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An investigation of the evolution of the anguimorph lizard venom system

Ivan Koludarov

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Abstract

Over the course of the last two decades significant advances were made in our understanding of the evolution of venom in squamate reptiles. Several studies looked at nuclear genes from various lizards and snakes and confirmed the hypothesis put forth in E. Kochva's article "Phylogeny of the oral glands in reptiles as related to the origin and evolution of snakes" – that anguimorph lizards form part of a monophyletic clade with all the snakes. This confirmation confounded decades of morphology based taxonomy, as did evidence of the inclusion of the Iguania lizard lineage into that group. The new group received the name Toxicofera, emphasizing the role of oral toxins in the evolution of the lineage. Despite being supported by genetic, anatomical and proteomic data, the Toxicofera hypothesis remains controversial. This strengthens the necessity for thorough investigation of toxicoferan reptile venom evolution. The oral secretions of anguimorph lizards demand particular attention not only because they are understudied, but also for the reason that Anguimorpha contains species with the considerable morphological diversity of venom glands – from incipient to advanced and almost snake-like.

Therefore the primary goal of this thesis is to address the lack of knowledge on anguimorph lizard oral secretions, in particular that of varanoid lizards which include various monitor species (genus *Varanus*) as well as *Heloderma* (gila monster) and *Lanthanotus* (Borneo earless monitor). This has been achieved through the implementation of several proteomic techniques (in particular, gel electrophoresis) as well as transcriptomic analysis and bioactivity testings.

Chapter One reviews all previously published information on anguimorph venom evolution, highlighting the data in support of the Toxicofera hypothesis. Chapter Two presents new data on *Heloderma* lizard venoms and the striking similarities between the venom profiles of specimens from different localities as well as between species. The data presented and discussed in Chapter Three represents the core finding of this study: the surprising complexity and diversity of varanid lizard oral secretions.

Taken together, this study present a compelling argument in favour of functional diversity and differential complexity of the venom of anguimorph lizards and discusses the evolutionary forces that helped generate this diversity.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature

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Contributor	Statement of contribution
Author IK (Candidate)	Designed experiments (70%) Conducted experiments (50%) Wrote and edited the paper (40%)
Author TNJ	Conducted experiments (30%) Wrote and edited the paper (30%)
Author KS	Conducted experiments (20%)
Author AN	Assisted with mass spectrometry experiments
Author IH	Collected venoms
Author BGF	Designed experiments (30%) Wrote and edited paper (30%) Formatted paper for submission Collected venoms

Contributions by others to the thesis

Chapter 1: TNJ co-wrote the manuscript (10%); BGF co-wrote the manuscript (10%).

Chapter 3: Bianca op den Brouw assisted with lab work; Nadya Panagides assisted with lab work; TNJ assisted with lab work and co-wrote the manuscript (30%); James Dobson assisted with lab work; Amanda Nowens assisted with mass spectrometry experiments; Peter Josh assisted with mass spectrometry experiments; Mary-Louise Roy Manchadi assisted with rat-ileum organ bath testings; Chip Cochran collected the venoms, BGF collected the venoms, helped designed the experiments and co-wrote the manuscript (20%).

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None.

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Introduction

After being neglected for a long period of time the field of lizard venomics has made significant progress in the 21 century. It is now an established fact that the oral glands of an array of Anguimorpha lizards (amongst others containing monitor lizards and beaded lizards) produce a complex mix of toxins, including toxins of families previously known only from snake venom.

Together with Iguania and Serpentes, Anguimorpha form the monophyletic clade Toxicofera, the common ancestor of which possessed oral glands that acted as the substrate for evolution of all known venom systems in reptiles. Several “core” (putatively ancestral) toxicoferan reptile toxin groups have been revealed, with homologs recovered from oral glands all across the clade.

Anguimorpha is a diverse group of lizards that contains several major lineages, of which helodermatid and varanid lizards are of special interest for modern venomics, as they provide an example, parallel to that of snakes, of the evolution of complex oral secretions and thus allow for evolutionary hypotheses to be formed based on differences and similarities in their venom systems.

Of all the anguimorph lizards, complex oral glands are found only amongst beaded lizards (*Heloderma*), and monitors (*Varanus* and *Lanthanotus*), with other species having relatively simple glands. This complexity evolved independently in the two lineages of venomous lizard. In both cases oral glands are covered by a thick membrane and have a structured lumen.

Chapter 1 discusses the Anguimorpha venom system in detail and reviews all the relevant information published prior to this thesis. The protein classes known or suspected to be present in anguimorpha lizard venoms are discussed with sufficient detail to provide the reader with the background information that subsequent chapters are built upon.

Chapter 2 presents a published proteomic study of *Heloderma* venom. Though helodermatid lizards have previously received significantly more attention than any other anguimorph lizard, including drug development research, our understanding of their venom is far from complete. The role of the venom in their evolutionary history and the extent to which they deploy it in their feeding or defense remains enigmatic. In our study we aimed

to breach some of this gap and conjectured evolutionary forces that may have shaped the extant *Heloderma* venom system.

On the other hand, venom in varanid lizards remains largely unstudied and some researchers remain skeptical as to whether varanid oral secretions possess any significant toxic activity despite the growing amount of evidence suggesting otherwise. Our study of this unique venom system is summarised in Chapter 3 that concludes the thesis.

All extant monitor lizards other than *Lanthanotus borneensis* belong to the *Varanus* genus. Their recent radiation in Australia resulted in great diversity of body size, ranging from 20 cm for dwarf monitor (*V. brevicauda*) to 3 m for Komodo Dragon, making the genus the most variable in body size of all extant vertebrate animals (except snakes). This diversity is even greater once the recently extinct 7m *Varanus* (*Megalania*) *prisca* is taken into consideration (Ast, 2001; Pianka and King, 2004). This diversity of form corresponds to diversity in habitat and diet: terrestrial monitors are large, while rock-dwelling lizards are small, with aquatic and arboreal species being somewhere in-between and morphologically specialised in their own ways.

To date no study has looked into the relationship between habitat, diet and venom composition in varanid lizards. Such a study will immensely contribute to our understanding of evolution of venom in reptiles as well as helping us to build a bigger picture of the evolution of venom in the animal kingdom.

Apart from tracing an evolutionary history, the study of monitor lizard oral secretions can lead to a drug discovery, since some of the toxins are exclusive to this lineage. A similar approach led to the discovery of exendin, found to be a peptide agonist of the glucagon-like peptide (GLP) receptor that promotes insulin secretion. It has been clinically used to treat type 2 diabetes and to enhance plasma insulin secretion.

Implementing an array of proteomic and transcriptomic methods, the present thesis aims to significantly increase the available data on venom complexity and content in Anguimorpha as well as to shed light on the evolutionary forces that shape them.

Chapter 1. Lizards: more than venomous. A review of past studies on Anguimorpha venom system.

Lizards: more than venomous. A review of past studies on Anguimorpha venom system.

Abstract

Venom research in reptiles is mostly focused on medically important front-fanged snakes that significantly contribute to animal-facilitated mortalities in humans. This is one of the reasons why venomous organisms that do not present an immediate threat to human health are severely understudied. However, improving our understanding of the role venom plays in non-medically important species could not only dramatically improve our understanding of venom and toxin evolution but may also lead to advances in drug design. This review puts together several decades worth of research on Anguimorph lizard venom systems in an attempt to demonstrate the sophistication and complexity of lizard venoms. At the same time it aims to show that the extent of current knowledge on the topic is only the tip of the iceberg, and future research is needed to fully uncover the properties and functions of lizard toxins, with all indicators pointing to the possibility of the high medicinal impact such research can foster.

Toxicofera

Until the beginning of the 21st century members of the genus *Heloderma*, most importantly the iconic gila monster, were considered to be the only venomous lizards. However, the recent expansion of scientific knowledge on the evolution of venom in reptiles as well as on the nature of oral glands in helodermatid lizards and their relatives revealed that reality is considerably more complicated.

Recent studies demonstrated that all members of the lizard clade Anguimorpha possess oral glands homologous to the venom glands of the front-fanged snakes (Vidal and Hedges, 2005; 2009; Fry et al., 2006; 2009b; 2010b). In addition, these studies demonstrated that all venomous lizards and all snakes share a common ancestor that may have possessed an incipient venom system, and that any modern day snakes lacking venom do so as the result of secondary loss.

This discovery was built upon two higher-level genetic studies aimed to solve phylogenetic conflicts within Squamata (Vidal and Hedges, 2004; Townsend et al., 2004). These studies suggested that previous morphology-based phylogenetic relationships are incorrect in positionining Iguania as a sister group to the rest of squamates.

Investigations shortly thereafter resolved aspects of the higher order relationships, finding snakes formed a clade with the two lizard lineages Anguimorpha and Iguania, and that the common ancestor of the clade lived some 170 million years ago. Based on the clade's principle synapomoprhy, toxin-secreting oral glands, this ancestor likely had oral secretory apparatus that acted as the basis for the subsequent evolution of venom systems in the Anguimorpha and Serpentes (Fry et al., 2006; Vidal and Hedges, 2005). The name Toxicofera was given in order to reflect the role venom played in the evolution of the clade.

Subsequent studies utilising a growing number of sampled taxa further corroborated the monophyletic origin of the Toxicofera and attempted to clarify relationships within it (e.g., Pyron et al., 2013, Reeder et al., 2015). The study by Reeder et al. compared molecular, morphological and fossil data and suggested that the perceived discordance between squamate phylogenies based on morphological and genetic data is largely due to inability of the former to discern between the multiple origins of limblesness in reptiles. If appropriately corrected, the morphological data is in fact concordant with the results drawn from molecular data. The study also placed Mosasauria and Polyglyphanodontia in Toxicofera as sister groups to Serpents (Reeder et al., 2015). A recent study (Hsiang et al., 2015) produced trees that were skewed by the inclusion of genetics, morphology and fossils (with the last two not always being present for a particular extant lineage). Hsiang et al. based their preferred tree on combined molecular and phenotypic data, but with many relationships constrained to match the phenotype-based tree, which was weakly supported. Since addressing it is beyond the scope of this chapter please refer to Streicher and Wiens for detailed discussion (Streicher and Weins, 2016). Thus recent reviews that relied on the Hsiang et al trees to reconstruct the evolutionary history of the organisms and also the associated venom systems inherit the problems of the original study (Mackessy et al., 2016; Sweet, 2016). Both Mackessy and Sweet also relied heavily upon Hargreaves tissue expression paper (Hargreaves et al., 2014) for their interpretation. For the exact differences between the traditional and Toxicofera phylogenetic approaches we recommend looking into dedicated studies (Streicher and Weins, 2016; Mackessy et al., 2016; Reeder et al., 2015; Jackson et al., 2016; Zheng and Weins, 2016; Vidal and Hedges, 2009; Townsend et al., 2004).

One of the major implications of the Toxicofera grouping in respect to the present study is that the presence of any venomous lizards outside of this clade is unlikely (though obviously not impossible) since the oral secretory apparatus that gave rise to all the known types of venom systems in reptiles is hypothesised to have evolved once within the clade.

Such a consideration does not, however, deplete the scope of possibilities considering that fossil evidence suggests that venom also evolved within the Sphenodontidae family during the Jurassic period (Reynoso, 2005). This putatively venomous squamate belongs to a family of which the only current extant representatives are the non-venomous New Zealand tuataras. However, in the absence of evidence for any other extant venomous lineage, the Toxicofera grouping also implies that any biodiscovery search for venom-derived components with a potential for therapeutic use should be focused on the unstudied members of the clade as they represent an untapped natural resource.

Evolution of the venom system in reptiles

The proposed ancestor of the Toxicofera clade had relatively simple serous dental glands in both upper and lower jaws (Fry et al., 2006), with the protein-secreting region being enlarged and distinct from the smaller mucus-secreting parts. These glands produced active substances that became the substrate for the evolution of all known reptilian toxins. For detailed discussion on types and evolution of reptile venom glands see “Endless forms most beautiful: the evolution of ophidian oral glands, including the venom system, and the use of appropriate terminology for homologous structures” (Jackson et al., 2016).

Iguania split off while this system was still developing and in most iguanian species the glands remain in the plesiomorphic state. The apparent lack of specialisation or refinement of the venom system within the Iguania is likely due to the fact that most species are insectivorous or herbivorous. However, toxin genes continue to be expressed in the venom glands of these lizard. It is also worth mentioning that bearded dragons and frilled dragons possess considerably larger glands than insectivorous and herbivorous species (Fry et al., 2006, 2013).

In contrast with iguanian lizards, venom became of greater importance within both the snakes and anguimorph lizards via differential evolutionary trajectories. Despite sharing a common origin, both groups have developed unique venom systems, presumably in accordance with differences in the evolutionary history and ecological role of venom between the two. In snakes the glands on the upper jaw became extensively diversified, while the lower jaw glands are atrophied or missing entirely in most species (Fry et al., 2006). However, some species of snakes do have active mandibular toxin-secreting glands (Fry, 2015).

On the other hand, the evolution of oral secretory apparatuses in anguimorph lizards resulted in extensive diversification of the glands on the lower jaw, with a correspondent regression of the maxillary glands, which are absent in most of the modern species studied (Fry et al., 2010b). The only anguimorph lizard currently known to retain maxillary glands in addition to mandibular glands is *Pseudopus apodus* (European legless lizard) (Fry et al., 2010b).

It has to be noted that both within snakes and anguimorph lizards there are differences in the location and types of secretory epithelia, as well as in the number and physical orientation of the gland compartments and the degree of encapsulation of the glands (Fry et al., 2006, 2010b), which strengthens the idea that venom system in reptiles exist in a continuum of forms and functions (Jackson et al., 2016).

Unlike the relatively simple venom glands of other anguimorph lizards, *Heloderma* and *Varanus* venom glands have independently evolved into complex organs with segregated protein and mucous secreting regions (Fry et al., 2010a,b). In both cases the glands are encapsulated by a thick membrane and have a reduced number of compartments, which are fused to increase storage space of the highly structured lumen (Russel and Bogert, 1981; Fry et al., 2006, 2010a,b).

The homology of lizard and snake venom glands was further corroborated by the data on expression of shared toxin genes. Several types of proteins were found to be expressed in the venom glands of both snakes and lizards: AVIT, CRiSP, kallikrein, nerve growth factor (Fry et al., 2006), hyaluronidase (Fry et al., 2010b) and kunitz peptides (Koludarov et al., 2012).

The complexity of varanid lizard oral gland transcriptomes is comparable to those of other reptile venom glands, containing a range of potentially active components: natriuretic peptides, type III PLA2, CRiSP and kallikrein (Fry et al., 2010b). Phospholipase A2 purified from varanid lizard venom causes platelet aggregation (Fry et al., 2006) – similar to the activity of PLA2 from *Heloderma* (Huang and Chiang, 1994). Intravenous injections of crude *Varanus varius* mandibular secretion to anaesthetized rats rapidly produced a sharp drop in blood pressure and specific analyses with precontracted rat aortic rings demonstrated relaxation of aortic smooth muscle, consistent with presence of natriuretic peptides in the venom and consistent with the testing of pure natriuretic peptides (Fry et al., 2006; 2009; 2010). Testing of other toxin types revealed a plethora of unique activities (Fry et al. 2010).

These data suggest that an ecological role of toxic oral secretions in lizards is not restricted to members of the *Heloderma* genus and might in fact be one of the evolutionary forces that shaped the Anguimorpha clade in a similar (though not as dramatic) way as in snakes.

Anguimorpha

The Anguimorpha clade has a Laurasian origin and currently includes slightly over 200 extant species (Pianka and King, 2004; Vidal and Hedges, 2009) separated into two lineages each characterised by its geographical distribution. Paleoanguimorpha inhabit the Old World while Neoanguimorpha primarily inhabit the New World. The Neoanguimorpha includes Anguidae, Anniellidae, Diploglossidae, Helodermatidae and Xenosauridae; while Paleoanguimorpha includes Shinisauridae, Lanthanotidae and Varanidae (Vidal and Hedges, 2009).

Heloderma

There are five extant species of helodermatid lizards: *H alvarezi*, *H. charlesbogerti*, *H. exasperatum, H. horridum* (gila monster) *and H. suspectum* (beaded lizard) (Reiserer et al., 2013). All of which are native to the south-western regions of the North American continent. Helodermatid lizards are large (up to 550 mm for gila monster), relatively slow-moving, largely nocturnal lizards. They are highly specialised for the rocky, semiarid habitat with scrub forestry that all of the species occupy. This is reflected in the extremely low-levels of morphological variation between the species. These lizards are also characterised by having a very low metabolic rate and showing a preference for low body temperatures, spending most of the year at temperatures lower than 25 °C. Helodermatid lizards are primarily nest feeders, raiding the nests of birds and mammals, but will readily kill and consume adult birds and rodents if they also happen to be in the nest (Pianka and King, 2004).

Species that belong to the *Heloderma* genus have been recognised as venomous for more than a century, which is reflected in their Hispanic name “el scorpio” (Russel and Bogert, 1981). Early accounts suggested the toxicity of gila monster venom to rattle snakes: “A lizard placed in a cage with a rattler shows no fear, and seems to recognize its superiority, while the snake, from the first, considers the monster a foe, and usually glides to the farthest corner of the cage. The lizard follows menacingly, and slowly moves up to the rattler until in a striking position. Then with a quick swing it imprisons the rattler's body in

its jaws and characteristically hangs on. The snake thrashes about, but seldom attempts to strike its deadly opponent. Three to five minutes are sufficient to quiet the average rattler and bring death, and then only does the victor loosen its grip on the snake's body. If the lizard battles an unusually large rattler, four and a half feet or over death may not result but for days the snake will appear very sick and almost lifeless" (Arrington, 1930).

The teeth of helodermatid lizards are deeply grooved in a manner convergent with those of some advanced snakes, in which grooved, venom-delivering teeth have independently evolved on multiple occasions and display extensive variation (Russel and Bogert, 1981; Fry et al., 2008), This is also convergent with various other extant and extinct venomous lineages including archosauriforms (Mitchell et al., 2010), conodonts (Szaniawski, 2009), sphenodons (Reyonso, 2005), insectivorous mammals such as shrews and solenodons (Cuenca-Bescos and Rofes, 2007; Dufton, 1992; Ligabue-Braun et al., 2012; Rofes and Cuenca-Bescos, 2009) and bird-like dinosaurs hypothesised to specialise in preying upon early birds (Gong et al., 2010).

Helodermatid lizard venom glands are very large (Russel and Bogert, 1981). A recent MRI study showed that paired glands have six compartments with each compartment having its own duct that terminates at the base of the grooved teeth (Fry et al., 2010a). The glands are located in the anterior half of the lower jaw just beneath the skin, separated from the latter by the connective tissue. The glands can be noted by the swelling underlying the lower jaw. Each duct terminates on the outer side of certain teeth of the lower jaw and the venom is carried into the groove solely by capillary action. It can be further mixed with the saliva and thus be carried into the grooves of the upper teeth as well (Russel and Bogert, 1981).

Most of human envenomation by *Heloderma* involve lizards biting and holding with their strong jaws, sometimes for hours. Envenomation can result in complex symptoms such as extreme nausea, fever, myocardial infarction, tachycardia, hypotension and inhibition of blood coagulation as well as pain, acute local swelling and faintness (Preston, 1989; Strimple et al., 1997; Cantrell, 2003). No antivenom is required, and standard treatment is effective though severe pain may last for up to 12 hours (Hooker et al., 1994) and lack of response by the victim to non-steroidal anti-inflammatory analgesics might complicate the situation (Ariano-Sánchez 2008).

Studies in mice found that subcutaneous injection of minimal lethal dose or similar amounts results in massive haemorrhages in the lungs, on the liver and intestinal organs,

on the epicardium, and especially in the various layers of the eyeball wall. Death occurred within 4-5 hours via pronounced asphyxia. In animals that were injected with higher doses, death occurred within 30-60 minutes without any observable haemorrhagic effect (Styblova and Karnalik, 1967).

Extensive studies of helodermatid venom have revealed a great diversity of components. Kallikrein-like activity of the venom was first observed by Mebs (1968, 1969a, 1969b) and subsequently attributed to the kallikrein-like toxins helodermatine (Alagon et al., 1986) and gilatoxin (Utaisincharoen et al., 1993) that also possess fibrinolytic and haemorrhagic activity (Utaisincharoen et al., 1993; Datta and Tu, 1997; Nikai et al., 1988). Other potent components recovered include phospholipases A2 type III (Dehaye et al., 1984), helothermine (Mochca-Morales et al., 1990), helokinestatins (Kwok et al., 2008), helofensis with neurotoxic activity (Komori and Nikai, 1988) and exendin peptides (Parker et al., 1984). Of these, exendin-4, isolated from *Heloderma suspectum* venom was found to be a peptide agonist of the glucagon-like peptide (GLP) receptor that promotes insulin secretion. It has been clinically used to treat type 2 diabetes and to enhance plasma insulin secretion (Drucker and Nauck, 2006).

Varanus

All extant varanid lizards are members of the genus *Varanus*. These lizards are unique in their body size diversity, with a range from 23 cm (*V. brevicauda*) to over 3 m (*V. komodoensis*). The extinct species *V. priscus* (Megalania) is estimated to have reached between 6 and 9 m in total length, making the *Varanus* genus the most variable in body size of all extant vertebrate genera, with the sole exclusion of snakes (Sweet and Pianka, 2007). Members of the genus have successfully colonised arboreal, terrestrial, rocky and aquatic habitats. Habitat preference is tightly linked with body size; body shape scales allometrically and otherwise is a subject of little to no change between the species (Openshaw and Keogh 2014, Clemente et al., 2013). Rock-dwelling varanids are usually small, whilst terrestrial and arboreal monitors are large and intermediate respectively (Collar et al., 2011).

Of the more than 60 species worldwide, 5 occur in Africa and 7 are distributed across mainland southern Asia. 30 species, grouped into two distinct clades, occur on islands in Southeast Asia (four of those species are also present on the mainland). Species richness reaches its peak in Australia, with 29 named species (5 shared with New Guinea) belonging to the single Indo-Australian clade (Pianka and King, 2004; Ast, 2001). The

smaller Australian species like the members of the *Odatria* clade represent unique Australian derivations, as do the giants including the extinct *V. prisca* and the extant *V. komodoensis.*

Most of the non-Australian varanids are large bulky lizards reaching more than 1.3 m in total length as adults. While the species diversity of large lizards does not change across Wallace's line, the amount of small varanid lizard species increases sharply to the east of the line. This has been linked with the fact that Wallace's line is a natural boundary for all small placental carnivores, such as cats, mustelids and viverrids. Apparently only large varanid species can coexist with placental carnivorous species, as they have big clutches and rapidly grow out of the vulnerable size range. Small varanid lizards always remain within the prey size range for carnivorous placental mammals that implement ambush hunting, unlike the Australian dasyurid marsupial carnivores that hunt by foraging (Sweet and Pianka, 2007). Consequently, of all the varanids lizards those in Australia have the most extreme variation in size and niche occupation.

While all varanid lizards will predate upon literally anything they can overpower, each species has a unique range of prey items, with some being primarily insectivorous (*V. gilleni*), and some feeding on larger animals such as birds and mammals (*V. giganteus*) (Pianka and King, 2004).

Similar to helodermatid lizards, monitors have large oral glands, homologous to those of front-fanged snakes. They also are divided into six compartments, even though the compartment structures are distinct from those of the helodermatids, and in varanids ducts terminate between successive bladelike teeth (Fry et al., 2010b). The helodermatid and varanid lineages thus represent independent derivations from the anguid lizard state, in which the protein and mucus secreting regions are divided and the protein secreting gland encapsulated to form a tube. The plesiomorphic anguimorph lizard state as seen in anguid lizards is characterised by the protein and mucus secreting cells being heterogenous, and presence of dorsal/ventral divisions within the gland lobules, with each lobule terminating between successive teeth along the jaw-line.

