Venoms of related mammal-eating species of taipans (*Oxyuranus*) and brown snakes (*Pseudonaja*) differ in composition of toxins involved in mammal poisoning

Jure Skejic[1,2,3*], David L. Steer[4], Nathan Dunstan[5], Wayne C. Hodgson[2]

[1] Department of Biochemistry and Molecular Biology, BIO21 Institute, University of Melbourne, 30 Flemington Road, Parkville, Victoria 3010, Australia
[2] Monash Venom Group, Department of Pharmacology, Monash University, 9 Ancora Imparo Way, Clayton, Victoria 3800, Australia
[3] Laboratory of Evolutionary Genetics, Division of Molecular Biology, Ruder Boskovic Institute, Bijenicka 54, 10000 Zagreb, Croatia
[4] Monash Biomedical Proteomics Facility, Monash University, 23 Innovation Walk, Clayton, Victoria 3800, Australia
[5] Venom Supplies Pty Ltd., Stonewell road, Tanunda, South Australia 5352, Australia
* Correspondence: jskejic@irb.hr

Abstract

Background

Differences in venom composition among related snake lineages have often been attributed primarily to diet. Australian elapids belonging to taipans (*Oxyuranus*) and brown snakes (*Pseudonaja*) include a few specialist predators as well as generalists that have broader dietary niches and represent a suitable model system to investigate this assumption. Here, shotgun high-resolution mass spectrometry (Q Exactive Orbitrap) was used to compare venom proteome composition of several related mammal-eating species of taipans and brown snakes.

Results

Venoms of the mammal-eating lineages of *Oxyuranus* and *Pseudonaja* differed greatly in the composition of proteins toxic to mammals. Venom of mammalivorous Western Desert taipan *Oxyuranus temporalis* consisted predominately of postsynaptic α-neurotoxins and was deficient in presynaptic phospholipase A_2 neurotoxins. In contrast, presynaptic PLA_2 neurotoxins (taipoxin and paradoxin) were abundant in the venoms of closely related mammal-eating specialists coastal taipan *O. scutellatus* and inland taipan *O. microlepidotus*. Significant expression of venom prothrombinase was not detected in *O. temporalis* venom despite the toxin being found at substantial levels in most other mammal-eating lineages. Presynaptic PLA_2 neurotoxins (textilotoxin) were present at high levels in the venoms of some mammal-consuming brown snakes, specifically the eastern *P. textilis* and northern brown snake *P. nuchalis*, but surprisingly were not discovered in the venom of a related mammal-eating species, Ingram's brown snake *P. ingrami*. Expression of an α-neurotoxin that is toxic to rodents (pseudonajatoxin b) was profoundly down-regulated in Queensland *P. textilis* venom and highly up-regulated in South Australian *P. textilis* venom despite both populations consuming this type of prey.

Conclusion

Very different expression patterns of mammal-toxic venom procoagulants and neurotoxins across *Oxyuranus* and *Pseudonaja* phylogeny suggest that prey type preference cannot fully account for interlineage differences in venom proteome composition.

Keywords

Snake venom, venom proteome evolution, predatory toxin expression, mammal prey, *Oxyuranus*, *Pseudonaja*

Background

Variability in snake venom composition has attracted a lot of research interest, but the causes of this phenomenon remain poorly known. A recent review of snake venom proteome profiles highlighted the presence of familial trends in venom composition – the venoms of viperid snakes generally abound in metalloproteinases, phospholipases A_2 and serine proteases, and those of elapids are dominated by three-finger toxins and phospholipases A_2 [1]. However, marked intrafamilial variation is also present. The venom of African mammal-eating elapid black mamba *Dendroaspis polylepis*, for instance, is predominately composed of kunitz-type dendrotoxins and 3FTx, whereas the venom of mammal-eating elapid from another continent – Australian coastal taipan *Oxyuranus scutellatus* – contains PLA_2 and venom prothrombinase proteins [1-3]. The types as well as the quantities of secreted venom proteins often vary among closely related species and even populations of the same species. Differences in protein composition of venoms between related species can be very large, for example in American coral snakes (genus *Micrurus*), palm pitvipers (*Bothriechis*) or African puff adders, Gaboon vipers and relatives (genus *Bitis*) [4-6]. In other cases, there seems to be little intragroup variation, for instance in North American pitvipers of the genus *Agkistrodon* [7]. Variation in venom composition is commonly observed between conspecific populations. A well-known example is the Mojave rattlesnake *Crotalus scutulatus*, which exhibits extraordinary large differences in the venom content of crotamine-myotoxins, metalloproteinases and PLA_2 proteins across populations [8].

The causes of variation in venom composition have been hypothesised to result from factors such as diet, phylogeny and geographic distance. A study that examined the effects of these factors on the venom composition of Malayan pitviper *Calosellasma rhodostoma* populations concluded that the intraspecific variation in venom composition was closely associated with diet and rejected the effects of geographic proximity and population phylogeny [9]. Another study conducted on North American rattlesnakes of the genus *Sistrurus* found interspecific variation in the abundance of some venom proteins to be related to diet [10]. Yet other studies, such as that on tiger snake *Notechis* populations in South Australia or a study on Afro-Asian carpet or saw-scaled vipers of the genus *Echis*, found no obvious link between venom composition and the kind of prey consumed [11, 12]. Research on neotropical palm pitvipers *Bothriechis* revealed large interspecific differences in venom proteome composition despite the genus members being reported to have similar generalist diets [6, 13].

