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Does the Saw-Scaled Viper (Echis carinatus sochureki) show phenotypic plasticity of venom composition in response to dietary change?

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(Image credit; Dr Wolfgang Wuster)

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Declaration to include in your thesis:

I hereby declare that this thesis is the results of my own investigations, except where otherwise stated. All other sources are acknowledged by bibliographic references. This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree unless, as agreed by the University, for approved dual awards.

Yr wyf drwy hyn yn datgan mai canlyniad fy ymchwil fy hun yw'r thesis hwn, ac eithrio lle nodir yn wahanol. Caiff ffynonellau eraill eu cydnabod gan droednodiadau yn rhoi cyfeiriadau eglur. Nid yw sylwedd y gwaith hwn wedi cael ei dderbyn o'r blaen ar gyfer unrhyw radd, ac nid yw'n cael ei gyflwyno ar yr un pryd mewn ymgeisiaeth am unrhyw radd oni bai ei fod, fel y cytunwyd gan y Brifysgol, am gymwysterau deuol cymeradwy.

Table of Contents

Chapter 1 – Literature Review 4

1 - Acknowledgements 4

2 - Abstract 4

3 – An Introduction to the Thesis 4

4 - A review of the Literature- Exploring phenotypic plasticity in the venom composition of snakes 5

4.1 - Introduction 5

4.2 – Venom in Snakes 7

4.2.1 – Functions of Venom in Snakes 8

4.2.2 – Origin and Evolution of Snake Venom 9

4.2.3– Composition of Venom in Snakes 12

4.2.4 – The Phenotype of Venom and Dietary Adaptation 15

4.2.5 – The Treatment of Snakebite 17

4.3 – Snake Venom Variation 17

4.3.1 – Genetic and Epigenetic Variation 17

4.3.2 – Interspecific Variation 18

4.3.3 – Intraspecific Variation 20

5 – Proposed Study 28

Chapter 2 – Experimental Study 29

1 - Methodology 29

1.1 – Ethics Statement 29

1.2 – Experimental Conditions 29

1.3 – Venom Collection 31

1.4 – Venom Composition Analysis 32

1.5 – Data Analysis 34

2 - Results 35

2.1 – Statistical Analysis 35

2.2 – Comparisons of Average Venom Compositions 37

3 – Discussion 46

3.1 – Conclusion 48

References 49

Chapter 1- Literature Review

1 - Acknowledgements

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2 - Abstract

While the plasticity of snake venom composition has been investigated in a wide variety of contexts from ontogenetic (Alape-Girón *et al.,* 2008; Cipriani *et al.,* 2017; Gibbs *et al.,* 2011; Guércio *et al.,* 2006; López-Lozano *et al.,* 2002; Madrigal *et al.,* 2012; Wray *et al.,* 2015; Zelanis *et al.,* 2007; Zelanis *et al.,* 2010) to seasonal variations (Antunes *et al.,* 2010; Brahma *et al.,* 2015; Sengupta *et al.,* 1994), there is very little research at present that describes the effect of dietary change on the composition of venom in snakes. Gibbs *et al.,* (2011) found some degree of plasticity in venom composition in response to dietary changes in adult Dusky Pygmy Rattlesnakes (*Sistrurus miliarius barbourin*). However, this study was primarily designed to investigate ontogenetic changes in venom composition in the absence of dietary changes and the sample size for adult snakes was small thus making it hard to draw any solid conclusions regarding the presence of phenotypic plasticity of venom composition in response to dietary change. In this thesis, I aim to explore the current literature surrounding this topic and then present my own research. In my research I carried out a controlled feeding experiment on neonatal *Echis carinatus sochureki,* raising some on an invertebrate only diet while raising others on a vertebrate only diet. I then analysed the protein composition of venoms from snakes belonging to each group. While I did not find any significant difference in the venom composition of vertebrate and invertebrate feeding snakes, I did see a pattern of slower ontogenetic development of venom composition amongst the invertebrate feeding snakes in comparison to the vertebrate feeding snakes. This was coupled with a higher mortality rate among invertebrate feeders and a significantly lower average weight of invertebrate feeders compared to vertebrate feeders.

3 - An Introduction to the Thesis

It is known that the diet of many snake species changes in response to a variety of factors. Many snakes show a season change in diet due to the changing availability of various food sources at different times of the year and ontogenetic changes in diet due to a change in the type of prey a snake is able to immobilize and swallow at different life stages (Antunes *et al.,* 2010; Brahma *et al.,* 2015; Brito, 2004; García & Drummond, 1988; Hirai, 2004; López *et al.,* 2013; Mackessy *et al.,* 2003; Natusch *et al.,* 2012; Pough, 1977; Santos *et al.,* 2000; Wray *et al.,* 2015). A change in venom composition is also often observed ontogenetically and seasonally. Snakes also show

plasticity in venom composition geographically. Snakes from the same species but living in different areas of their range show different venom phenotypes (Cipriani *et al.,* 2017; Gren *et al.,* 2017; Modahl *et al.,* 2016; Neale *et al.,* 2017; Williams and White, 1992). It is also safe to assume that there is likely to be at least some difference in prey communities across the range of most snake species. Thus, with all this considered, it is logical to ask the question 'does diet directly affect venom composition?'.

In this thesis I aim to investigate the effects of dietary change on venom composition by conducting a controlled feeding experiment on juvenile Pit Vipers (*Echis carinatus sochureki*), whereby I will raise some on an invertebrate diet and some on an invertebrate diet and analyse the changing compositions of venom in both groups.

4 - A review of the Literature- Exploring phenotypic plasticity in the venom composition of snakes

4.1 - Introduction

Venoms are complicated mixtures of toxic compounds honed by natural selection to attack another organism at molecular levels for the primary purposes of prey subjugation or predator defence (Juárez *et al.,* 2004; Zancolli *et al.,* 2017). They contain a vast array of different compounds. For example, the Forest Cobra (*Naja melanoleuca*) produces venom containing 52 different proteins (Lauridsen *et al.,* 2017) and the Australian Scorpion (*Hormurus waigiensis*) produces a venom containing 182 distinct molecules (Housley *et al.,* 2020). The compounds which make up venoms include peptides and proteins, amino acids, organic molecules, salts and minerals and amines and alkaloids (Fry *et al.,* 2009). Venoms have evolved convergently across many taxa (figure 1), from jellyfish (Remigante *et al.*, 2018) to mammals such as Shrews and Slow Loris' (Ligabue-Braun, 2015).

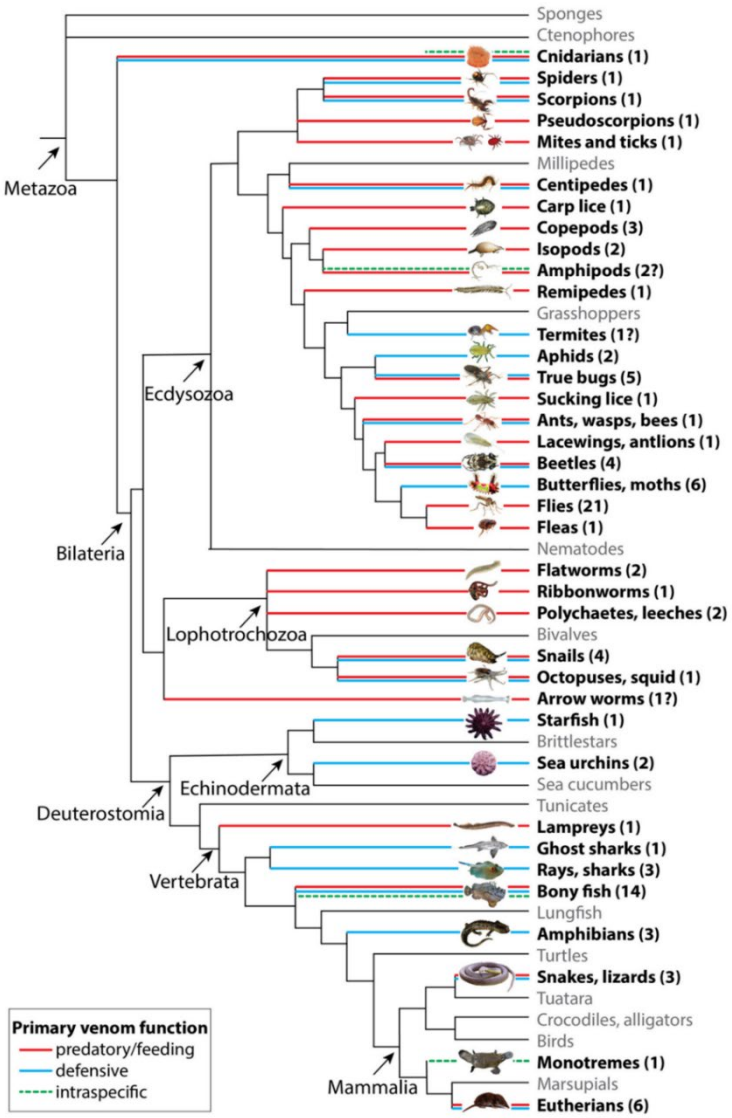

Figure 1. A pruned and schematic phylogenetic tree summarising taxonomic diversity and primary functions of venom (Schendel *et al.,* 2019).