The previous lack of sufficient knowledge of the role of venom in the Anguimorpha clade contributed to some misinterpretations of the feeding ecology of varanid lizards, in particular those of the iconic *Varanus komodoensis* (Komodo Dragon or ora). This species evolved in Australia around 4 million years ago but now can be found only on several Indonesian islands, mainly Komodo, Rinca, Flores, Gili Motang, and Gili Dasami (Hocknull

et al., 2009). The islands' megafauna became extinct around 12,000 years ago, and for 5,000 years *V. komodoensis* fed only on relatively small prey until the introduction, by humans, of pigs from Sulawesi. In recent history deer and water buffalo were also introduced on the islands, and now oras occupy a novel ecosystem, with their potential prey consisting of feral pigs, deer and buffalo. This situation has led to a unique ecological position for the Komodo Dragon, enabling it to function as an entire vertebrate predator guild, by going through rapid ontogenetic niche shifts from preying on relatively small arthropods as a juvenile to big ungulate mammals as an adult (Purwandana et al., 2016).

V. komodoensis have light-weight skulls whose biting force is relatively weak compared to the mass of the animal (D'Amore et al., 2011; Moreno et al., 2008; Fry et al 2009). Instead of bite force, *V. komodoensis* utilise their large, serrated teeth as the primary weapon, inflicting deep parallel wounds in a 'bite and slice' form of a prey capture. Mechanical damage dealt to a prey may alone result in rapid death from blood loss, especially if major arteries are affected.

While previously the long-lasting effects of ora's bites were largely attributed to pathogenic bacteria this has since been suggested to be misleading as no pathogenic oral flora was recovered from *V. komodoensis* saliva or gingiva that would set it apart from any other carnivorous animal (Goldstein et al., 2013). While the effect might be attributed to the pathonogenic bacteria getting into the wound after the bite itself, that only seems plausible in case of attacked water buffalo that seek shelter in stagnant pools of water. Other prey animals do not display similar behaviour and their bite wounds do not exhibit the effects of pronounced sepsis like those of water buffalo. The overall clinical picture, including prolonged blood loss, suggests toxin action (Auffenberg, 1981), which is concordant with recent studies revealing that *V. komodoensis* venom contains anti-coagulant toxins that increase blood loss and other toxins that induce hypotension and shock (Fry et al., 2006, 2009, 2010b).

Though many bites by monitor lizards have been reported by the lay press, including a lethal attack of Komodo dragon on a 5-year old boy (Fry et al., 2010a), only a few cases of *Varanus* bites were published in peer-reviewed journals. A case of lethal bite attributed to *V. bengalensis* reports that a 55-year old female victim experienced severe local pain, blood loss, as well as nausea, diaphoresis, dizziness, and breathlessness (Vikrant and Verma, 2014). Though bleeding time was considered normal, the whole blood clotting time was prolonged. Patient was treated with injections of antihistaminic, hydrocortisone and

tetanus toxoid. 72h after the bite, patient succumbed to a sudden cardiac arrest. Post mortem kidney biopsy revealed that morphological features are consistent with acute tubular injury associated with pigment nephropathy.

From the clinical picture and morphological features seen on kidney biopsy, authors concluded that this varanid lizard venom is capable of producing intravascular haemolysis, coagulopathy, and rhabdomyolysis. These pathogenic effects were responsible for causing the acute kidney injury. It was suggested that the direct nephrotoxicity of the lizard venom might have played a direct role, as it is known for other biological nephrotoxins (Strimple et al., 1997; Cantrell, 2003; Ariano-Sanchez, 2008; Piacentine et al., 1986; Preston 1989). The pathogenic mechanisms observed in the case were found very similar to those seen with snakebite-related acute kidney injury (Sitprija, 2006) and other authors have doubted the attribution to *V. bengalensis*, instead suggesting that the victim was bitten by a Russel's viper (White and Weinstein, 2015). According to White and Weinstein, the vernacular name for *V. bengalensis* is ‘‘goh’’ (a contraction of ‘‘gohera’’ or ‘‘ghorpad’’) and is quite similar to ‘‘ghonas’’ and ‘‘gunas’’, local names for *D. russelii*. It has to be noted, that though initial effects such as prolonged blood loss, nausea and pain are consistent with the action of toxins recovered from varanid venom (Fry et al 2009), it remains to be elucidated whether varanid venom can cause acute kidney injury.

In contrast, Ducey et al. report a much less dangerous bite by a juvenile Komodo dragon that resulted in a faintness, prolonged bleeding and transient hypotension (Ducey et al., 2016). At 8 months postinjury, the victim still had tingling and numbness in the distal aspect of her right fifth finger where the tooth had been retained after the bite. Though authors admitted that there might be multiple reasons for the main hypotensive effect of the bite, they concluded that it was likely due to a vasovagal reaction – an autonomic nervous system response unspecific to the trigger – despite the effects being consistent with laboratory studies of Komodo dragon venom (Fry et al 2009).

To date a number of toxin types have been recovered from varanid lizard oral secretions, most of them sharing type similarity with those recovered from helodermatid lizard venoms, reinforcing their shared evolutionary origin. Different forms of kallikrein toxins, CRiSP, natriuretic peptide, AVIT and phospholipases A2 reveal the ongoing evolution of varanid lizard venom systems, though few components have been functionally characterized (Fry et al., 2010b, 2013; Fry, 2015).

Other anguimorphs

Most anguid lizards possess oral glands homologous to those of *Heloderma* and *Varanus*, though usually their glands are much less developed. Anguid lizard glands are usually of the mixed type, with a serous portion occupying the bottom of the gland and a mucous part above it, leading to the duct, which opens at the base of a tooth (Fry et al., 2010b). Of all the anguimorpha species studied to date, only *Pseudopus apodus* has retained the ancestral condition of having both maxillary and mandibular glands which have unstructured central lumens. In contrast, *Lanthanotus borneensis* – the closest extant relative of Varanidae – possesses serous protein-secreting glands with a well-structured lumen, encapsulated in a thick membrane in a similar manner to *Varanus* glands and thus also convergent with helodermatid lizard glands (Fry et al., 2010b).

Though most of the anguimorph lizards are medically unimportant, they still secrete a number of plesiotypic toxicoferan toxin types. CRiSP and kallikrein are present in the venom gland transcriptomes of *Pseudopus apodus*, *Gherronotus infernalis* and *Celestus warreni*. The latter two species also share similar forms of helokinestatin and natriuretic peptides. Apart from that the three species have different toxin profiles: *P. apodus* expresses lectin, *G. infernalis* hyaluronidase, and *C. warreni* PLA2. *C. warreni* expresses a potential novel toxin type of its own – celestoxin, entirely unrelated to other Toxicofera or Anguimorpha toxins (Fry et al., 2010b).

In our recent study of the venom gland trnascriptome of the small arboreal lizard *Abronia graminea* (Koludarov et al., 2012), we showed that members of Anguidae sometimes have robust and serous mandibular glands – traits usually considered reflective of a gland in active use. A wide array of recovered transcripts were shown to be homologous to those of helodermatid lizards, some of which are evidently under rapid diversification – in particular helokinestatin peptides. Interestingly, natriuretic peptides that are encoded in tandem with helokinestatins were shown to be largely under the influence of negative selection with 27% of the sequence being under constraint. We were also able to sequence kunitz peptides for the first time from a lizard toxin-secreting gland, suggesting a potential ancestral role of this toxin in the Toxicofera clade.

Toxins

Venom proteins are likely the result of ‘toxin recruitment events’ (Fry, 2004), whereby genes encoding normal body proteins are duplicated and the new copy preferentially

expressed in the venom glands (Fry, 2005). Toxins are often exquisitely targeted and typically characterised by specific scaffold highly cross-linked with disulphide bonds that allow for greater proteolytic resistance than that of typical body proteins.

The ancestral toxicoferan reptile is hypothesised to possess a core set of toxin genes, with additional genes added to the venom gland arsenal at different points in the evolutionary history of the clade (Fry et al., 2006, 2009, 2010a,b, 2013; Koludarov et al., 2012). Such toxins as AVIT, kallikreins, CRiSPs, NGF and hyaluronidase are present in different lineages of Toxicofera and their sequences have high similarity, with phylogenetic analyses recovering monophyletic origin of toxin forms (Fry et al., 2005 and 2010b; Koludarov et al., 2012).

Additional toxins identified as present exclusively in lizard venoms are: helofensin (lethal toxin isoforms), exendin, B-type natriuretic peptides, and type III phospholipase A_2 (Fry et al., 2006; 2009; 2010b). In addition, helokinestatin peptides are unique proline-rich *de novo* derivations within the precursor region of the natriuretic peptides. Post-translational cleavage liberates the helokinestatin peptides to act as independent toxins. Currently they are thought as unique to *Heloderma* and anguid lizards, for varanid natriuretic peptides studied to date lack these derivatives (Fry et al., 2010a,b; Koludarov et al., 2012). The full list of toxins currently known from anguimorph lizards is presented in Table 1.

Table 1 Toxins found in anguimorph lizards oral secretion

Protein type/toxin class	**Toxin action**
Exendin	Induces hypotension via relaxation of cardiac smooth muscle.
Kallikrein	Increase of vascular permeability and production of hypotension in addition to stimulation of inflammation
CRiSP (cysteine rich secretory protein)	Paralysis of peripheral smooth muscle and induction of hypothermia through blockage of various channels including ryanodine and L-type calcium channels
B-type Natriuretic peptide/helokinestatin	Natriuretic peptides produce hypotension through the relaxation of aortic smooth muscle. The helokinestatin peptides are antagonists of

precursor	bradykinin at the B2 bradykinin receptor.
Phospholipase A_2 type III	Block of platelet aggregation
Nucleosides	Potentially hypotension, paralysis, necrosis
Vespryn	Induces hypolocomotion and hyperalgesia.
Hyaluronidase	Enhancement of tissue permeability
Kunitz peptide	Protease inhibtion
Goannatyrotoxin	Induces hypertension followed by hypotension
Helofensin	Lethal toxin which possesses an inhibitory effect on direct electrical stimulation of the isolated hemi-diaphragm.
Celestoxin	Induces hypotension
C-type lectin	Platelet aggregation mediated by galactose binding.
AVIT	Toxic activity currently uncharacterised.
Cholecystoxin	Toxic activity currently uncharacterised.
Veficolin	Toxic activity currently uncharacterised.
NGF	Toxic activity currently uncharacterised.
VEGF	Toxic activity currently uncharacterised.
Hepatocyte growth factor	Toxic activity currently uncharacterised.

Traditionally, reptile venom research has been focused on clinically important species. As a result, our understanding of the evolution of venom systems in most toxicoferan reptiles is limited. Previous studies of venomous but harmless to human lizard species showed that a wide range of venom components were present in the venom-gland transcriptomes

of such lizards (Fry et al., 2006, 2010b; Koludarov et al., 2012). Consistent with use in prey capture, some components showed an evidence of rapid evolution. Helokinestatin peptides in particular accumulated residues that underwent episodic diversifying selection. In contrast, natriuretic peptides have evolved under evolutionary constraints despite being encoded in tandem with helokinestatins by the same gene precursor.

Exendins

Exendins are secretin hormone-like peptides that were isolated from the venom of helodermatid lizards. Exendins-1 and -2 are vasoactive intestinal peptide (VIP)-like, both in sequence and function, while exendins-3 and -4 are glucagon-like peptide-1 (GLP-1)-like (Irwin 2012). Previously names helospectin and helodermin were used for exendin-1 and exendin-2 respectively.

Occurrence of different exendin forms seems to differ between *Heloderma* species: exendin-2 and exendin-4 is apparently restricted to *H. suspectum* venom (Vandermeers et al., 1987; Eng et al., 1990). There is also a concentration difference between the forms: in *H. horridum* venom exendin-3 is 5 times more abundant in molar concentration than exendin-1 (Eng et al., 1990). Helodermatid adenylate cyclase activating peptide (PACAP), VIP and GLP show high sequence similarity to exendin peptides (Vandermeers et al., 1987).

There is evidence that exendin-4 is present in *Heloderma* plasma and therefore has a potential role as a metabolic hormone. However, it has been shown that its levels increase only when the feeding strategy involves bitting (Christel and Denardo 2006). Though the exact role exendin plays in plasma is yet to be uncovered, it is highly plausible that its occurrence in the blood is a byproduct of its primary function in subjugation of a prey. Given that it is one of the dominant components of the venom, it is not unlikely that it enters the stomach along with the meal and then is filtered into the blood due to its relatively small size. Of course it cannot be ruled out that it has a metabolic function, given its known ability to increase the circulation of insulin.

Apart from occurring in helodermatid lizards' venom, exendin-like peptides (in particular similar to exendin-1) were identified in high concentration in the thyroid C cells and in the noradrenaline containing cells of the adrenal medulla in many mammals (Sundler et al., 1988; Grunditz et al., 1989; Bjartell et al., 1989; Grundemar and Högestätt, 1990; Uddman et al. 1999).

Structure

Exendin-1 occurs as two isoforms in the venom of *Heloderma suspectum*. One isoform (dubbed helospectin-I) is a 38-residue peptide and another (helospectin II) is a 37-residue peptide identical to first one except that it lacks serine in 38 position.

Exendin-2 is a 35-residue peptide. Both forms of exendin-1 as well as exendin-2 have high similarity with VIP with 15 of the first 28 residues being identical (Hoshino et al., 1984; Parker et al., 1984; Grundemar and Högestätt, 1990) and significant similarity with human pituitary adenylate cyclase activating peptide (PACAP) with exendin-2 being 42% identical to it (Vandermeers et al., 1987). The precursor transcript of an exendin-2 isoform was shown to have high sequence similarity to the precursors of exendins-3 and -4. The signal peptide differed only in 3 residues from that of exendin-3 (Fry et al., 2012)

Exendin-3 is a 39-residue peptide with an amide at the C-end. It has greater similarity with glucagon and human glugaonlike peptide-1 (48% and 50% respectively) than with exendin-1 and -2 (32% and 26% respectively) (Eng et al., 1990).

Exendin-4 is a 39-residue peptide. Its cDNA is similar to exendin-2 both in size and structural organisation (in particular, the respective positions of the peptide and N-terminal extension peptide), suggestive of their evolution from a common ancestral gene through gene duplication event (Vandermeers et al., 1987; Eng et al., 1992). It was shown to have a high stability to degradation mediated by DPPIV and ectopeptidases (Montrose-Rafizadeh et al., 1997). NMR-study revealed a well-converged helix over residues 18-27 in both GLP-1 and exendin-4 in the conditions mimicking that just prior to binding to the receptor. Exendin-4 was shown to display a greater intrinsic propensity toward the formation of a monomeric helix than GLP-1, which can be attributed to the absence in exendin-4 of the helix-interrupting glycine residue at position 16 of GLP-1 (Neidigh et al., 2001). This allows for the helix to go much further toward the N-terminus in exendin-4 that, along with other structural differences between GLP-1 and exendin-4, may reduce the entropic cost of binding and be responsible for the higher potency of exendin-4 in some organisms.

Function

Crude venom of *Heloderma suspectum* was shown to be a potent pancreatic secretagogue (Eng et al., 1990). Exendin-1 and -2 were found to have immunological and

biological properties similar to mammalian VIP while exendins-3 and -4, in contrast, have biological functions most similar to mammalian GLP-1 (Irwin, 2012).

Both exendin-1 and -2 mimic VIP action in reducing blood pressure in the rat, however exendin-2 was shown to be less effective than VIP in the low dose-range, as well as VIP having longer duration that either of the peptides. In accordance with the vasodepressor effect, both exendin-1 and -2 were shown to relax precontracted femoral arteries, with exendin-1 being less potent than VIP or exendin-2. The maximum relaxation induced by the either of the three was of similar magnitude and further addition of any of the other peptides did not produce a further relaxation (Grundemar and Högestätt, 1990).

Exendin-2 is at least partially responsible for the tachycardia seen in human envenomations (Naruse et al., 1986; Konturek et al., 1989).

Similar to VIP, intraarterial infusion of exendin-2 causes a dose dependent increase in femoral blood flow and intravenous injection produces systemic hypotension and tachycardia in the dog models, though exendin-2 produced more long-lasting increase in canine femoral blood flow than VIP (Naruse et al., 1986; Konturek et al., 1989).

Exendin-4 inhibits glucagon secretion (Silvestre et al., 2003), stimulates insulin synthesis (Alarcon et al., 2006) and secretion (Silvestre et al., 2003; Parkes et al., 2001), protects against b-cell apoptosis (Kawasaki et al., 2010; Wang et al., 2010) and promotes b-cell proliferation (Kwon et al., 2009). On an organismal level it was shown to promote satiety, reduce food intake, fat deposition and body weight both in normal animals and experimental models (Scott and Moran, 2007; Williams et al., 2009; Primeaux et al., 2010; Washington et al., 2010).

Exendin-4 was shown to stimulate secretion of insulin in the presence of elevated blood glucose concentrations, but not during hypoglycemic periods, which led to its pharmacological use in the treatment of type 2 diabetes (Neidigh et al., 2001). Treatment with exendin-4 increases cell proliferation and neuroblast differentiation in the dentate gyrus, potentially through promoting structural plasticity (Li et al., 2010).

The very first studied effects of exendin-1 and -2 was their ability to activate adenylate cyclase in plasma membranes of the rat pancreas (Vandermeers et al., 1984), but it is only exendin-2 that also induces amylase secretion (Konturek et al., 1989). Exendin-3 was shown to be similar in action to exendin-2, inducing the increase of cellular adenylate cyclase and releasing amylase in guinea pig pancreatic acini (Vandermeers et al., 1987).

Though exendin-3 amylase releasing activity was less potent that that of exenind-1, exendin-2 or VIP (Raufman et al., 1991).

On the guinea pig pancreatic acini models exenndin-3 was shown to interact with VIP receptors at high concentration and with specific exendin receptor in lower concentrations. The former action is responsible for its amylase releasing activity through increase of cellular cAMP, while the latter activity results only in increase of acinar cAMP without stimulating amylase release (Raufman et al., 1991; Uddman et al. 1999).

Exendin-4 was likewise shown to stimulate adenylate cyclase production with a potency and efficacy equal to or greater than that of GLP-1 and binding with a similar (in humans) and higher (w7.5 fold in rats) affinity to the mammalian GLP-1 receptor (Eng et al., 1990; Edwards et al., 2001; Egan et al., 2002; Mann et al., 2010).

The adenylate cyclase activating effect is attributed to N-terminal regions as was shown for exendins-3 and -4, while middle and C-terminal regions influence binding affinity to the receptor (Eng 1992). N-terminal regions of exendin-3 and -4 are different by two amino acids, which were shown to be responsible for the lack of exendin-4 ability to interact with VIP receptors. This might be the reason why exendin-3 causes the biphasic increase in adenylate cyclase, while exendin-4 action is monophasic (Eng et al., 1992).

The function of the N-terminal region is more than that though as an exendin-4 version truncated beyond the second N-terminal amino acid acts as a potent antagonist, rather than agonist, of the GLP-1 receptor (Eng 1990; Montrose-Rafizadeh et al., 1997).

C-terminal sequence of 9 amino acids in exendin-4, absent in GLP-1 structure, is responsible for its high affinity to GLP-1 receptor in rats, its removal was shown to reduce the potency of binding and addition of the sequence to GLP-1 resulted in increase of potency (Doyle et al., 2003). However this sequence does not contribute to the binding of exendin-4 to human GLP-1 receptor (Runge et al., 2007), which might explain the aforementioned differential affinity to the receptor between exendin-1 and GLP-1 in rat in human models.

Evolution

The existence of exendin-like peptides in mammals, especially in the adrenal medulla (Bjartell et al., 1989; Grundemar and Högestätt, 1990), suggests a potential plesiotypic role of exendins in cardiovascular regulation.

Despite the earlier speculation of exendin-2 and -4 being evolutionary precursors to the mammalian members of glucagon-like peptide family (PACAP, VIP and GLP-1) based on similarity in activity between those groups, it was revealed that they are not evolutionary closely related (Vandermeers et al., 1987; Pohl and Wank, 1998). The finding is also corroborated by the fact that exendins' expression in *Heloderma* seems to be limited to venom glands only (Vandermeers et al., 1987).

Differences in biological activity within exendins, in particular similarity between VIP and exendins-1 and -2 and between GLP-1 and exendins-3 and -4 led to uncertainty weather exendins are derived versions of VIP or GLP-1 or even a result of convergent evolution.

Genetic studies ruled out the last possibility and showed that exendins are in fact closely related to each other and the signal and pro-peptide sequences have greater similarity between themselves then to the corresponding regions of any other secretin-like hormone precursor (Irwin, 2012). Since the glucagon and VIP genes are situated on different chromosomes in various vertebrates, it is very likely that the genomic separation between the two occurred in the early evolution of the common vertebrate ancestor and therefore the interchromosomal shuffling of exons was the unlikely reason for the differences between the exendins.

Two most likely scenarios of exendins' evolution were proposed with one involving duplication of a glucagon gene followed by an additional duplication event and convergent evolution of exendins-1 and -2 sequences to the VIP sequence, and another involving duplication of the VIP gene with a subsequent duplication event and convergence of the exendin-3 and -4 sequences with that of GLP-1.

A glucagon-like gene identified in anole lizard suggested that a first scenario is more likely than the second. Not only the gene sequence was most similar to proglucadon-derived sequences of other vertebrates as well as exendin sequences from *Heloderma*, it also had intron that was homologous to those of the proglucagon and GLP genes. The altogether position of introns and exons, including flanking splice donor and acceptor sequences was found similar to that of other proglucagon genes (Irwin, 2012).

Comparison of the exendin sequence of helodermatid lizard with that of anole lizard, zebra finch, turkey and *Xenopus* suggested that exendin genes exist in a conserved neighborhood, strongly indicating that they are orthologous. Phylogenetic trees built with just the exendin precursor sequences showed that there is an extremely long ancestral

lineage leading to the *Heloderma* exendins. Phylogenies generated with just the N-terminal signal peptides produced identical topologies for all the different exendins used, however the length (amount of sequence change) leading to *Heloderma* was much shorter. Exendin sequences showed the length (amount of sequence change) of the ancestral lineage leading to *Heloderma* exendins becoming exaggerated, and disturbed the monophyletic groupping of reptilian and avian sequences. All of that indicated a more rapid evolution of exendin sequence in *Heloderma* lineage as compared to other vertebrate lineages. At the same time the rate of evolution of pro-peptide and signal-peptide regions stayed the same across all studied lineages (Fry et al., 2012; Irwin, 2012).

Reconstruction of the ancestral sequence of the *Heloderma* exendins further corroborated proglucagon-like origin scenario. When compared to the extant exendins, it was found to be most similar to GLP-like exendins-3 and -4 with 5 and 7 substitutions respectively, while showing 15 and 17 replacements with exendins-1 and -2 (Irwin, 2012).

These findings suggest that *Heloderma* exendins diversified by gene duplication after *Heloderma* lineage diverged from other reptiles. As the peptides were adapting to a new role as toxins, their sequence rapidly evolved – a phenomenon well associated with the recruitment of body protein for the use as a toxin.

It was shown that VIP hormones are more potent vasodilators than endogenous glucagon hormones (Ezawa et al. 2006) making it likely for the selection to favor the evolution of a toxin that mimics VIP action then for the VIP-like peptide to evolve into a less potent glucagon-like form. These suggests that while exendin-3 and -4 retained the ancestral structure of proglucagon-derived family, exendin-1 and -2 convergently evolved the structure similar to that of VIP, resulting in similar bioactive properties and a long-lasting confusion of their origin (Fry et al 2010b).