Venomous snakes from Australia and New Guinea, which include taipans (*Oxyuranus*) and brown snakes (*Pseudonaja*), are a suitable group to study divergence of venom composition in closely related lineages (Fig. 1). This is a medically important snake group, responsible for a number of serious and fatal envenomation cases [14-16]. The advantages of choosing this model system are: (i) the group includes multiple lineages of dietary specialists and generalists (ii) comprehensive studies of the dietary niches are available, including identification of prey items from museum specimens and scats of live specimens, and (iii) the toxic activities of a few protein families in the studied prey class (small mammals) have been reasonably well-characterised. Brown snakes and taipans are swift moving, medium to large-sized terrestrial snakes, which form a monophyletic group within the oxyuranine elapids

[17]. The majority of species eat small mammals as adults, including rodents and small marsupials. The coastal taipan *Oxyuranus scutellatus* and inland taipan *O. microlepidotus* are specialists, feeding mainly on mammals [18]. Dietary records for the Western Desert taipan *O. temporalis* show that this species, like its relatives, eats mammals [19]. Brown snakes such as the eastern brown snake *P. textilis*, northern brown snake *P. nuchalis*, speckled brown snake *P. gutt*ata and most other congeneric species have generalist vertebrate diets as adults, consuming mammals, lizards and frogs [20]. A study by Shine recovered only rats from the preserved Ingram's brown snake *P. ingrami* specimens [20], but based on anecdotal reports the species may feed on frogs as well (N. Dunstan, personal communication). A small-bodied member of the group, ringed brown snake *Pseudonaja modesta*, is saurophagous i.e. predating on lizards [20].

The venoms of taipans and brown snakes contain proteins belonging to several protein families [21-23]. This project focused on the expression patterns of the proteins that have been shown experimentally to be toxic to rodents and are presumed to have a role in mammal prey incapacitation. These include procoagulant venom prothrombinase and neurotoxins from two protein families: phospholipases A_2 and three-fingered toxins. Venom prothrombinase is a protein complex consisting of coagulation factor X-like serine protease and fV-like multicopper oxidase-related protein [24-27]. Functionally, it is one type of a prothrombin-activator [28]. Upon injection in rodents, this toxin induces coagulopathy and collapse of the circulatory system, which manifests in a rapid fall in systemic blood pressure [29]. Neurotoxins target the neuromuscular system and induce paralysis. They belong to two major protein families: phospholipases A_2 (PLA_2) and three-finger toxins ($3FT_X$). Not all PLA_2 are neurotoxic, but some induce potent presynaptic neurotoxicity and are called β-neurotoxins [30, 31]. Neurotoxic PLA_2 from Australian elapid venoms include textilotoxin, taipoxin and paradoxin and display variable protein complex composition [32-34]. 3FTx are a large protein family that includes members with diverse functions. Those that target the acetylcholine nicotinic receptors are called postsynaptic or α-neurotoxins [35, 36]. Within this family there are three major clades of α-neurotoxins, including: short-chain (also called type I), long-chain (type II) and oxyuranine (type III) [37, 38].

Venom proteins were identified using bottom-up shotgun proteomics and their relative abundances were obtained using label-free quantification (iBAQ). In order to examine the evolution of venom composition in closely related mammalivorous lineages, the toxin expression profiles and prey classes were plotted onto the phylogenetic tree.

Figure 1. Australian taipans and brown snakes represent an attractive model system to study divergence in venom composition. Phylogenetically closely related mammal-eating species displayed above secrete venoms that differ in the composition of mammal-targeting toxins: (a) coastal taipan *Oxyuranus scutellatus*, (b) inland taipan *O. microlepidotus*, (c) Ingram's brown snake *Pseudonaja ingrami*, (d) Western Desert taipan *O. temporalis* and (e) eastern brown snake *P. textilis.* Photos taken by Scott Eipper (a and c), Akash Samuel (b), Brian Bush (d) and Stewart Macdonald (e).

Results and Discussion

Venom of mammal-eating Western Desert taipan *Oxyuranus temporalis* consists predominately of three-finger α-neurotoxins and is deficient in PLA_2 neurotoxins and venom prothrombinase

O. temporalis venom was found to contain abundant short-chain and some long-chain α-neurotoxins (Fig. 3). Cumulatively, these neurotoxins comprised most of the identified proteins (relative iBAQ abundance = 97.9%), making this taipan species distinct in producing venom that consists mainly of postsynaptic neurotoxins (Fig. 2). The chains forming presynaptically-neurotoxic phospholipase A_2 protein complexes were not found, with exception of one protein match that is homologous to paradoxin alpha chain (Additional files 1-3). The expression level of this chain was so low (iBAQ = 0.28%) that it is unlikely to play a major role in venom neurotoxicity. The proteomic data presented here confirm that the very rapid neurotoxic activity of *O. temporalis* venom in chick biventer cervicis physiological assay, reported by Barber et al [39], is due to an extremely high concentration of α-neurotoxins. A very significant finding is a lack of venom prothrombinase in *O. temporalis* venom. Specifically, MaxQuant search did not detect tryptic peptides matching fX serine protease in this venom sample, indicating that the enzyme is either extremely scarce or absent. Four peptides corresponding to fV were detected in *O. temporalis* venom, but the overall expression of the protein was negligible (iBAQ = 0.00033%) (Additional files 1-3). The venom protein family relative abundances for this and other species are listed in Table 1.

