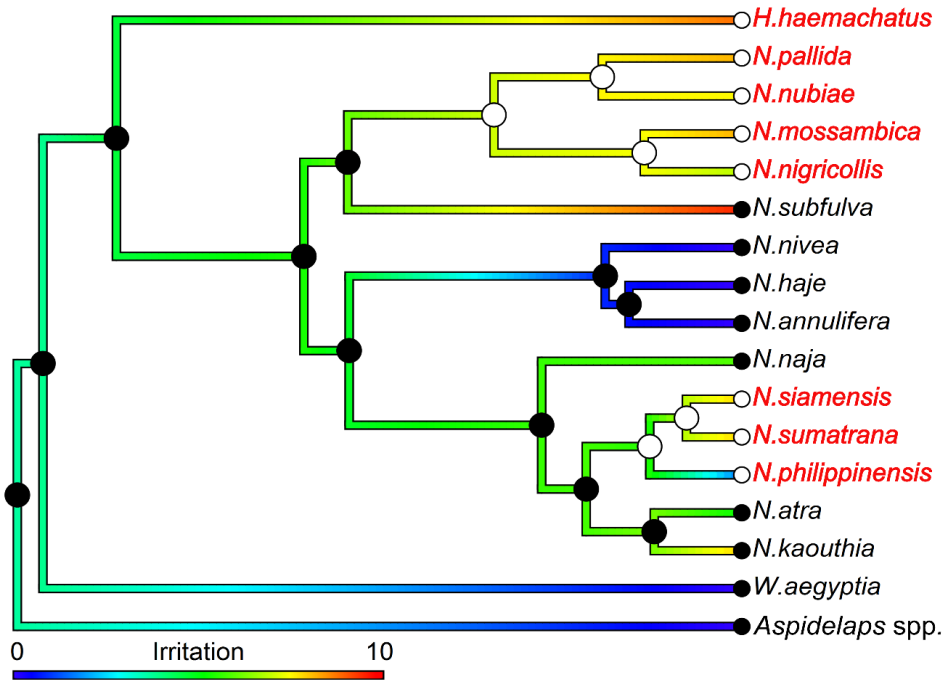


Fig. S6. Ancestral state estimation of eye-irritation scores, determined in the hen's egg test-chorioallantoic membrane assay, demonstrate no significant association with the emergence of venom spitting. The Phylogenetic Generalized Least Squares (PGLS) analysis revealed no association between the extent of venom-induced eye irritation and the emergence of spitting ($t = 1.08$, $df = 15$, $p = 0.30$). Colored branches are scaled according to extent of irritation (blue, low abundance; red, high abundance). At each node and tip of the tree, filled or empty circles represent the estimated ancestral state of non-spitting or spitting, respectively, and red tip labels are used to highlight spitting lineages.