Aside from predator defence and prey subjugation, various taxa have also evolved a number of alternative uses for venom. Many invertebrates such as spiders use venom for the purposes of digestion as well as prey immobilisation (Kardong, 1996; Langenegger *et al.,* 2019). Digestive properties of venom have also been suggested for various species of Squamate (an order of reptiles consisting of snakes, lizards and worm lizards) including the extant species of the family Helodermatidae (Gila Monsters etc), several species from the family Varanidae (monitor lizards) as well as species representing the three major families of venomous snake; Colubridae, Viperidae and Elapidae (Bottrall *et al.,* 2010; Koludarov *et al.,* 2017; Rodríguez-Robles & Thomas, 1992, Thomas & Pough, 1979). Far less commonly, some species have developed the use of their venom for mate competition. The Duck-Billed Platypus (*Ornithorhynchus anatinus*) injects venom from spurs positioned on its hind legs, these spurs are only present in the males of the species and enlarge during the breeding season due to their use in intraspecific mate

competition (Whittington & Belov, 2016). Slow Lorises (genus: *Nycticebus*) produce a venom by combining a secretion from the branchial gland on the arm with their saliva. This venom is used for defence and possibly intraspecific competition, but it also has another purpose; ectoparasite control. Species of Slow Loris groom their venom through their fur and through the fur of their offspring in order to reduce harm caused by ectoparasites such as fleas and ticks (Nekaris *et al.,* 2013). Most venomous animals pose minimal risk to humans due to lack of lethality, lack of interaction or lack of an adequate venom delivery system (Vetter & Visscher, 1998). However, one group of venomous animals have representatives which possess venom lethal to humans, come into regular contact with humans and have a delivery system capable of envenoming humans. These are the snakes. In Brazil (the most complete set of data present in the literature) snakes cause significantly more human deaths than any other venomous taxa (see table 1).

Table 1- Yearly number of deaths caused by envenomation by taxa in Brazil, 2001-2012 (Chippaux, 2015)

Year	Unknown	Snake	Spider	Scorpion	Caterpillar	Bee	Total
2001	6	70	9	44	0	5	134
2002	3	114	2	58	0	15	192
2003	9	120	5	51	0	7	192
2004	4	114	5	42	4	9	178
2005	4	113	9	48	3	13	190
2006	2	76	9	28	0	13	128
2007	13	132	20	66	0	19	250
2008	17	122	22	88	4	11	264
2009	14	131	21	94	1	33	294
2010	12	132	17	67	2	30	260
2011	17	143	19	86	4	31	300
2012	10	127	16	97	2	30	282
Total	111	1,394	154	769	20	216	2,664
Mean ±95% CI	9.25 ± 3.1	116.17 ± 12.5	12.83 ± 4	64.08 ± 12.9	1.7 ± 0.9	18 ± 5.8	222 ± 34.2

4.2 - Venom In Snakes

Snakes (suborder; Serpentes) are perhaps the most medically significant clade to evolve venom. It is estimated that between 1.2 and 5.5 million people are bitten by snakes annually, these bites are estimated to result in around 400,000 amputations and anywhere between 20,000 and 125,000 deaths a year (see table 2; Chippaux, 1998; Kasturiratne *et al.,* 2008; Williams *et al.,*2010). The World Health Organisation (WHO) recognises about 250 snake species as “of medical importance” and recently recognised snakebite as a highest priority Neglected Tropical Disease in 2017 (WHO, 2019). The composition of a venom refers to the proteins and peptides present within the venom and the ratios at which they occur. The venom from a single species of snake can vary in composition for a variety of reasons from range (Girón *et al.,* 2018) to ontogeny (Amazonas *et al.,* 2018) to sex (Amorim *et al.,* 2018; Augusto-de-Oliveira *et al.,* 2016).

Table 2- Estimated numbers of bites, envenomation and deaths caused by snakes worldwide (Chippaux, 1998). * Population at risk.

	Population ($X10^6$)	Total number of bites	No. of envenomations	No. of deaths
Europe	730	25,000	8,000	30
Middle East	160	20,000	15,000	100
USA and Canada	270	45,000	6,500	15
Central and South America	400	300,000	150,000	5,000
Africa	760	1,000,000	500,000	20,000
Asia	3,500	4,000,000	2,000,000	100,000
Oceania	20*	10,000	3,000	200
Total	5,840	5,400,000	2,682,500	123,345

4.2.1 - Functions of Venom In Snakes

The venom of snakes predominantly has a single purpose; the immobilization (not necessarily death) of prey (Calvete *et al.,* 2005). This is reflected by the common occurrence of specific prey lethality in snake venoms. Coral snake (*Micrurus sp.*) venoms show higher lethality for natural prey than for non-prey species (da Silva and Aird, 2001). Similarly, several species from the families Viperidae and Colubridae show strong evidence of prey specificity in venom composition (Casewell *et al.,* 2013; Daltry *et al.,* 1996; Modhal *et al.,* 2016). Despite prey capture being the primary function of venom in snakes, the majority of snake species (including most venomous snakes) bite defensively (Hayes *et al.,* 2002). Therefore, it can be argued that snake venom has the secondary function of predator defence. The most extreme cases of snakes using venom as a defence come from the spitting cobras. Spitting is a behaviour which occurs in several species from the genus *Naja.* Spitting cobras use very sophisticated aiming techniques and morphologically adapted fangs (figure 2; Berthé *et al.,* 2009; Paterna, 2019; Westhoff *et al.,* 2005) in order to spray venom into a would-be predator's eyes and thus cause extreme discomfort and often blindness (Berthé *et al.,* 2013; Westhoff *et al.,* 2010).

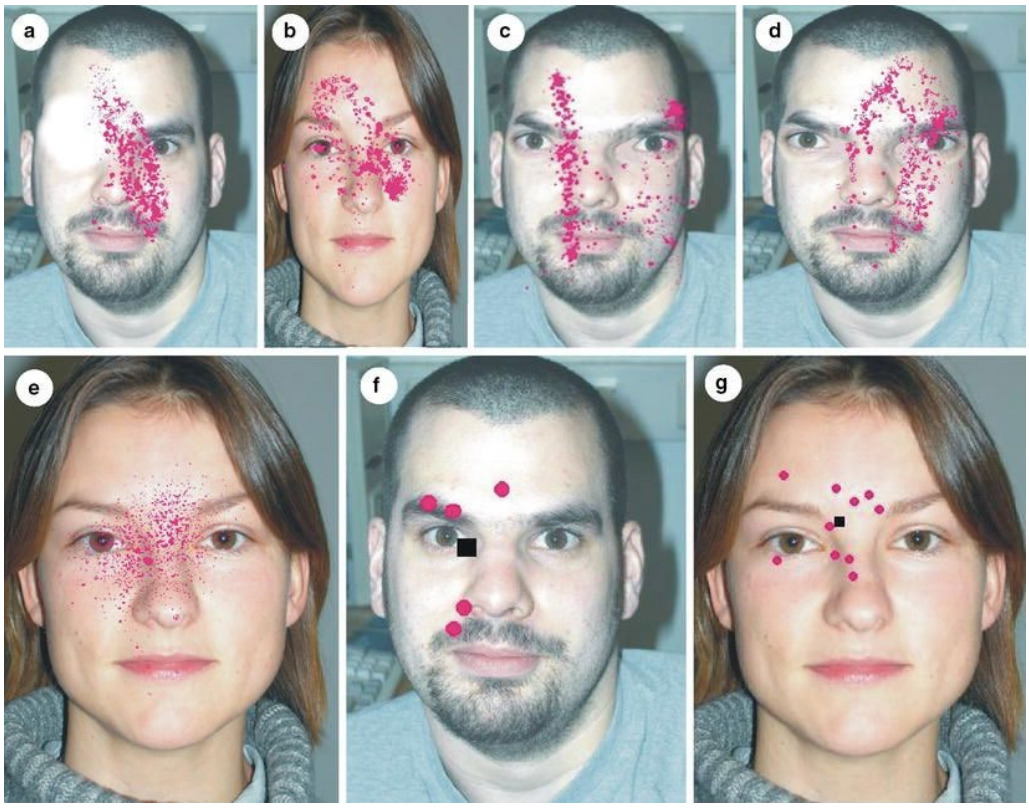

Figure 2. Examples of venom spay aiming by the spitting cobra, *Naja pallida* (a–d) on real-size pictures of human faces with altered eye positions. The spitting pattern of a different species of Spitting Cobra, *Naja nigricollis* to a real face protected with a visor that was covered with a transparent foil, is shown (e). Center of individual spitting patterns (*dots*) and average of all spitting pattern centres (*square*) are shown for *N. pallida* (f) and *N. nigricollis* (g). This figure shows eye aiming behaviour exhibited by Spitting Cobras.

Similarly, it has been suggested that other species have also developed a defensive function for venom. The Texas Coral Snake (*Micrurus tener tener*) has a component in its venom known as MitTx, which acts entirely for the purpose of inflicting pain (Bohlen *et al.,* 2011). Pain serves no real purpose in the restraining of prey; in fact, it could be argued that inflicting pain will cause prey to retaliate more and so inhibit a snake's ability to restrain a prey item. Thus, it appears that the *M. t. tener* has evolved a venom toxin which serves an anti-predator function. With the primary function of snake venom being prey capture, a less specific and more generic venom would be more useful as it would not limit possible prey items. However, if a snake species feeds almost exclusively on a single prey type, then specific venom may be more effective for the subjugation of its prey and may also be energetically cheaper to produce. Also, as a snake grows, the prey it feeds off as well as the predators which prey upon it are likely to change. Therefore, an ontogenetic change in venom composition may occur in order to deal specifically with new prey items or predators. These points will be discussed in depth in the sections ahead.

4.2.2 - Origin and evolution of Snake Venom

Venom glands in snakes (figure 3) are thought to have evolved from salivary glands in non-venomous ancestors (Gibbs *et al.,* 2011; Kochva, 1987).