Mammal-eating specialists coastal taipan *O. scutellatus* and inland taipan *O. microlepidotus* possess venoms that abound with PLA_2 presynaptic neurotoxins

The venoms of mammalivorous specialists *O. scutellatus* and *O. microlepidotus* contained copious amounts of PLA_2 presynaptic neurotoxins, that is – chains belonging to taipoxin and paradoxin clades (Fig 2, Additional files 1-4). In *O. scutellatus* venom these neurotoxins comprised more than half of the identified venom proteome and almost a third of *O. microlepidotus* venom proteome (iBAQ = 56.6% and 31.8%, respectively). The venoms of these two taipan species differed greatly in the relative abundances of postsynaptic neurotoxins, *O. microlepidotus* secreting a higher quantity of α-neurotoxins (iBAQ = 50.2% of identified *O. microlepidotus* venom proteome vs only 5.8% in *O. scutellatus*). It is important to point out that the examined venom of *O. scutellatus* was from Queensland (Julatten area). There is an indication that the venoms of this species may contain different proportions of α-neurotoxins in different parts of its geographic range. For example, a more reduced effect of Saibai Island *Oxyuranus scutellatus* venom on chick biventer cervicis contractile response to acetylcholine suggests that this population would have even lower expression of α-neurotoxins than the specimens from the mainland [39]. *O. scutellatus* and *O. microlepidotus* venoms also contained venom prothrombinase, but in lower proportions than in some *Pseudonaja* venoms (Table 1).

Textilotoxin or related PLA_2 neurotoxins were not detected in the venoms of some mammal-eating brown snakes

In common with most other brown snake species, venoms of *Pseudonaja ingrami* and *P. gutatta* contained prothrombinase toxins and abundant α-neurotoxins (Fig. 2). Interestingly,

Table 1. Expression levels of major venom protein families

Protein family	*O. temporalis*	*O. scutellatus*	*O. microlepidotus*	*P. guttata*	*P. modesta*	*P. ingrami*	*P. nuchalis*	*P. textilis* SA	*P. textilis* QLD
fX serine protease		2.28	0.68	2.02	0.0035	4.82	4.58	1.88	11.02
fV multicopper oxidase	0.00033	2.41	1.08	3.39	0.0022	8.77	6.27	2.60	10.64
PLA_2 β-neurotoxins	0.28	56.61	31.79				12.36	1.93	17.27
other PLA_2		3.88	5.98	1.89	1.37	11.38	6.38	3.98	12.28
α-neurotoxins short	80.35		32.85			42.43			
α-neurotoxins long	17.55	0.026	12.23	77.24	0.42	0.046	48.52	40.73	0.24
α-neurotoxins oxy A				0.053	84.40		0.022	29.90	0.37
α-neurotoxins oxy B		5.78	5.02			0.71	0.45		
kunitz inhibitors	0.16	23.30	0.33	0.98	8.92	12.82	19.62	13.14	43.15
lectins				0.051	0.45	15.53	0.00094	2.89	1.08
natriuretic peptides	1.57	4.30	4.49	0.18	0.49	0.69	0.74		0.070
waprins			4.16						
CRISP		0.63	0.41	0.33	2.35	0.62		0.42	0.78

Relative iBAQ (%) of the total identified venom proteome. Based on UniProt database search: taxon / Serpentes, 70,087 sequences. Details on the examined venom samples are noted in the Methods.

the examined venom samples from these species lacked matches to textilotoxin or other related β-neurotoxin clades (Additional files 1-4). *P. ingrami* has been reported to feed on rats, and a lack of detection of potent presynaptic toxins in its venom adds another example of variable expression of mammal-targeting toxins among related species.

Venom prothrombinase is a major component of mammalivorous brown snake (*Pseudonaja*) venoms

Venom prothrombinase is composed of a serine protease toxin homologous to coagulation factor X and its co-factor homologous to coagulation factor V of multicopper oxidase family. These proteins were found in the venoms of all examined mammalivorous *Pseudonaja* species, including the eastern *P. textilis*, northern *P. nuchalis*, Ingram's *P. ingrami* and speckled *P. guttata* brown snake. Particularly high levels of venom prothrombinase were recorded in the first three mentioned species (Table 1). For instance, in *P. textilis* venom sample from Queensland (Mackay area), the expression level of venom prothrombinase toxins was about one-fifth of the identified venom proteome (iBAQ for fX = 11.0% and fV = 10.6%). A recent study recorded high fX activity of the western brown snake *P. mendgeni* venom, suggesting that high expression of venom prothrombinase can be expected to be found in the venom of this species as well [40].