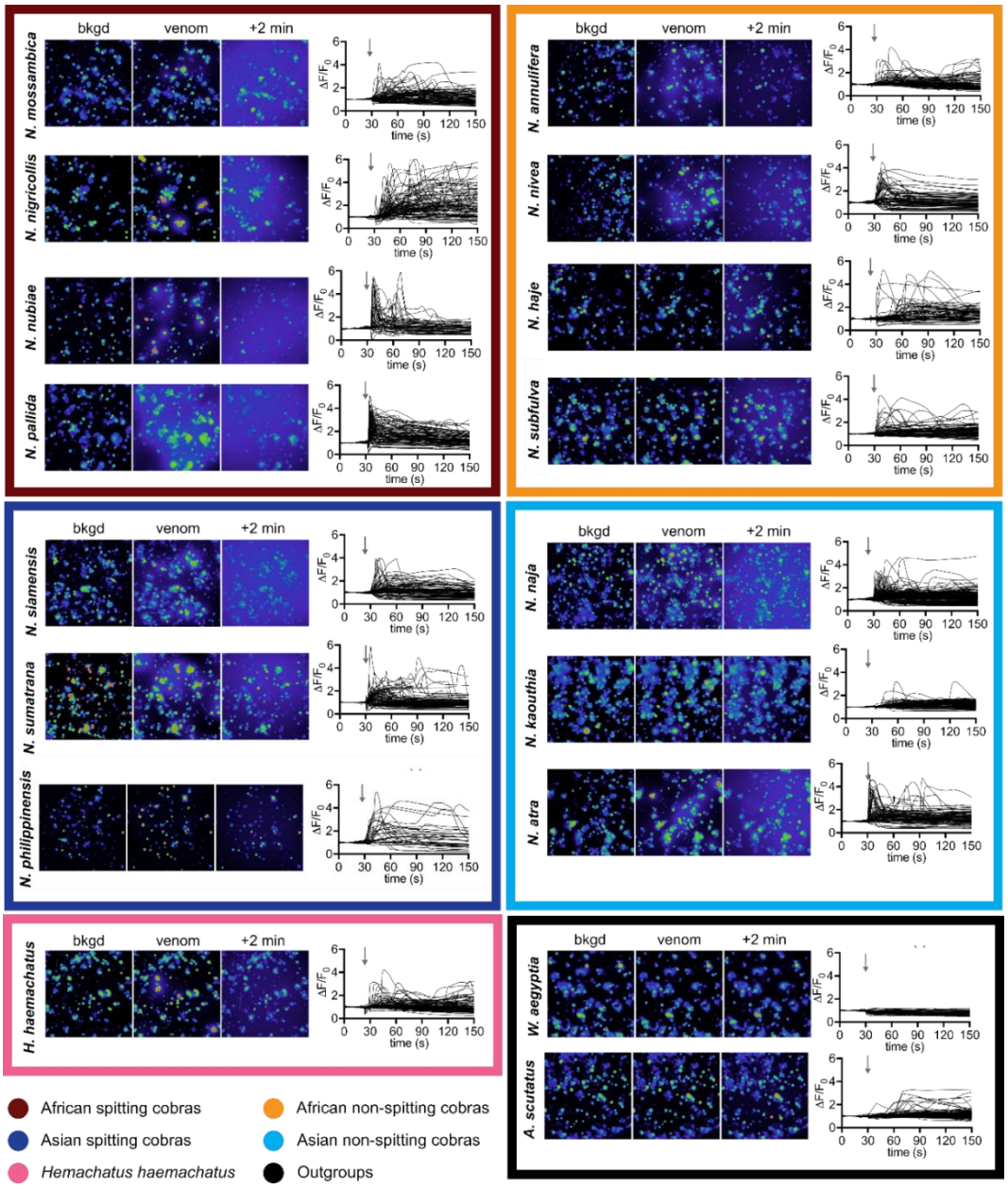


Fig. S7. *Naja* and *Hemachatus* venoms activate sensory neurons *in vitro*. Application of *Naja* and *Hemachatus* venoms (100 ng/µL) to dissociated mouse trigeminal ganglion cells caused, over the 2 min recording, increases in $[Ca^{2+}]_i$ in both neurons and non-neuronal cells, which was followed by a decrease in $[Ca^{2+}]_i$ reflecting dye leakage from the cells into the media. These data indicated that components of the venom permeate cell membranes allowing a massive influx of Ca^{2+} (and likely other ions) followed by leakage of cellular contents. Such an action on trigeminal ganglion neurons is consistent with the painful effects reported for envenomation.

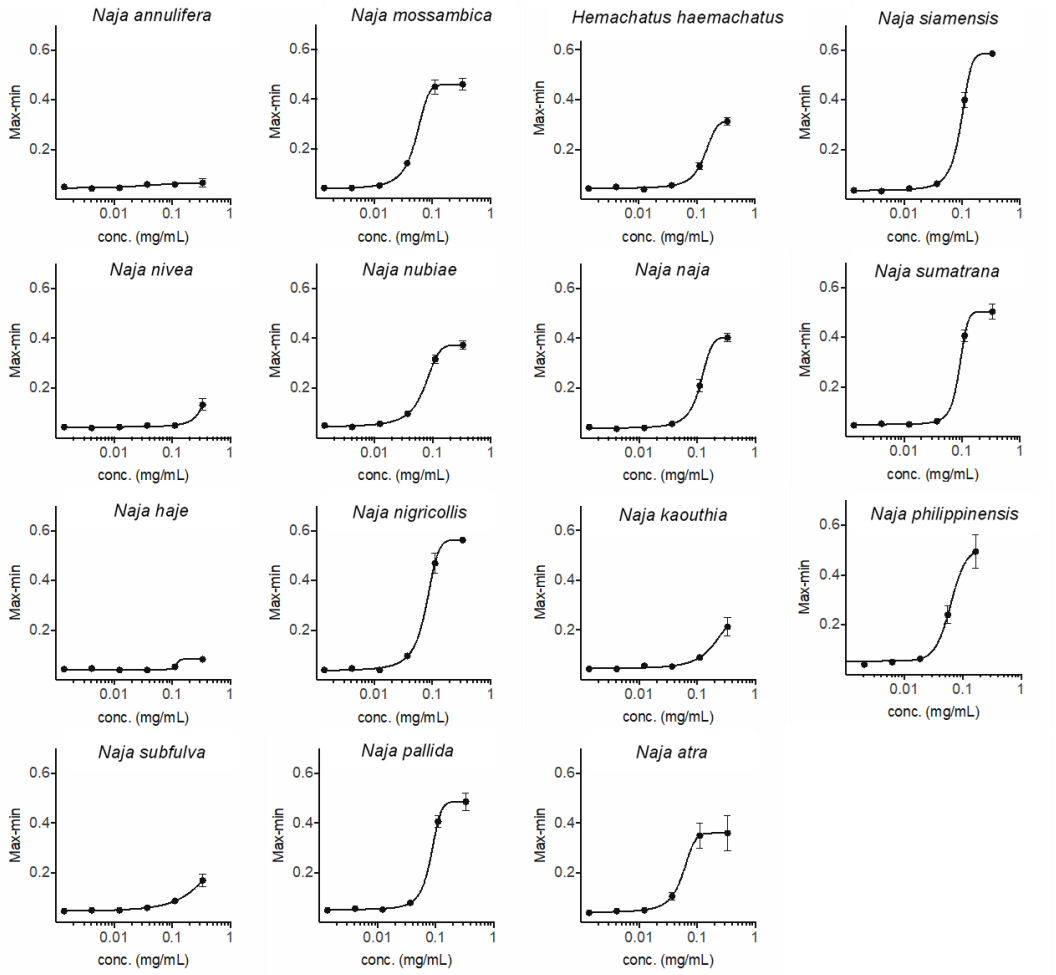


Fig. S8. Potency of *Naja* and *Hemachatus* venoms on activation of sensory neuron-derived F11 cells. Values are max-min (n = 3), and error bars represent standard error of the mean (SEM). Curves were fitted using a four-parameter Hill equation (Variable slope) in Graphpad Prism (version 8.02).