Medicinal use of exendin-4

The discovery of GLP-1 like action of exendin-4 rapidly led to numerous studies on its potential anti-diabetic effects. They show that continuous administration of exendin-4 in diabetic mice, diabetic and obese-diabetic rats, diabetic rhesus monkeys result in increases in concentration of circulating insulin accompanied by reductions in blood glucose and glycosylated haemoglobin (Greig et al., 1999; Young et al., 1999; Gedulin et al., 2005). Exendin-4 was shown to stimulate b-cell neogenesis in some type 2 diabetes

animal models, while stimulating the replication and differentiation of islet cells in others (Xu et al., 1999; Tourrel et al., 2002).

In humans, exendin-4 reduced fasting and postprandial blood glucose in healthy volunteers and improved b-cell sensitivity to glucose and reduced blood glucose in type 2 diabetes patients (Edwards et al., 2001; Egan et al., 2003; Kolterman et al., 2003; Buse et al., 2004, 2009; DeFronzo et al., 2005; Moretto et al., 2008; Preumont et al., 2010; Bunck et al., 2010).

Despite its ability to cause antibody formation similar to any other subcutaneously injected peptide, no safety and efficacy concerns were shown in exendine-naive patients. Study on anti-exendine antibody formation and the incidence of immune-related and hypersensitivity reactions after exendine re-exposure found no increase in treatment-emergent adverse events (Faludi et al., 2009).

Synthetic exendin-4 was released as a type 2 diabetes treatment under the market name “Byetta” (Furman, 2012).

Kallikrein

Kallikrein-scaffold forms of S1 peptidase family are broadly represented in reptile venoms with 250 toxin forms, a majority of which were recovered from viperid snakes. Toxicoferan venom kallikreins (TV-kallikreins) mostly affect blood pressure and coagulation through their proteolytic activity, most importantly liberating kinins from plasma kininogen or depleting plasma of fibrinogen by cleaving it to facilitate blood loss. Only half of toxicoferan venom kallikreins have been functionally characterized. A number of kallikreins were recovered from Anguimorpha lizards with two major examples being helodermatine and gilatoxin, both isolated from helodermatid lizards’ venom. TV-kallikreins have a potential for medicinal use due to their ability to alter hemostasis with one instance of snake kallikrein developed into an anticoagulant under the commercial name “Arvin” (Fry et al., 2009a; Fry, 2015)

Structure

Toxicoferan venom kallikreins have evolved from glandular/tissue kallikreins and are similar to them in structure and most of their functions. They are single-chain proteins with a sequence length of usually about 230 amino acids, preceded by 6 residues of activation peptide and 18 residues of signal peptide. Proteolytic domain contains Ser/Gly189 or

Asp189 (α-chymotrypsin numbering) and is located between two antiparallel six-stranded β-barrels. All toxicoferan venom kallikreins have 5 or 6 cystines stabilizing their structure. Viperid and some elapid forms of toxin kallikreins possess C-terminal 7 amino acid extension with an additional cysteine residue, while anguimorph lizards' kallikreins lack the extension and instead have a cysteine-carrying insertion of 6-9 amino acids in-between positions 93-95. Other structural variations occur mainly in surface loops. TV-kallikreins are usually heavily glycosylated and that can significantly increase their predicted Mw of 26-28 kDa. The isoelectric point is usually between 5 and 7 or 8 and 9, but can be as low as 4 (Fry, 2015).

Gilatoxin isolated from the venoms of Helodermatid lizards is a monomeric acidic kallikrein of Mw in the range of 30-37 kDa and an isoelectric point of 3.9-4.25 (Hendon and Tu 1981; Nikai et al., 1988). The complete sequence includes 246 residues. Gilatoxin is highly glycosylated, containing approximately 8 mole of monosaccharide per mole of toxin, but appears to lack O-glycosylation sites. Sequence alignments of gilatoxin with batroxobin, crotalase, thrombin and trypsin revealed its considerable similarity to those enzymes, particularly in the catalytic site. The highest sequence identity (40%) was with batroxobin (Utaisincharoen et al., 1993).

Previously, gilatoxin was reported to have an additional 7 residues extension (including Asp-96, in the basic specificity pocket of thrombin), which was thought to contribute to the unusual substrate specificity of the toxin (Utaisincharoen et al., 1993). Recent studies demonstrated that sequence used in 1993 analysis is not authentic and all other gilatoxins lack this extension (Fry et al., 2010b). Gilatoxin from *Heloderma horridum* venom was shown to have Mw of 31 kDa and isloelectric point of 3.9. It was found to be similar to porcine pancreatic kallikrein with homologous sequences in N-termial region (Nikai et al., 1988).

Similar to other TV-kallikreins, varanid forms show a conservation of functional residues and cysteine spacing. However two forms from *V. acanthurus* and *V. scalaris* were shown to evolve new instance of cysteine absent in the ancestral condition. At the same time kallikrein toxins recovered from *Celestus warreni* and *Gherronotus infernalis* show not only a newly derived cysteine not present in other sequences but also the loss of the last ancestral cysteine (Fry et al., 2010b).

Function

Toxicoferan venom kallikreins perform a range of functions in the bite and largely contribute to the profound, rapidly developing swelling characteristic of viperid snakes and anguimoprh lizards' bites. Similar to tissue kallikreins they evolved from, TV-kallikreins' main activity lies in processing of low-Mw kininogens into kallidin (Lys-bradykinin). This leads to inflammation, smooth muscle contraction, lowered blood pressure and increased vascular permeability. Other major activities of TV-kallikreins include angiotensinolytic activity, resulting in hypotension and fibrinolytic activity, resulting in unstable clots, depletion of fibrinogen and clotting inhibition (Fry et al., 2015).

Kinin-generating activity of the *Heloderma suspectum* venom was discovered by Mebs (Mebs, 1969a) and was later attributed to helodermatine (Alagon et al., 1986). Helodermatine was shown to cleave synthetic peptide substrates with similar kinetics to serum kallikrein, realeasing 75% of the amount of kinin from human high-Mw-kininogens similar to human plasma kallikrein. This might be indicative of another function of helodermatine in venom, potentially converting toxins precursors into active forms (Alagon et al., 1986). Since several tissue kallikreins are known to process insulin and glucagon, it is not unlikely that helodermatine or gilatoxin play a role in processing of exendins in *Heloderma* venom.

Helodermatine was found to produce hypotensive effect in rabbits and is responsible for hypefibrinolytic state at the site of the *Heloderma* bite (Alagon et al., 1986). Similarly, gilatoxin lowered blood pressure in rats and induced contraction of isolated rat uterus smooth muscle.

In accordance with plesiotypic functions for TV-kallikreins, gilatoxin was shown to release bradykinin from kininogen. It catalyzed the hydrolysis of various substrates for trypsin and thrombin and degraded both angiotensin I and II by cleaving the dipeptide Asp-Arg from the N-terminus. It was shown to have higher substrate specificity than trypsin and being very similar to plasma kallikrein. Gilatoxin degrades fibrinogen without clot formation (Utaisincharoen et al., 1993), which should lead to further clotting inhibition.

Toxicity of anguimorph kallikreins is thought to be moderate. For instance, gilatoxin's toxicity toward Swiss-Webster mice is 2.5 pg/g body weight (IV LD50) (Utaisincharoen et al., 1993).

Structure-function mechanics of TV-kallikreins are poorly understood. However the variation between Gly/Ser189 and Asp189 in catalytic site is thought to correspond to difference in substrate specificity (Fry et al., 2015).

CRiSP

Cysteine rich secretory proteins (CRiSPs) are part of CAP (**c**ysteine-rich secretory proteins, **a**ntigen 5, and **p**athogenesis-related 1 proteins) superfamily and act as inhibitors of a number of ion channels. Reptilian venoms are rich in CRiSPs, in particular venoms of non-front-fanged snakes and anguimorph lizards, but the toxic role of these proteins remains enigmatic. It is most likely that they disrupt homeostasis through the blockage of cyclic nucleotide-gated and voltage-gated ion channels, as well as inhibition of smooth-muscle contraction. A number of CRiSPs were recovered from Anguimorpha and Iguania lizards' oral secretion (Fry et al., 2009a; Fry, 2015).

Structure

CRiSPs are single chain proteins with a sequence of approximately 230 amino acid residues and Mw of 20-30 kDa. All CRiSPs have universally conserved 16 cysteine residues that form 8 cystines responsible for the great stability of the structure (Lyns, 2007). CRiSP molecule has two distinct domains separated by a hinge region: 21 kDa structurally conservative CAP (PK-1) at the N-terminus and 6 kDa CRD (cysteine rich domain) at the C-terminus, with the latter containing 10 out of 16 cysteine residues. Even though CRiSPs lack transmembrane domain, they can associate with membrane components through interactions with membrane proteins (Fry et al., 2015)

Helothermine, isolated from *Heloderma horridum* (Mochca-Morales, Martin and Possani, 1990), has a Mw of 25 kDa and consists of 223 amino acids, its structure stabilized by eight disulfide bridges. It has significant similarity with CRiSPs from human (49,6%) and mouse (46.7%) testes, as well as CRiSPs found in mouse salivary glands and mouse and rat epididymis (Morrissette et al., 1995).

While most details of CRiSPs' function remain to be elucidated, it is hypothesized that their distinct two-domain structure allow for the dual function, with CRD being responsible for the ion-channel binding activity.

Varanid CRiSPs were shown to have the loop I doublet (KR) that is thought to be an essential part of the blockage of cyclic-nucleotide-gated calcium channels. Helothermine

as well as most varanid CRiSPs also have the loop I motif (EXXF) that is thought to facilitate blockage of the L-type Ca channels and contribute to the inhibition of smooth muscle contraction (Fry et al., 2006).

Anguimorph CRiSPs were shown to have variation in structural and functional domains, including in the ion channel-binding region (CRD). Similar to kallikreins, their cysteine pattern remains highly conserved, with only *Celestus warreni* and *Varanus indicus* so far revealed to possess newly evolved cysteines (Fry et al., 2006).

Function

Organismal effects of CRiSP toxins are diverse. Injections of the purified helothermine produced lethargy, rear limb paralysis, hypothermia, and death in rodents (Mochca-Morales et al., 1990).

Helothermine was found to produce a strong and constant inhibition of ryanodine binding to cardiac and skeletal sarcoplasmic reticulum with a more pronounced effect on skeletal receptor. It was shown that helothermine interacts with ryanodine receptors in a very selective manner, most likely lowering the Ca sensitivity of the channel through selectively decreasing the number of release sites (Morrissette et al., 1995). Helothermine was also shown to block K+ and Ca2+ currents in rat cerebellar cells (Nobile et al., 1994, 1996).

While the physiological targets of helothermine are unknown, its toxic action in rodents likely includes either assistance by other venom components (such as PLA2) or cleavage of the whole protein into a smaller fragment in order to facilitate penetration of the muscle cells to produce a direct block of ryanodine receptor (Morrissette et al., 1995).

Evolution

Toxicoferan venom CRiSPs form two distinct lineages with a high likelihood of separate events leading to their recruitment. Snakes' CRiSPs are more diverse and have more sites under the influence of positive selection than their lizards' counterparts (Sunagar et al., 2012).

Natriuretic/helokinestatin peptides

Natriuretic peptides are a vast group of molecules performing diverse physiological roles. They have been independently recruited as toxins in mammalian (platypus) and reptilian venoms. Anguimorpha lizards' venom natriuretic peptides belong to a group B of

natriuretic peptides and are thus called LV-BNP. There are three target guanylyl cyclase receptors associated with the activity of natriuretic peptides. GC-A and GC-B are the primary targets of LV-BNPs, they trigger secondary pathways of intracellular guanylyl cyclase. GC-C is a secondary target mediating the activity through G-protein dependent pathways (Fry et al., 2009a; Fry, 2015).

Helokinestatins are a family of proline-rich peptides originally isolated from *H. suspectum*, they were later found to be present in venoms of other members of Anguimorpha family – *Gherronotus infernalis* and *Celestus warreni* (Fry et al., 2010b). Helokinestatins act as inhibitors of bradykinin action on smooth muscle (Kwok et al., 2008) and represent one of the apotypic lineages of evolution of toxic natriuretic peptides in reptiles, another being snake venom BPPs (Ma et al., 2011).

Structure

All natriuretic peptides are expressed as a long precursor containing signal peptide, propeptide and natriuretic peptide domain that is post-translationally cleaved to liberate individual NP. All natriuretic peptides have 17-amino-acid disulfide loop, all lizard natriuretic peptides have a C-terminal tail, that sets them apart from snake NP (Fry, 2015).

Helokinestatins are usually small peptides with Mw of 1 to 1.5 kDa. To date seven isoforms have been identified in the *Heloderma* venoms with their nomenclature reflecting their position on the precursor. All helokinestatins are rich in prolyl residues, which impose certain rigidity and spatial order on their structure and makes them resistant to non-specific proteolysis (Fry et al., 2010a; Zhang et al., 2010; Ma et al 2011). All helokinestatins possess a Pro-Arg motif at C-terminus as opposed to Ile/Val-Pro-Pro motif of snake venom BPPs. All helokinestatins are encoded as tandem repeats within the natriuretic peptide precursor (Fry et al., 2010a,b, 2012; Zhang et al., 2010).

Helokinestatins have a highly conserved structure among anguimorph lizards, which is suggestive of their fundamental function in the venoms of this clade (Ma et al., 2011). Between the species of *Heloderma* several helokinestatins are fully-conserved in primary structure while others are different.

Differences between the species occur also in the length of the precursor. One precursor sequence was identified to have an open-reading frame of 196 amino acids in *H. horridum* and 178 residues in *H. suspectum*. The difference is due to the insertion of an additional domain of 18 residues coding helokinestatin-3 molecule. In *H. horridum* precursor

sequence encodes one copy of helokinestatin-1, one copy of helokinestatin-2, two tandem copies of helokinestatin-3 (that set it apart from *H. suspectum* version), a single copy of helokinestatin-6 and a single copy of C-type natriuretic peptide (Ma et al., 2011).

Function

Helokinestatins antagonize the actions of bradykinin on mammalian smooth muscle (Kwok et al., 2008) unlike the bradykinin-potentiating peptides (BBPs) isolated from venoms of certain snakes.

Synthetic replicates of helokinestatins 1–3 and 5 were all found to antagonize the relaxation effect observed following bradykinin application to a rat arterial smooth muscle preparation in a manner that suggested a non-competitive mode of action. This effect was also more pronounced than that recorded for guinea pig ileum smooth muscle (Zhang et al., 2010). However, unlike all other members of the family, Helokinestatin-7H induces a dose-dependent contraction of rat arterial smooth muscle (Ma et al., 2012).

Due to the tandem-repeat nature of the evolution of this peptide family, most forms are just structurally slightly different versions of each other. At the same time, even single substitution can have a dramatic effect on the peptide's bioactivity. A helokinestatin-2 isoform from *H. horridum* has a single phenylalanine substitution which can be attributed to the differential potency of both forms (with and without substitution) in attenuating bradykinin responses on arterial smooth muscle, with the substitution causing an almost 50% reduction in activity. Another example is helokinestatin-6 that has an unusual C-terminal sequence of EPR that results in it being the least potent of helokinetsatins in bradykinin inhibition (Ma et al., 2011).

This feature is further elucidated by helokinestatin-7 forms that differ in having Arg/Thr at position 6 and Lys/Glu at position 7. These changes increase the net negative charge of the molecule with helokinestatin-7H having -1 and helokinestatin-7S having -4, at the same time most other helokinestatins exhibit a net positive charge of +2 which, along with the higher presence of prolyl residues, might explain the lower potency of helokinestatin-7 forms as bradykinin antagonists since their physiochemical properties are closer to that of the appropriate ligand (Ma et al., 2012).

To date no varanid helokinestatin forms were recovered, and varanid natriuretic peptides were shown to have retention of the residues necessary for natriuretic activity (Fry et al., 2006), which might be indicative of low importance of this toxins in varanid lizards' venom.

Natriuretic peptide from *G. infernalis* was shown to have hypotensive effect, with aspartate at ring-position 7 and isoleucine at ring-position 9 being important for this activity. While aspartate 7 replacement was affecting it more than isoleucine 9, both were shown to be important for full activity (Fry et al., 2010b).

Evolution

Snake venom BPPs were shown to encode a single copy of a peptide exhibiting a closer structural similarity with helokinestatins, in addition having similar propeptide convertase site cleavage specificity – properties otherwise outlining the differences between helokinestatins and BPPs (Zhang et al., 2010). However, phylogenetic studies show that lizard venom natriuretic peptides are the result of a recruitment event independent to that of the snake venom natriuretic peptides. In phylogenetic studies, lizard venom natriuretic peptides are nested within the BNP clade, while snake forms are nested within the CNP clade – two different types of natriuretic body peptides (Fry et al., 2010b).

In this line of evidence helokinestatins are likely to occur early in the Neoanguimorpha clade, given their wide present in the oral secretion of relatively-distantly related neoaguimorph lizard lineages. This is further corroborated by the absence of novel helokinestatin domains within the natriuretic precursor gene of varanid lizards (Fry et al. 2006, 2009b) and the differential rate of evolution between the scaffold of natriuretic peptides and helokinestatins in *Abronia graminea* (Koludarov et al., 2012).

Medicinal potential of helokinestatins

Helokinestatins were found to act synergistically with some anticancer drugs and evoke prostaglandin production (Kwok et al., 2008), which might lead to the development of a medicinal drug based on helokinestatin.

Phospholipase A2 type III

Phospholipases A2 are low-molecular weight enzymes that catalyze the hydrolysis of ester bond in glycerophospholipids. Being one of the largest groups of lipid-hydrolyzing enzymes, phospholipases have been convergently recruited into toxin arsenals of cephalopods, cnidarians, insects, arachnids, reptiles and other lineages. There are three classes of PLA2s, with groups I and II finding their way into snakes' venom and group III present in the venom of anguimorphs (Fry et al., 2009a; Fry, 2015).

Initially phospholipases A2 type III were thought to be present only in the venom of helodermatid lizards (Dehaye et al., 1984; Sosa et al., 1986), however subsequent studies revealed their presence in a broader spectrum of anguimorph lizards' oral secretion (Fry et al., 2006; Fry et al., 2009b; Fry et al., 2010b; Koludarov et al., 2012), thus suggesting its plesiotypic role in Anguimorpha clade.

Structure

Anguimorph lizards' secretory anionic phospholipases A2 belong to unique class III of this protein family, with the snake phosopholipases compromising class I and mammalian pancreatic ones as well as some snakes' belonging to class II. They usually have a sequence of around 140-160 amino acids with Mw around 16-19 kDa. Their cysteine pattern (usually 10 half-cystine residues), short hydrophopic N-terminus and C-terminal region sequence making them closely related to bee venom phospholipase A2 than to any other (Gomez et al., 1989; Vandermeers et al., 1991; Huang and Chiang, 1994).

Within *Heloderma* alone several forms of PLA2s were identified with up to 15% difference in sequence that results in differential bioactivity. The N-terminal part, including the lipid-binding area remains invariant. Active site and C-terminus also remain quite conserved with differences between the forms mostly occurring in the region separating the two (Vandermeers et al., 1991).

Function

PLA2s from *H. horridum* were shown to exclusively inhibit thromboxane-induced platelet aggregation of human platelet-rich plasma probably by virtue of their phospholipase activity on plasma phospholipids, converting them into lysophospholipids, which then interfere with the coupling of TXA, receptor and its signalling transduction system. However they had little effect on collagen- and ADP-induced platelet aggregation (Huang and Chiang, 1994).E

Evolution

Similarities between honeybee PLA2s and that of *Heloderma* lizards and the differences in PLA2 structure between anguimorph lizards and other toxicoferans hint at the possibility of ancient gene duplication event occurring in a provertebrate ancestor. One branch then putatively led to bee and lizard forms with 4 and 5 disulfide bonds and another after experiencing additional duplication event within the reptiles led to type I and II PLA2s with

7 and 6 disulfide bonds respectively (Gomez et al., 1989). Though it might be that *Heloderma* PLA2 type III evolved from ancestral reptilian PLA2 of uncertain type, the striking similarity between PLA2 from *Heloderma* venom and arthropodian venom type III PLA2s all of which form monophyletic clade (Ghomashchi et al., 1998; Hariprasad et al., 2009) and the wide-range study showing that at least two PLA2 genes existed in the vertebrate line before divergence of reptiles and mammals (Davidson and Dennis, 1990) point towards the model of PLA2 type III evolution proposed by Gomez.

Nucleosides and nucleoside-releasing enzymes

Nucleosides are a known component of toxicoferan venoms, corresponding to as much as 8.7% of the dry weight in some cases (Aird, 2005). Nucleosides were found to be present in venoms of *Heloderma horridum* and *Heloderma suspectum* though in smaller amounts than in many elapid or viperid venoms (Aird, 2008). Both exogenous venom nucleosides and endogenous nucleosides released from prey tissue have a complex function in reptilian venom, simultaneously immobilizing the prey via both hypotension and paralysis, and contributing to prey digestion via apoptosis and fast necrotic cell death (Aird, 2002). Since they have similar pharmacological roles in all vertebrates they are useful against all types of vertebrate prey. Many non-toxic venom components may exert a toxic role via meditating release of endogeneous nucleosides in the prey.

Structure

Aird (2008) reported presence of adenosine, cytidine, guanosine, hypoxanthine, inosine, and uridine in venoms of both *Heloderma horridum* and *Heloderma suspectum*.

Fry et al. (2010b) reported ribonuclease sequences from *G. infernalis* that lack two ancestral cysteines as opposed to body form. Two of those isoforms had a frameshift mutation in the C-terminal region, which resulted in aformention lack of ancestral cysteines and presence of two new ones.

Function

Helodermatid nucleosides are likely to act in concert with kallikreins, exendins and helothermine in inducing hypotension in prey. At the same time no significant nucleoside-releasing activity was found in *Heloderma* venoms (Aird 2008).

Other toxins

A number of other toxins and potentially toxic enzymes were identified in anguimorph lizards' venom glands or oral secretion.

Serotonin was reported as a minor component in *Heloderma* venoms, but its toxic role remains unclear (Strimple et al., 1997). Likewise the functions of vespryn (Fry et al., 2010a), NGF, VEGF, hepatocyte growth factor, peroxiredoxin, semaphoring 3E and other minor components discovered recently in Anguimorph venom (Sanggaard et al., 2015) remain to be elucidated. Since most of those proteins were recovered indirectly, they are yet to be properly charachterised.

A lethal toxin-1, a protein of 28 kDa was shown to have inhibitory effect on direct electrical stimulation of the isolated mouse hemidiaphragm (Komori et al., 1988). Later study recovered the entire precursor gene of lethal toxin-1 orthologs, showing it to be constructed of beta-defensin domain repeats, therefore naming the toxin group "helofensins" (Fry et al., 2012). Helofensins evolved from an ancestral beta-defensin precursor through three domain duplication events, making it a third known independent recruitment of beta-defensin as a toxin with the other two being crotamine and platypus toxin.

AVIT peptides are a plesiotypic group of toxicoferan toxins with a significant similarity with bioactive components from firebellied toads' (genus *Bombina*) defensive skin secretions. They are potent agonists of mammalian prokineticin receptors and their effect mimics that of an endogenous prokineticin overdose leading to a rapid constriction of intestinal smooth muscles. AVIT have been isolated from snakes' venom and several members of *Varanus* genus (Fry et al., 2010b).

Fry et al. (2010b) report three new peptides with their toxin function inferred from their structure and location: cholecystoxin from *V. varius*, celestoxin from *C. warreni*, and goannatyrotoxin from *V. glauerti*. Cholecystoxin was found to be homologous to normal body cholecystokinin, while goannotyrotoxin homologous to YY peptides. Goannotyrotoxin had a potent hypertension activity followed by a prolonged hypotension. Celestoxin was found to be hypotensive and did not show any similarity with any known protein type. The same study recovered lectin from *Psedopus apodus* and lizard veficolin whose sequences formed a monophyletic group with snake forms, thus suggesting its plesiotypic toxicoferan role (Fry et al., 2010b).