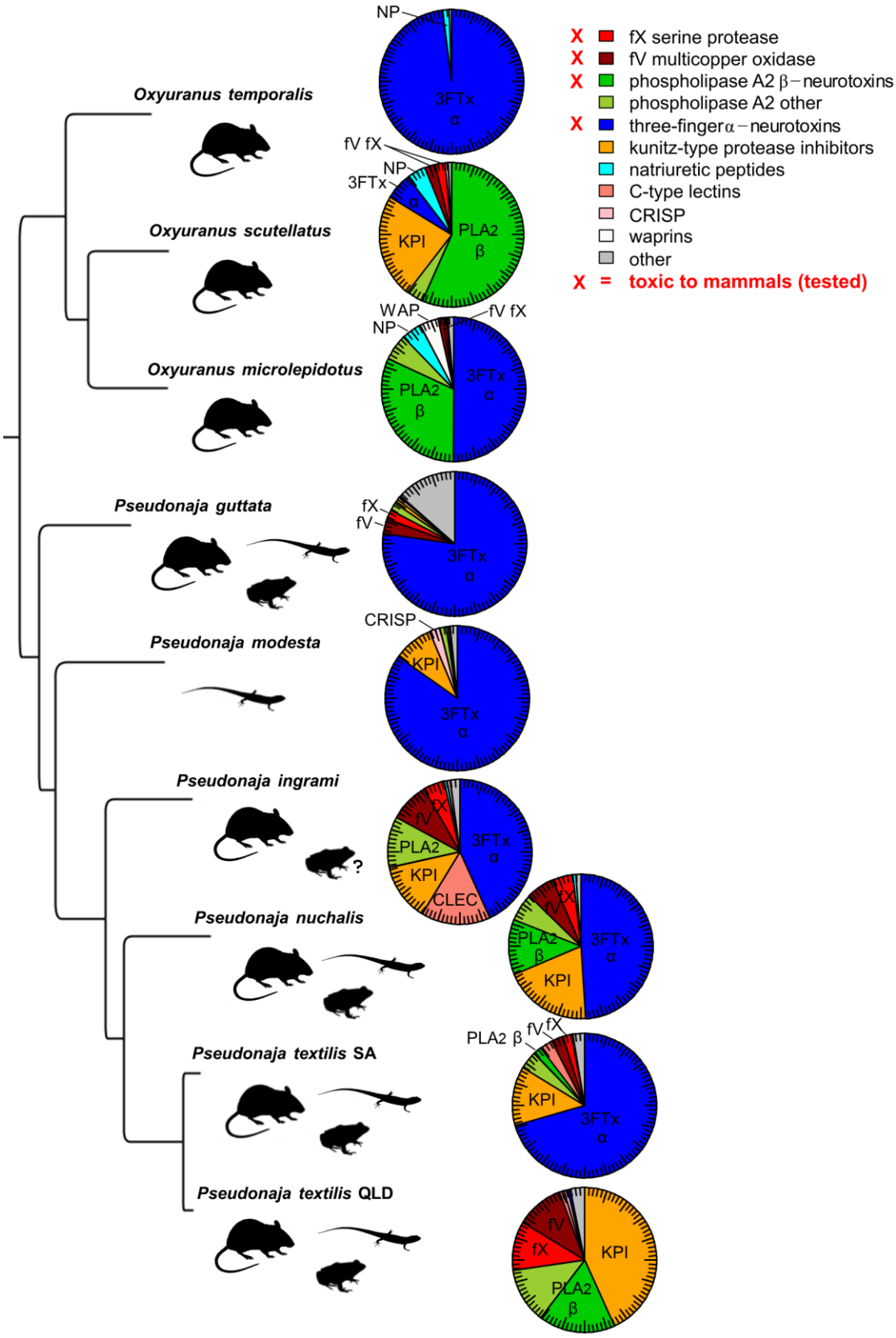
Oxyuranus temporalis
Oxyuranus scutellatus
Oxyuranus microlepidotus
Pseudonaja guttata
Pseudonaja modesta
Pseudonaja ingrami
Pseudonaja nuchalis
Pseudonaja textilis SA
Pseudonaja textilis QLD
NP
3FTx α
fV fX
KPI
PLA2 β
WAP
CRISP
CLEC
PLA2
?
X fX serine protease
X fV multicopper oxidase
X phospholipase A2 β−neurotoxins
phospholipase A2 other
X three-finger α−neurotoxins
kunitz-type protease inhibitors
natriuretic peptides
C-type lectins
CRISP
waprins
other
X = toxic to mammals (tested)

Figure 2. Venom protein family expression profiles of adult individuals mapped over the species phylogeny. Australian elapid venom proteins demonstrated to possess potent toxicity towards mammals (rodents) are marked with the red X symbol. Protein expression was quantified using the relative iBAQ method (major tick = 5%, minor tick = 1% of identified venom proteome). Recorded dietary items include mammals, squamate reptiles and amphibians (see the Diet data section).