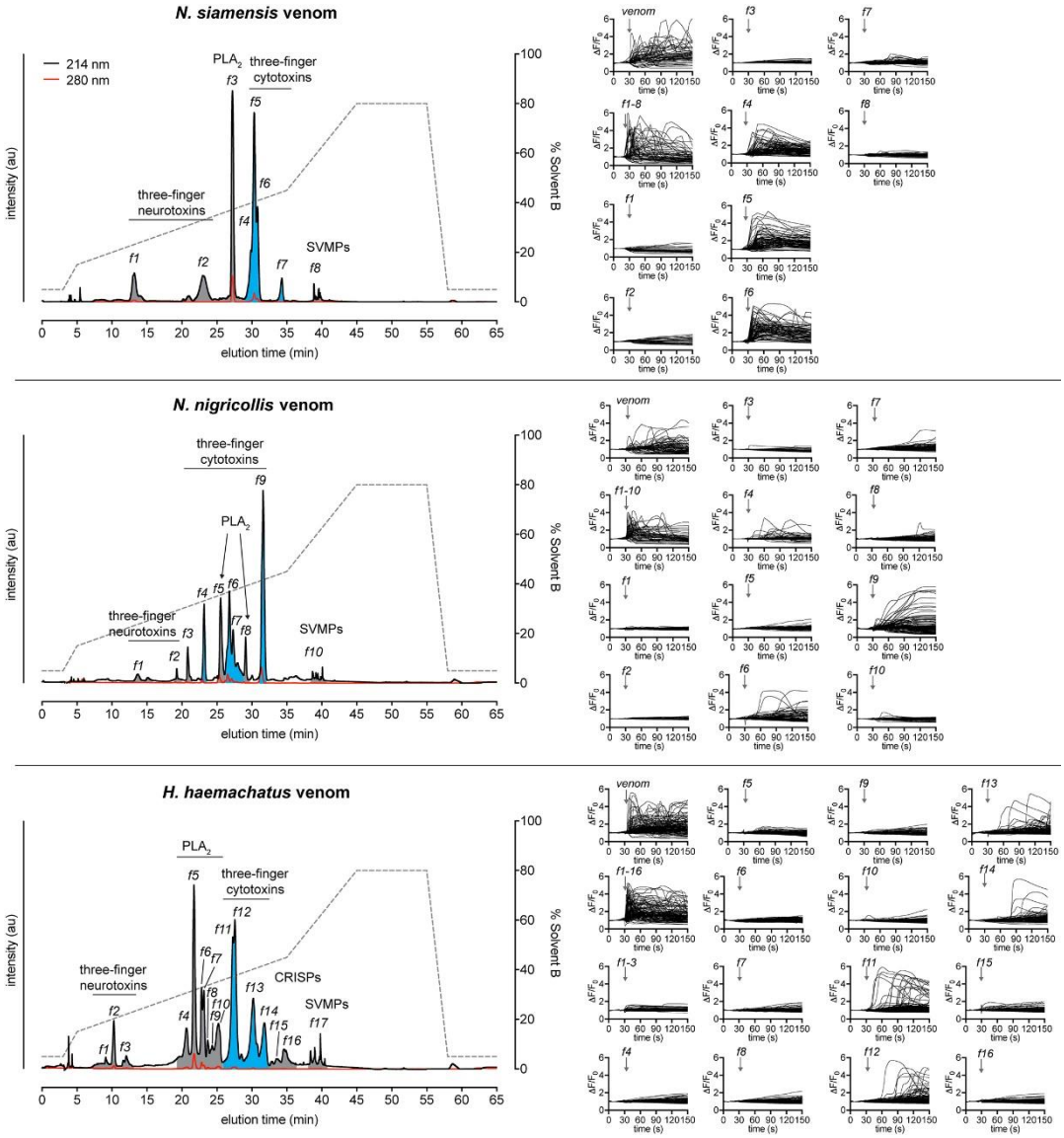


Fig. S9. Separation of *Naja siamensis, Naja nigricollis* and *Hemachatus haemachatus* venoms and activity of fractions on sensory neurons. *N. siamensis, N. nigricollis* and *H. haemachatus* venoms (500 µg) were each separated by RP-HPLC using a Phenomenex Gemini NX-C18 column (250 x 4.6 mm, 3 µm particle size, 110 Å pore size; gradient of 15–45% solvent B (90% ACN, 0.05% TFA) over 30 min; flow rate of 1 mL min^{-1}). Fractions were collected according to absorbance at 214 nm. Bottom-up proteomics was used to confirm the identity of the major component/s of each fraction. Individual fractions were assessed for activity on sensory neurons (mouse DRG). Active fractions are highlighted in blue, inactive in grey. Each set of traces represents all cells of one experiment. Only the pooled fractions and those individual fractions containing three-finger cytotoxins were able to activate sensory neurons.