Recent studies revealed presence of kunitz peptides in oral secretion of Anguimorpha venom (Koludarov et al., 2012; Fry et al., 2013). Kunitz peptides are present in a variety of snake venoms and are known to function as protease inhibitors and channel-blocking neurotoxins. Most reptilian venom kunitz peptides only known only through their primary sequence, with groups like textilins from *Pseudonaja textilis* being a stark exception (Fry, 2015). Their role in Anguimorpha oral secretion as well as their evolutionary history within the Toxicofera remains to be elucidated.

A presence of another core toxicoferan toxin – hyaluronidase was first inferred from the bioactivity of *Heloderma* venom (Tu and Hendon, 1983), with further study recovering hyaluronidase sequences from both *H. suspectum* and *G. infernalis* and showing their homology with snake venom hyaluronidases (Fry et al., 2010b). As can be inferred from their name, hyaluronidases catalyze hydrolysis of hyaluronic acid, a major component of interstitial barriers, thus increasing tissue permeability to allow for a more efficient spreading of other toxins. These enzymes are widely present in animal venoms with little sequence diversity across the taxa (Fry et al., 2009a; Fry, 2015)

Concluding remarks

For a long time venom research has had its focus fixed upon medically important species, resulting in a subtle anthropocentric bias in purely biological endeavours such as the application of the function concept and the study of evolutionary trends. The tides are changing now, with an ever-increasing amount of evidence outlining the role venom systems have played in the diversification of some reptilian lineages.

If we put lizard venom research in perspective and take into consideration the fact that it is only four or five decades old, then we should view any criticism based on scarce evidence of functional role as a criticism of the insufficient number of studies. The aim of this review was partially to facilitate the emergence of new studies in the area of functional characterization of lizard venoms by supplying an outline of the current knowledge on the topic.

It has to be noted that the role venom plays in the evolutionary and life history of anguimorph lizards is likely drastically different to that which it plays in front-fanged or even non-front-fanged snakes. In many venomous serpents venom is the only way to subdue a prey, while lizards usually have other ways of securing a meal, whether through the large blade-like teeth of varanid lizards or the powerful jaws of gila monsters.

Nevertheless, the reality of the presence of myriad toxins in the oral secretions of the Anguimorpha is inescapable, as is the homology of those molecules to their snake counterparts and their toxic activities – for those few that have been tested. It might be that venom plays largely a defensive role in the ecological realities of anguimorph lizards, it might be that it gives them a slight edge, marginally increasing their predatory success rates – in nature a slight effect may be enough to favour the selection of a functional trait.

It is also likely that not all proteins secreted by venom glands have toxic role, perhaps acting as antimicrobial agents or digestive enzymes. However, a surplus of evidence is telling us that anguimorph lizards possess a unique and complicated oral secretory apparatus that produces myriad molecules with diverse exogenous effects. Such an array requires resources for its production, suggesting these molecules have been playing and continue to play an important role in the evolutionary reality of anguimorph lizards.

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Chapter 2: Fossilized venom: the unusually conserved venom profiles of *Heloderma* species (beaded lizards and gila monsters)

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Fossilized venom: the unusually conserved venom profiles of Heloderma species (beaded lizards and gila monsters)

Abstract

Research into snake venoms has revealed extensive variation at all taxonomic levels. Lizard venoms, however, have received scant research attention in general, and no studies of intraclade variation in lizard venom composition have been attempted to date. Despite their iconic status and proven usefulness in drug design and discovery, highly venomous helodermatid lizards (gila monsters and beaded lizards) have remained neglected by toxinological research. Proteomic comparisons of venoms of three helodermatid lizards in this study has unravelled an unusual similarity in venom-composition, despite the long evolutionary time (~30 million years) separating *H. suspectum* from the other 2 species included in this study (*H. exasperatum* and *H. horridum*). Moreover, several genes encoding the major helodermatid toxins appeared to be extremely well-conserved under the influence of negative selection (but with these results regarded as preliminary due to the scarcity of available sequences). While the feeding ecologies of all species of helodermatid lizard are broadly similar, there are significant morphological differences between species, which impact upon relative niche occupation.

Introduction

There are five extant species of helodermatid lizards: *Heloderma alvarezi*, *H. charlesbogerti*, *H. exasperatum, H. horridum and H. suspectum* (Douglas et al., 2010; Reiserer et al., 2013). *H. suspectum* last shared a common ancestor with the other extant species approximately 30 million years ago. *H. exasperatum* and *H. horridum*, the other two species included in this study, last shared a common ancestor approximately 4 million years ago. All are native to the south-western part of the North American continent and inhabit rocky, semiarid and scrubland habitats. Such an absence of appreciable intraclade diversity in the ecology is reflected in the extremely overall morphological similarity of the species. These lizards are also characterised by having a very low metabolic rate and are known to exhibit a preference for low-body temperatures, spending most of the year at temperatures lower than 25 °C (Pianka and King, 2004). Helodermatid lizards raid the nests of birds and rodents but may also predate upon adult rodents (Beck, 2005; Fry personal observations).

The genus *Heloderma* has been recognised as venomous for more than a century. The teeth are deeply grooved and the glands are very large. Most cases of human envenomation involve lizards biting and holding with their strong jaws, sometimes for hours. Envenomations by helodermatid lizards may be clinically complex, with symptoms including extreme pain, acute local swelling, nausea, fever, faintness, myocardial infarction, tachycardia, hypotension, and inhibition of blood coagulation (Bogert and del Campo, 1956; Bouabboud and Kardassakis, 1988; Cantrell, 2003; Hooker and Caravati, 1994; Miller, 1995; Strimple et al., 1997). Studies of helodermatid lizard venom have identified several components (Table 1). Of these, exendin-4, isolated from *Heloderma suspectum* venom, is a peptide agonist of the glucagon-like peptide (GLP) receptor that promotes insulin secretion. It has been clinically used to treat type 2 diabetes and to enhance plasma insulin secretion (Drucker and Nauck, 2006).

Table 1 Heloderma venom peptides/proteins which have been proteomically characterised (based on previous studies)

Protein type/toxin class	**Toxic action**	**Uniprot accession #(s)**
14-3-3	Possible disruption of signal transduction, possible disruption of neuronal cells homeostasis	by similarity
CRiSP (cysteine rich secretory protein)	Paralysis of peripheral smooth muscle and induction of hypothermia through blockage of various channels including ryanodine and L-type calcium channels.	Q91055
Exendin	Induces hypotension via relaxation of cardiac smooth muscle.	C6EVG1, C6EVG2, P04203, P04204, P20394, P26349
Helofensin	Lethal toxin that inhibits direct electrical stimulation of the isolated hemi-diaphragm.	C6EVG6, D2X5W3, D2X5W4, Q7LZ31
Hepatocyte growth factor-like protein	Toxin action unknown	predicted
Hyaluronidase	Increase of membrane permeability	gi|190700985
Kallikrein	Increase of vascular permeability, production of hypotension, stimulation of inflammation in addition to cleavage of fibrinogen.	P43685, C6EVG4, C6EVG5
B-type Natriuretic peptide/helokinestatin precursor	Natriuretic peptides produce hypotension through the relaxation of aortic smooth muscle. The helokinestatin peptides are antagonists of bradykinin at the B2 bradykinin receptor.	C6EVG7, D7FB56, D7FB57, E8ZCG5
Neuroendocrine convertase 1	Potentially involved in activation of exendins	predicted
Peroxiredoxin-4	Toxin action unknown	predicted
Phospholipase A2 (Type III)	Inhibition of platelet aggregation via the epinephrine-	C6EVG9, C6EVH0

	induced pathway	
Semaphorin-3E	Toxin action unknown	predicted
Vascular Endothelium Growth Factor	Potentially increases vascular permeability	based on similarity
Venom Nerve Growth Factor	Toxin action unknown	based on similarity

Variation in venom profiles has been extensively documented between snake species of the same genus (Angulo et al., 2008; Calvete et al., 2007; Fry et al., 2005, 2002, 2003; Gutierrez et al., 2008; Lomonte et al., 2008; Mackessy, 2010; Salazar et al., 2009; Sanz et al., 2008, 2006; Tashima et al., 2008; Wagstaff et al., 2009) and between individuals of the same species, with intraspecific differences found among different geographic localities (Fr et al., 2002; Boldrini-Franca et al., 2010; Castro et al., 2013; Daltry et al., 1996; Forstner et al., 1997; French et al., 2004; Sunagar et al., 2014), and between juveniles and adults (Daltry et al., 1996; Calvete et al., 2009; Lopez-Lozano et al., 2002; Mackessy, 1988). Such taxonomic, geographic and ontogenetic variation has been linked to strong natural selection in response to differing prey species (Fry et al., 2003a,b,c, 2008; Daltry et al., 1996; Sunagar et al., 2012, 2014; Brust et al., 2013; Casewell et al., 2013; Gibbs and Mackessy, 2009; Pawlak et al., 2009).

Traditionally, reptile venom research has focused mostly on clinically important snake species. As a result, our understanding of the evolution of helodermatid lizard venom is limited. In this study, we compare the venom proteomes of *H. exasperatum, H. horridum* and *H. suspectum* in order to gauge the extent of the diversification in venom composition that has occurred over 30 million years since these species last shared a common ancestor (Douglas et al., 2010).

Results and Discussion

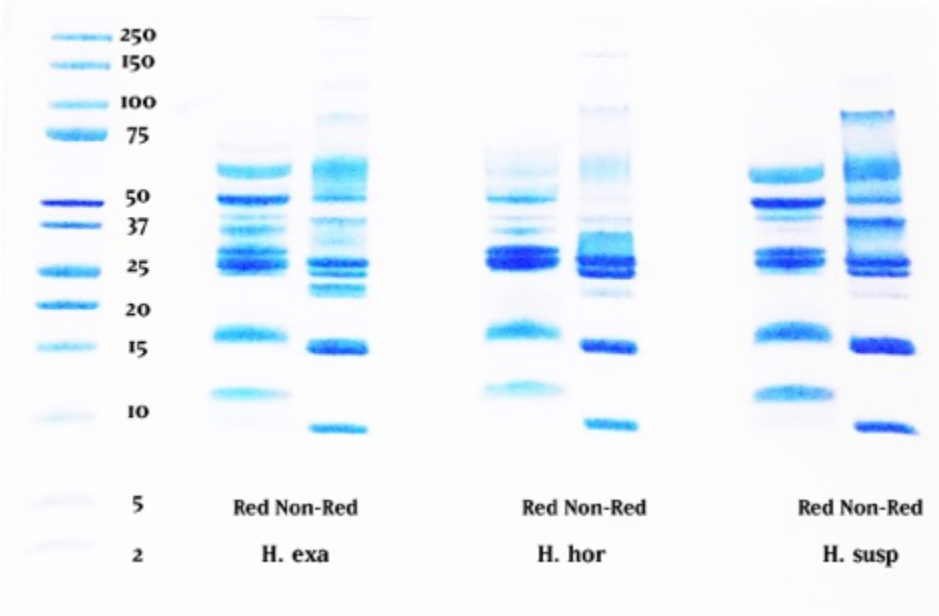

Figure 1: Reduced and non-reduced tris-tricine 1D-gel comparison of H. exasperatum, H. horridum and H. suspectum.

Shotgun sequencing recovered toxin types previously known from the *Heloderma* venom proteome: CRiSP, exendin, kallikrein, helokinestatin and Type III phospholipase A2. In addition, this analytical technique recovered types previously known only from transcriptome studies: hyaluronidase, natriuretic peptide and nerve growth factor.

One-dimensional gel electrophoresis (1D-GE) utilizing the tris-tricine method indicated a gross overall similarity between the three venoms (Figure 1). For each species there was a notable difference between non-reduced (NR) and reduced (R) samples. Most notably, a 100 kDa band was present in the non-reduced lanes but absent from the reduced lanes. Conversely, the reduced lanes exhibited a dark band at 50 kDa not present in the non-reduced, indicating that the 100 kDa band was a disulfide-linked dimer.

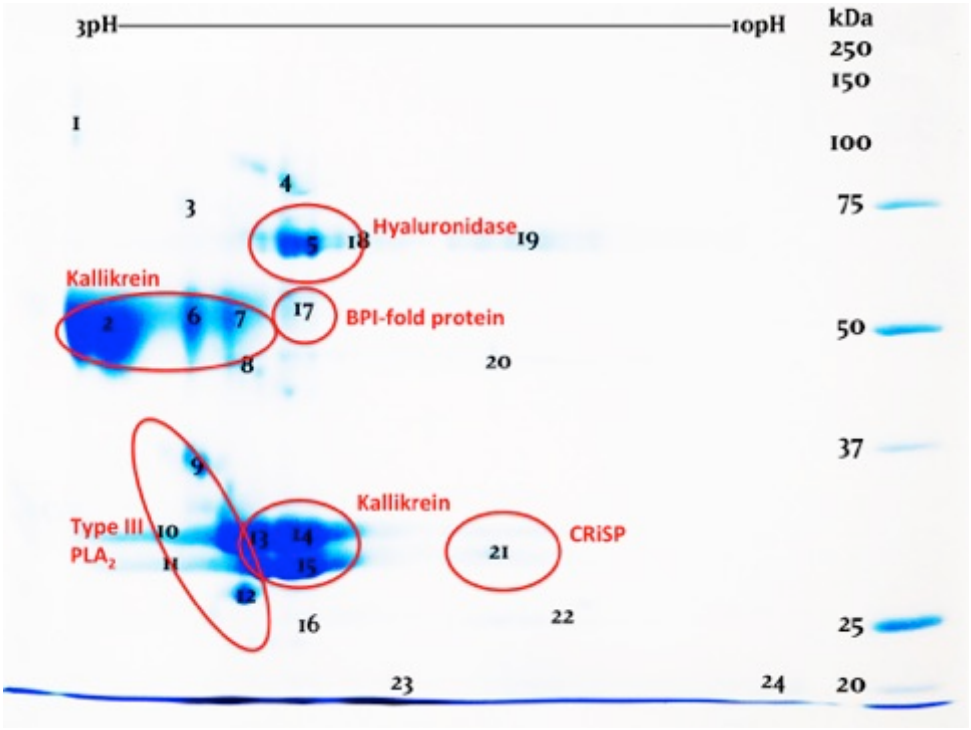

Figure 2: 2D-gel examination of H. exasperatum venom.

2D gels (2D-GE) confirmed the striking similarity of the three venoms (Figures 2-4). While the venoms are broadly similar in overall protein composition, it is clear that there are significant differences in relative expression levels. This was most apparent in the PLA_2 region. While these variations may point towards differential evolution, they may be also the result of intergel variation or simply arbitrary variation in venom gland content between individuals at the time of milking. More extensive comparative sampling is required to elucidate individual variation versus species level variations. Regardless, the overall protein composition was vastly more conserved than has been noted even for closely related species of snake (*c.f.* Ali et al., 2014).

2D-GE revealed in all venoms previously unknown venom components which were identified as bactericidal/permeability-increasing (BPI)-fold (all species) and semaphorin proteins by searching the LC-MS/MS results against our previously constructed *H. suspectum* venom gland cDNA library (Fry et al., 2010a,b). While semaphorin was identified in *H. suspectum* only, it must be noted that light spots located in similar regions of the *H. exasperatum* and *H. horridum* gels, for which mass spectrometry analysis was unable to provide an identity, suggest that this component is likely present in the other *Heloderma* venoms. mRNA sequences are given in supplemental file 1 and genbank accession numbers are KP224275 (BPI-fold) and KP224276 (semaphorin). Phylogenetic

analysis for both protein types identified *Anolis* genome sequences as the nearest known relative (Figures 5 and 6). The fact that both of these were identified as being transcribed by the venom gland indicates that they are indeed secreted by this gland and are not mucus contaminants. Therefore, they may play an as yet unidentified role in envenomation.

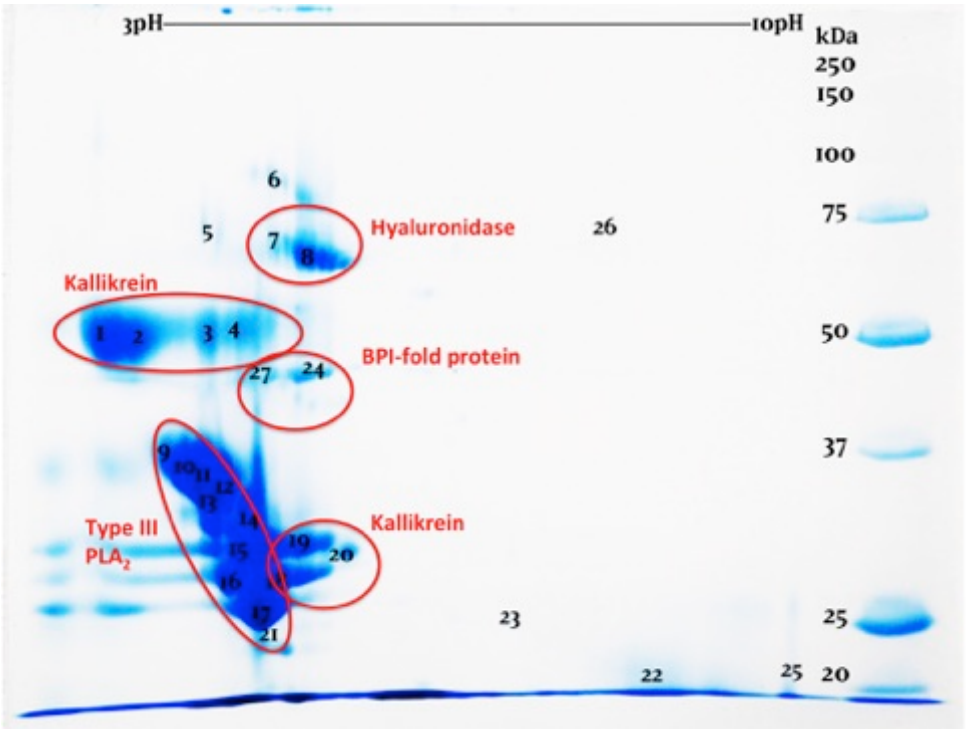

Figure 3: 2D-gel examination of H. horridum venom.

The precise role of venom in the ecology of helodermatid lizards remains unknown. Beck (Beck, 1990) considered it a “paradox” that helodermatid lizards hold on “with bulldog tenacity” when biting in apparent defence, thus increasing the lizard’s chance of injury or death. But this assertion merely reflects a common fallacy of evolutionary thinking – that the individual lizard is the “unit of selection”. As selection takes place at the level of the gene, the death of the individual lizard does not preclude strong selection for “bulldog tenacity” in defensive bites, as presumably this tactic maximises the unpleasantness of the encounter for the aggressor, thus ensuring it avoids such encounters in future. However the overall pharmacological profile of the venom includes components with lethal neurological effects or other severe physiological targeting, actions not consistent with a purely defensive role for the venom as defensive toxins are typically pain inducing (Casewell et al., 2013). Rather such lethal effects point towards the venoms having at least some role in predation.

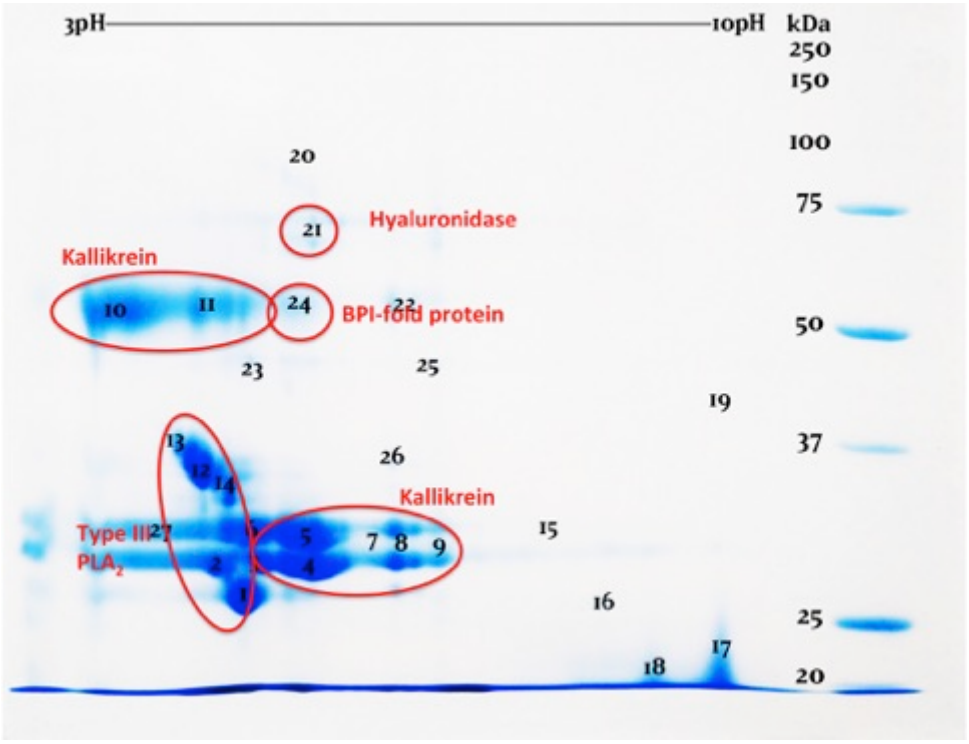

Figure 4: 2D-gel examination of H. suspectum venom.

Very few sequences are available and most are from *H. suspectum* and thus selection analyses must therefore be regarded as extremely preliminary. Regardless, pairwise-estimation of omega [non-synonymous (dN) to synonymous (dS) ratio] in this study revealed a greater influence of negative selection on the major toxin-encoding genes of *Heloderma* lineage, even for comparisons between *H. horridum* and *H. suspectum* sequences. Although for the in of intraspecific comparisons, dn/ds values lower than 1 can mean both weak negative selection or very strong positive selection, we chose to interpret these results as a sign of negative selection, since it is consistent with the proteomic data. However we acknowledge the possibility that future studies with larger sampling size might prove our decision wrong. The absence of diversity in venom-composition and toxin-encoding genes within the sister *Heloderma* lineages suggest that the venoms are not evolving under the diversifying selection pressure characteristic of the predator-prey "chemical arms races" in which venomous organisms utilising their venom for prey subjugation find themselves (Sunagar et al., 2014; Brust et al., 2013; Casewell et al., 2013; Sunagar et al., 2012). However, as noted above, there are obvious variations in relative expression levels within toxin classes and thus these relative expression levels may be a novel form of diversification.

While the venom has actions consistent with predatory use, a defensive venom role is also supported by the aposematic colouration of helodermatid lizards, the fact that these lizards are slow moving and vulnerable above ground (the osteoderms in their skin are another line of defence against would-be predators), and the fact that the lizards often feed on “defenceless” prey such as eggs and nestlings (Pianka and King, 2004). That being said, the results of the present study do not refute the hypothesis that helodermatid lizard venom may be used (at least partially) for predation, as all species occupy similar ecological niches and therefore feed on similar prey items. It must be emphasised that some toxins have actions consistent with predatory effects including lethal effects upon blood pressure, coagulation and neurological function. More in-depth analyses of the venom gland transcriptomes of helodermatid lizards to mine ‘enough’ nucleotide sequences for evolutionary selection analyses may shed light in this regard.

Table 2. Relative toxin molecular evolutionary rates.