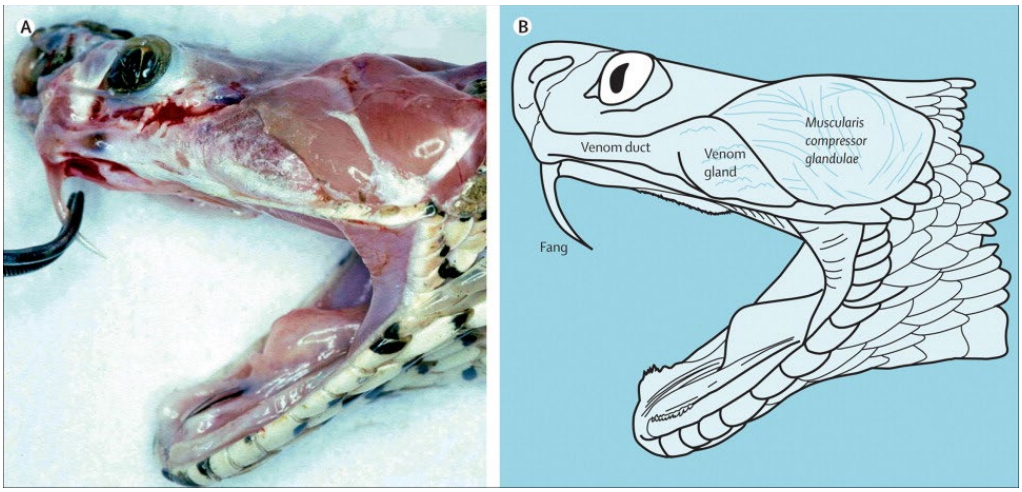

Figure 3. A dissection photograph and scientific diagram showing the derived fang, venom duct, venom gland and compressor muscle of a Russell's Viper (*Daboia siamensis*). Vipers and Elapids have more derived venom glands than rear-fanged colubrids (Warrell, 2010).

Some extant mammal species show the evolution of toxic protein production from the salivary gland, namely species among the order Eulipotyphla (Shrews, Moles, Solenodon etc) belonging to the genera *Solenodon, Blarina* and *Neomys* (Kowalski and Rychlik, 2018). Furthermore, the venom of many snake species may play a role in digestion similar to many proteins found in saliva (Bottrall *et al.,* 2010; Koludarov *et al.,* 2017; Rodríguez-Robles & Thomas, 1992, Thomas & Pough, 1979). Mexican Beaded Lizards (*Heloderma horridum*) and Northern Short-Tailed Shrews (*Blarina brevicauda*) have shown convergent evolution of serine protease toxins (BLTX in *B. brevicauda,* GTX in *H. horridum*) by the process of acquiring small insertions followed by rapid sequence evolution (Aminetzach *et al.,* 2009). This shows the process by which the salivary gland may begin to produce a variety of toxin proteins and thus supports the theory that venom glands in snakes are a descendant of salivary glands in non-toxic ancestors. Furthermore, two venom protein families; CRISPs (cysteine-rich secretory proteins) and kallikrein toxins both have been shown to have derived via modifications of existing salivary proteins (Fry, 2005).

However, a venom is useless without a delivery system. Snakes have developed 3 different forms of derived dentition in order to deliver their venom (figure 4; Vonk *et al.,* 2008). Venomous Colubrids have developed fixed fangs in the rear of their mouth with a groove for venom to run down into a wound inflicted by the fangs (opisthoglyphous fangs) this is a fairly basic method of venom delivery as the venom needs to be 'chewed' into a wound in order to be delivered. However, Vipers and Elapids have both evolved far more derived and sophisticated venom delivery systems. Both Elapids and Vipers have evolved hollow syringe-like fangs (proteroglyphous and solenoglyphous fangs respectively) however the fangs of Elapids are fixed in place in contrast to the fangs of Vipers which are hinged. The Fangs of the Vipers are the most derived (Vidal, 2002), the joint between the fangs and the maxilla allows the fangs to be tucked back and so, far larger fangs can develop (Cundall, 2009). Extremely large fangs are useful due to the physical trauma that a bite can cause as well as the shock caused to prey via a bite. In many instances, it is very possible that the physiological damage inflicted by a bite will cause death or

immobilization before venom takes effect. This means that potentially dangerous prey such as large rodents are less likely to be able to injure the snake during a struggle (Glaudas *et al.,* 2017).

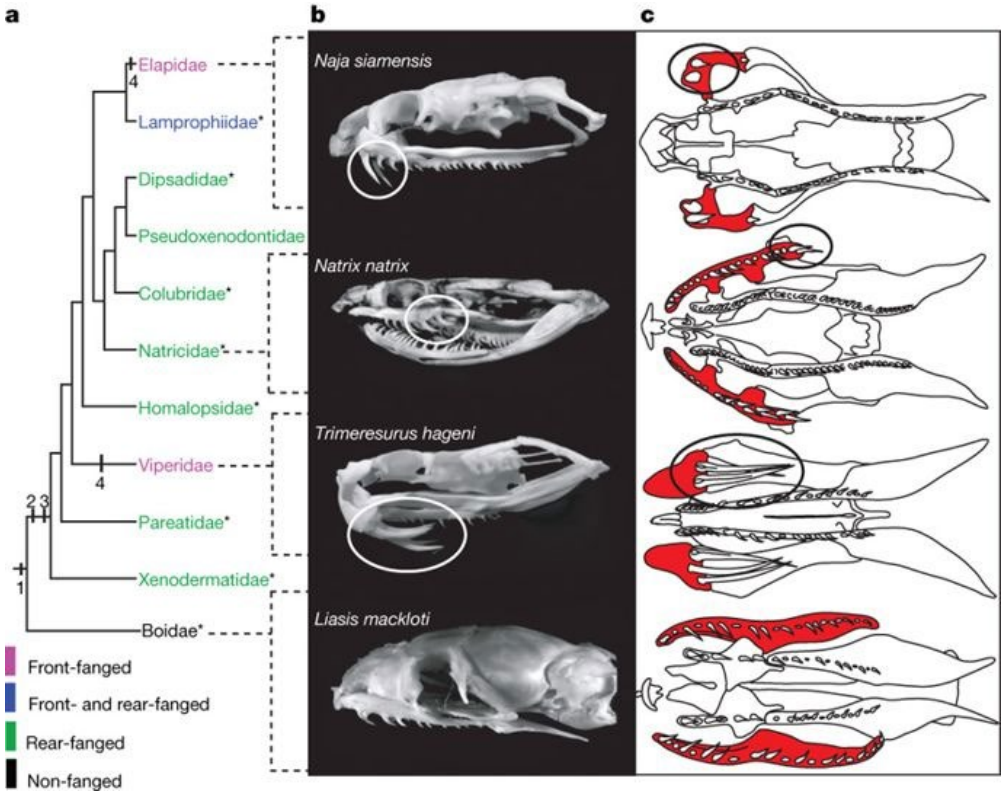

Figure 4. The venom delivery systems for a Colubrid (*Natrix natrix*), an Elapid (*Naja siamensis*) and a Viper (*Trimeresurus hageni*). Also present is the dentition of a Boid, which have no venomous representatives and so possess no venom delivery system. Also show are the evolutionary relationships between the three venom clades of snake as well as Boids (Vonk *et al.,* 2008).

Many snake venom proteins seem to have an evolutionary origin in other common house-keeping proteins (Fry, 2005; Fry & Wüster, 2004). This concept informs the hypothesis that snake venom proteins are recruited via the duplication and mutation of genes coding for house-keeping proteins (Vonk *et al.,* 2013; Wong & Belov, 2012). In Pythons, venom gene homologues were found to be expressed widely throughout tissues outside of oral glands (Reyes-Velasco *et al.,* 2015). All Pythons are considered to be entirely non-venomous. Therefore, the presence of homologous venom genes supports the idea that venom coding genes in Caenophidians (clade including Colubrids, Elapids and Vipers) derived from these homologues via duplication and mutation (Casewell *et al.,* 2012) and were recruited to the salivary gland (Lomonte & Rangel, 2012). However, gene duplication is known to be a rare event, in eukaryotes gene duplication is estimated to occur at a rate of 1 gene per million years (Lynch & Conery, 2000). Additionally, the recruitment of these mutated gene duplications to new tissues (neofunctionalization) is even rarer. Another theory was proposed by Hargreaves *et al* (2014) who found that many proposed venom proteins are expressed in a wide range of tissues including the salivary glands of non-venomous reptiles. This theory suggests that genes present in salivary glands duplicate and mutate into venom protein coding genes, these genes are then restricted to salivary glands post-duplication (sub-functionalization) as opposed to being recruited to salivary glands from other

tissues in the body. Regardless of theory it seems that venom toxins originate from proteins present in a wide variety of tissues in non-venomous ancestors via gene duplication and mutation.

4.2.3 - Composition of Venom in Snakes

As previously mentioned, snake venoms are very complex mixtures of bioactive proteins and peptides comprising of at least 63 different protein families each containing several specific toxins (Tasoulis & Isbister, 2017). The venom of the Indian Cobra (*Naja naja*) has been found to contain as many as 81 different proteins and peptides (Choudhury *et al.,* 2017). Peptides consist of two or more amino acids in a chain whereas proteins refer to one or more polypeptides and so are larger molecules. Similarly, the venom of the Saw-Scaled Viper (*Echis carinatus carinatus*) contains up to 90 different proteins from 15 different protein families (Patra *et al.,* 2017). In contrast the venom of the Brazilian wasp, *Polybia paulista* contains only 23 venom proteins (de Souza *et al.,* 2019) similarly, the venom of the Blue-Ringed Octopus (*Hapalochlaena maculosa*) was also found to contain only 23 toxins (Whitelaw *et al.,* 2016). This illustrates the comparative complexity and diversity of snake venoms and their components. However, the medically significant Indian Red Scorpion (*Mesobuthus* tamulus) also has a highly complex venom containing up to 110 venom toxins (Das *et al.,* 2020). Furthermore, the venom of the medically significant wandering spider (*Phoneutria nigriventer*) is estimated to contain up to 150 peptides (Peigneur *et al.,* 2018). Some of the most complex venoms belong to the Cone Snails (family: Conidae), this family contains over 800 species which each produce over 1000 venom peptides although a lot of these peptides are shared across species (Himaya & Lewis, 2018).

The effects of snake venom proteins can be broadly split into three major categories; neurotoxic, cytotoxic and haemotoxic (Munawar *et al.,* 2018).