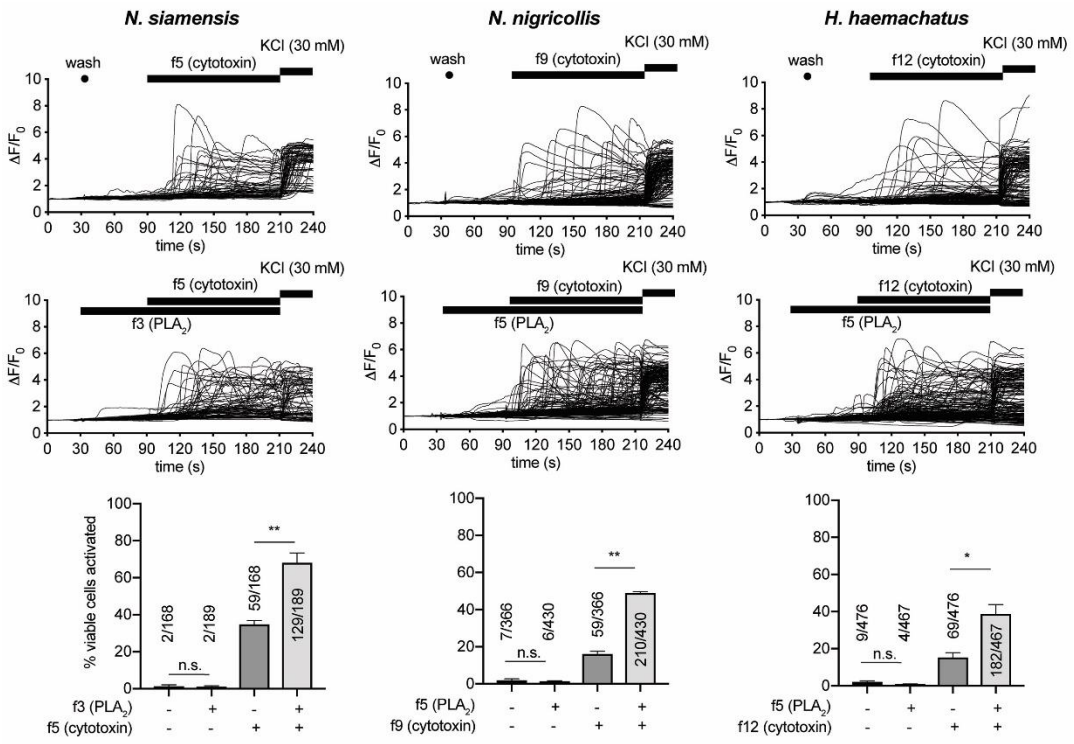


Fig. S10. Activation of sensory neurons by spitting cobra three-finger cytotoxins is potentiated by PLA_2s. For all venoms tested, there was no significant difference in cell (mouse DRG) activation between buffer/wash and a PLA_2 fraction alone, while cytotoxin fraction activation was potentiated in the presence of a corresponding PLA_2 fraction. Each set of traces represents all cells of one representative experiment. Data are derived from 2-3 independent experiments. Statistical comparisons were made using unpaired parametric *t*-tests in Graphpad Prism (version 8.02). *, $P < 0.05$; **, $P < 0.01$.

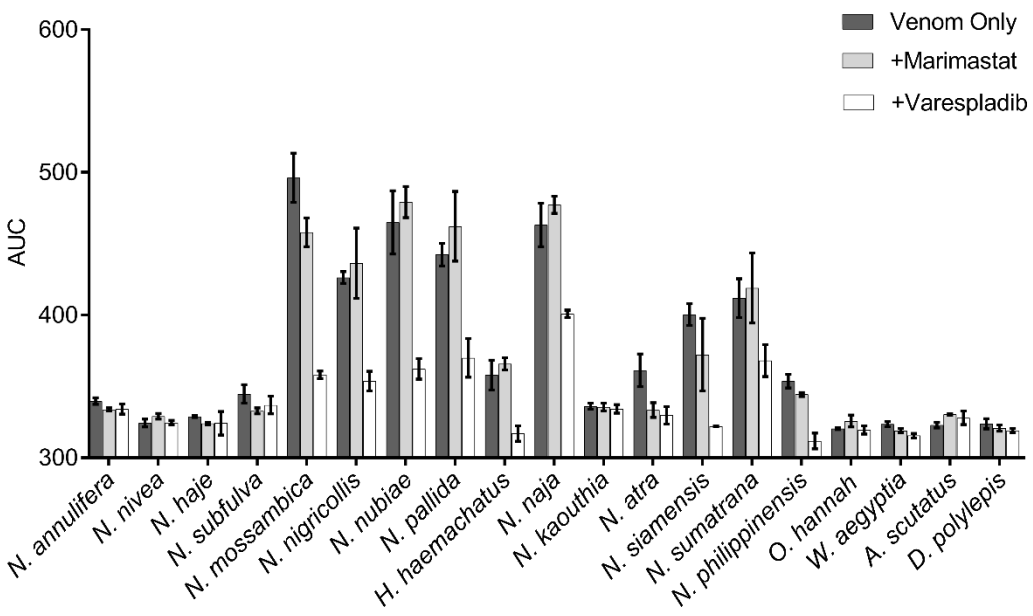


Fig. S11. The PLA_2 inhibitor varespladib reduces the activation of sensory neurons caused by spitting cobra venoms. 80 µg/mL of venoms (or 160 µg/mL in the case of *H. haemachatus* and *N. philippinensis* venom) were added to F11 cells. Values are area-under-curve (n = 3), and error bars represent standard error of the mean (SEM), with either venom only (negative control), the PLA_2 inhibitor varespladib (13 µM) or the metalloprotease inhibitor marimastat (15 µM).