Toxin Type	**Sequence pairs**	**Estimates**
Kallikrein	EU790962.1 (H. suspectum) vs. HM437246.1 (H. horridum)	dN: 0.180; dS: 0.225; dN/dS: 0.80
	EU790963.1 (H. suspectum) vs. HM437246.1 (H. horridum)	dN: 0.242; dS: 0.450; dN/dS: 0.53
	EU790962.1 (H. suspectum) vs. EU790963.1 (H. suspectum)	dN: 0.081; dS: 0.173; dN/dS: 0.47
	Average	dN: 0.167; dS: 0.282; dN/dS: 0.60
CRiSP	EU790958.1 (H. suspectum) vs. U13619.1 (H. horridum)	dN: 0.011; dS: 0.022; dN/dS: 0.49
Helofensin	GQ918270.1 (H. suspectum) vs. EU790964.1 (H. suspectum)	dN: 0.030; dS: 0.036; dN/dS: 0.84

	GQ918271.1 (H. suspectum) vs. EU790964.1 (H. suspectum)	dN: 0.052; dS: 0.065; dN/dS: 0.80
	GQ918271.1 (H. suspectum) vs. GQ918270.1 (H. suspectum)	dN: 0.020; dS: 0.027; dN/dS: 0.74
	Average	dN: 0.034; dS: 0.042; dN/dS: 0.80

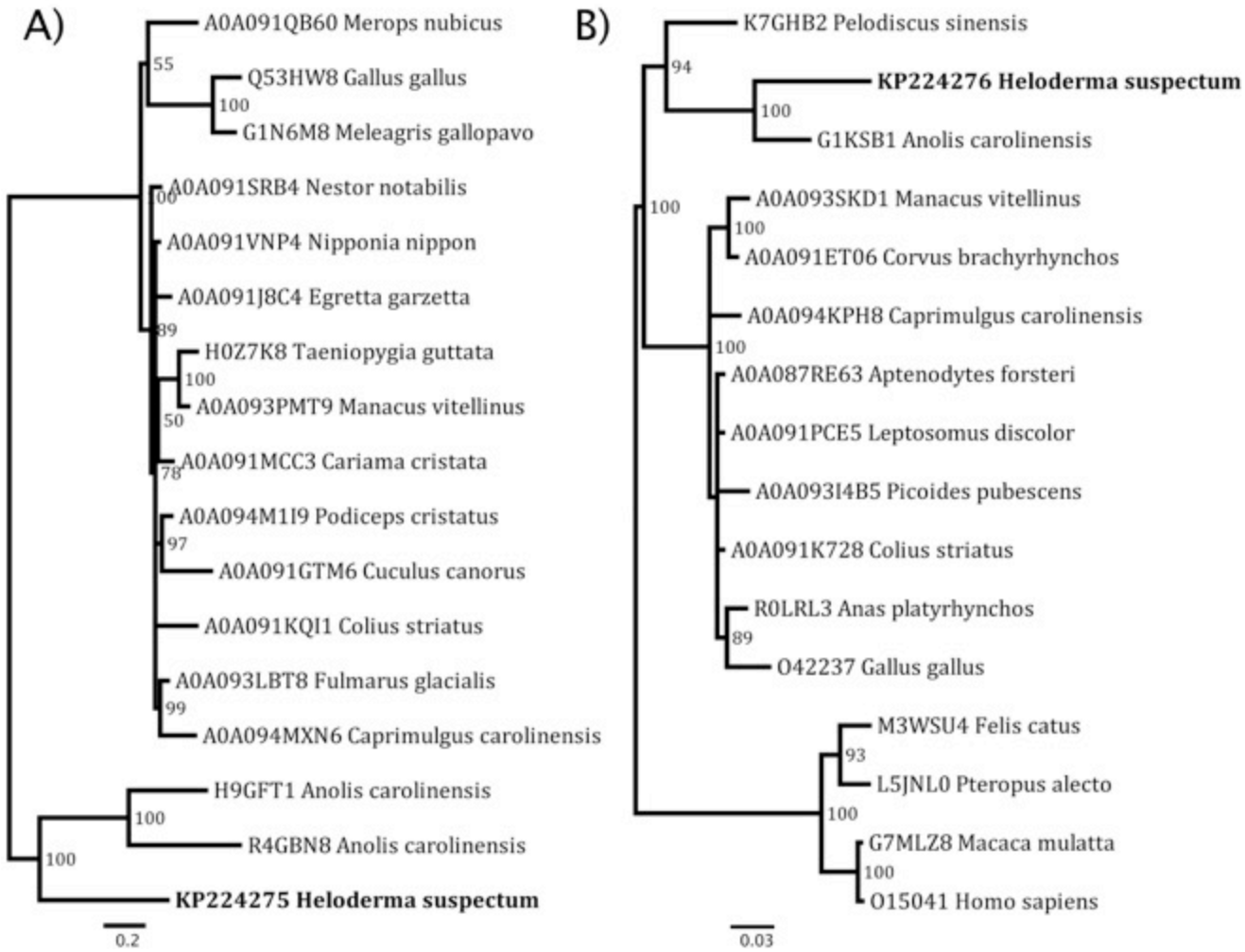

Figure 5: Phylogenetic reconstruction of A) BPI-fold and B) semaphorin proteins. Previously known sequences are referred to by their uniprot accession codes while Heloderma suspectum sequences obtained in this study are referred to by their genbank codes.

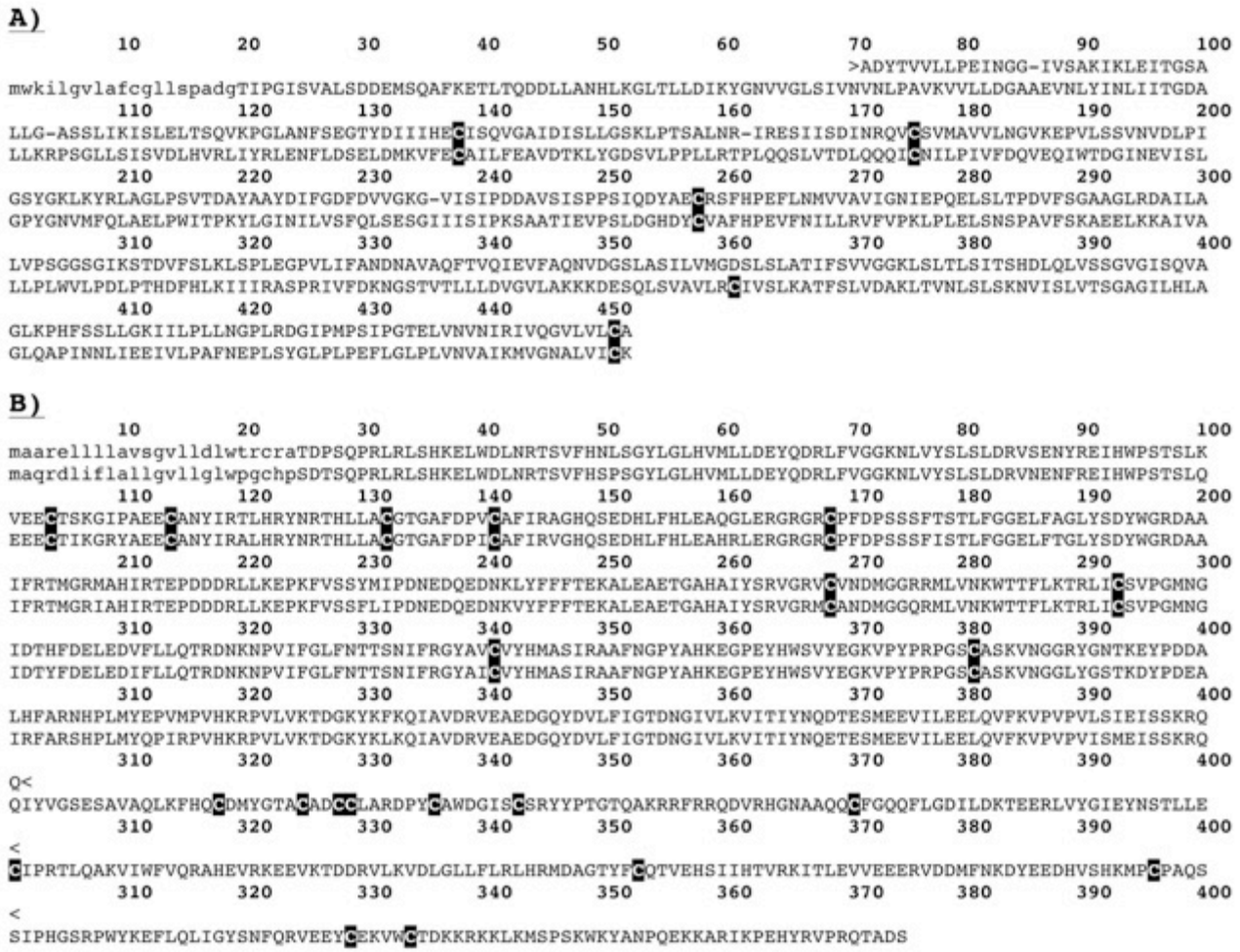

Figure 6: Sequence alignment of A) the BPI-fold proteins from Heloderma suspectum venom (KP224275) and Anolis caronlinensis genome (R4GBN8) and B) the semaphorin proteins from Heloderma suspectum venom (KP224276) and Anolis caronlinensis genome (G1KSB1).

Materials and Methods

Venom collection

Venoms were obtained from captive bred adult male specimens of *Heloderma exasperatum* (Rio Fuerte, Mexico founder stock)*, Heloderma horridum* (Colima, Mexico founder stock) and *Heloderma suspectum* (Phoenix, Arizona founder stock).

Shotgun sequencing

In order to identify low molecular weight peptides that do not resolve well on 1D or 2D gels, shotgun sequencing was used. 3 µg of crude venom sample was dissolved in 50 µL of 100 mM ammonium carbonate to reduce and alkylate cysteine bonds with subsequent addition of 50 µL of 2% iodoethanol/0.5% triethylphosphine in acetonitrile. The sample was

afterwards resuspended in 20 µL of 40 mM ammonium bicarbonate, before overnight incubation (at 37 °C) with 750 ng of sequencing grade trypsin (Sigma-Aldrich). To stop digestion 1 µL of concentrated formic acid was added to each of the samples. Samples were lyophilised then resuspended in 20 µL of 5% ACN/0.5% FA, put into MS vials and subjected to LC–MS/MS analysis.

One-dimensional gel electrophoresis

In order to compare venom proteomes between species, 1D gradient gels were run under both reducing and non-reducing conditions using the manufacturer (BioRad) protocol. Gels were prepared as follows: 0.05 mL Milli-Q H2O, 2.5 mL 30% acrylamide mix, 1.5 mL 1.0 M Tris-HCl, pH 8.45, 0.48 glycerol, 20 µL 10% APS, 2 µL TEMED (spreading gel); 0.76 mL Milli-Q H_2O, 0.76. mL 30% acrylamide mix, 0.76 mL 1.0 M Tris-HCl, pH 8.45, 15 µL 10% APS, 2 µL TEMED (spacer gel); 1.56 mL Milli-Q H_2O, 0.34 mL 30% acrylamide mix, 0.63 mL 1.0 M Tris-HCl, pH 8.45, 15 µL 10% APS, 2 µL TEMED (stacking gel). Spreading gel was cast first. After it set the spacer gel was slowly layered atop it, and after the spacer gel set the stacking gel was layered atop it. Running buffers were: 0.2 M Tris-HCl, pH 8.9 (anode buffer); 0.1 M Tris-tricine-HCl pH 8,45. The gels were run at 100 V for three hours at room temperature. 30 µg of venom was reconstituted in Tricine loading buffer (Bio-Rad) with 10 mM DTT added to provide reduce conditions. Gels were stained overnight with colloidal Coomassie brilliant blue G250 (34% methanol, 3% phosphoric acid, 170 g/L ammonium sulphate, 1 g/L Coomassie blue G250). After the staining was complete, gels were destained using MilliQ water.

Two-dimensional gel electrophoresis

In order to further investigate the proteomics variation, particularly that of isoelectric variation, 2D gels were run. 0.3 mg of venom sample were solubilized in 125 µL of rehydration buffer (8 M urea, 100 mM DTT, 4% CHAPS, and 0.5% ampholytes (Biolytes pH 3–10, Bio-Rad Lab)) with 0.01% bromophenol blue. The sample was mixed with shaking and centrifuged for 5 min at 4 °C, 14, 000 rpm. This was done to remove any insoluble material. The supernatant was loaded onto IEF strips (Bio-Rad ReadyStrip, non-linear pH 3–10, 7 cm IPG) and left overnight for passive rehydration. Protein focusing was achieved via PROTEAN i12 IEF CELL (Bio-Rad Lab). The IEF running conditions were as follows: 100 V for 1 h, 500 V for 1 h, 1,000 V for 1 h and 8,000 V until 98,400 V/h. Actual current in the final step of the run varied in accordance to resistance. To each strip a constant current of 50 µA was applied. After the run IPG strips were incubated for 10 min

in a reducing equilibration buffer (50 mM Tris–HCl, pH 8.8, 6 M urea, 2% SDS, 30% glycerol, 2% DTT) to reduce cysteine bonds. To alkylate reduced bonds IPG strips were further incubated for 20 min in an alkylating equilibration buffer (50 mM Tris–HCl, pH 8.8, 6 M urea, 2% SDS, 30% glycerol, 2.5% iodoacetamide). After rinse with SDS-PAGE running buffer, IPG strips were positioned on top of 12% polyacrylamide gels (Protean-II Plus, 18 × 20 cm, Bio-Rad Lab) using 0.5% agarose. Gels were run with a current of 10 mA/per gel for 20 min followed by 20 mA/per gel for the rest of the run until the bromophenol dye front was within 0.5 cm of the base of the gel. After the run, gels were briefly washed with water and stained with 0.2% colloidal Coomassie brilliant blue G250 overnight. Water was used to remove the excess of the dye after staining was complete. Visible spots were subsequently picked from gels and digested overnight at 37 °C with the use of sequencing grade trypsin (Sigma-Aldrich). Afterwards gel spots were washed with MiliQ water, destained (40 mM NH_4CO_3/50% acetonitrile (ACN)) and dehydrated (100% ACN); rehydration occurs in 10 µL of 20 µg/ml TPCK trypsin with subsequent incubation at 37 °C overnight. To elute peptides following solutions were used per each spot: 20 µL of 1% formic acid (FA), followed by 20 µL of 5% ACN/0.5% FA. Collected peptides were put into MS vials and subjected to LC–MS/MS analysis.

LC–MS/MS

In order to identify the toxin types present, digested gel spots and digested whole venom (shotgun) samples were processed using an Agilent Zorbax stable bond C18 column (2.1 mm by 100 mm, 1.8 µm particle size, 300 Å pore size) at a flow rate of 400 µL per minute and a gradient of 1–40% solvent B (90% acetonitrile, 0.1% formic acid) in 0.1% formic acid over 15 minutes or 4 minutes for shotgun samples and 2D-gel spots respectively on a Shimadzu Nexera UHPLC coupled with an AB SCIEX 5600 Triple TOF mass spectrometer. MS2 spectra are acquired at a rate of 20 scans per second with a cycle time of 2.3 seconds and optimised for high resolution. Precursor ions were selected between 80 and 1800 m/z with a charge state of 2–5 and of an intensity of at least 120 counts per second with a precursor selection window of 1.5 Da. The isotopes within 2 Da were excluded for MS2. MS2 spectra were searched against known translated transcriptome libraries or UniProt database with Proteinpilot v4.0 (ABSciex) using a thorough identification search, specifying iodoacetamide as an alkylation method, trypsin digestion and allowing for biological and chemical modifications (ethanolyl C or deamidated N in particular) and amino acid substitutions, including artefacts induced by the preparation or

analysis processes. This was done to maximize the identification of protein sequences. Spectra were inspected manually to eliminate false positives.

Phylogenetic analysis and alignment

We performed phylogenetic analyses in such a way so as to allow reconstruction of the molecular evolutionary history of each toxin type for which transcripts were bioinformatically recovered. We identified toxin sequences by comparison of the translated DNA sequences with previously characterised toxins using a BLAST search of the UniProtKB protein database. Molecular phylogenetic analyses of toxin transcripts were conducted using the translated amino acid sequences.

In each dataset we included comparative sequences from other venomous reptiles and physiological gene homologs previously identified from non-venom gland transcriptomes to suit as outgroup sequences. All sequences obtained in this study are referred to by their Genbank accession numbers (http://www.ncbi.nlm.nih.gov/sites/entrez?db=Nucleotide) and sequences from previous studies are referred to by their UniProtKB accession numbers (www.uniprot.org).

For sequence alignment we used CLC Mainbench. In sequence alignments, the leader sequence is shown in lowercase and cysteines are highlighted in black. > and < indicate incomplete N/5’ or C/3’ ends, respectively and * is used to indicate the end of a sequence.

To analyse datasets we used Bayesian inference implemented on MrBayes, version 3.2.1 using lset rates=invgamma with prset aamodelpr=mixed, which enables the program to optimize between nine different amino acid substitution matrices. The analysis was performed by running a minimum of 107 generations in four chains, and saving every 100th tree. The log-likelihood score of each saved tree was plotted against the number of generations to establish the point at which the log likelihood scores reached their asymptote, and the posterior probabilities for clades established by constructing a majority-rule consensus tree for all trees generated after completion of the burn-in phase.

Pairwise-estimation of dN/dS

Pairwise-estimates of dN/dS were obtained for *Heloderma* Kallikreins, CRiSPs and lethal toxins using the Codeml program of PAML package.

Conclusion

Due to the limited amount of previously available proteomic data, these results significantly contribute to our understanding of helodermatid lizard venoms. Despite the *H. suspectum* having been separated from all other extant species for 30 million years, the venoms have a significant overall level of similarity in regards to protein/peptide types present but with variable expression within these conserved classes. This suggesting that their venoms experienced a diversifying selection pressure different from that often governs the evolution of venom in other squamate reptiles such as snakes which display significant differences in the types of proteins expressed, even at low taxonomical levels. This variation pattern is likely a consequence of the fact that all helodermatid lizards intrinsically occupy the same ecological niche and thus feed upon the similar prey items. However, the discovery of novel components represents an exciting opportunity for biodiscovery and reinforces the basic premise that poorly investigated venomous lineages represent untapped resources of molecules with potential for utilization in drug design and development.

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Chapter 3. Taming the dragon: investigating evolutionary forces that shape the complexity of monitor lizards' venom

Taming the dragon: investigating evolutionary forces that shape the complexity of monitor lizards' venom

Abstract

For almost a century the intriguing consequences of the Komodo dragon's bite have demanded a thorough scientific investigation. Several hypotheses have been proposed and a mounting pile of evidence indicates that the oral glands of varanid lizards secrete various toxins homologous to those of venomous snakes. Here we present a first broad scale investigation into the composition of varanid lizard venom, and compare proteomic and bioactivity data with published reports of monitor lizard ecology. Our results not only reveal the remarkable complexity of varanid lizard venom, but are also indicative of a tripartite relationship between their venom content, size and habitat. Altogether, our findings portray *Varanus* oral secretions as a multifunctional tool, likely to play an active role in either defence, predation or digestion, or perhaps a combination of these.

Introduction

Monitors, varanid lizards or goannas are mostly carnivorous squamate reptiles belonging to the genus *Varanus*. Their body size ranges from 23 cm for the adult *V. brevicauda* to over 3 m for *V. komodoensis* and they occupy diverse habitats throughout Africa, Asia and Australia, with the latter having the highest number of varanid species, apparently due to the lack of indigenous placental predators (Sweet and Pianka, 2007; Pianka and King, 2004).

Varanid lizards belong to the lizard clade Anguimorpha that also contains anguid lizards and helodermatids of which the gila monster is the most well-known species (Pyron et al., 2013, Vidal et al., 2012; Reeder et al., 2015). The closest extant relative of *Varanus* is the Borneo earless monitor *Lanthanotus borneensis*, also included in this study.

Despite their unique appearance as giant terrestrial reptiles and stories that blow their notoriety out of proportion (Auffenberg, 1981), for a long time only scant scientific information was available on the basic ecology, feeding behaviour, anatomy and physiology of varanid lizards. Only relatively recently have varanid lizards started to get attention they deserve.

In the 21st century studies on monitor lizard diets (Guarino, 2001), foraging behaviour (Guarino, 2002), shape variation (Collar et al., 2011; Openshaw and Keogh, 2014), bite force (D'Amore et al., 2011; Fry et al 2009), movement strategies and others began to form the basis of scientific understanding of *Varanus*. However, these studies were largely biased towards easily accessible species that spend most of their adult life on the ground, leaving out cryptic arboreal species.

At the same time, studies demonstrating the uniqueness of varanid oral glands began to appear. Morphological studies by Elazar Kochva revealed similarities between *Heloderma* and *Varanus* glands and put them on continuum with anguid lizard glands, naming them “glands of Gabe” (Kochva, 1978). Further studies revealed that the oral glands of the Komodo dragon are of sophisticated structure with separated protein and mucus parts, structured lumen and a thick membranous cover (Fry et al., 2010a,b).

Morphological as well as molecular evolutionary studies suggested that these glands are homologous not only to *Heloderma* venom glands, but also to the venom glands of front-fanged snakes (Vidal and Hedges, 2005; Fry et al., 2006, 2009b,c 2010a). At the same time it was shown that within the Anguimorpha complex oral glands are relatively uncommon. Only beaded lizards (*Heloderma*) and monitors (*Varanus* and *Lanthanotus*) have independently evolved complex oral secretory apparatus, with others mostly having simple-structured glands.

On the other hand anecdotal data on complications following Komodo dragon bites triggered scientific interest and was at first explained via the potential of pathogenic bacteria unique to the lizards’ oral flora. The origin of this idea dates back to folk myths, however Auffenberg is often quoted as the originator of it. In his monumental 1981 study he reports the presence of *Staphylococcus sp.*, *Providencia sp.*, *Proteus morgani* and *Proteus mirabilis* in mucoid samples from the external gum surface of the upper jaw of two freshly captured “oras” (the local name for Komodo dragon). In the same study, the specimen from the San Diego Zoo possessed none of these bacteria, allowing for the conclusion that oras may depend on frequent reinfestation from carrion to replenish their “weaponised bacteria”. Though Auffenberg admits that *Proteus*-dominated infection could be responsible for the consequences of some of the recorded bites and could potentially play an adaptive role in Komodo dragon ecology, he himself concludes “that the infectious feature of an ora bite is a folk myth” (Auffenberg, 1981).

It was not until very recently, however, that the idea was definitively discarded, since Komodo dragon oral flora turned out to be not at all dissimilar from that of any other carnivorous animal (Goldstein et al., 2013).

By that time, multiple phylogenetic studies had reassessed morphology-based classifications of squamate reptiles and positioned anguimorph lizards in a clade with Iguania and Serpents (Vidal and Hedges 2005, 2009; Fry et al., 2006). The clade was given the name “Toxicofera” to reflect the presence of toxin-secreting oral glands as one of the defining synapomorphies of the group. Genetic studies further corroborated close evolutionary relationships between snakes and anguimorph lizards, showing that Anguimorpha oral glands express proteins homologous to toxins found in the venom of front-fanged snakes (Fry et al., 2010b).

Such toxin groups as kallikrein, CRiSP, natriuretic peptides and phospholipase A2 type III have been recovered from varanid lizard venom gland transcriptomes (Fry et al., 2006, 2010b), some of which might be responsible for the hypotensive effect of the crude venom intravenous injections in rats (Fry et al., 2006).