Neurotoxins-

Neurotoxins affect synaptic junctions and so either promote or inhibit synaptic function. Synaptic junctions are where two nerve cells connect and signalling chemicals known as neurotransmitters are released by one cell and received by the other in order to produce a reaction to a stimulus. Due the nature of their purpose, neurotoxins are highly specific to particular biological pathways and so have radiated into a vast array of different toxin families, which act on specific biological pathways. Here we provide a brief summary of the different types of neurotoxins present in snake venom and the functions they serve. Neurotoxic venom proteins and peptides often affect neuromuscular synapses (synapses between nerves cells and muscle cells) causing symptoms such as general muscular paralysis, respiratory paralysis and rapid muscle contraction (figure 5; Aminoff *et al.,* 2014; Silva *et al.,* 2016). Three-finger toxins (3FTxs) are a large family of polypeptides which bind to a huge variety of receptors and acceptors causing an enormous variety of effects in prey (Kini and Doley, 2010). Several polypeptides from the 3Txs family exhibit neurotoxic effects. Curaremimetic Toxins, Muscarinic Toxins and K-Neurotoxins each act on different aspects of the cholinergic system thus inhibiting nerve cells that rely on the neurotransmitter Acetylcholione. Acetylcholinesterase Inhibitors inhibit acetylcholinesterase at neuromuscular junctions causing involuntary muscle contractions (Tu, 2012). Non-Conventional 3FTxs (previously known as weak neurotoxins) bind to

acetylcholine receptors at micromolar concentrations, these toxins can cause an increase in blood pressure and a decrease in heart rate (Kini and Doley, 2010). Ion Channel blockers often inhibit acid-sensing ion channels causing analgesic effects such as drowsiness and dizziness (Munawar *et al.,* 2018). Neurotoxins can also affect homeostasis by influencing the release and regulation of various hormones thus inhibiting or promoting systems reliant on endocrine signalling. Natriuretic Peptides in vertebrates are housekeeping compounds, which play a vital role natriuresis, in mammals these peptides are used to regulate cardiovascular and renal functions in an endocrinal manner. After envenomation, Natriuretic peptides interfere with pressure-volume homeostasis of body fluids and often cause a reduction in blood pressure, which often results in loss of consciousness in prey (Vink *et al.,* 2012). Bradykinin Potentiating peptides have been found to function as natriuretic peptides in the brains of some snakes. They are also natural inhibitors of angiotensin-converting enzymes, which are used to raise blood pressure. When injected into prey they cause a severe drop in blood pressure often resulting in loss of consciousness (Sciani and Pimenta, 2017). Crotamines (AKA myoneurotoxins) are a toxin family found in Rattlesnake venoms, which show both neurotoxic and cytotoxic effects. These toxins have been shown to display analgesic properties far more potent than morphine, causing a lack of coordination in prey. Crotamines also penetrate muscle tissue causing necrosis (Munawar *et al*., 2018). Cystine-rich secretory proteins (CRISPs) are another major family of venom neurotoxins which inhibit the smooth contraction of muscle and cyclic nucleotide-gated ion channels (Yamazaki & Morita, 2004).

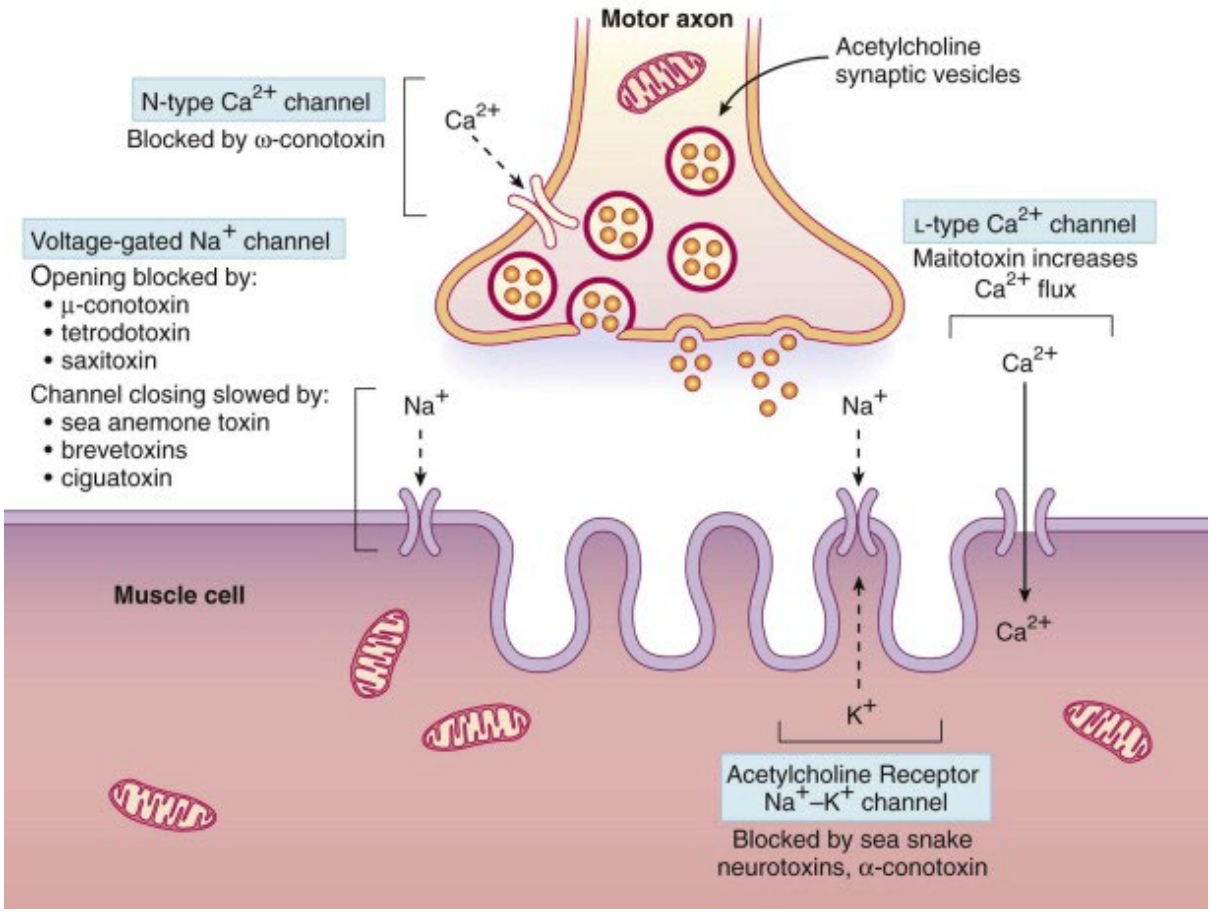

Figure 5. Schematic representation of a neuromuscular junction and the sites of action of various neurotoxins. Neurotoxins work on a variety of aspects of the neuromuscular junction to cause either paralysis or muscular spasm.

Cytotoxins-

Cytotoxins act at the cellular level by breaking down cell membranes, often resulting in local tissue damage and necrosis (Gasanov *et al.,* 2014). Cytotoxins such as phospholipases A_2 (PLA_2) often damage muscle tissue around the bite site this is referred as a myotoxic affect (figure 6). Cytotoxins can also depolarise cells as in the case of cardiotoxins. Cardiotoxins have a high membrane perturbation ability and so cause cellular injury and are often related to local muscular and tissue damage/necrosis in bite victims. These effects can also lead to cardiac arrhythmias due to the depolarisation of cardio-muscular cells (Hedge *et al.,* 2016). Many cytotoxic proteins can also be classed as haemotoxic as they affect and break down blood cells and the cells of blood vessels causing mass haemorrhaging. Cytotoxins are also often associated with digestive properties in venom due to their cell membrane perturbation properties, often envenomed prey items have been shown to take less time to be digested by the envenomating snakes (Cedro *et al.,* 2018).

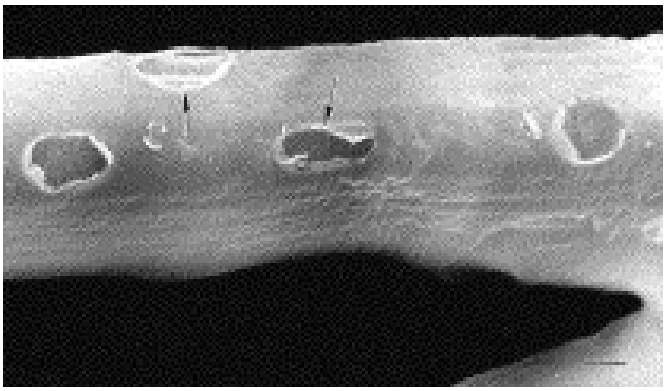

Figure 6. Degeneration of skeletal muscle tissue induced by venom phospholipases A_2 (Gutiérrez & Ownby, 2003).

Haemotoxins are toxins which affect the circulatory system of an animal. As mentioned above, many haemotoxins work by the lysis of the cell membrane in blood cells. Cathelicidins, are an important aspect of the innate immune system and are commonly documented in mammals. These peptides are also now known to be produced in the venom glands of certain snakes and are very common in the venom glands of Elapids. In snakes these toxins provide a similar function but have also been seen to show haemolytic activity when injected into prey (Zhao *et al.,* 2008). Snake venom metalloproteinases (SVMPs) are a common family of haemotoxins which cause lysis of blood cells leading to haemorrhaging effects (see figure 7; Katkar *et al.,* 2015; Knight *et al.,* 2019) as well as having fibrinolytic, prothrombotic, pro-coagulatory and platelet aggregatory inhibiting affects (Markland & Swenson, 2013). Platelet Aggregation Inhibitors inhibit the activity of fibrinogen and so prevent coagulation of blood thus causing excessive bleeding (Teng and Huang, 1991). Kunitz-Type Serine Protease Inhibitors are a very common family of toxins found in the venoms of several Elapids and Vipers. These toxins are thought to affect a prey's homeostasis and interfere with the blood coagulation cascade (Munawar *et al.,*

2018). Disintegrins are a peptide family believed to serve the function of distribution of other toxins throughout tissues after envenomation. The toxins are thought to bind to integrins and inhibit platelet aggregation from occurring immediately after envenomation (Cesar *et al.,* 2019). This prevents thrombosis in prey and so allows blood to continue to flow thus distributing venom more thoroughly throughout the prey's tissues and circulatory system. These toxins are found mainly in Viperidae snakes and make up close to a fifth of total venom proteins in this clade (Munawar *et al.,* 2018).