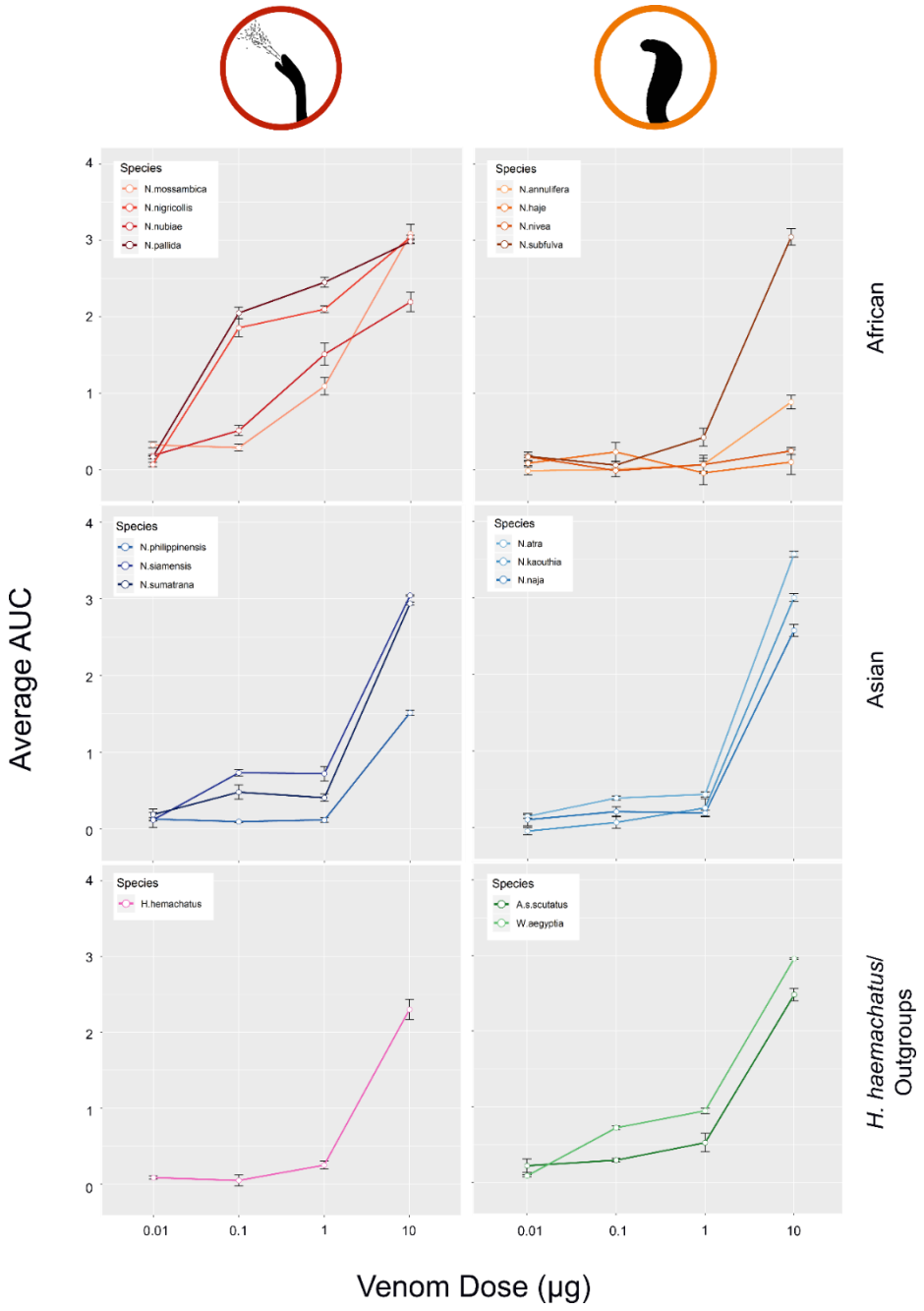


Fig. S12. Concentration curves displaying the enzymatic PLA_2 activity of the various cobra venoms. The PLA_2 activity of each venom was characterized using kinetic measurement of an *in vitro* colorimetric assay (Neumann et al., 2020). Venom doses of 0.01 µg, 0.1 µg, 1 µg and 10 µg were quantified in quadruplicate alongside Tris Buffer negative controls. The mean area under the curve (AUC) of the resulting control profile at 10 min was calculated from the four replicates and used to subtract from each individual venom measurement. The mean of the resulting venom data was then plotted, with error bars representing the standard error of the mean of the AUC values at each dose tested.

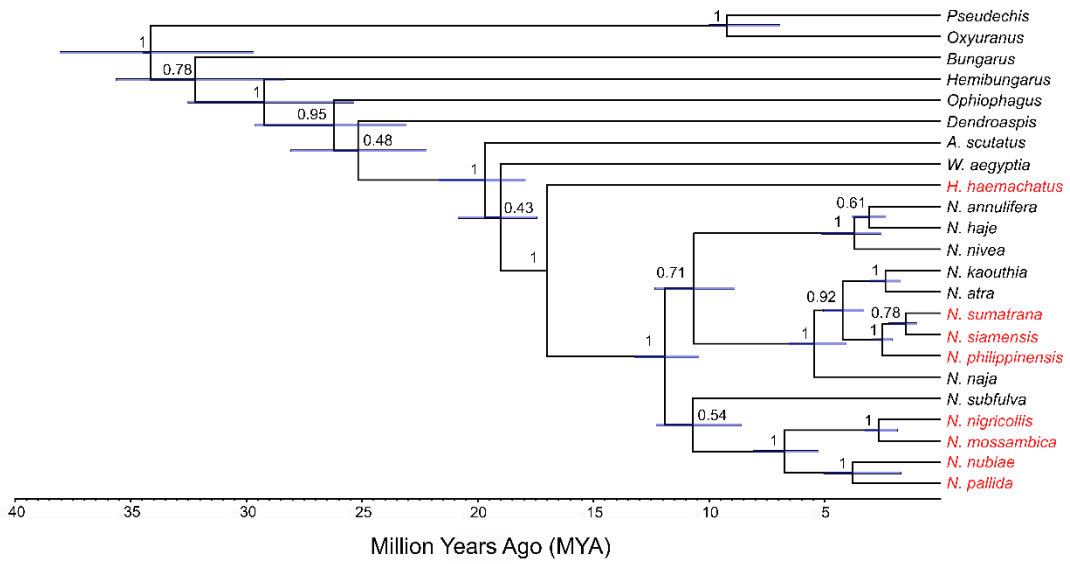


Fig. S13. Time-calibrated species tree of elapids, with the *Naja* genus expanded to show the phylogenetic position of the species used in this study. Node labels denote posterior probabilities and blue bars at each node represent age range estimations. Red tip labels indicate spitting species.

Supplementary Tables

Table S1. The relative representation of phospholipases A_2 (PLA_2) and three-finger toxins (3FTX) in elapid venom proteomes described in the literature. The data displayed represents the percentage of each toxin family in the whole venom, and note that the method of quantification applied varies among studies.