Recent reports of human envenomations by monitor lizards seem to be inconclusive as to whether their bite possesses any threat to human health other than pure mechanical damage. Vikrant and Verma report a lethal bite by *Varanus bengalensis* that induced local pain, blood loss, as well as nausea, diaphoresis, dizziness, and breathlessness in victim and eventually led to an acute kidney injury and cardiac arrest (Vikrant and Verma, 2014); however, the actual culprit responsible for this bite has been questioned by clinical toxinologists (Weinstein and White, 2015). In contrast, Ducey et al. report a bite by a juvenile Komodo Dragon that led to faintness, prolonged bleeding and transient hypotension that the authors attributed to a vasovagal reaction (Ducey et al., 2016). Eventually, the only lasting effect of the bite was numbness in the finger where the monitor’s tooth had been retained after the bite. Obviously, these two incidents cannot be properly compared due to the species differences as well as age of the lizards. Anecdotally, a great many varanid lizard bites to biologists, zookeepers and amateur reptile enthusiasts have resulted in little that could be attributed to the action of toxins; however, some bite victims do report burning sensations and inflammation disproportionate the the mechanical damage inflicted (Sweet 2016).

When compared with much better studied gila monster bites, one clear aspect of difference becomes apparent: a helodermatid lizard will stay attached, continuously

chewing in more venom into the bite site, while a monitor lizard is unlikely to hold onto something that is not a food item. This obviously should lead to difference in amount of oral fluids administered to the victim. Interestingly, while feeding on large prey items, varanid lizards seem to have a tendency to shake it violently, prompting subjugation (Loop, 1974).

Given that both varanid and helodermatid venom glands are the most developed within the Anquimorpha, and that a number of studies have revealed remarkable complexity and medical potential of *Heloderma* venom (Ma et al., 2011; Furman, 2012; Irwin, 2012), it became a point of interest to study the venom system of varanid lizards in detail.

The evolution of a complex venom system is likely only possible under certain contingent circumstances – i.e., when both environmental conditions select for it and a species' overall evolutionary trajectory facilitates it. For example, in Iguania the incipient venom glands never developed any significant complexity probably due to the mainly insectivorous/herbivorous nature of these lizards. In addition, in at least some cases when animal develops a method of subduing prey that renders the venom system excessive, or switches to defenceless prey, the system degrades – as seen in numerous snake species (Fry et al., 2012). The cost of venom production is presumably high enough to justify the presence of active secretory and delivery apparatuses only when it is contributing to a functional role in the life of the organism (Morgenstern and King, 2013).

The fact that varanid lizards possess highly developed oral glands suggests that those glands in one way or another play an important role in their life. They may have functions different to that of aiding in subjugation of prey: they may help in digestion, providing specific enzymes, or aid in maintaining oral health by secreting antimicrobial agents. And even if they are indeed toxin-secreting glands, those toxins might not be necessarily for prey capture, they may be equally important in defence against predators (Arbuckle, 2009).

It goes without saying that no varanid lizard would rely entirely on its venom (if it indeed is venomous) for subduing its prey, and the role of this hypothetical venom would be remarkably different to that which it plays in the lives of most venomous snakes. The remarkable speed at which monitors charge at food items, their sharp teeth and claws all seem to guarantee a successful meal. However, no predator will have a 100% success rate in hunting, so a system that can increase this rate even by a margin will be selected for – as long as its benefits outweigh its costs.

All monitor lizards share the same morphological features; their body shape scales allometrically with only minor exceptions that we mention below (Collar et al., 2011; Openshaw and Keogh, 2014; Thompson et al., 2009). Given how plastic the body plan generally is in reptiles and how easily it is shaped by changes in habitat and diet, it does not seem to be a coincidence that all varanid lizards rely on more or less the same strategy for securing a meal – the strategy appears to work remarkably well.

The strategy in question seems to be “eat everything you can grab”. That might be the reason why varanid lizards tend to occupy any carnivorous niche the conditions allow for and only cold climate and carnivorous placental mammals can hinder their progress. Some monitors function as an entire predator guild, undergoing an ontogenetic niche shift from being strictly arboreal in juvenile stage all the way to strictly terrestrial when they are adults (Purwandana et al., 2016). At the same time, monitor lizards also scavenge a lot – from carrion to even fruit in some species (Pianka and King, 2004). Both of these strategies benefit from monitor’s stamina and chemosensory apparatus as well as “bite and tear” build. That means that habitat will affect monitor’s diet in two ways – directly, by providing prey items of specific type and indirectly, by putting constraints on size.

Given all of that, if oral secretions are evolutionarily important for varanid lizards we would expect to see some tripartite relationship between size, habitat and oral secretion composition.

A previous study (Fry et al., 2010b) revealed the presence of kallikrein transcripts in venom gland transcriptome of varanid lizards. Toxicoferan venom kallikreins are known for inducing fibrinogen depletion in the prey organism and thus prompting prolonged bleeding (see Chapter 1 for more information on toxic functions of kallikreins). It is not unforeseeable that, if given a chance, a predator would benefit from inducing blood loss or altering blood pressure in its prey, for this will increase the chance of successful subjugation by weakening the prey. However, in the case of some monitor lizards, in particular the big species like the lace monitor (*Varanus varius*) or Komodo dragon, this type of toxic action might be beneficial even if a prey manages to escape the intial attack but succumbs to blood loss in the aftermath. Big monitors are known for relying on scavenging to the point that for some of them carrion can be considered a staple food item (Guarino, 2001), and in most areas these species inhabit, they are one of the most if not the most prominent scavengers (Auffenberg, 1981; Pascoe et al., 2011; Pianka and King, 2004). At the same time they are known to have wide foraging areas (Guarino, 2002) and

to be capable of tracking food items (especially carrion) from miles away due to their extremely refined chemosensory apparatus (Auffenberg, 1981; Pianka and King, 2004; Guarino, 2002). Given all of that and the tendency to bite everything that looks like a prey item, a scenario in which a victim escapes but subsequently succumbs to blood loss will be beneficial for the monitor lizard.

Of course, all of that does not mean that aiding in scavenging is the primary role of venom in big varanid lizards, but it helps to illustrate the point that even slow-acting venom will still have a positive impact on the monitor's wellbeing, playing into both of the feeding strategies. In order to be selected for and maintained, this hypothetical venom system would not have to be a nuclear arsenal aimed to kill the victim on instant, it would only have to give the lizards' hunting techniques a certain edge (Jackson and Fry, 2016).

In the present study we used a number of proteomic techniques to reveal the complexity and content of oral secretion from representative monitor species (Table 1). In addition we screened for a key bioactivities that might be reflective of the role these secretions play in the dietary ecology of monitor lizards.

Table 1. Species studied.

Code	Species	Clade	Habitat	Diet	Total Length
ACA	*Varanus acanthurus*	*Odatria*	Rocky areas throughout northern Australia, except eastern Queensland.	Invertebrates like grasshoppers and beetles, also takes skinks and geckoes	60 to 78 cm
BAR	*Varanus baritji*	*Odatria*	Rocky outcrops and stony hills with wild vegetation in the extreme parts of Northern Territory from the Adelaide River district east to near Borroloola.	The species is poorly studied, but is closely related to *V. acanthurus* and is likely to be similar in feeding behaviour	60 to 72 cm.
GIG	*Varanus giganteus*	*gouldii*	Arid inland from western Queensland to the coastal areas of Western Australia.	Feeds on anything it can overpower: reptiles, birds and mammals	150 to 250 cm.
GIL	*Varanus gilleni*	*Odatria*	Dried parts of inland Australia, mostly in *Acacia*, *Casuarina* and *Eucapyptus* woodlands.	Wide range of prey items: arthropods, lizards, bird eggs and small mammals	34 to 40 cm.
GRI	*Varanus griseus*	African	Sand dunes, clay steppes, riverbeds and other mostly arid and semiarid areas from Rio De Oro throughout the Sahara desert, to Egypt and northern Sudan, Arabian peninsula, central Asian deserts, Pakistan and northwestern India	Mainly rodents, snakes and agamid lizards. Large arthropods are hunted intensively only when vertebrate prey is scarce. Egs and chicks are frequently eaten.	120 to 150 cm.
JOB	*Varanus jobiensis*	Indo-Asian B	Mixed alluvium and hill forests of New Guinea. Terrestrial and arboreal, seeks refuge by climbing trees.	Tarantulas, insects, frogs and reptile eggs. Most prey items are insects. Can also eat fish or shrimps.	up to 120 cm.

KOM	*Varanus komodoensis*	*varius*	Monsoon forests, grasslands, savannas and mangrove forests of Komodo, Rinca, Gili Dasami, Flores and other islands in Indonesia. Hatchlings are arboreal, juveniles and subadults are both arboreal and terrestrial. Adults are strictly terrestrial.	Juveniles are active predators, adults ambush their prey. Diet includes insects, bird and sea turtle eggs, carrion and small to large vertebrates: lizards, snakes, rodents, monkeys, wild boars, deer and water buffalo.	up to 300 cm.
MEL	*Varanus melinus*	Indo-Asian B	Sawmps and tropical forests of Sula islands	Insects, birds's eggs, tree frogs, potentially prawns and fish.	120-150 cm
MER	*Varanus mertensi*	*gouldii*	Tropical northern Australia near water, from Kimberly to Cape York Peninsula.	Water invertebrates, fish, frogs, turtle eggs, lizards and small mammals	110 to 130 cm.
MIT	*Varanus mitchelli*	*Odatria*	Northern Australia, from Kimberley region to extreme north-western Queensland in habitats with permanent or semi-permanent water.	Fish, frogs, invertebrates, crabs, reptile and their eggs, small mammals and birds	60 to 96 cm.
PRA	*Varanus prasinus*	Indo-Asian B	Mostly highly arboreal. Monsoon, rain and palm forests, mangrove swamps and coca plantations of mainland New Guinea.	Mostly insects. Occasionally spiders and rodents.	up to 85 cm
RUB	*Varanus panoptes rubidus*	*gouldii*	Coastal and inland Western Australia in a variety of habitats with the preference for sandy or stony soils	Preys on virtually anything it can overpower: insects, small mammals, reptiles, frogs, eggs	120 to 160 cm.
SAL	*Varanus salvadorii*	*varius*	Delta estuaries of big rivers in southern New Guinea. Arboreal and terrestrial.	Little known. Belived to eat deer, pigs (hauled into canopy and eaten there), insects, lizards, birds, mammals. No precise information.	up to 250 cm.

SCA	*Varanus scalaris*	*Odatria*	Mostly woodland, as well as subhumid and humid rainforest habitats from Kimberley region to the far north-eastern coastal Queensland.	Invertebrates, small skinks, geckoes, frogs, bird eggs and fledglings	55 to 60 cm.
TRI	*Varanus tristis*	*Odatria*	Wide range of habitats all over Australia except some parts of south and south east.	Lizards, invertebrates, birds and their eggs	60 to 80 cm.
VAR	*Varanus varius*	*varius*	Forested areas from the north-eastern coast of Queensland through coastal and inland New South Wales to Victoria and South Australia	Preys on everything it can overpower	150 to 200 cm.

Materials and Methods

Milking

Venoms and tissues were collected by A/Prof Fry. Specimens were milked into sterile jars. In order to remove mucous, all samples were filtered through 0.2 micron syringe filters prior to lyophilisation.

1D gel electrophoresis

In order to establish the proteomics variation, 1D gradient gels were run under both reducing and non-reducing conditions using the manufacturer (BioRad) protocol.

Gels were prepared as follows: 0.05 mL Milli-Q H2O, 2,5 mL 30% acrylamide mix, 1.5 mL 1.0 M Tris-HCl, pH 8.45, 0.480 glycerol, 20 µL 10% APS, 2 µL TEMED (spreading gel); 0.760 mL Milli-Q H2O, 0.760 mL 30% acrylamide mix, 0.760 mL 1.0 M Tris-HCl, pH 8.45, 15 µL 10% APS, 2 µL TEMED (spacer gel); 1.560 mL Milli-Q H2O, 0.340 mL 30% acrylamide mix, 0.630 mL 1.0 M Tris-HCl, pH 8.45, 15 µL 10% APS, 2 µL TEMED (stacking gel).

Spreading gel was cast first. After it was set the spacer gel was slowly layered atop of it, and after spacer gel was set the stacking gel was layered atop of it. Running buffers were:

0.2 M Tris-HCl, pH 8,9 (anode buffer); 0.1 M Tris-tricine-HCl pH 8,45. The gels were run at 100 V for three hours at room temperature.

30 µg of venom was reconstituted in Tricine loading buffer (Bio-Rad) with 10 mM DTT added to provide reduce conditions.

Gels were stained overnight with colloidal Coomassie brilliant blue G250 (34% methanol, 3% phosphoric acid, 170 g/L ammonium sulphate, 1 g/L Coomassie blue G250). After the staining was complete, water was used to remove excess of the dye.

2D gel electrophoresis

In order to further investigate the proteomics variation, particularly that of isoelectric variation, 2D gels will be run using protocols previously optimised in the Fry lab (Ali et al., 2013, Low et al., 2013). 0.3 mg (7 cm gels) and 2 mg (17 cm gel) of venom sample were solubilized in 125 µL (7 cm gels) and 1 ml (17 cm gels) of rehydration buffer (8 M urea, 100 mM DTT, 4% CHAPS, and 0.5% ampholytes (Biolytes pH 3–10, Bio-Rad Lab)) with 0.01% bromophenol blue. The sample was mixed with shaking and centrifuged for 5 min at 4 °C, 14 000 rpm. This was done to remove any insoluble material.

The supernatant was loaded onto IEF strips (Bio-Rad ReadyStrip, non-linear pH 3–10, 7 cm and 17 cm IPG) and left overnight for passive rehydration. Protein focusing was achieved via PROTEAN i12 IEF CELL (Bio-Rad Lab). The IEF running conditions were as follows: 100 V for 1 h, 500 V for 1 h, 1000 V for 1 h and 8000 V until 98,400 V/h. Actual current in the final step of the run varied in accordance to resistance. To each strip a constant current of 50 µA was applied. After the run IPG strips were incubated for 10 min in a reducing equilibration buffer (50 mM Tris–HCl, pH 8.8, 6 M urea, 2% SDS, 30% glycerol, 2% DTT) to reduce cysteine bonds. To alkylate reduced bonds IPG strips were further incubated for 20 min in an alkylating equilibration buffer (50 mM Tris–HCl, pH 8.8, 6 M urea, 2% SDS, 30% glycerol, 2.5% iodoacetamide). After rinse with SDS-PAGE running buffer, IPG strips were positioned on top of 12% polyacrylamide gels (Protean-II Plus, 18 × 20 cm, Bio-Rad Lab) using 0.5% agarose.

Gels were run at 4 °C with a current of 10 mA/per gel for 20 min followed by 20 mA/per gel for the rest of the run until the bromophenol dye front was within 0.5 cm of the base of the gel. After the run, gels were briefly washed with water and stained with 0.2% colloidal Coomassie brilliant blue G250 overnight. Water was used to remove the excess of the dye after staining was complete.

Visible spots were subsequently picked from gels and digested overnight at 37 °C with the use of sequencing grade trypsin (Sigma-Aldrich).

Afterwards gel spots were washed with MiliQ water, destained (40 mM NH4CO3/50% acetonitrile (ACN)) and dehydrated (100% ACN); rehydration occurs in 10 µL of 20 µg/ml TPCK trypsin with subsequent incubation at 37 °C overnight. To elute peptides following solutions were used per each spot: 20 µL of 1% formic acid (FA), followed by 20 µL of 5% ACN/0.5% FA. Collected peptides were put into MS vials and subjected to LC–MS/MS analysis.

Shotgun sequencing

In order to identify low molecular weight peptides that do not resolve well on 1D or 2D gels, shotgun sequencing was used. 3 µg of crude venom sample was dissolved in 50 µL of 100 mM ammonium carbonate to reduce and alkylate cysteine bonds with subsequent addition of 50 µL of 2% iodoethanol/0.5% triethylphosphine in acetonitrile. The sample was afterwards resuspended in 20 µL of 40 mM ammonium bicarbonate, before overnight incubation (at 37 °C) with 750 ng of sequencing grade trypsin (Sigma-Aldrich). To stop digestion 1 µL of concentrated formic acid was added to each of the samples. Samples were lyophilised then resuspended in 20 µL of 5% ACN/0.5% FA, put into MS vials and subjected to LC–MS/MS analysis.

LC–MS/MS

In order to identify the toxin types present, digested gel spots and digested whole venom (shotgun) samples were processed using an Agilent Zorbax stable bond C18 column (2.1 mm by 100 mm, 1.8 µm particle size, 300 Å pore size) at a flow rate of 400 µL per minute and a gradient of 1–40% solvent B (90% acetonitrile, 0.1% formic acid) in 0.1% formic acid over 15 minutes or 4 minutes for shotgun samples and 2D-gel spots respectively on a Shimadzu Nexera UHPLC coupled with an AB SCIEX 5600 Triple TOF mass spectrometer. MS2 spectra are acquired at a rate of 20 scans per second with a cycle time of 2.3 seconds and optimised for high resolution. Precursor ions were selected between 80 and 1800 m/z with a charge state of 2–5 and of an intensity of at least 120 counts per second with a precursor selection window of 1.5 Da. The isotopes within 2 Da were excluded for MS2. MS2 spectra were searched against known translated transcriptome libraries or UniProt database with Proteinpilot v4.0 (ABSciex) using a thorough identification search, specifying iodoacetamide as an alkylation method, trypsin digestion

and allowing for biological and chemical modifications (ethanolyl C or deamidated N in particular) and amino acid substitutions, including artefacts induced by the preparation or analysis processes. This was done to maximize the identification of protein sequences. Spectra were inspected manually to eliminate false positives.

Bioactivity studies

Phsopholipase A2 activity

We assessed the continuous phospholipase A2 (PLA2) activity of the venoms using a fluorescence substrate assay (EnzChek® Phospholipase A2 Assay Kit, ThermoFisher Scientific). A working stock solution of freeze dried venom was reconstituted in a buffer containing 50% MilliQ/50% glycerol (99.9%, Sigma) at a 1:1 ratio to preserve enzymatic activity and reduce enzyme degradation with the final venom concentration of 0.1 mg/ml, and then stored at -20°C. Venom solution (0.1 µg in dry venom weight) was brought up to 12.5 µl in 1X PLA 2 reaction buffer (250 mM Tris-HCL, 500 mM NaCl, 5 mM CaCl 2, pH 8.9) and plated out in triplicates on a 384 well plate. Triplicates were measured by adding 12.5 µL quenched 1mM EnzChek® Phospholipase A2 substrate per well (total volume 25µL/well) over 100 cycles at an excitation of 485 nm and emission of 520 nm, using a Fluoroskan Ascent (ThermoFisher Scientific). The negative control consisted of PLA 2 reaction buffer and substrate only.

Matrix mettaloprotease and kallikrein activity

A working stock solution of freeze dried venom was reconstituted in a buffer containing 50% MilliQ/50% glycerol (>99.9%, Sigma) at a 1:1 ratio to preserve enzymatic activity and reduce enzyme degradation with the final venom concentration of 0.1 mg/ml, and then stored at -20°C. Venom solutions (1 µg in dry venom weight) were plated in triplicates on a 384 well plate and measured by adding 90µL quenched fluorescent substrate per well (total volume 100µL/well; 10µL/5mL enzyme buffer - 150 mM NaCl and 50 mM Tri-HCl pH 7.3, Fluorogenic Peptide Substrate, R & D systems, Cat#ES001 & ES011, Minneapolis, Minnesota). Fluorescence was monitored (Cat#ES001 excitation at 320 nm and emission at 405 nm; Cat#ES011 excitation at 390 nm and emission at 460 nm) over 400 min or until activity has ceased.

Rat ileum organ bath testings

The rat ileum muscle preparations were isolated from adult male rats. The rats were killed by CO2 asphyxiation. The isolated preparations were individually mounted in 15 ml parallel

organ baths containing a Krebs solution with the following constituents (mM): NaCl, 118.4; KCl, 4.7; MgSO4, 1.2; KH2PO4, 1.2; CaCl2, 2.5; NaHCO3, 25 and glucose, 11.1). The Krebs solution was continuously bubbled with carbogen (95% O2 and 5% CO2) to maintain a pH between 7.2-7.4 at a temperature of 32-34°C. A resting tension between 1 and 3g was found to be the optimal starting baseline.

Stimulation was performed with 200 µg/ml of crude venom; Milli-Q H2O (170 µl) was used as a control. The venom was left in the organ bath with the preparation for approximately 30 minutes or until the twitch response was completely abolished.

Results and Discussion

1D PAGE results (fig. 1-3) revealed differential complexity of oral secretion within the genus. Compared to the published results on *Heloderma* venom, which shows little variation across the genus (cf: Chapter 2), this data is suggestive of the differential role oral secretion plays in different varanid species and that it is evolving under selection pressure.

2D PAGE results (fig. 4-8) further elucidated the clear differences in venom complexity across the genus. *V. mertensi* (fig. 5) has the most streamlined profile, with essentially a single acidic protein group of approximately 40 kDa, whereas the most complex profile recovered is that of *V. varius* (fig. 7) with various protein groups dispersed all across molecular weight range and pI gradient. All other species profiles form a continuum of complexity between the two. The 40 kDa acidic protein group is present in all specimens with the sole exception of *V. griseus* (fig. 7) which exhibits a 2D profile with only a few low-molecular weight protein groups. Several profiles exhibit a specific pattern usually indicative of different isoforms of the same protein (most likely due to difference in glycosylation), and this is especially notable in the *V. salvadorii* profile (fig. 7).

MS-analysis of excised gel spots revealed that while different, all venoms but that of *V. griseus* are mainly composed of kallikrein and CRiSP proteins – the approximately 40 kDa spot in the acidic region. This finding was further corroborated by crude venom MS that portrayed varanid oral secretion as largely based on kallikrein, CRiSP, lysosomal acid lipase, phospholipase A2 and natriuretic peptides, with individual species and phylogenetic groups having their unique components (fig. 9). Altogether more than 20 different protein classes were recovered in oral secretion of studied species, all of which have the potential to play a functional role in defence or feeding (including predation and scavenging).

CRiSP toxins are reported to have various channel-blocking activities resulting in smooth muscle paralysis and hypothermia. Toxicoferan venom kallikreins increase vascular permeability and reduce blood pressure as well as stimulating inflammation and blood loss through fibrinogenolysis. Toxic natriuretic peptides also induce hypotension, while PLA2 block platelet aggregation (Fry, 2015).

On the other hand, chitinase and chitotriosidase have a well-defined function of cleaving chitin, which might potentially play a role in digestion of arthropods as well as provide defence against chitin-coated pathogens such as *Plasmodium* or *Cryptococcus* (Hamid et al., 2013).