Venom prothrombinase is negligibly expressed in the venom of lizard-eating ringed brown snake *P. modesta*

Although the study focused on mammal-eating species, it was useful to examine the expression patterns of toxins that are known to be lethal to mammals in the venom of a related lizard-eating species. Two peptides unique to fX serine protease and four peptides unique to fV were detected in the venom of *Pseudonaja modesta*, confirming presence of the prothrombinase complex components (Additional files 1-3). However, the expression levels of fX and fV proteins in the venom of this lizard-feeding species were extremely low (iBAQ = 0.0035% and 0.0022%, respectively). Their detection indicates that *P. modesta* apparently has functional genes to code for the venom prothrombinase protein complex, but the toxin expression is so low that it is insufficient to induce a physiological effect. This is supported by the studies that found no procoagulant activity of this venom [40, 41]. Our study provides evidence of very low expression of venom prothrombinase fX and fV in this lizard-feeding snake at the protein level (Table 1). A transcriptomic study on West Australian *P. modesta* lineage did not find transcripts belonging to fX and recorded low-level expression of fV [38]. Down-regulated expression of prothrombinase proteins may be related to possible ineffectiveness of the toxin to subdue squamate reptiles. A presumed role of diet is further supported by the study that recorded a lack of prothrombin activation and factor Xa activity by the venoms of various juvenile brown snake species, which also feed on lizards [20, 40].

Eastern brown snakes *Pseudonaja textilis* from Queensland and South Australia have similar diets but distinct venom proteomes, differing greatly in α-neurotoxin content

Coastal Queensland populations of the eastern brown snake *Pseudonaja textilis* belong to the northeastern mtDNA clade, whereas South Australian populations in the vicinity of Adelaide belong to the southeastern clade [42]. *P. textilis* venoms from these regions of Australia show considerable differences in protein composition. Queensland *P. textilis* venom is characterised by higher abundances of kunitz-type serine protease inhibitors, PLA_2 neurotoxins (textilotoxin) and venom prothrombinase proteins than the South Australian venom of the same species (Table 1). The most striking difference in venom composition was high abundance of three finger α-neurotoxins in South Australian *P. textilis* venom (iBAQ = 70.6%) and very low levels of these neurotoxins in Queensland *P. textilis* venom (iBAQ = 0.61%). In our prior physiological study [29], Queensland *P. textilis* venom did not induce noticeable postsynaptic neurotoxicity, which is concordant with down-regulated expression of α-neurotoxins. This difference is of particular interest considering that the populations of *P. textilis* from these regions have been reported to have similar diets, eating mainly rodents

and lizards [20]. A study conducted in South Australia showed that the protein family composition of *P. textilis* venom does not seem to vary significantly among individuals within the population [43]. Nonetheless, in future studies it would be desirable to obtain additional samples and from different populations.

Pseudonajatoxin b, a lethal long-chain α-neurotoxin, is found only in certain mammal-eating lineages

Our previous study found high levels of pseudonajatoxin b in South Australian *P. textilis* venom and very low levels in Queensland *P. textilis* venom [44]. Pseudonajatoxin b is long-chain α-neurotoxin that is highly lethal to mice [45]. It is remarkable that despite the capacity of this neurotoxin to incapacitate rodents, its expression is profoundly down-regulated in rodent-feeding Queensand *P. textilis*. This study reveals that this lethal neurotoxin is also expressed in high quantity in the venom of mammal-eating *Pseudonaja nuchalis* (Additional files 1-3).

Interspecific variation in α-neurotoxin groups

Three-finger toxins found in *Pseudonaja* and *Oxyuranus* venoms included short-chain α-neurotoxins (also called type I), long-chain α-neurotoxins (type II), and the third α-neurotoxin clade unique to oxyuranine elapids (type III). A maximum likelihood tree of these toxins distinguished two subclades within the oxyuranine α-neurotoxin group, provisionally labelled as A and B (Additional file 4). Short-chain α-neurotoxins (type I) and oxyuranine α-neurotoxins of the subclade B were found in substantial quantities in species that eat mainly mammals (*Oxyuranus* members), while oxyuranine α-neurotoxins of the subclade A were found in species that feed chiefly or partially on lizards (*P.modesta* and South Australian *P. textilis*) (Fig. 3). A striking difference is seen between a mammal-eating specialist *O. temporalis* and a lizard-eating specialist *P. modesta*. The venom proteomes of both species were found to be composed predominately of α-neurotoxins. However, the venom proteome of *O. temporalis* contained abundant short- and some long-chain α-neurotoxins, whereas that of *P. modesta* was dominated by an entirely different group – oxyuranine α-neurotoxins of the subclade A (Fig 3.). Hypothetically, certain α-neurotoxins could be adapted to more efficiently target the neuromuscular system of a specific prey class, but this remains unknown. Few α-neurotoxins from the venoms of Australian elapids have been characterised. A recent study sequenced α-Elapitoxin-Ot1a short-chain α-neurotoxin and found it to be a potent and abundant constituent of *O. temporalis* venom [46]. It should be noted that the finding of a large concentration of short-chain (type I) α-neurotoxins in the venom of *Oxyuranus temporalis* may be medically important, requiring further investigation.