Species	3FTX		PLA_2	Citation
	CTX	Other		
Aipysurus laevis	0	25.3	71.2	(Laustsen, Gutiérrez, et al., 2015)
Aspidelaps lubricus cowlesi	4.9	71.2	4.9	(Whiteley et al., 2019)
Aspidelaps lubricus lubricus	2.1	75.7	5.7	(Whiteley et al., 2019)
Aspidelaps scutatus intermedius	33.7	49.0	6.1	(Whiteley et al., 2019)
Bungarus caeruleus	0	19.0	64.5	(Oh et al., 2017)
Bungarus candidus	0	30.1	25.2	(Rusmili et al., 2014)
Bungarus fasciatus	0	1.3	66.8	(Ziganshin et al., 2015)
Bungarus fasciatus	0	17.4	44.2	(Rusmili et al., 2014)
Bungarus multicinctus	0	32.6	66.4	(Shan et al., 2016)
Bungarus multicinctus	0	27.5	15.3	(Ziganshin et al., 2015)
Bungarus sindanus	0	50.3	32.6	(Oh et al., 2019)
Calliophis intestinalis	15.9*	5.1	43.8	(K. Y. Tan et al., 2019)
Dendroaspis angusticeps	0	69.2	0	(Lauridsen et al., 2016)
Dendroaspis angusticeps	0	64.2	0	(Ainsworth et al., 2018)
Dendroaspis jamesoni jamesoni	0	65.5	2.2	(Ainsworth et al., 2018)
Dendroaspis jamesoni kaimosae	0	74.8	1.8	(Ainsworth et al., 2018)
Dendroaspis polylepis	0	31.0	0	(Laustsen, Lomonte, et al., 2015)

Dendroaspis polylepis	0	40.0	0	(Ainsworth et al., 2018)
Dendroaspis viridis	0	76.7	0.2	(Ainsworth et al., 2018)
Hydrophis curtus	0	26.3	62.0	(C. H. Tan, Tan, et al., 2019)
Hydrophis cyanocinctus	0	81.1	18.9	(Calvete et al., 2012)
Hydrophis platurus	0	49.9	32.9	(Lomonte et al., 2014)
Hydrophis schistosus	0	70.5	27.5	(C. H. Tan, Tan, Lim, et al., 2015)
Laticauda colubrina	0.3	66.1	33.3	(C. H. Tan et al., 2017)
Micropechis ikaheka	0	9.2	80.0	(Paiva et al., 2014)
Micrurus alleni	0	77.3	10.9	(Fernández et al., 2015)
Micrurus altirostris	0	79.5	13.7	(Corrêa-Netto et al., 2011)
Micrurus corallinus	34.8		38.5	(Aird et al., 2017)
Micrurus dumerilii	0	28.1	52.0	(Rey-Suárez et al., 2016)
Micrurus frontalis	0	42.4	49.2	(Sanz et al., 2019)
Micrurus lemniscatus carvalhoi	2.3		48.6	(Aird et al., 2017)
Micrurus lemniscatus lemniscatus	34.3		19.4	(Aird et al., 2017)
Micrurus mipartitus	0	61.1	29.0	(Rey-Suárez et al., 2011)
Micrurus mipartitus	0	83.0	8.2	(Rey-Suárez et al., 2011)
Micrurus mosquitensis	0	22.5	55.6	(Fernández et al., 2015)
Micrurus nigrocinctus	0	38.0	48.0	(Fernández et al., 2011)
Micrurus paraensis	12.3		65.9	(Aird et al., 2017)

Micrurus pyrrhocryptus	0	27.0	17.0	(Olamendi-Portugal et al., 2018)
Micrurus spixii spixii	9.5		64.0	(Aird et al., 2017)
Micrurus spixii spixii	0	56.5	37.4	(Sanz et al., 2019)
Micrurus surinamensis	90.1		6.6	(Aird et al., 2017)
Micrurus surinamensis	36.8		33.0	(Aird et al., 2017)
Micrurus surinamensis	0	95.4	4.2	(Sanz et al., 2019)
Micrurus tschudii	0	95.2	4.1	(Sanz et al., 2016)
Naja atra	52.9	23.5	16.8	(H.-W. Huang et al., 2015)
Naja atra	59.4	20.5	14.0	(H.-W. Huang et al., 2015)
Naja atra	65.3	19.0	12.2	(Shan et al., 2016)
Naja haje	54.0	6.0	4.0	(Malih et al., 2014)
Naja kaouthia	45.7	18.0	23.5	(Kae Yi Tan et al., 2015)
Naja kaouthia	27.6	50.7	12.2	(Kae Yi Tan et al., 2015)
Naja kaouthia	44.9	31.5	17.4	(Kae Yi Tan et al., 2015)
Naja kaouthia	27.9	28.6	26.9	(Xu et al., 2017)
Naja katiensis	62.7	4.4	29.0	(Petras et al., 2011)
Naja melanoleuca*	25.2	31.9	12.9	(Lauridsen et al., 2017)
Naja mossambica	67.7	1.6	27.1	(Petras et al., 2011)
Naja naja	63.8		11.4	(Dutta et al., 2017)
Naja naja	69.3	4.8	21.4	(Sintiprungrat et al., 2016)
Naja naja	71.6	8.9	14.0	(Sintiprungrat et al., 2016)

Naja nigricollis	72.8	0.5	21.9	(Petras et al., 2011)
Naja nubiae	58.3	12.6	26.4	(Petras et al., 2011)
Naja pallida	64.9	2.8	30.1	(Petras et al., 2011)
Naja philippinensis	21.3	45.3	22.9	(C. H. Tan, Wong, Chong, et al., 2019)
Naja sputatrix	48.1	16.1	31.2	(N. H. Tan et al., 2017)
Notechis scutatus	0	5.6	74.5	(C. H. Tan et al., 2016)
Ophiophagus hannah	9.0	55.2	2.8	(Petras et al., 2015)
Ophiophagus hannah	0.5	42.5	4.0	(C. H. Tan, Tan, Fung, et al., 2015)
Oxyuranus scutellatus	0	4.2	68.3	(Herrera et al., 2012)
Oxyuranus scutellatus	0	1.5	79.4	(Herrera et al., 2012)
Pseudechis papuanus	0	3.1	90.2	(D. Pla, Bande, et al., 2017)
Toxicocalamus longissimus	0	92.1	6.5	(Calvete et al., 2012)

* BlastP analysis of the proteomic fragments reported in this study reveals that six of the seven proteins annotated as cytotoxins do not match with cytotoxins as their closest match in the NCBI database. The remaining sequence showed only 44% identity with a cytotoxin.