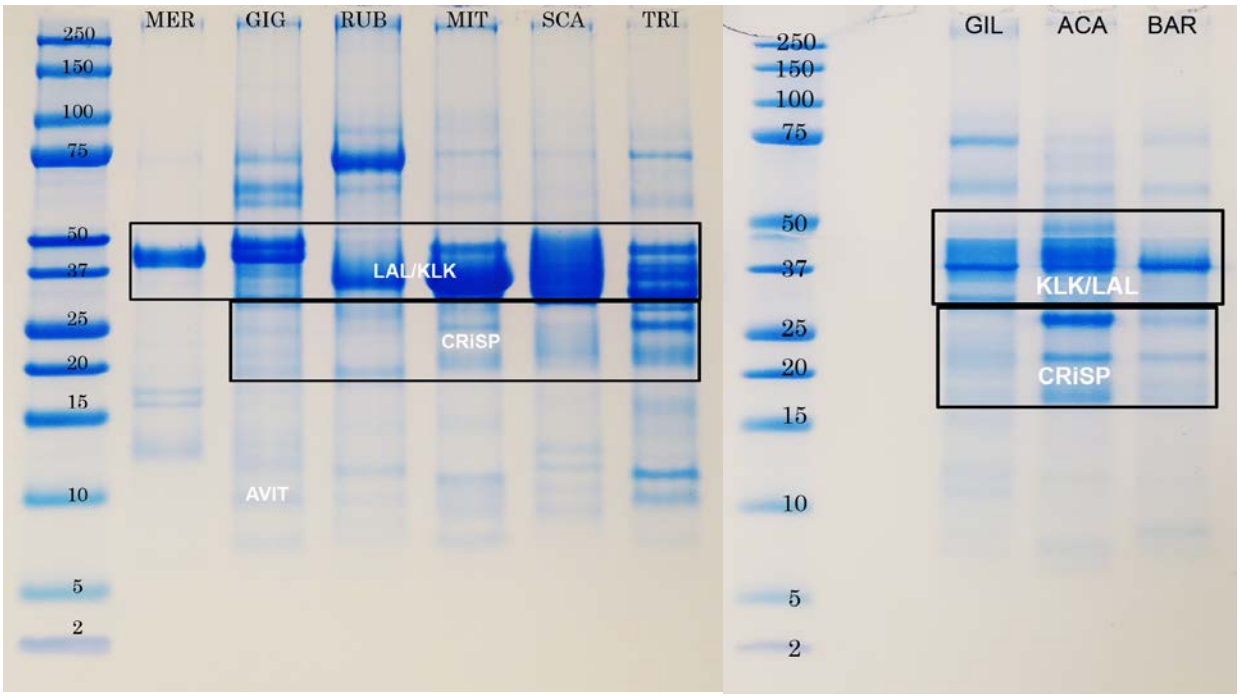


Figure 1. 1D PAGE reduced results. MER – V. mertensi, GIG – V. giganteus, RUB – V. panoptes rubidus, MIT – V. mitcheli, SCA – V. scalaris, TRI – V. tristis, GIL – V. gilleni, ACA – V. acanthurus, BAR – V. baritji. LAL – Lysosomal acid lipase, KLK – kallikrein.

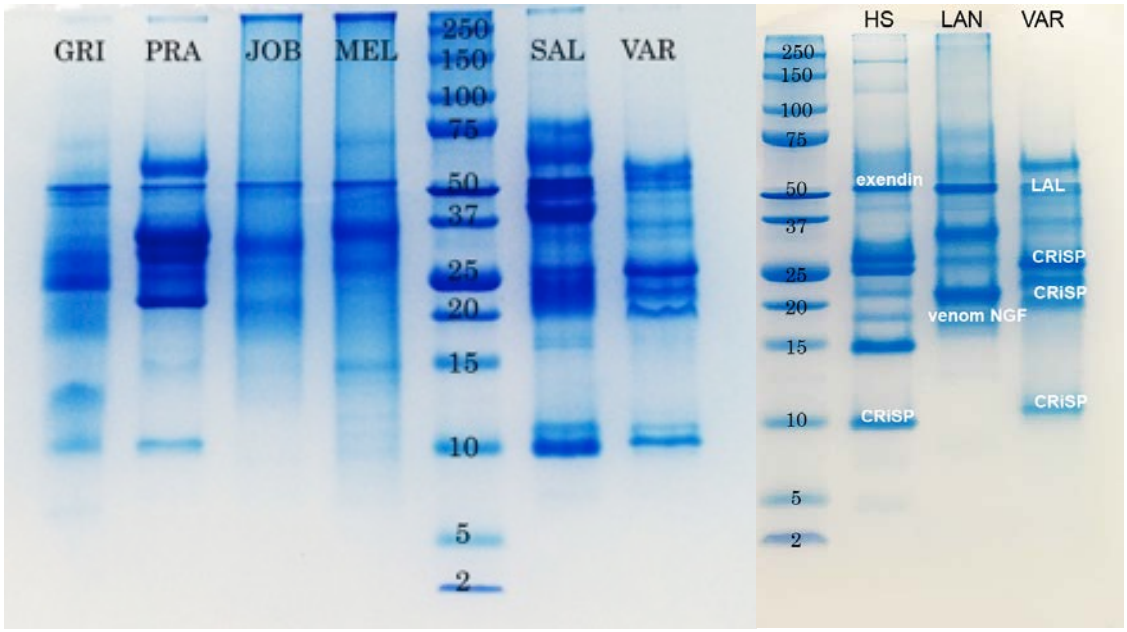


Figure 2. 1D PAGE reduced results. GRI – V. griseus, PRA – V. prasinus, JOB – V. jobiensis, MEL – V. melinus, SAL – V. salvadorii, VAR – V. varius, HS – H. suspectum, LAN – L. borneensis. LAL – Lysosomal acid lipase, KLK – kallikrein.(Notice that positioning of exendin in HS might be an artefact, for it is a small peptide, though it is not unknown for the proteins to have modifications that would increase its MW almost tenfold).

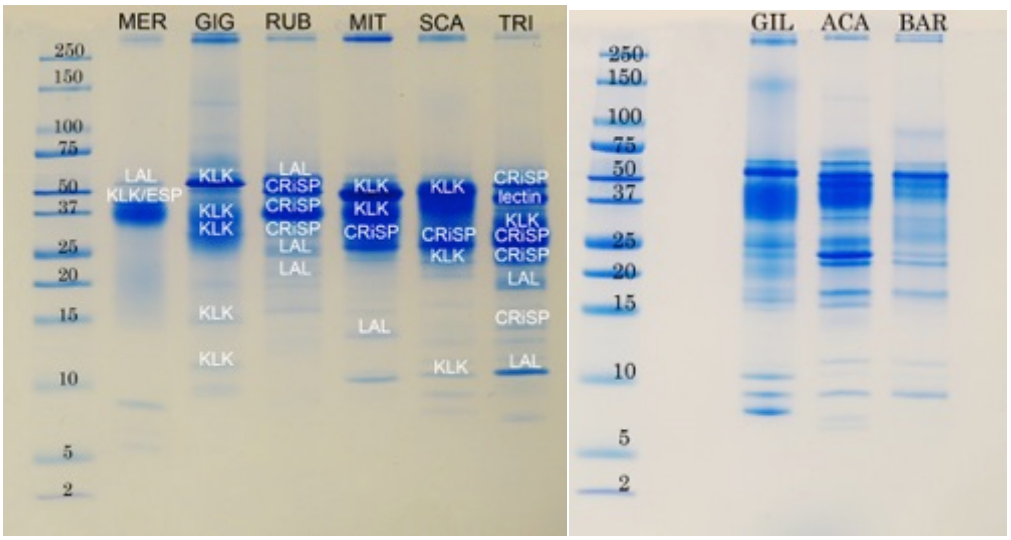


Figure 3. 1D PAGE non-reduced results. MER – V. mertensi, GIG – V. giganteus, RUB – V. panoptes rubidus, MIT – V. mitcheli, SCA – V. scalaris, TRI – V. tristis, GIL – V. gilleni, ACA – V. acanthurus, BAR – V. baritji. LAL – Lysosomal acid lipase, KLK – kallikrein.

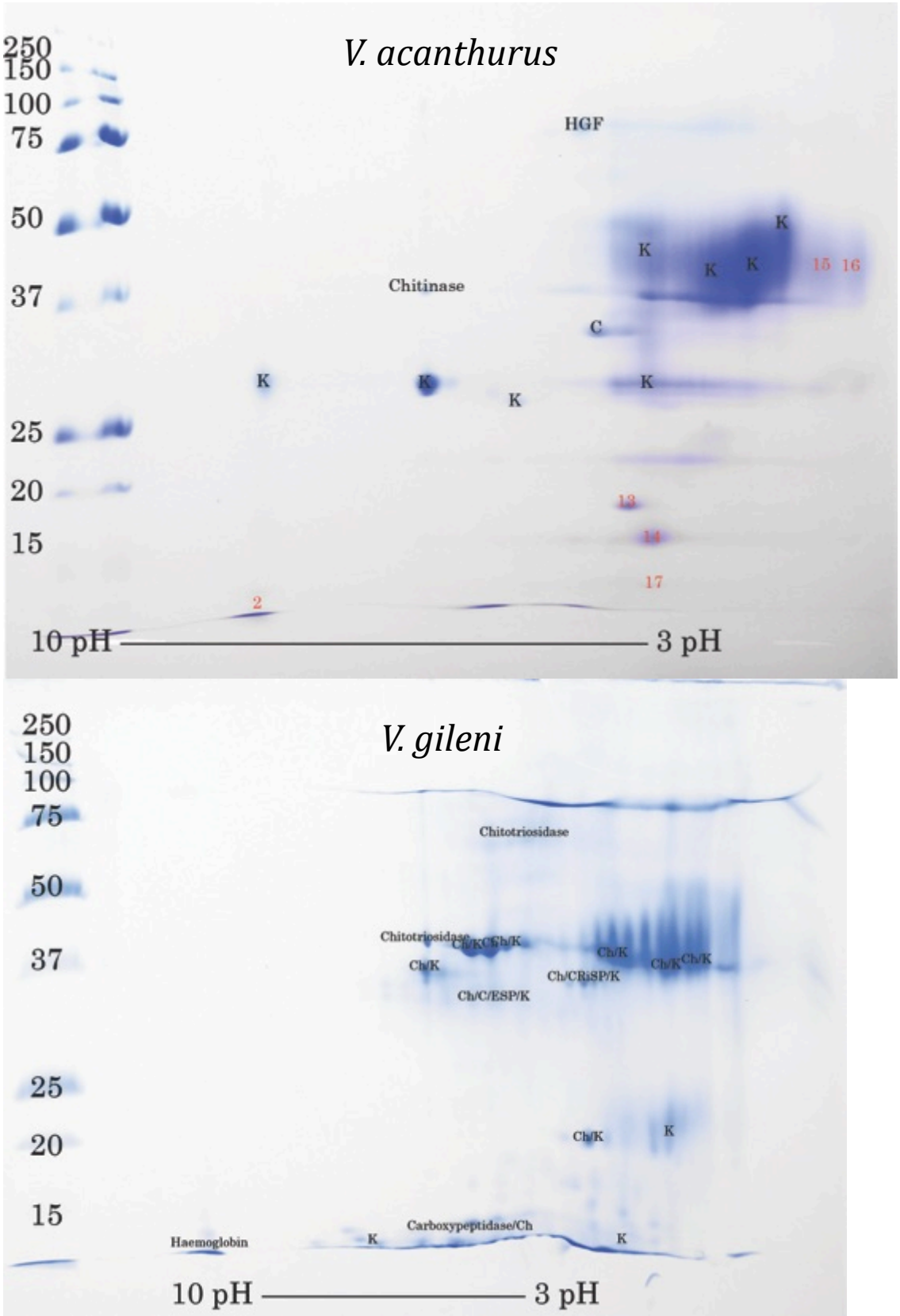
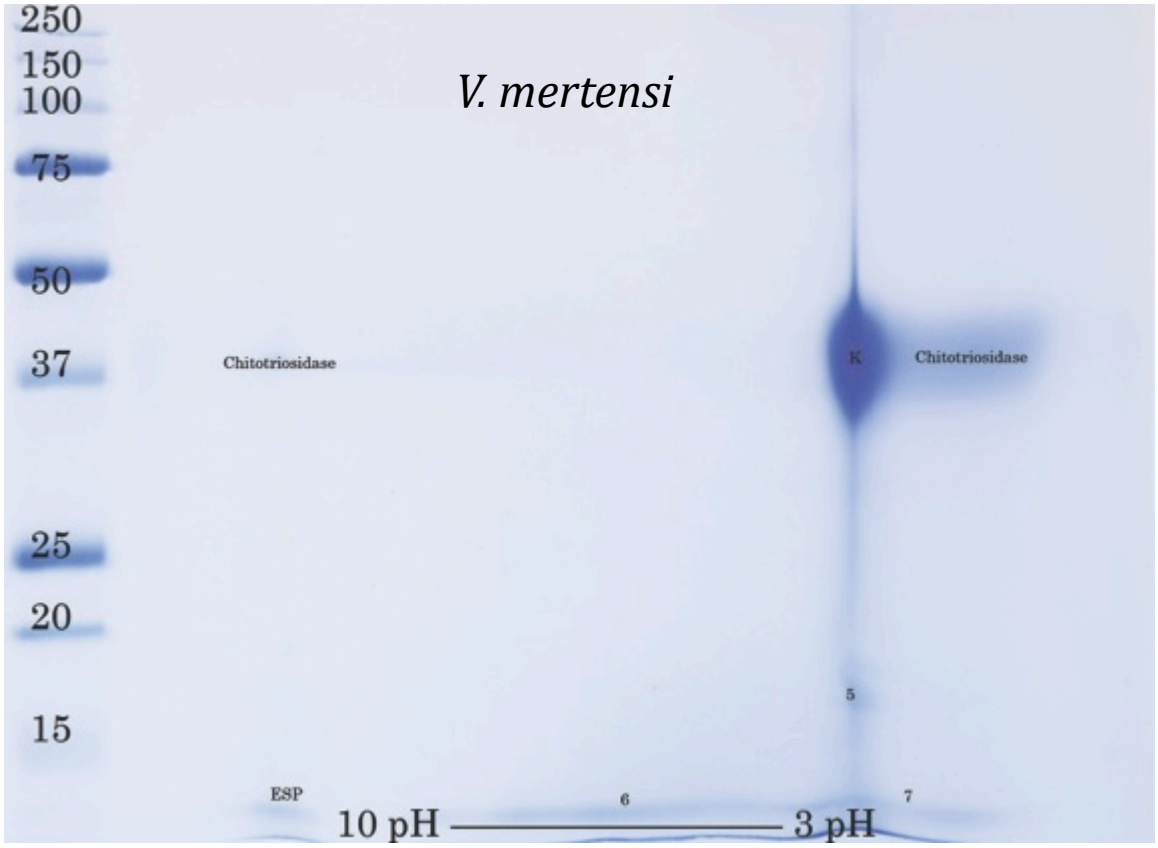


Figure 4. 2D PAGE results. C – CRiSP, L – Lysosomal acid lipase, K – kallikrein, HGF – Hepatocyte growth factor, A – AVIT, Ch – Chitotriosidase



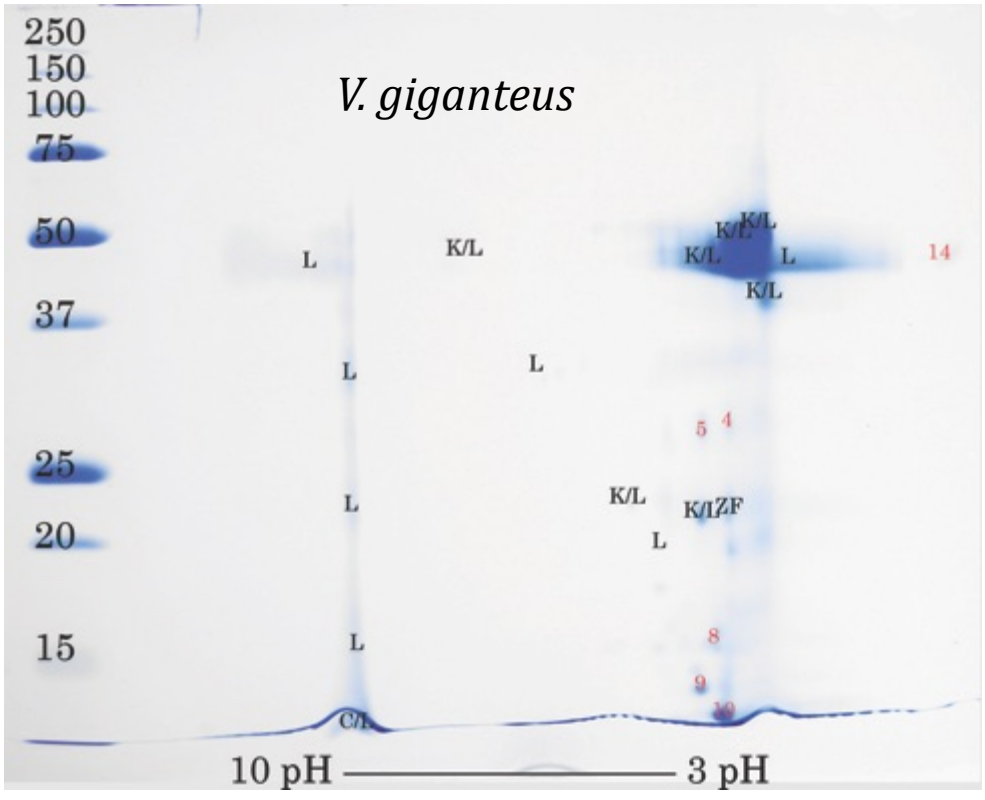


Figure 5. 2D PAGE results. C – CRiSP, L – Lysosomal acid lipase, K – kallikrein, HGF – Hepatocyte growth factor, A – AVIT, Ch – Chitotriosidase

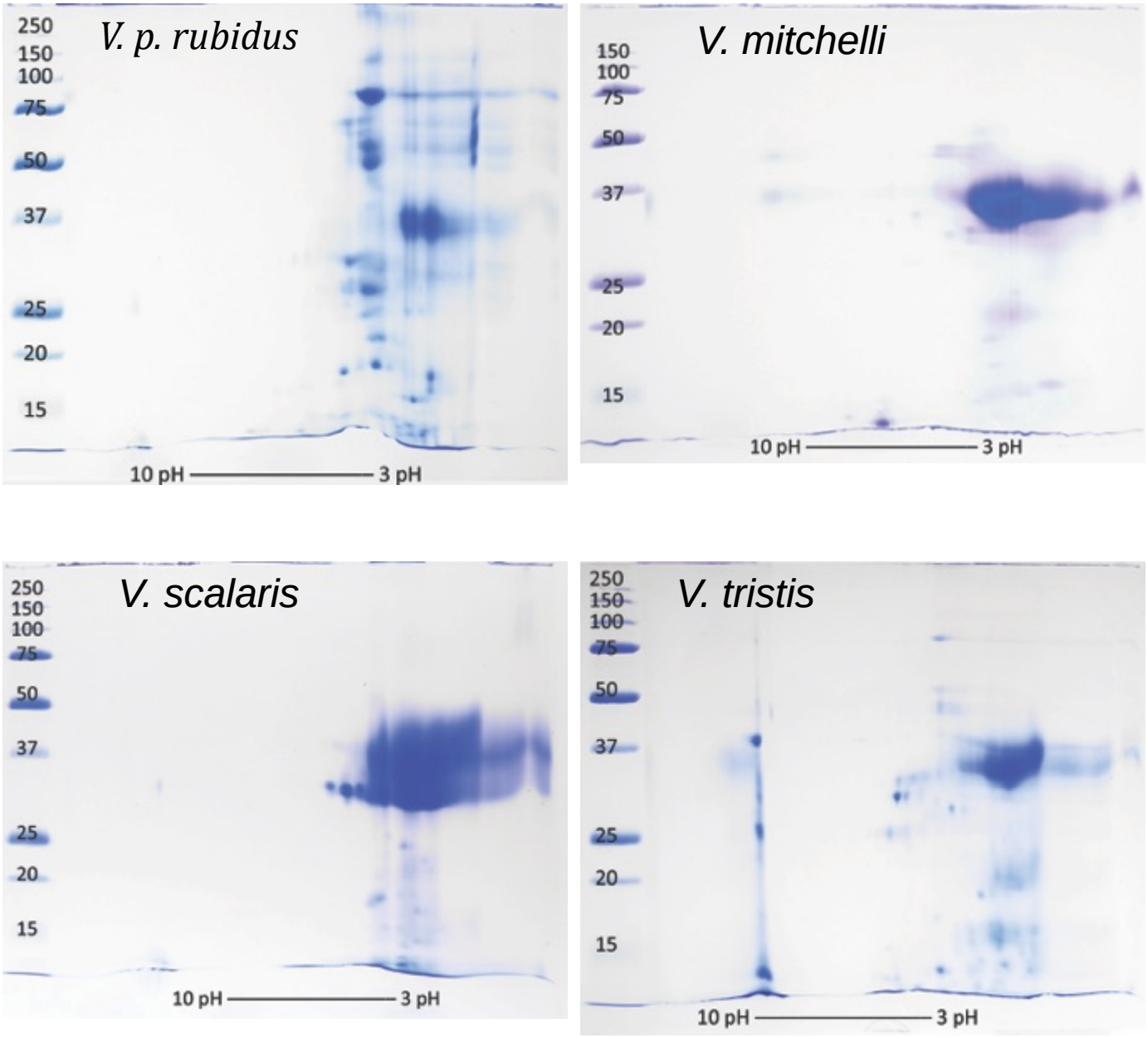


Figure 6. 2D PAGE results.

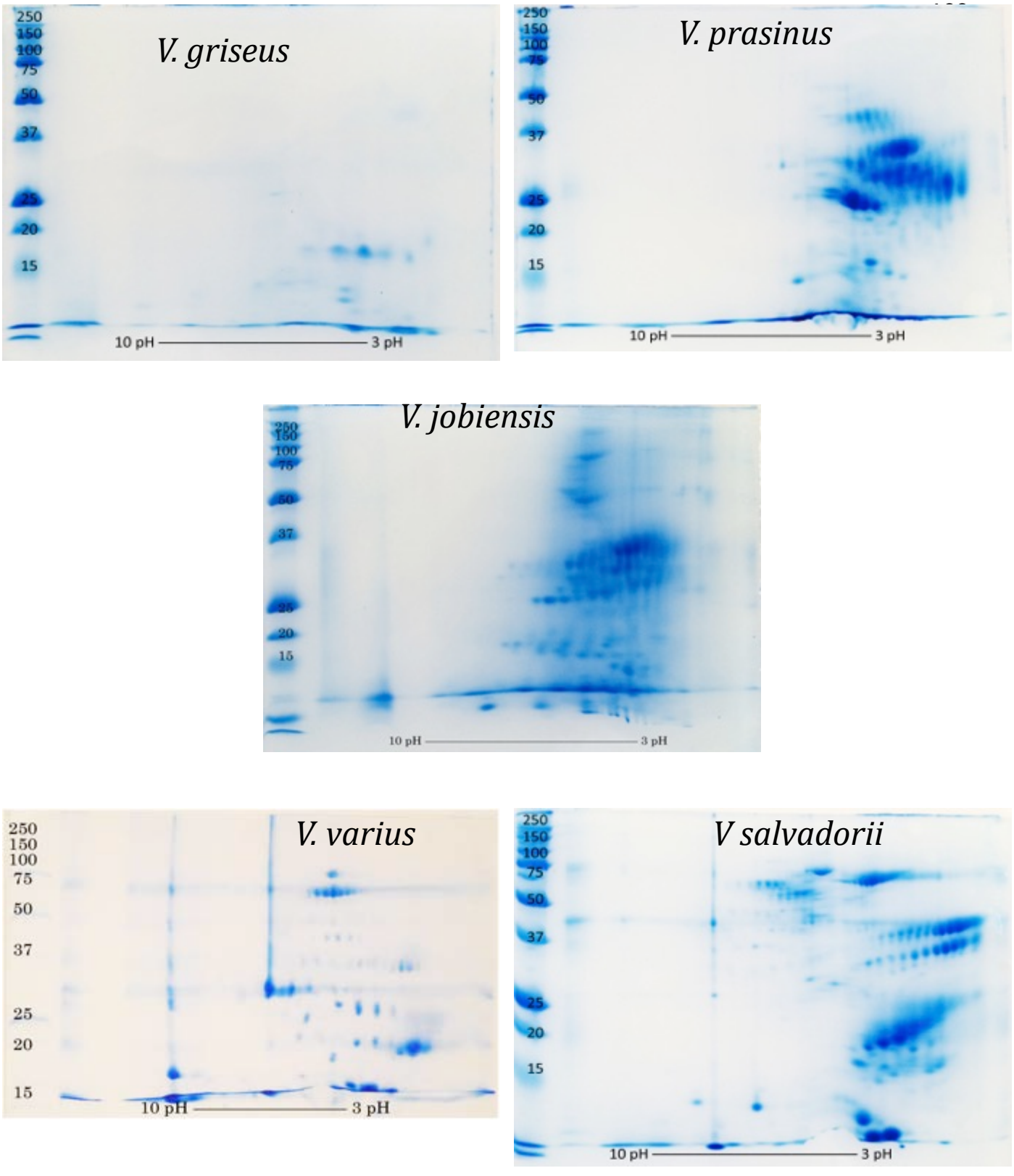


Figure 7. 2D PAGE results.