Variable expression patterns of other main components

Kunitz-type serine protease inhibitors were determined to be one of abundant venom proteome components of most *Pseudonaja* species and *Oxyuranus scutellatus* (Fig. 2, Table 1). This was by far the most abundant protein family comprising the venom of *Pseudonaja textilis* from Queensland (iBAQ = 43.2%). Natriuretic peptides were found in significant quantities in the venoms of all three *Oxyuranus* species, with the iBAQ abundance of around 4% in *O. scutellatus* and *O. microlepidotus,* and a somewhat lower value in *O. temporalis* venom (1.6%). *Pseudonaja ingrami* venom is interesting in expressing high levels of C-type

lectin proteins, which make up 15.5% of the identified venom proteome. Waprins were found at a significant level in *O. microlepidotus* venom, constituting about 4.2 % of iBAQ-quantified proteins. CRISP were expressed in the highest relative proportion in the lizard-feeding *Pseudonaja modesta*. Some of these families such as kunitz-type serine protease inhibitors and natriuretic peptides have been shown to possess potent bioactivities with potentially toxic effects in prey [47-50].

Other identified low abundance proteins

This analysis identified a myriad of proteins that were expressed in lower quantities (relative iBAQ expression was < 1% in all studied species). Some of these include: nerve growth factor, cystatin, cathepsin, calreticulin, transferrin, 5'nucleotidase, aminopeptidase, snake venom metalloproteinase (SVMP), carboxypeptidase, protein-disulfide isomerase, phospholipase B-like, sulfhydryl oxidase, complement C3, vascular endothelial growth factor (VEGF), hyaluronidase, glutathione peroxidase, PLA_2 inhibitors, phosphatidylethanolamine-binding protein, alkaline phosphatase, dipeptidase, venom dipeptidylpeptidase and others (Additional files 1-3).

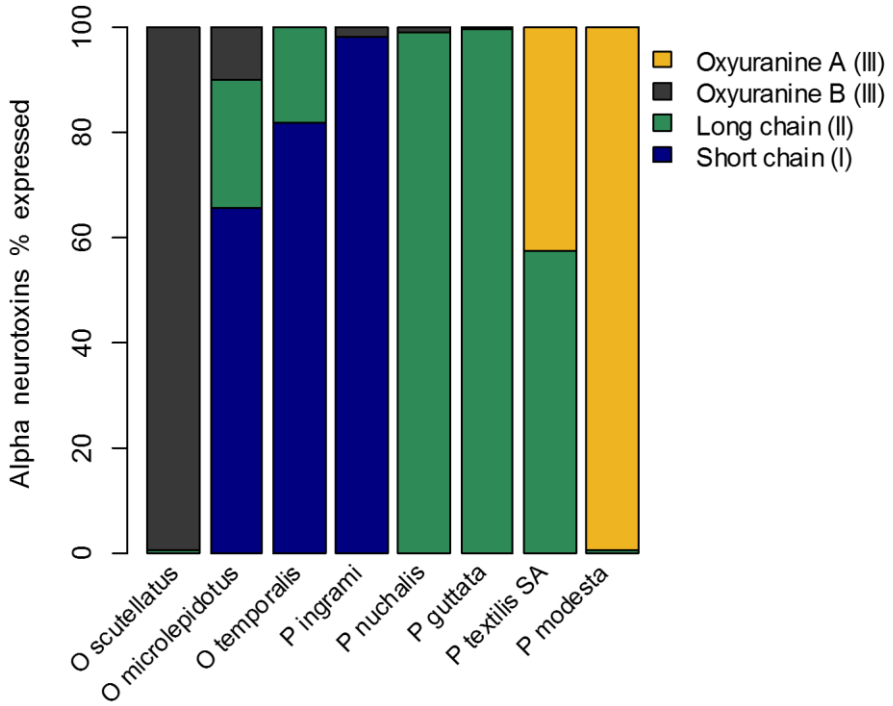

Figure 3. Venom samples show differences in the relative abundances of identified α-neurotoxin groups. Short-chain and oxyuranine α-neurotoxins of one subgroup (labelled A on the tree) were common in the venoms of mammalivorous *Oxyuranus* species, whereas oxyuranine α-neurotoxins of the other subgroup (labelled B) predominated in the venom of saurophagous *P. modesta* (α-neurotoxin type nomenclature that uses Roman numerals is shown in the parentheses).

Divergence in venom proteome composition

Venom prothrombinase is a lethal procoagulant protein complex that has been found to induce coagulopathy and circulatory collapse in rodents [29, 51]. Another group of potent toxins, neurotoxic phospholipases A_2, have been shown to paralyse the diaphragm in rodent nerve-muscle preparations and kill rodents when injected into them [52-55]. Further, pseudonajatoxin b, which is a long-chain α-neurotoxin belonging to the three-finger toxin family, has been demonstrated to be highly lethal to rodents [45]. It is remarkable that despite the capacity of these toxin families to incapacitate small mammals, they show very different expression patterns in closely related mammal-eating species of Australian elapid snakes. For example, the proteomic data suggest that *Oxyuranus temporalis* relies principally on postsynaptic α-neurotoxins to subdue mammal prey, while a related species *Oxyuranus scutellatus* injects a different lethal protein set, containing abundant neurotoxic phospholipases A_2 and venom prothrombinase, to accomplish the same goal.