** This study was published before the recent reclassification of *N. melanoleuca* into five distinct species (Wolfgang Wüster et al., 2018). Based on the locality provided in this publication, the venom used in this study was likely sourced from either *N. melanoleuca* or *N. subfulva*.

Table S2. A summary of the Phylogenetic Generalized Least Squares (PGLS) tests performed under the scenario of *Naja atra* and *Naja kaouthia* being coded as non-spitting species. All tests were performed using the monophyletic grouping of *Naja* and *Hemachatus*, with the outgroup species of *Walterinnesia aegyptia* and *Aspidelaps scutatus* used to provide additional phylogenetic context. Significant values (*P* <0.05) are represented by bold red font. Degrees of freedom = 15.

Test Variable	t	P-value
PLA_2 proteomic abundance	**4.24**	**0.0007**
CTX proteomic abundance	-0.83	0.42
'Other 3FTX' proteomic abundance	-1.21	0.25
Venom lethality by murine lethal dose 50 (LD_{50})	0.86	0.40
Potency of neuronal cell activation (EC_{50})	**-4.48**	**0.0004**
PLA_2 enzymatic activity	**2.24**	**0.04**
Venom cytotoxicity via HET-CAM assay	1.08	0.30
Neuronal cell activation (EC_{50}) + PLA_2 activity	**-4.45**	**0.0004**

Table S3. Time-dependent irritation scores described for Luepeke's *in vivo* hen's egg test-chorioallantoic membrane (HET-CAM) assay (Luepke, 1985).

Effect	**Time (min)**		
	0.5	2.0	5.0
Hyperaemia	5	3	1
Haemorrhage	7	5	3
Coagulation	9	7	5

Table S4. The previously described relationship between the cumulative irritation score of Luepeke's *in vivo* then's egg test-chorioallantoic membrane (HET-CAM) assay (see Table S3) and 'irritation potential' (Luepke, 1985).

Cumulative score	**Irritation assessment (based on Luepke** (Luepke, 1985)**)**	**Irritation potential**
0.0-0.9	Practically none	No irritation
1.0-4.9	Slight	Slight irritation
5.0-8.9	Moderate	Moderate irritation
9.0-21.0	Strong	Severe irritation

Table S5. The murine lethality of the elapid snake venoms used in this study represented by intravenous median lethal dose (LD_{50}) values and corresponding 95% confidence intervals (CI).

Species	Group	Lineage	LD_{50} (µg/mouse)	95% CI
H. haemachatus	Spitter	*H. haemachatus*	57.96	47.28-80.00
N. mossambica	Spitter	African spitter	24.48	19.68-29.51
N. nubiae	Spitter	African spitter	23.65	19.17-27.37
N. pallida	Spitter	African spitter	9.29	3.76-13.23
N. nigricollis	Spitter	African spitter	27.49	22.55-38.21
N. annulifera	Non-spitter	African non-spitter	33.75	29.72-37.18
N. subfulva	Non-spitter	African non-spitter	5.84	3.27-7.47
N. nivea	Non-spitter	African non-spitter	15.05	10.20-18.70
N. haje	Non-spitter	African non-spitter	8.15	6.55-9.89
N. philippinensis	Spitter	Asian spitter	2.36	0.64-4.20
N. siamensis	Spitter	Asian spitter	18.43	14.02-22.17
N. sumatrana	Spitter	Asian spitter	17.43	13.29-20.97
N. atra	Non-spitter	Asian non-spitter	17.86	14.16-27.89
N. kaouthia	Non-spitter	Asian non-spitter	6.69	3.89-8.54
N. naja	Non-spitter	Asian non-spitter	11.1	7.90-13.10
A. scutatus	Non-spitter	Outgroup	5.75 *	5.29-6.26
W. aegyptia	Non-spitter	Outgroup	15.08	11.78-20.68

* Previously determined by Whiteley et al. (Whiteley et al., 2019).

Table S6. A summary of the Phylogenetic Generalized Least Squares (PGLS) tests performed under the scenario of *Naja atra* and *Naja kaouthia* being coded as spitting species. All tests were performed using the monophyletic grouping of *Naja* and *Hemachatus*, with the outgroup species of *Walterinnesia aegyptia* and *Aspidelaps scutatus* used to provide additional phylogenetic context. Significant values ($P < 0.05$) are represented by bold red font. Degrees of freedom = 15.

Test Variable	t	P-value
PLA_2 proteomic abundance	**2.86**	**0.01**
CTX proteomic abundance	-0.57	0.58
'Other 3FTX' proteomic abundance	-0.76	0.46
Venom lethality by murine lethal dose 50 (LD_{50})	1.25	0.23
Potency of neuronal cell activation (EC_{50})	**-4.59**	**0.0004**
PLA_2 enzymatic activity	1.80	0.09
Venom cytotoxicity via HET-CAM assay	**3.29**	**0.004**
Neuronal cell activation (EC_{50}) + PLA_2 activity	**-4.57**	**0.0004**

Table S7. The snake species, localities of origin, and number of specimens that contributed to the venom pools used in venom proteomic analyses and murine lethality tests.