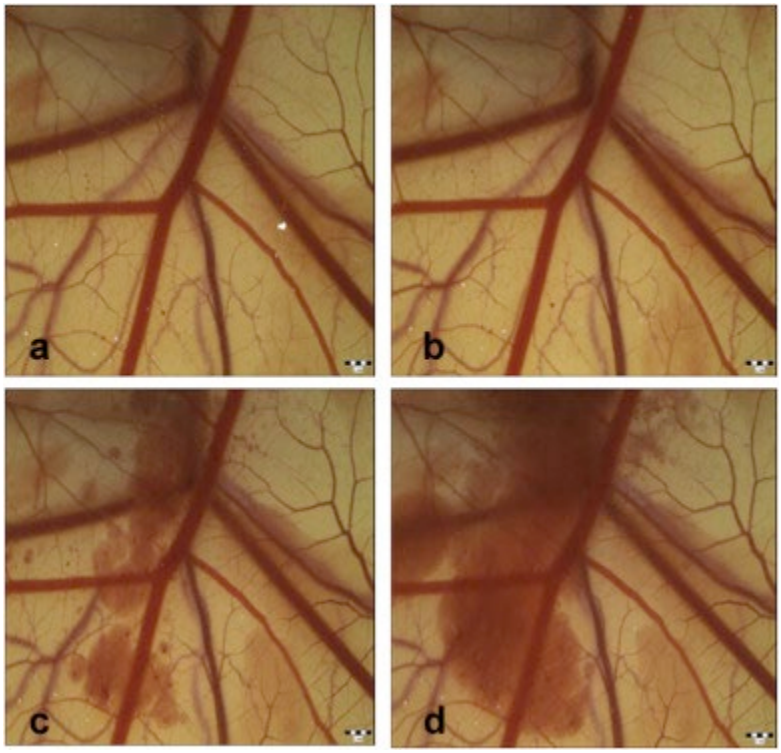

Figure 7. The haemorrhagic effect of haemotoxic venom proteins on blood vessels as seen when venom from various viper species was introduced to the chick chorioallantoic membrane (Knight *et al.,* 2019).

4.2.4 - The Phenotype of Venom and Dietary Adaptation

As may be assumed, given the diversity of different snake venom toxins which exist, the mass taxonomic radiation of Snakes and wide variety of diets present in snakes, the composition of their venom varies across clades (Sousa *et al.,* 2013). Different housekeeping proteins have been weaponised (Richards *et al.,* 2011) by different species and combined in different ratios to produce a massive variety of venom phenotypes observed across venomous snakes in order to subdue different prey types (figure 8). Snakes as a clade, have a vast arsenal of venom toxins at their disposal and thus, it is to be expected that the phenotype of venom composition in snakes is likely to be a plastic one (Amazonas *et al.,* 2018; Amazonas *et al.,* 2019; Gibbs *et al.,* 2011). This is illustrated by the presence of variation in the venom phenotype at all taxonomic levels from family to individual (Mackessy *et al.,* 2018; Nawarak *et al.,* 2003; Petras *et al.,* 2019; Sousa *et al.,* 2013). However, not all toxins within a snake's venom are functional. It is likely that some components of venoms do not play a role in prey subjugation and are simply left over from the evolutionary origins of the venom. For example, the Marbled Sea Snake (*Aipysurus eydouxii*),

have a venom which is fairly non-functional given its diet of fish eggs (Li *et al.,* 2005). It is also possible that non-functional venom proteins may still be present in some species due to leaky transcription. Leaky transcription refers to transcription which still takes place even when an inhibitor is present or promoter is absent (Alarcon *et al.,* 1999). Perhaps, also the presence of non-specific/non-functional venom proteins may support the overkill hypothesis which suggests that snakes produce far more venom containing far more compounds than are necessary for the capture of prey (Gangur *et al.,* 2018).

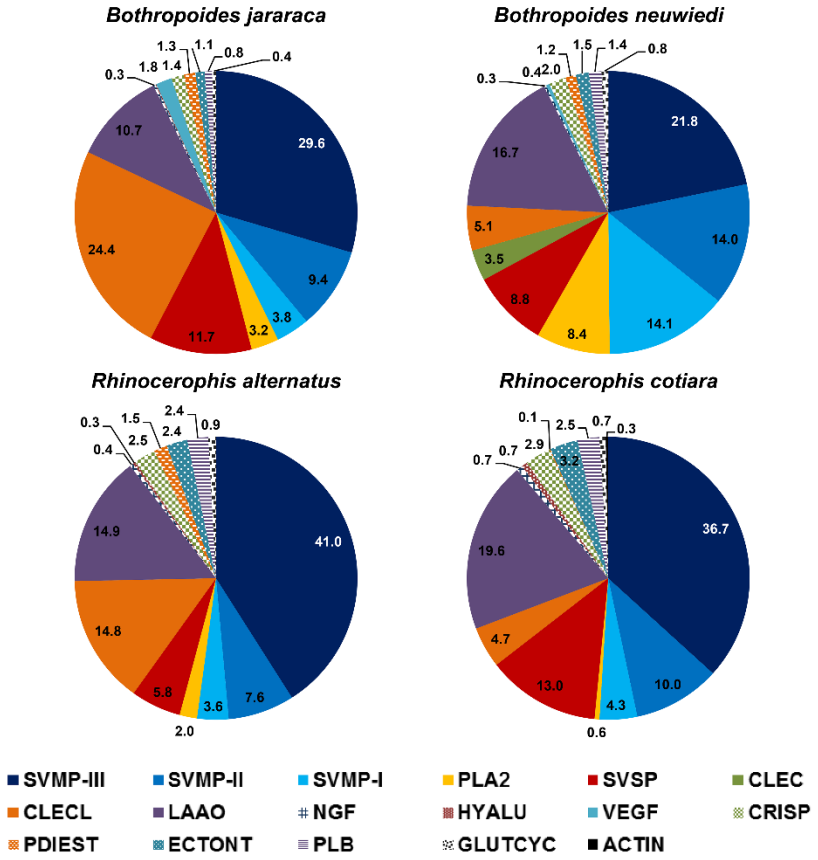

Figure 8. Protein family distribution (venom phenotype) for the venoms of four species of snake from two genera (Sousa *et al.,* 2013). The venom of all four species have SVMP-III (snake venom metalloproteinases) as their most abundant venom protein family. The overall composition of each of the snakes venom does however vary to a large degree.

Phenotypic plasticity refers to an organism's ability to change a phenotype in response to environmental or internal changes undergone by the organism (Price and Qvarnström, 2003). For example, it has been observed that juvenile shore crabs (*Carcinus maenas*) are able to lighten or darken the colour of their carapace in a matter of hours in response being placed on either a white or a black background (Stevens *et al.,* 2014). In terms of behavioural phenotypes, a species of nematode worm (*Pristionchus pacificus*) has been observed to change its feeding behaviour between predatory and bacterivorous (Sommer *et al.,* 2017). This plasticity is controlled by developmental switch genes which are, in turn, under epigenetic control. This form

of phenotypic plasticity is relevant because it doesn't suggest differential gene duplication and loss as the cause of phenotypic change. Instead, it suggests that the genome remains the same but different genes/combinations of genes can be expressed in order to change a trait. Maybe, in snakes, gene expression can be controlled in response to a stimulus in order to cause a change in venom phenotype. Perhaps more relevantly, mice were found to change their saliva composition in response to tannin levels in their food (Da Costa *et al.,* 2008). Mice given a diet with higher concentrations of tannins produce more tannin-precipitating proteins within their saliva this is another example of phenotypic plasticity. Regarding venom composition, the phenotype refers to the toxins present within the venom and the ratios in in which they appear (Núñez *et al.,* 2009). Snake venom composition is known to show phenotypic plasticity in response to a variety of factors from geographical range to season and age of an individual (Cipriani *et al.,* 2017; Gren *et al.,* 2017; Modahl *et al.,* 2016; Neale *et al.,* 2017; Williams and White, 1992). However, as we aim to develop the treatment of snakebite, it is of the greatest importance that we fully understand all the causes of variation in the venom composition of medically significant snakes. In order to further our understanding, it is vital that we conduct research to determine which environmental factors may influence the phenotype of venom in snakes.

4.2.5 - The Treatment of Snakebite

Anti-venom is the only current effective treatment for snakebite (Williams *et al.,* 2019). Anti-venoms are produced by injecting animals (usually horses) with sublethal doses of snake venom. The animal's plasma is then harvested, and the antibodies produced to counter the effects of the venom are refined (Theakston *et al.,* 2003). These antibodies can then be used to treat the effects of snakebite in human cases. As such, anti-venoms are generally species specific as different venoms require specific antibodies to counter their effects (Visser *et al.,* 2008). The antivenom produced will only be truly effective at treating a bite from a snake with the same venom composition as the venom used to produce the antibodies. Therefore, it is important to develop anti-venoms which are affective at treating bites from snakes with different venom compositions within a species. Thus, research into the mechanisms driving variation in snake venom composition is vital to better understand how to treat snakebite.

4.3 - Snake Venom Variation

4.3.1- Genetic and Epigenetic variation

There are, of course, a variety of possible factors which may cause variation in the composition of snake venoms. Some papers have linked abiotic factors such as climatic changes (precipitation and temperature) and elevation across a snake's range to variation in venom composition (Holding *et al.,* 2018; Strickland *et al.,* 2018; Sunagar *et al.,* 2014). Although these abiotic factors are often found to have a fairly small effect on venom variation. Holding *et al.,* (2018) found that abiotic factors accounted for only 19% of variation seen in the venom composition of Northern Pacific Rattlesnakes (*Crotalus oreganus*). In that same study genetic differentiation between populations was found to account for 46% of variation seen in the venom of *C. oreganus.* Similarly, several other studies have found that genetic differentiation is responsible for differences in venom composition (Dagda *et al.,* 2013; Dowell *et al.,* 2018; Strickland *et al.,* 2018; Wooldridge *et al.,* 2001). Genetic differentiation between populations caused by natural

selection is likely to be driven by differences in prey communities. However this evidence suggests that diet does not have a direct influence on venom composition but rather causes a strong selective pressure and thus allows the successful inheritance of certain venom coding genes.