What are the processes leading to the observed differences in venom composition? The data presented here and in our previous study that looked at the expression patterns of individual α-neurotoxic proteins in Queensland and South Australian *P. textilis* venoms (see [44]) suggest that the processes underlying up/down regulation of venom protein expression have an important role in determining venom proteome composition and associated toxicity. In particular, low-level expression of α-neurotoxins in Queensland *P. textilis* venom indicates that the genes coding for these neurotoxins are functional even though α-neurotoxin concentration is too low to induce noticeable postsynaptic neurotoxicity. Similarly, low-level expression of prothrombinase fX and fV proteins in *Pseudonaja modesta* venom indicates that the species possesses the genes coding for these toxins, but they are expressed at functionally insignificant levels. This conclusion is supported by the studies that recorded no procoagulant activity of *P. modesta* venom [40, 41]. In addition to protein expression, genomic mechanisms can have a profound impact on venom composition differences in related lineages. For example, a recent study on the evolution of PLA_2 toxins in rattlesnakes (genus *Crotalus*) suggested that the interspecific differences in the content of PLA_2 toxins in *Crotalus* are due to gene losses [56]. A role of gene duplication in generating neurotoxin diversity has been demonstrated by a genomic study on *P. textilis*, which identified five paralogous genes encoding oxyuranine α-neurotoxins [57]. Currently, little is known about the extent to which genomic mechanisms and phylogeny contribute to variation in venom composition in Australian elapids.

Diet should intuitively be regarded as an important factor in venom protein evolution because toxins need to efficiently disable physiological functions of targeted prey organisms. However, the results of the current study on *Oxyuranus* and *Pseudonaja* show that diet cannot be considered the sole factor contributing to venom proteome differentiation among closely related lineages. This inference is also supported by the finding that the venom of the black-speckled palm pitviper *Bothriechis nigroviridis* has unique toxin composition despite the species being observed to eat the same varied vertebrate prey like its congeners, consisting of small rodents, lizards, frogs and small birds [13].

Conclusion

This study revealed very different expression patterns of the procoagulant toxins and neurotoxins involved in mammal poisoning among closely related mammal-eating lineages of Australian elapid snakes. The proteomic data gathered strongly suggest that venom composition differences among related snake lineages cannot be explained fully by prey type selection. The results reiterate the need for research focusing on the molecular mechanisms that regulate venom protein expression.

Methods

Venom sampling

Venoms were pooled from adult individuals kept at Venom Supplies (Tanunda, SA, Australia). Adults of *O. temporalis* were housed at Adelaide Zoo (SA, Australia). Multiple individuals representing each lineage were obtained, with the exception of a single *P. modesta* specimen. The geographic origin of the individuals examined is as follows: *Pseudonaja textilis* (southeastern lineage) Barossa Valley, South Australia (3 adults); *Pseudonaja textilis* (northeastern lineage) Mackay, Queensland (2 adults); *Pseudonaja ingrami*, Boulia area, Queensland (3 adults); *Pseudonaja nuchalis,* Darwin region, Northern Territory (3 adults); *Pseudonaja modesta*, Queensland (1 adult); *Pseudonaja guttata* Queensland (multiple adults); *Oxyuranus microlepidotus*, South Australia (6 adults); *Oxyuranus scutellatus* Julatten, Queensland (3 adults); *Oxyuranus temporalis* Ilkurlka, Western Australia (2 adults). The venoms were freeze-dried.

Liquid chromatography and high-resolution mass spectrometry

The venom protein samples were weighed to approximately 1 mg and each sample was reconstituted to a concentration of 5 mg/ml in a solution of 10% acetonitrile, 20 mM ammonium bicarbonate. An aliquot containing the equivalent of 100 µg of the protein sample was reduced in 2.5 mM DTT at 50°C for 15 min followed by alkylation with 10 mM Iodoacetamide for 60 min in the dark at room temperature. Following alkylation, a solution containing 1 µg Trypsin (Promega corp., Madison, WI, USA) in 20 mM ammonium bicarbonate was added and the samples incubated at 37°C overnight. The final concentration of the protein digest mixture was 2 µg/µl.

For analysis by LC-MS, the samples were diluted to 0.2 µg/µl using a buffer of 2% acetonitrile and 0.1% formic acid, and centrifuged at 16000 g for 5 min before transfer to an autosampler vial. The mass spectrometry instrument used for analysis was Q-Orbitrap, model Q Exactive, Thermo Scientific (Bremen, Germany), software: Thermo Excalibur 2.2. The LC instrument used was nano RS-HPLC, Ultimate 3000, Thermo Scientific (Bremen, Germany), software Thermo Excalibur 2.2 DCMS link.