Species	Locality	Number of individual snakes contributing to the venom pool
Hemachatus haemachatus	Captive bred	2
Naja annulifera	Captive bred	2
Naja atra	Captive bred	2
Naja haje	Uganda	6
Naja kaouthia	Captive bred	3
Naja mossambica	Tanzania	4
Naja naja	Captive bred	2
Naja nigricollis	Nigeria	3
Naja nivea	South Africa	3
Naja nubiae	Captive bred	3
Naja pallida	Tanzania	2
Naja philippinensis	Captive bred	2
Naja siamensis	Captive bred	2
Naja subfulva	Cameroon	2
Naja sumatrana	Captive bred	1
Walterinnesia aegyptia	Captive bred	2

Table S8. PCR primers and typical thermocycling conditions for the genes sequenced for the phylogenetic analysis. The denaturing step involved 30-45 s at 94°C and the extension step 1 min at 72°C, with a final extension step of 5 min at 72°C followed by cooling to 4°C for 15 min.

Gene	Sense	Name	Sequence	Citation	Annealing temp.	Cycles
CytB	Forward	Gludg	5'– TGACTTGAARAACCAYCGTTG – 3'	(Palumbi, 1996)	47°C	39
	Reverse	ATRCB3	5'– TGAGAAGTTTTCYGGGTCRTT – 3'	(M. B. Harvey et al., 2000)		
		H16064	5'– CTTTGGTTTACAAGAACAATGCTTTA – 3'	(Burbrink et al., 2000)		
ND4	Forward	NADH4	5'– CACCTATGACTACCAAAAGCTCATGTAGAAGC – 3'	(Arévalo et al., 1994)	57°C	39
	Reverse	H12763V	5'– TTCTATCACTTGGATTTGCACCA – 3'	(Arévalo et al., 1994)		
C-MOS	Forward	AV CMOSF	5'– AAGCACATCAAGGATTCGTCG – 3'	(Laing, 2019)	60.5°C	44
	Reverse	AV CMOSR	5'– TCTGCCTTGGGTGTGATTTTCT – 3'			
	Forward	G303	5'– ATTATGCCATCMCCTMTTCC – 3'	(Hugall & Lee, 2004)	57°C	35
	Reverse	G708	5'– GCTACATCAGCTCTCCARCA – 3'			
NT3	Forward	NTF3 F1	5'– ATGTCCAATCTTGTTTTATGTGATATTT – 3'	(Townsend et al., 2008)	42°C	39
	Reverse	NTF3 R1	5'– ACRAGTTTRTTGTTYTCTGAAGTC – 3'			
PRLR	Forward	PRLR F1	5'– GACARYGARGACCAGCAACTRATGCC – 3'	(Townsend et al., 2008)	48°C	39
	Reverse	PRLR F3	5'– GACYTTGTGRACTTCYACRTAATCCAT – 3'			
RAG1	Forward	AV RAG1F	5'– AAATGTGACAGGGTCTCT – 3'	(Laing, 2019)	59°C	44
	Reverse	AV RAG1R	5'– GGGCATCTCAAAACCAAATTGT – 3'			
UBN1	Forward	BaUBN F	5'– CCTCTGGTTACTCAGCAGCA – 3'	(Nicholas R. Casewell, Wagstaff, Harrison, & Wüster, 2011)	40°C	39
	Reverse	BaUBN R	5'– ATTGGCCACTCCTTGTGTTC – 3'			

Table S9. The snake species, localities of origin, and number of specimens that contributed to the venom pools used in the hen's egg test-chorioallantoic membrane (HET-CAM) assay.

Species	Locality	Number of individual snakes contributing to the venom pool	Same venom sample as that used for proteomic analysis?
Naja kaouthia	Captive bred	1	No
Naja mossambica	Captive bred	1	No
Naja nivea	Captive bred	1	No
Naja atra	Captive bred	1	No
Naja sumatrana	Captive bred	1	No
Naja siamensis	Captive bred	1	No
Naja pallida	Captive bred	1	No
Naja annulifera	Captive bred	1	No
Naja haje	Captive bred	1	No
Naja subfulva	Captive bred	1	No
Naja naja	Captive bred	1	No
Naja nubiae	Captive bred	1	No
Walterinnesia aegyptia	Captive bred	2	Yes
Hemachatus haemachatus	Captive bred	2	Yes
Naja philippinensis	Captive bred	2	Yes
Naja nigricollis	Nigeria	3	Yes
Aspidelaps lubricus cowlesi	Captive bred	1	N/A (proteomics previously performed)

Supplementary Data Files

Data S1.

Top-down MS analyses of the venom proteomes described in this study: including African and Asian *Naja* species, the desert black snake (*Walterinnesia aegyptia*), and the rinkhals (*Hemachatus haemachatus*), as summarized in Fig. 1A and Fig. S2.

Data S2.

Nucleotide sequence alignment for the three-finger toxin (3FTX) family, extracted from venom gland transcriptomes from *Naja* spp., the desert black snake (*Walterinnesia aegyptia*) and the rinkhals (*Hemachatus haemachatus*) described in this study, along with data from related elapid snake species (*Aspidelaps scutatus intermedius* (Whiteley et al., 2019), *Bungarus flaviceps* (Siang et al., 2010), *Bungarus multicinctus* (52), *Dendroaspis* spp. (Ainsworth et al., 2018), *Micrurus fulvius* (Margres et al., 2013) and *Ophiophagus hannah* (53, 54)) sourced from previous, aligned against a sequence from the outgroup species (*Python regius*; NCBI accession number GBIC00000000, from (A. D. Hargreaves et al., 2014)).

Data S3.

Nucleotide sequence alignment for the cytotoxin (CTX) subset of the three-finger toxin (3FTX) family, extracted from venom gland transcriptomes from *Naja* spp., the rinkhals (*Hemachatus haemachatus*), the shield-nosed cobra (*Aspidelaps scutatus intermedius*, from (Whiteley et al., 2019)) and the king cobra (*Ophiophagus hannah*, from (Li,J., Zhang et al., 2006)), aligned against a sequence from the outgroup elapid, the red-headed krait (*Bungarus flaviceps*, NCBI accession number GU190795, from (Siang et al., 2010)).