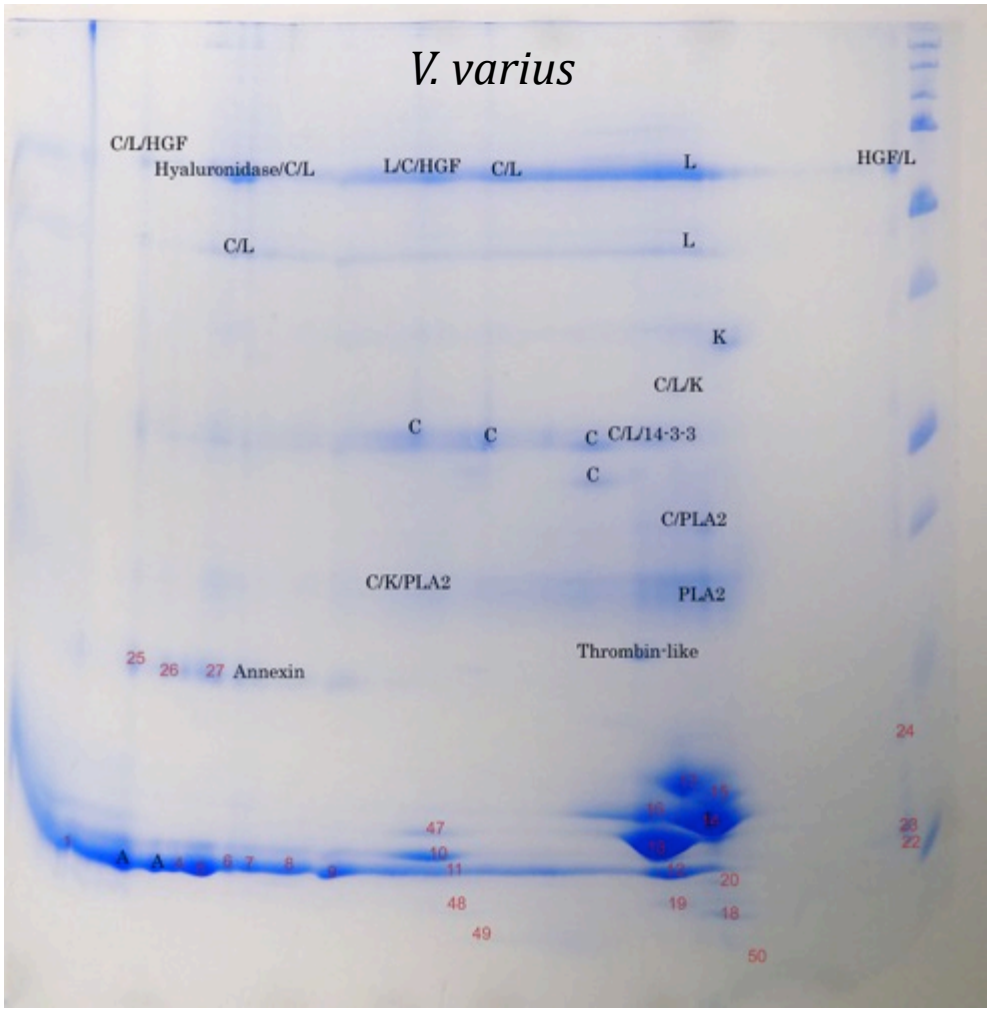


Figure 8. 17cm 2D PAGE results. C – CRiSP, L – Lysosomal acid lipase, K – kallikrein, HGF – Hepatocyte growth factor, A – AVIT, Ch – Chitotriosidase, PLA2 – phsopholipase A2 type III

	14-3-3	AVIT	Beta-cardiotoxin	C-type lectin domain family	Carboxypeptidase	Chitinase	Chitotriosidase	Cholechstoxin	CRiSP	Cytotoxin homolog	Disintegrin domain-containing protein	ESP	Exendin	Gilatoxin
Lanthonotus borneensis										1	1			
Varanus griseus		1	1						1					
Varanus prasinus									1	1	1			
Varanus jobiensis		1							1	1		1	1	1
Varanus melinus		1	1						1				1	1
Varanus salvadorii		1							1		1			
Varanus komodoensis									1			1		
Varanus varius	2	1, 2						1	1, 2			1		
Varanus mertensi							2					2		
Varanus giganteus									1, 2			1		
Varanus rubidus									1			1		
Varanus gilleni					2	1, 2	2		1, 2		1	1, 2		
Varanus acanthurus						2	2		1, 2					
Varanus baritji						1	2		1					
Varanus tristis						1			1					
Varanus scalaris						1			1					
Varanus mitcheli				1					1			1		

1 Components of oral secretion identified with crude venom MS
2 PAGE
3 activity
Lanthonotus borneensis
V. griseus (African monitor clade)
members of the Indo-Asian B monitor clade
members of the *varius* clade
members of the *gouldii* clade
members of the Odatria clade

	Hepatocyte growth factor-like protein	Hyaluronidase	Kallikrein	L-amino-acid oxidase	Lectin-like protein	Long neurotoxin	Lysosomal acid lipase	Metalloproteinase	Natriuretic peptide	Nerve growth factor beta polypeptide	PLA2	Short neurotoxin	Vespryn	Zinc finger
Lanthonotus borneensis	1			1		1	1			1				
Varanus griseus	1		1				1							1
Varanus prasinus	1		1				1				1			1
Varanus jobiensis			1						1		1		1	1
Varanus melinus	1		1			1			1		1	1		1
Varanus salvadorii	1		1				1	1	1		1			1
Varanus komodoensis			1				1		1		1			
Varanus varius	2	2	1, 2				1, 2		1, 2		1, 2			1
Varanus mertensi			1, 2				1		1		1			1
Varanus giganteus		1	1, 2				1, 2		1		1			1, 2
Varanus rubidus			1		1									
Varanus gilleni			1, 2				1		1		1			
Varanus acanthurus	1, 2		1, 2				1				3			1
Varanus baritji	1		1, 2				1							1
Varanus tristis			1				1		1					
Varanus scalaris	1		1				1		1		3			1
Varanus mitcheli			1				1		1					1

Figure 9. All components of varanoid oral secretion recovered in this study.

Bioactivity studies further strengthened the differences between the oral secretion profiles, with *V. varius* having the highest of all phospholipase A2 activity rate – almost three times higher than that of *V. melinus* and *V. scalaris* and an order of magnitude higher than the rest, most of which had no significant activity (fig. 10). No phospholipase A2 activity was recovered for *V. mitchelli*. Ability to cleave kallikrein substrate was equally variable between the species: *V. mitchelli* activity rate was more than twice than that of *V. scalaris*, *V. panoptes rubidus*, *V. melinus* and *V. prasinus* and almost ten-fold that of other species. *V. gilleni* and *V. griseus* had the least potency in that respect amongst all the species studied.

The observed differences in activity rates, especially with respect to kallikrein, might be the consequence not only of the structural differences between the proteins of different species, but also a result of differential component complexity, as is clearly the case with *V. mitchelli* whose 2D profile is largely composed of different kallikrein isoforms.

At the same time, differences in activity rate might be related to the dramatic size difference between the studied species – almost seven-fold between the smallest (*V. gilleni*) and biggest (*V. varius*) in SVL (snout to vent length). The prey-predator size ratio is drastically different for those species, and venom replenishment costs may also vary considerably.

Experiments of the effect of *V. varius* venom on rat ileum contractility showed a pronounced effect of increased contractility induced by the administration of the crude venom (fig. 11). This may be attributable to the presence of AVIT and natriuretic peptides, as recovered by the MS analysis.

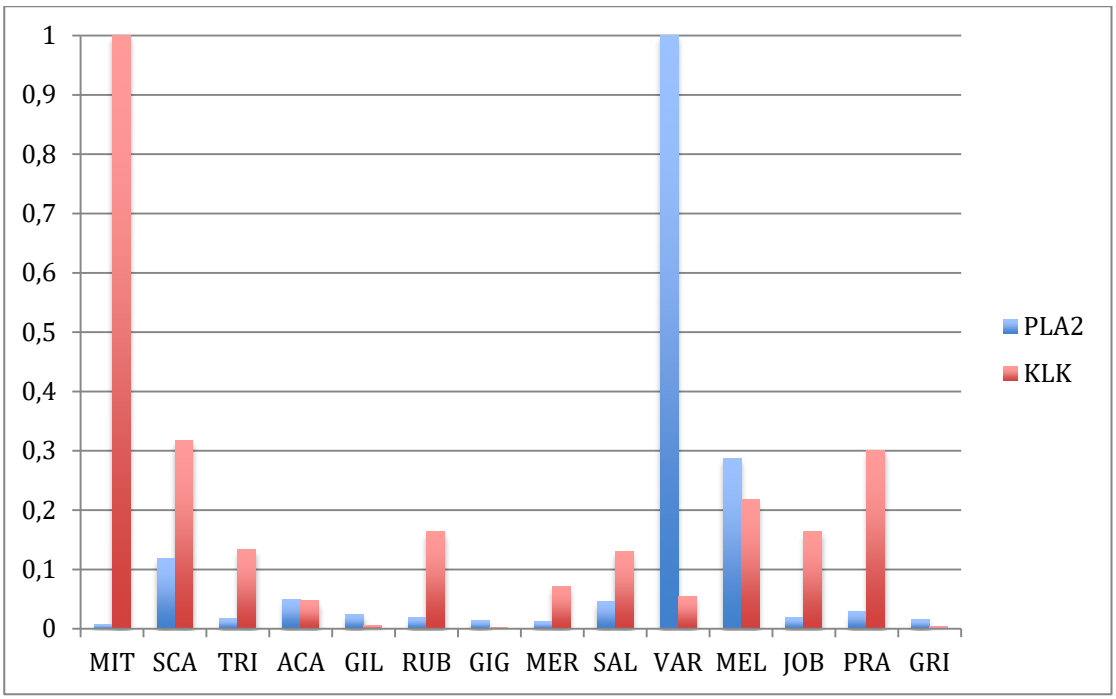


Figure 10. Normalised results of bioactivity testings. On the Y-axis is the initial activity rate normalised within the study group, with the highest rate being assign to 1 and the others calculated as a fraction of it. MIT – V. mitcheli, SCA – V. scalaris, TRI – V. tristis, ACA – V. acanthurus, GIL – V. gilleni, RUB – V. panoptes rubidus, GIG – V. giganteus, MER – V. mertensi, SAL – V. salvadorii, VAR – V. varius, MEL – V. melinus, JOB – V. jobiensis, PRA – V. prasinus, GRI – V. griseus.

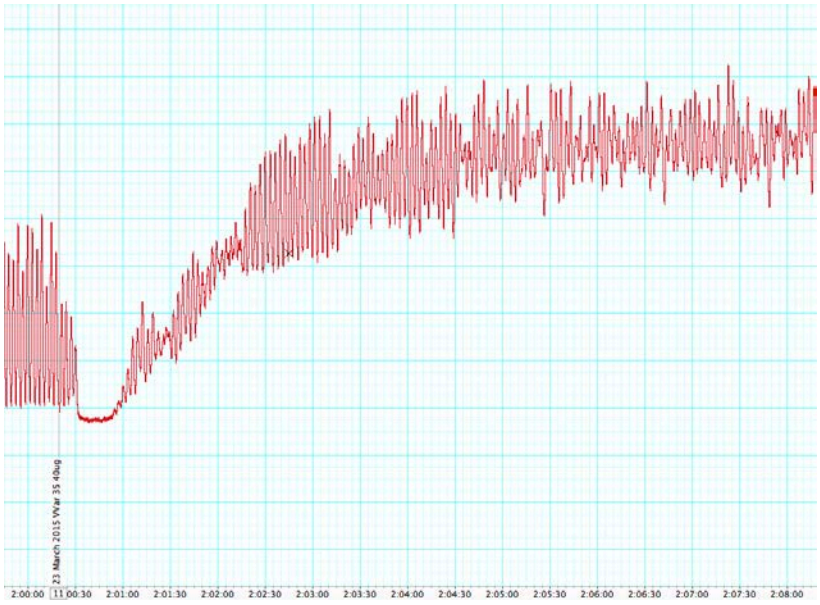


Figure 11. Change in rat ileum smooth muscle contractility after administration of crude V. varius venom.On the Y-axis is the pulling force in relative units; on the X-axis is time.

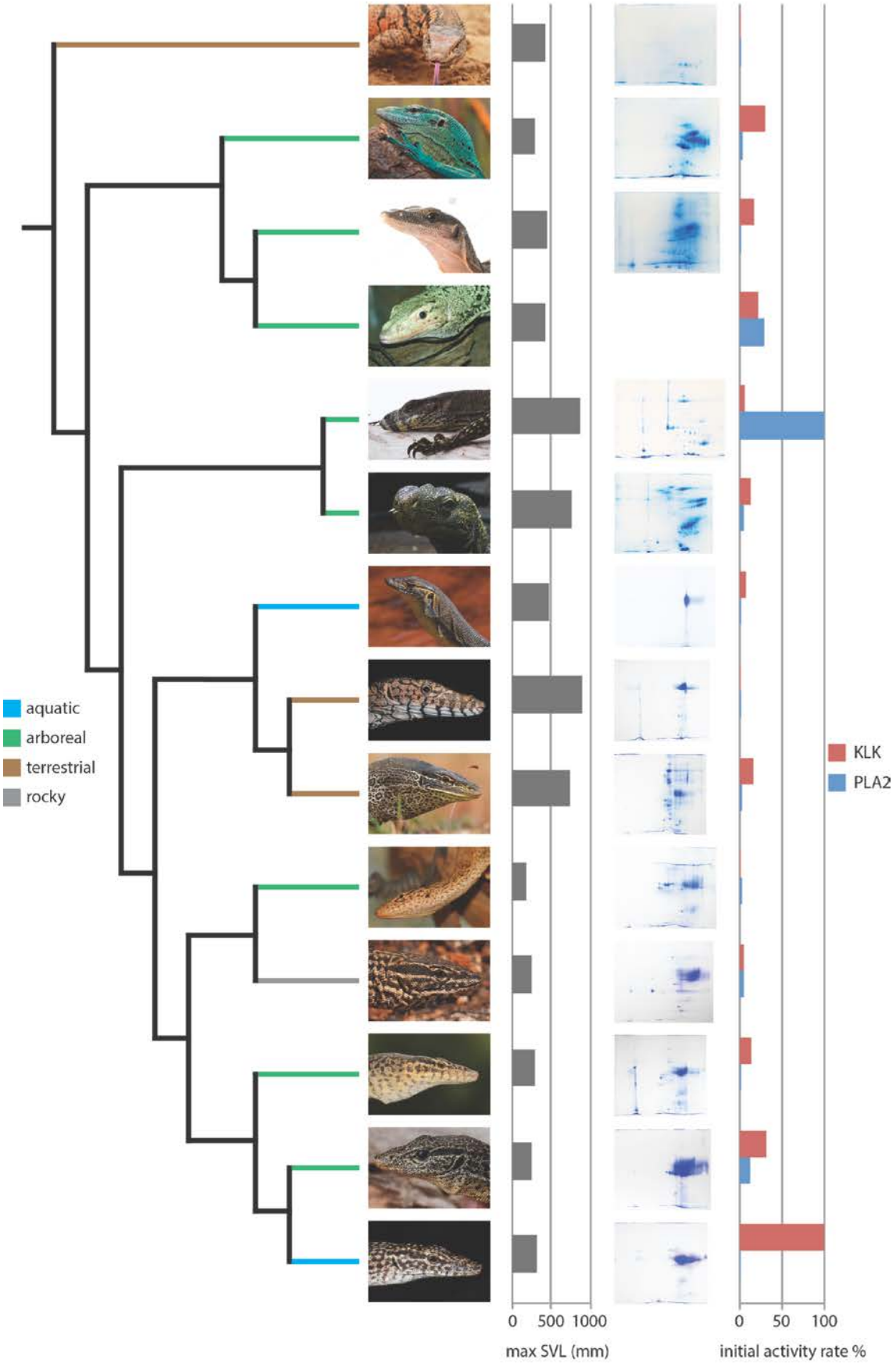


Figure 12. Phylogenetic relationships between the studied species (based on Ast, 2001) with SVL meassurments (based on Pianka and King, 2004) and main findings of the present study.

The higher complexity of the venom of large species of monitor, in comparison to their smaller congeners, may be explained by the broader dietary range in larger species, both of the adult animals and across the life history (see fig. 12 for reference on size and phylogenetic relationships). As dietary studies of the lace monitor show (Pascoe et al., 2012), it has the broadest possible dietary range, feeding on everything from small invertebrates and eggs to medium-sized mammals, most-likely experiencing ontogenetic niche shifts, which further necessitate adaptations to different prey items. At the same time smaller species (members of the *Odatria* group) predominately feed on lizards and insects throughout their life (Pinka and King, 2004).

The dietary connections seems especially clear with respect to the presence of chitinase and chitotriosidase in all the venoms of *Odatria* monitors (fig. 10) (with the sole exception of *V. mitcheli*) and the complete absence of these enzymes in monitors of other groups. *Odatria* or dwarf monitors are unique to Australia, presumably due to the absence of eutherian mammal carnivores. No member of the clade grows bigger than 600 mm SVL and most of them are exclusively arboreal or rock-dwelling. Chitinase enzymes are likely helpful in the digestion of thick carapaces of arthropods, which are their substantial food source. The potential digestive role of the varanid venom was previously set up as a hypothesis (Arbuckle, 2009) and our data seems to corroborate it.

The greater complexity of the *V. gilleni* venom profile in comparison to other dwarf monitors might be reflective of a plesiotypic condition, since it is the most basal species in the study from that group and it probably did not undergo an evolutionary habitat shift from the putatively ancestral arboreal condition (Collar et al., 2011; Thompson et al., 2009) – the transition to a rock-dwelling lifestyle might be associated with the streamlining of the *V. acanthurus* and *V. baritji* venom profiles.

Habitat might likewise be responsible for the relative simplicity of *V. mitchelli* and especially *V. mertensi* profiles, since both of the monitors are predominantly aquatic and might have both a reduced need for defensive venom due to their ability to seek refuge in water and a reduced need for predatory venoms due to a diet dominated by fish and aquatic arthropods.

Since rock-dwelling may be the only lifestyle that imposes significant constraints on body shape – in particular on the shape of the head (Openshaw and Keogh, 2014; Thompson et al., 2009), it is possible that rock-dwelling monitors like *V. acanthurus* and *V. baritji*

experience increased selection pressure on the size and therefore function of their venom glands and have reduced venom complexity as a result.

If that is indeed the case, then from the perspective of the evolution of oral secretion in varanids those species would represent the most remote state from the ancestral condition, having undergone firstly an adaptation to dwarfism (for instance in the form of chitin-digesting enzymes) and secondly an adaptation to rock-dwelling that purified secretion of all that was not essential.

In this line of argument, the *gouldii* group would represent the venom system in its plesiotypic state for the Australasian monitors, retaining the kallikrein/CRiSP-dominated profile, which is not dissimilar from Indo-Asian species examined in this study. On the other hand, the *varius* group, which contains the lace monitor (*V. varius*), crocodile monitor (*V. salvadorii*) and Komodo dragon (*V. komodoensis*) has the most diverse venom with protein groups across the entire molecular-weight range, and is less rich in kallikrein and CRiSP than other clades. This might be explained as an adaptation to the large body size and increased dietary range and habitat shifts those lizards experience across their life histories.

Amongst those studied, the *V. griseus* profile might reflect the most plesiotypic condition of varanid oral secretions in general, since it was the most basal species examined, although it should be noted that basal species are not necessarily the closest to ancestral states. Its oral secretion contains kallikrein, CRiSP, AVIT and lysosomal acid lipase, three of which are present in most of the venoms studied and all four are present in venoms of other non-Australian monitors. However, none of those components are heavily expressed in the venom if 2D PAGE results are of any indication. If not a sampling artefact, that might be either reflective of the condition of the venom system that the ancestor common to *V. griseus* and other monitors had, or could reflect subsequent changes the system underwent through adaptation to desert life-style of this monitors.

Conclusion

The oral secretions of varanoid lizards represent a heavily understudied area. Multiple sources of evidence point to an ongoing evolutionary process shaping the secretion content that is very likely to be playing a crucial role in the lives of varanid lizards. The present study reveals the differential complexity of oral secretion in *Varanus* and argues for the evolutionary causes behind it.

According to our results, varanid venom is largely based on kallikrein and CRiSP toxins that previous studies have shown to be homologous to that of venomous snakes (Fry et al., 2010a). Additional components are present in various species with profile complexity seemingly being a function of size and habitat with the bigger arboreal species having the most complex venom and the small rock-dwelling and aquatic monitors the least.

Some components recovered in the study are very likely to be an adaptation for arthropod-based diet, which strengthens the point that venom glands in varanid lizards have more than a single function. That is, in addition to its potential role in prey subjugation or defence (the primary roles of any venom system), varanid lizards` oral secretion can potentially aid in digestion.

With 16 varanid species studied out of more than 60 species in existence, this study is far from being an attempt to paint a complete picture of evolution of oral secretions in *Varanus*, and future studies are very likely to expand our knowledge on the topic. For example it will be very interesting to see the venom profile of *V. salvator* – a big, but predominantly aquatic lizard, as well as frugivorous species (such as *Varanus olivaceus*) from Indo-Asian A clade (classification according to Ast, 2001).

It is abundantly clear that the unique nature of venom in varanid lizards presents interesting evolutionary questions that will require a systems approach to be solved in any satisfying way. Turning away from this fruitful area of research by denying the biochemical reality of their secretions will only hinder our progress.

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Conclusions

Anguimorpha venoms are diverse and reflect the variety of ecological niches occupied and feeding strategies implemented by these lizards. The current study provides insight into the evolutionary trajectories of defensive and predatory venoms that share a relatively recent common origin.

Venoms of *Heloderma* lizards appear to have remained relatively uinchanged for more than 30 mya, their proteomic composition showing no difference between the individuals from different localities or members of different species. Several major helodermatid toxin genes appear to be under the heavy influence of negative selection.

At the same time varanid lizards have evolved a plethora of different venom profiles, some of which are extremely streamlined while others are remarkably complex, in particular that of the *Varius* clade, which may be tailored to suit their ontogenetic niche shift. Some of the components of monitor oral secretions suggest that venom in varanids serve several purposes. Most notably, chitinase, present in small monitors of *Odatria* clade, hints that venom might aid in digestion as well as being used in defence or prey subjugation.

Altogether our findings suggest a great role that habitat and diet play in evolution of venom composition. Though it is very hard to discern the environmental effect from purely phylogenetic one, juxtaposing *Heloderma* (no environment or venom diversity across the genus) and *Varanus* (great diversity both in environment and venom across the genus), as well as comparing different species within *Varanus* (despite belonging to different clades, both aquatic monitors *V. mertensi* and *V. mitcheli* have streamlined venom profiles) allow for potential insight on the forces that shaped venoms in reptiles.

The present study also highlights the importance of biodiscovery research being driven by advances in phylogenetics as well as the cost of biased conceptions about nature. There are more than 50 described species of varanid lizard, of which only 16 have had their venom investigated, with most of that only over the course of this study. African varanids are completely unstudied in this respect and, judging by the venom complexity of their Australian counterparts, there is incredible potential for further research out there. Reality will always outperform our expectations, which is why we should always prefer to study nature before making assumptions about it.