Epigenetic inheritance refers to inheritance which occurs 'outside the gene'. This generally means that although the genes inherited by offspring are the same, their expression is affected by the parents of a snake. This could refer to the inheritance of inhibitors or promoters of certain genes. In Eastern Diamondback Rattlesnakes (*Crotalus adamanteus*) and in Cottonmouths (*Agkistrodon piscivorous piscivorous*) variation in venom which enables the non-lethal subjugation of frogs was found to be passed on through epigenetic mechanisms (Gennaro *et al.,* 2007).

4.3.2 - Interspecific variation

There is debate within the literature regarding when, in the evolutionary history of snakes, the venom system first evolved. Some argue that the venom system evolved once, very early in squamate radiation (Fry *et al.,* 2017), this is known as the toxicofera hypothesis. Whereas others believe that venom has evolved many times far more recently in many reptiles (Hargreaves *et al.,* 2014; Hargreaves *et al.,* 2017). Both theories go a long way to explain the high degree of variation of venom composition and apparatus in snakes. If the toxicofera hypothesis is to be believed, then reptiles have possessed venom for a very long evolutionary period thus allowing for extensive radiation and variation of venom systems through the loss or recruitment of certain genes. Additionally, if the toxicofera hypothesis is not to be believed then we can assume that venom systems have evolved several times in various lineages in novel ways, this would also explain the high levels of variation in squamate venom systems. However, the presence of venom coding genes in a snake species' genome doesn't necessarily mean that the same venom toxin will be present within the venom of all snakes from that species. The process by which a specific gene loci is used to synthesise a specific protein is a multi-level process with several stages (figure 9) where the process can be halted by gene regulation (Casewell *et al.,* 2014; Dowell *et al.,* 2016). A section of DNA coding for a specific venom protein may not be transcribed into RNA in certain species or even in individuals within a species (Hargreaves *et al.,* 2014). Similarly if a gene is transcribed into RNA it may not then be translated into a protein. Even if a protein is synthesised it may not end up in the venom of the snake. This means that in two species with very similar genomes and lots of similar gene loci for specific venom proteins, not all venom coding genes may be expressed and so the venom phenotype of the two species may vary (Gibbs *et al.,* 2009).

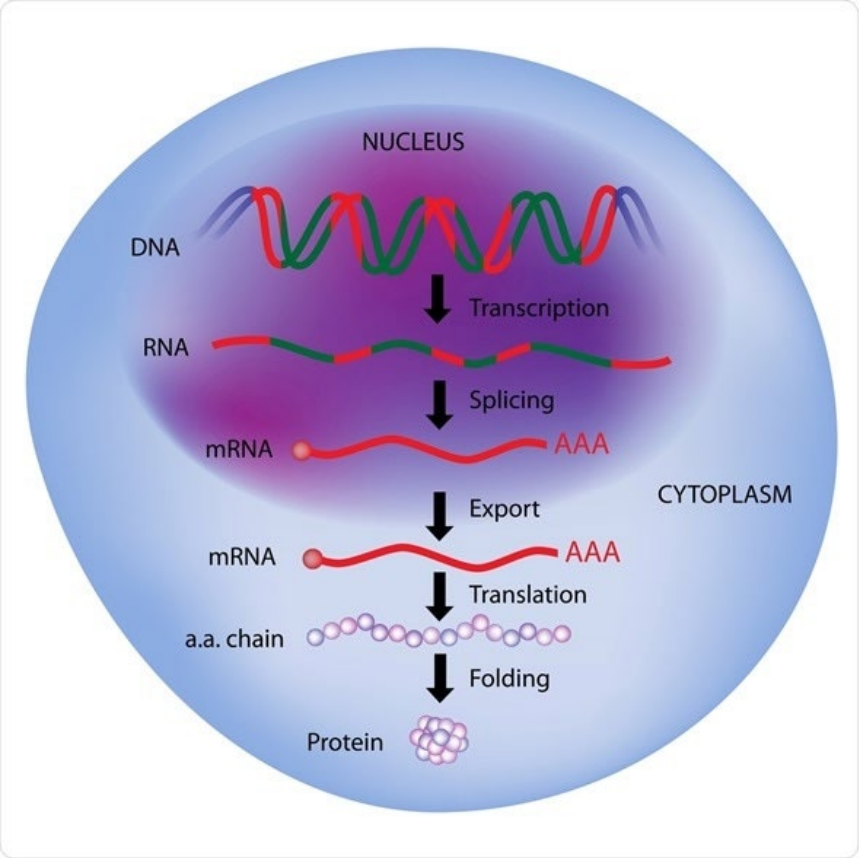

Figure 9. The stages involved in the expression of proteins from DNA. *Image Copyright: Alila Medical Media / Shutterstock.*

A lot of this interspecies venom variation (Gregory-Dwyer *et al.,* 1986) is often put down to genetic divergence and gene regulation (Zancolli *et al.,*2019), however it is also likely that prey specificity is at least partially responsible for venom variation. Different snake species feed on a huge variety of prey, from eggs to slugs to mammals. Diversity in the physiology of prey means that different venom toxins will work at various levels of effectiveness on specific prey groups. A good model of this is the Marbled Sea Snake (*Aipysurus eydouxii*) which feeds exclusively on fish eggs. Such a diet does not require the use of venom and so the toxicity of venom in *A. eydouxii* has decreased by up to 100-fold compared to other species of the same genus (Li *et al.,* 2005) thus illustrating the direct relationship between diet composition and venom toxicity. This is exemplified by the wide pattern of specific lethality of venom on natural prey types observed across many snake species (da Silva *et al.,* 2001; Barlow *et al.,* 2009; Healy *et al.,* 2019). This suggests that natural selection in relation to prey type is in part responsible for a great deal of the interspecies variation we see in the venom systems of snakes.

Other than Darwinian evolution, there are possible molecular contributors to variation in venom composition. Hybridisation has been shown to dramatically affect the composition of snake venom within species which are able to inter-breed. Changes in venom composition as a result of hybridisation have been documented in the offspring of inter-breeding *Crotalus scutulatus scutulatus* and *Crotalus oreganus helleri.* These hybrids show a different venom composition

phenotype to both parents and exhibit characteristics of the venom composition of both *C. s. scutulatus* and *C. o. helleri* (Smith and Mackessy, 2016). Hybridisation allows cross-species gene flow thus allowing for new venom genes to be introduced from one species to another. However, this gene flow via hybridisation is not an example of plasticity as the venom composition is changing due to changes in the genome.

4.3.3 - Intraspecific variation

As previously mentioned, snake venom varies at every level, including the species level. The variation of a snake species venom can be linked to a variety of factors which are outlined ahead.

Geographical Venom Variation-

As previously mentioned, snake venom can vary at all taxonomic levels. Venom composition can vary between two closely related species of the same genus (Petrilla *et al.,* 2014). Interestingly, venom variation does not stop at the generic level. A single species of snake can also exhibit multiple different venom phenotypes across its geographical range (Girón *et al.,* 2018; Kalita *et al.,* 2018; Zancolli *et al.,* 2019). This is particularly well documented for the Lancehead (*Bothrops asper*) which is found in central America. It is known that the venom of *B. asper* varies significantly across its range (figure 10). Venom composition varies between populations of *B. asper* found in the north of their range, on the Caribbean coast and the south of their range on the Pacific coast (Alape-Girón *et al.,* 2008) similar patterns can be seen in Venezuelan Lanceheads (*B. venezuelensis*) another species of the same medically significant genus (Girón *et al.,* 2018). However, it is important to consider that the two populations of *B. asper* sampled by Alape-Girón *et al.,* (2008) are geographically isolated by a mountain range (figure 11) and so it could be argued that these two species are currently undergoing evolutionary divergence which may explain the difference in venom composition found. Perhaps the variation of venom composition across a snake's range is the result of sub-speciation. Differential duplication or loss of genes related to venom composition across sub-species would likely cause variation in the venom phenotype between sub-species. For example, venom phenotype has been found to vary between sub-species of the Anatolian Meadow Viper (*Vipera anatolica anatolica* and *V. a. senliki*) as well as between sub-species of the Desert Rattlesnake (*Crotalus scutulatus scutulatus* and *C. s. salvini*). These differences in venom composition are only minor but do corelate with differences in range (Dobson *et al.,* 2018; Hempel *et al.,* 2020). However, in the case of the Brazilian Tropical Rattlesnake (*Crotalus durissus*) two sub-species are present (*C. d. cascavella* and *C. d. collilineatus*). These sub-species share 90% of their venom proteome yet produce venoms which vary significantly in composition due to differences in the ratios at which certain proteins are expressed (Boldrini-França *et al.,* 2010). This suggests that venom variation between sub-species may be the result of phenotypic plasticity in response to changing conditions across a geographical range (i.e. variation in prey community), differential gene duplication or loss or a combination of these two factors. In fact, it's not unreasonable to assume that a variation in prey community may cause phenotypic plasticity in venom composition which may, in time, lead to differential gene duplication or loss in response to a new survival pressure.