The tryptic digests were injected at a volume of 2 µl and concentrated on a 100 µm, 2 cm nanoviper pepmap100 trap column with loading buffer (2% acetonitrile, 0.1% formic acid) at a flow rate of 15 µl/min. The peptides then eluted and separated with a 50 cm Thermo RSLC pepmap100, 75 µm id, 100 Å pore size, reversed phase nano column with a gradient starting at 97% buffer A (0.1% formic acid), between 4 and 5 min the concentration of buffer B (80%

acetonitrile, 0.1% formic acid) increased to 10%, followed by a 30 min gradient to 40% B then to 95% B in 2 min, at a flow rate of 300 nl/min. The eluant was nebulized and ionized using the Thermo nano electrospray source with a distal coated fused silica emitter (New Objective, Woburn, MA, USA) with a capillary voltage of 1800 V.

The MS data were acquired with the following parameters: resolution 70000, AGC target 3e6, 120 ms Max IT. Peptides were selected for MSMS analysis in Full MS/dd-MS^2 (TopN) mode with the following parameter settings: TopN 10, resolution 17500, MSMS AGC target 1e5, 60 ms Max IT, NCE 27 and 3 m/z isolation window. Underfill ratio was at 10% and dynamic exclusion was set to 15 s.

Shotgun proteomics

Raw mass spectra were processed with MaxQuant software (version 1.6.0.1), Max Planck Institute of Biochemistry [58-60]. The searched database included 70,087 available protein sequences from UniProt, taxon: Serpentes (downloaded on 17/8/2017). The parameters were: digestion enzyme: trypsin, missed cleavages: 2, fixed modifications: carbamidomethyl (C), variable modifications: oxidation (M), instrument: Orbitrap, first peptide search tolerance: 20 ppm, main peptide search tolerance: 6 ppm, fragment mass tolerance: 0.02 Da. Peptide spectrum matches were filtered with high confidence FDR of 0.01 and protein FDR was set to 0.05.

Venom protein expression was estimated using iBAQ label-free quantification method, which is implemented in MaxQuant [61]. iBAQ is calculated by dividing the total intensity of a protein by the number of theoretically observable peptides, produced by *in silico* digestion of proteins with trypsin. The iBAQ values of individual proteins were summed to obtain the iBAQ value of the whole protein family. This value is then divided by the sum of all iBAQ values to give an estimate of the expression level of a protein family within the venom proteome. The expression profiles were graphed using the statistical computing environment R [62].

Species and toxin trees

For the purpose of plotting the diet and venom protein expression profiles onto the *Pseudonaja-Oxyuranus* phylogeny, the species tree was constructed using the maximum likelihood method with SMS automatic model selection implemented in PhyML (online version 3.0) [63, 64]. Mitochondrial DNA sequences were available from NCBI GenBank deposited by Skinner et al [42] and Brennan et al [19]. This method produced a tree congruent with the established Bayesian species phylogenies of *Pseudonaja* and *Oxyuranus* [42, 65]. In order to sort identified α-neurotoxins according to their 3FTx subfamily, a maximum likelihood tree was generated using PhyML's SMS model selection and protein sequences from UniProt. Approximate likelihood ratio test (aLRT) was used for branch support [66]. The same method was applied to assign PLA_2 matches to textilotoxin, taipoxin and paradoxin neurotoxin clades. The trees were graphed with FigTree software (Andrew Rambaut lab).

Diet data

The data on the species' diets were obtained from the published studies that identified prey items recovered from the guts of preserved adult specimens and scats of captured live adults

[18-20]. In addition to mammals, *P. ingrami* has also been observed to feed on frogs (N. Dunstan, personal communication).

Animal ethics statement
The freeze-dried snake venoms used in this study were obtained by the Monash Venom Group, the Department of Pharmacology, Monash University, Australia, in accordance with the procedure approved by the Monash University Animal Ethics Committee (MARP/2012/008). The venoms were used for the analysis of their composition by mass spectrometry, which was conducted at Monash Biomedical Proteomics Facility, Monash University, Australia.

Supplementary Information

Additional file 1: MaxQuant proteinGroups files (xlsx)
Additional file 2: MaxQuant peptide files (xlsx)
Additional file 3: MaxQuant evidence files (xlsx)
Additional file 4: Supplementary figure S1. Phospholipase A_2 maximum likelihood tree; Supplementary figure S2. Three-finger toxin maximum likelihood tree (pdf)

Declarations

Ethics approval and consent to participate
The freeze-dried snake venoms used for mass spectrometry analysis were obtained by the Monash Venom Group, the Department of Pharmacology, Monash University, Australia, following the procedure authorised by the Monash University Animal Ethics Committee (MARP/2012/008).

Consent to publish
Not applicable.

Availability of data and materials
The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [1] partner repository with the dataset identifier PXD008887.

Competing interests
The authors declare that they have no competing interests.

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Authors' contributions
JS conceived the study concept, analysed and interpreted the data and wrote the manuscript, DLS designed the LC-MS protocol and performed the LC-MS sample runs, ND conducted

venom sampling, contributed expertise and funds, and WCH contributed expertise and funds and reviewed the manuscript. All authors read and approved the final manuscript.

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