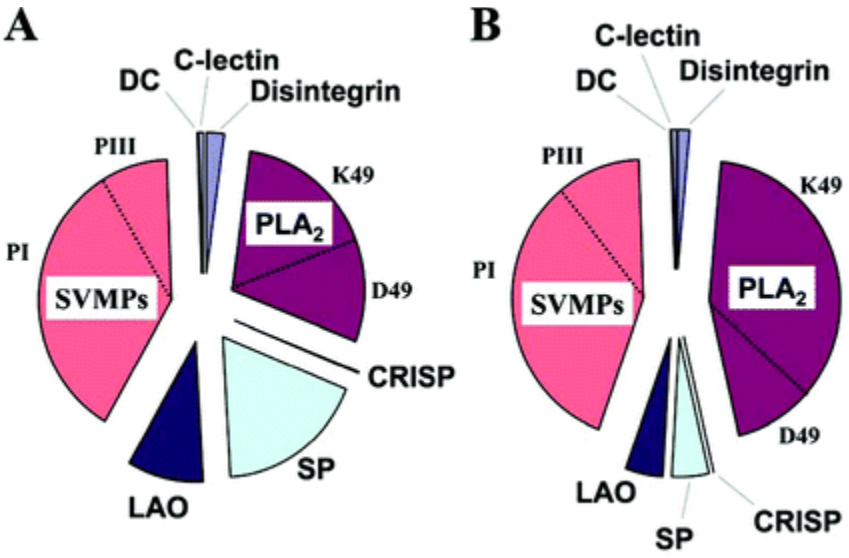

Figure 10. venom protein compositions of pooled venom from adult *B. asper* from the Caribbean region of their range (A) and adult *B. asper* from the Pacific region of their range (B) (Alape-Girón *et al.,* 2008).

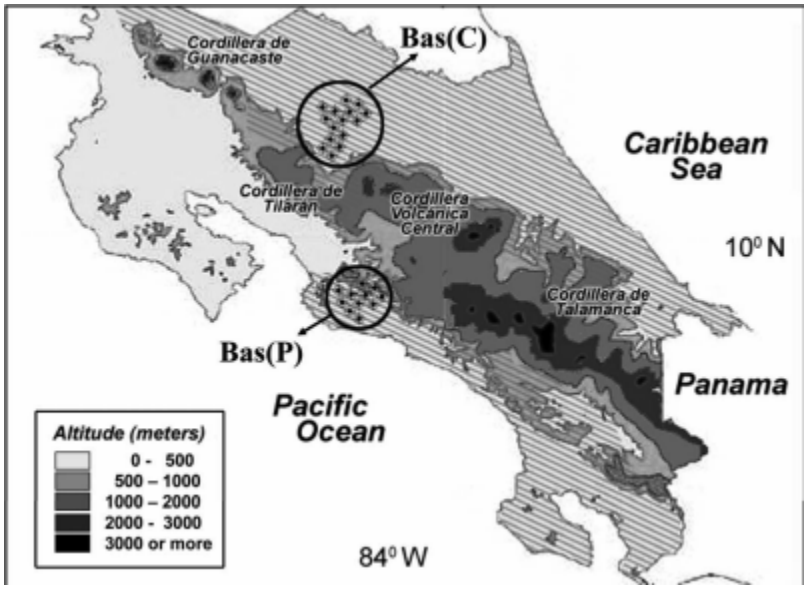

Figure 11. Physical map of Costa Rica showing the sampling sites for snakes in the Caribbean region (Bas(P)) and the Pacific region (Bas(C)).

Sexual Venom Variation-

It has also been suggested that venom composition may vary between sexes within a species (Furtado *et al.,* 2006; Menezes *et al.,* 2006; Pimenta *et al.,* 2007). This may support a genetic

basis for variation in snake venom composition; perhaps certain venom coding genes are found on sex chromosomes. Alternatively, certain venom-coding genes could be regulated endocrinologically via hormones only present in one sex. In snakes, females are heterogametic (ZW sex-determining chromosomes), and males are homogametic (ZZ sex-determining chromosomes). Therefore, if any venom-coding genes are present on the W chromosome only, then only females will possess that gene. Thus, if that gene is expressed, the venom of female snakes could possess a venom toxin not present in the venom of the male snakes. Alternatively, if there is a venom coding gene present on the Z chromosome then the male will have two copies of the gene and so may produce the venom toxin in greater quantities thus increasing in the concentration of that toxin the venom of male snakes.

Seasonal Variation-

The composition of venom has also been known to vary seasonally in snakes (Antunes *et al.,* 2010; Brahma *et al.,* 2015; Sengupta *et al.,* 1994). In Long-Nosed Vipers (*Vipera ammodytes*) two venom proteins which were expressed in the venom during summer months were not expressed at all during winter months (Gubenšek *et al.,* 1974). However, a study on Southern Pacific Rattlesnakes (*Crotalus viridis helleri*), Northern Black-Tailed Rattlesnakes (*C. molossus molossus*) and Western Diamondback Rattlesnakes (*C. atrox*) showed the opposite. Venom composition did not change in individuals when natural seasonal weather was replicated in a lab using artificial lighting and heating over a 20-month period (Gregory-Dwyer *et al.,* 1987). However, this study did not control for the possibility that seasonal venom variation could possibly be triggered by seasonal changes in humidity, or seasonal fluctuations in prey abundance.

The diets of snakes have been known to vary seasonally as prey communities and food availability varies (Brito, 2004; García & Drummond, 1988; Hirai, 2004; Santos *et al.,* 2000). therefore, it is possible that seasonal variations in venom composition are the result of seasonal dietary changes in snakes. However, it may not be the case that venom composition changes in order to better suit dynamic prey communities. Rather, it could be the case that seasonal reductions in food availability reduce the health or metabolic function (Christian *et al.,* 2007; Crews *et al.,* 1987) of snakes thus reducing the production rates of metabolically expensive compounds such as venom proteins.

It is also possible that venom may vary seasonally in line with mating seasons. Hormonal signals may be used to down regulate the production of venom proteins during mating seasons. This may occur due to the costly nature of mating, both the synthesise of gametes and courtship behaviours are often metabolically costly processes (Aubret *et al.,* 2002; Friesen *et al.,* 2017; Olsson *et al.,* 1997). However, no research has yet been done to test whether or not venom composition varies in line with mating seasons. It is unlikely that this is the case given the vital importance of venom in prey subjugation and the direct survival benefits associated with having effective venom. Conversely, some snakes are known to fast while eggs are developing (Brischoux *et al.,* 2011; Lourdais *et al.,* 2002), this could lead to a down regulation in the production of venom proteins in female snakes. This down regulation (if present) could be for the purposes of energy conservation due to the lack of use of venom during the incubation of eggs. Again, it is important to acknowledge that there is no evidence present in the literature to suggest that venom composition changes in line with breeding seasons.

Ontogenetic Variation-

Snake venom composition is a truly plastic phenotype and so varies at the intraspecific level. A commonly studied pattern of intraspecific variation of venom composition in snakes is ontogenetic variation. Many snakes show a change in venom composition as they grow. In the aforementioned study by Alape-Girón *et al.,* (2008), ontogenetic variation of venom was also investigated in the lancehead viper *B. asper* (figure 12). Ontogenetic variation in snake venom composition is well documented (table 3) and recorded for many species in several studies (Cipriani *et al.,* 2017; Gibbs *et al.,* 2011; Guércio *et al.,* 2006; López-Lozano *et al.,* 2002; Madrigal *et al.,* 2012; Wray *et al.,* 2015; Zelanis *et al.,* 2007; Zelanis *et al.,* 2010).

Table 3- A brief summary of the findings of 8 studies investigating ontogenetic variation of venom composition in a variety of vipers and elapids

Paper-	Study species-	Ontogenetic diet shift-	Ontogenetic venom shift-
Alape-Girón *et al.,* 2008	Fer-de-lance (*Bothrops asper*)	None recorded	Shift from PIII-SVMP rich to PI-SVMP rich venom as well as an increase in venom complexity.
Cipriani *et al.,* 2017	9 spp of Australian Brown Snakes (*Pseudonaja*)	Refers to previously documented shift from reptilian diet to more generalized diet	Shift from non-coagulopathic to coagulopathic venoms in all 9 spp
Gibbs *et al.,* 2011	Dusky Pygmy Rattlesnakes (*Sistrurus miliarius barbouri*)	Diet was controlled so no natural shift	No major shift in venom composition, but fine-scale changes in the abundance of several toxins
Guércio *et al.,* 2006	Common Lancehead (*B. atrox*)	None recorded	Differential expression of polypeptides caused shift of venom composition at each developmental stage
López-Lozano *et al.,* 2002	Common Lancehead (*B. atrox*)	None recorded	Human plasma coagulant activity was higher in juveniles, 23kDa protein found in adults but not juveniles
Madrigal *et al.,* 2012	Central American Bushmaster (*Lachesis stenophrys*)	None recorded	Major shifts in toxin composition involving changes in the concentration of vasoactive peptides and serine proteinases

Wray *et al.*, 2015	Timber Rattlesnakes (*Crotalus horridus*) and Eastern Diamondback Rattlesnake (*C. adamanteus*)	Study carried out on neonates prior to first shed so before the first meal	All snakes showed significant shifts in venom composition after postnatal ecdysis
Zelanis *et al.*, 2007	Golden Lancehead (*B. insularis*)	Results are likely correlated with dietary habits, but no shift was recorded in this study	Venoms from younger specimens showed higher coagulant activity
Zelanis *et al.*, 2010	Jararaca (*B. jararaca*)	Shift from ectothermic to endothermic prey	Adult venom was more lethal towards mice and juvenile venom was more lethal towards chicks. Juvenile venom showed human coagulant activity 10X stronger than in the venom of adults

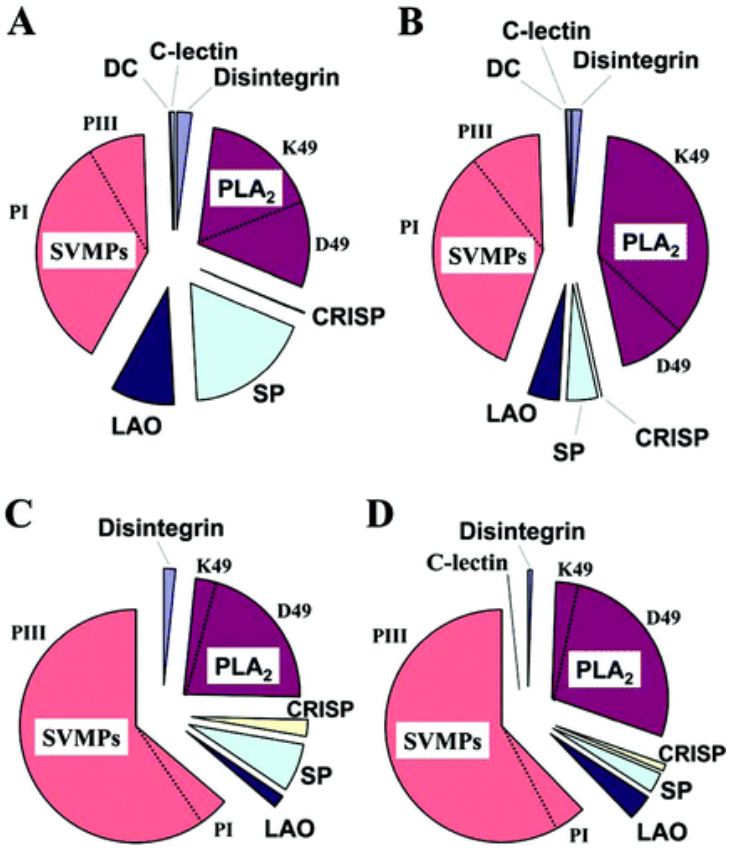

Figure 12. Comparison of venom composition of *B. asper* neonates (C, D) and *B. asper* adults (A, B) from the Caribbean region of their range (A, C) and the Pacific region of their range (B, D). In both *B. asper* populations, neonate venom contained notably more SVMPs (Snake Venom Metalloproteinases) and notably fewer PLA_2 (Phospholipase A2) proteins.

These ontogenetic variations in snake venom composition are likely the result of the aforementioned process of gene regulation whereby certain genes are present but not expressed (Casewell *et al.,* 2014; Dowell *et al.,* 2016; Gibbs *et al.,* 2009; Hargreaves *et al.,* 2014). This regulation can happen at any one of three stages; transcription, translation or expression of the protein in the venom itself. Although the same venom coding genes will always be present throughout a snake's development, the expression of said genes changes as the snake grows, causing an ontogenetic variation in snake venom composition (Durban *et al.,* 2013; Gibbs *et al.,* 2009). This ontogenetic change in venom composition could be caused by a feedback-loop in the automatic nervous system in response to hormonal changes which occur at different life stages of the snake. This is the case for the regulation of several other protein coding genes in other animals (Álvarez-Campos *et al.,* 2019; Enjapoori *et al.,* 2017; Hirose *et al.,* 2019; Mello *et al.,* 2019; Savino *et al.,* 2016). This is supported by Durban *et al* (2013) who concluded that ontogenetic changes in venom composition were more likely to be caused by molecular gene regulation mechanisms than by a reaction to dietary change.

However, another possible cause of ontogenetic shifts in venom composition is diet. A large array of snake species show ontogenetic shifts in diet composition (López *et al.,* 2013; Mackessy *et al.,* 2003; Natusch *et al.,* 2012; Pough, 1977; Wray *et al.,* 2015), as they grow different prey items become available to them. Certain venom proteins are effective against specific prey types (Gibbs & Mackessy, 2009; Lyons *et al.,* 2020; Pawlak *et al.,* 2009; Starkov *et al.,* 2007) and so a change in venom composition is likely to aid in the subjugation of particular prey items. Therefore, it is possible that the change in diet is responsible for the change in venom composition although it is difficult to determine a cause and effect here. This effect may be due to gene regulation as a result of a feed-back loop in the automatic nervous system in response to the presence of different proteins within a snake's diet. This would be similar to the response shown in mice to tannin rich foods (Da Costa *et al.,* 2008). Alternatively, the change in venom composition could be the result of a more enriched diet. Larger snakes are likely to tackle larger prey and therefore have a more nutritious diet thus allowing for the production of more energetically costly compounds in the venom (McCue, 2006; Pintor *et al.,* 2010; Smith *et al.,* 2014).

Throughout a snake's different life-stages, predation risks and predator response behaviours vary (Glaudas *et al.,* 2006; Roth & Johnson, 2004). It is therefore possible that this is linked to ontogenetic shifts in venom composition. The Australian Rainforest Scorpion (*Liocheles waigiensis*) shows a shift in venom composition from offensive to primarily defensive in the presence of a predation risk (Gangur *et al.,* 2017). However, this pattern is unlikely to be replicated in snakes as their venom is thought to be almost exclusively used for prey subjugation, not anti-predator defence (Calvete *et al.,* 2005). This is illustrated by other ontogenetic adaptations shown by snakes for the purposes of predator defence such as changes in behaviour or colouration. The Eastern Racer (*Coluber constrictor*) changes from a blotched pattern as a juvenile (harder to detect when stationary) to a stripped/banded pattern as an adult (harder to

determine speed and direction when moving). This ontogenetic change in patterning in *C. constrictor* is accompanied by a change in anti-predator behaviour from aggressive fight responses in hatchlings to flight responses in adults (Creer, 2005).

Furthermore, a study showed venom composition in captive Pacific Rattlesnakes (*Crotalus oreganus*) was not affected by researcher induced disturbance (Claunch *et al.,* 2017). Researcher induced disturbance (enclosure shaking) is likely to mimic potential predator presence. The study species showed no changes in venom composition thus implying that predation risk does not influence changes in the venom composition of snakes, ontogenetically or otherwise.

Diet Controlled Venom Variation-

Phenotypic divergence as seen in snake venom composition is predominantly driven by a difference in selective pressures present in each location within a range (Schluter & Nagel, 1995). In the case of snake venom, it can therefore be assumed that different venom compositions provide survival benefits in specific locations. In certain locations across a snakes range it is likely that the prey community will vary. Given that the primary use of venom in snakes is prey subjugation and that specific prey lethality of venom is common in snakes it can be assumed that prey community is a predominant factor in intraspecies variation of venom composition. There is a multitude of research supporting this hypothesis. Differences in the composition of the venom in the Malayan Pit Viper (*Calloselasma rhodostoma*) across its range are likely due to variation in prey community and thus diet of the snake (Daltry *et al.,* 1996). This study dismissed both contemporary gene flow (estimated from geographical proximity) and phylogenetic relationships (assessed by analysis of mitochondrial DNA) as possible causes of geographical venom variation in *C. rhodostoma*. Instead concluding that venom variation across range is instead likely to be directly related to a geographical difference in prey community, as different prey species show differential susceptibility to different venom proteins. Given that the primary function of venom in snakes is prey subjugation, this explains the need for different venom phenotypes in order to subdue different prey species. A link between prey community and venom composition variation has also been suggested by a few other studies in a few other species. In the *Echis* genus (the study subject of this project), species with a more arthropod based diet had a venom with a higher specific lethality towards arthropods than species with a more vertebrate rich diet (Barlow *et al.,* 2009). The Asian pit viper *Trimeresurus stejnegeri* was shown to have geographically variable venom composition. Research found that this variation was not the result of neutral molecular evolution, as previously assumed, but was in fact likely due to natural selection for regional diet (Creer *et al.,* 2003). Gibbs *et al* (2011) found that Dusky Pygmy Rattlesnakes (*Sistrurus miliarius barbouri*) showed fine scale changes in venom composition when moved from a varied diet to a diet consisting entirely of either frogs, lizards or mice. Northern Pacific Rattlesnakes (*Crotalus oreganus*) also show significant venom variation across their range. A study found that although most of this variation could be explained by genetic differentiation (46% of total variation), differences in prey community composition were still responsible for 23% of total variation in venom composition (Holding *et al.,* 2018). Additionally, the diet of several taxa has been shown to affect saliva composition (Da Costa *et al.,* 2008; Méjean *et al.,* 2015). Given the likely evolutionary origin of venom glands being salivary

glands, this supports the theory that diet can directly influence venom composition as it can saliva composition.

Contrary to this, it is thought that the venom of the Southern Pacific Rattlesnake (*Crotalus oreganus helleri*) is under the constraints of negative selection regarding diet and its venom is in fact evolving to become less specific (Sunagar *et al.,* 2014). This makes evolutionary sense as venom which effectively subdues a greater variety of prey will allow a snake to take advantage of more potential food sources. However, this change could still be a response to dietary shift, perhaps several regular changes in prey community around a particular species of snake will drive the evolution of less prey-specific venom.

Furthermore, it is likely that changes in prey community and diet will affect the venom composition of an individual snake throughout its lifetime. Differing prey communities are found in different areas of a snake's rage, potentially relating to interspecific venom variation (Barlow *et al.,* 2009; Creer *et al.,* 2003; Gibbs *et al.,* 2011; Holding *et al.,* 2018). At the intraspecific level diet is also likely to have a significant effect on venom composition in snakes. As previously discussed, both ontogenetic and seasonal changes in venom composition can be easily linked to dietary changes (Antunes *et al.,* 2010; Brahma *et al.,* 2015; Brito, 2004; García & Drummond, 1988; Hirai, 2004; López *et al.,* 2013; Mackessy *et al.,* 2003; Natusch *et al.,* 2012; Pough, 1977; Santos *et al.,* 2000; Wray *et al.,* 2015). However, it is very hard to determine whether or not dietary change has an immediate effect on venom composition. It may be that these changes are an innate trait, evolved in order maximise the effectiveness of a snake's venom at different life stages or times of the year. These changes may occur regardless of dietary change. Gibbs *et al.,* (2011) found that Dusky Pygmy Rattlesnakes (*Sistrurus miliarius barbourin*) showed ontogenetic changes in venom composition despite not experiencing a change in diet. On the contrary, the study also found that adults of the same species showed some degree of plasticity when exposed to a change in diet. Perhaps venom varies without dietary change, but dietary change determines how it varies. It is likely that the highly plastic phenotype of venom composition is influenced by a wide variety of factors (Holding *et al.,* 2018).

As well as the supporting evidence and the instances where venom composition variation coincides with dietary change, it seems highly plausible that the two are linked. Furthermore, it is widely accepted that snake venom glands have evolved from salivary glands in non-toxic ancestors (Gibbs *et al.,* 2011; Kochva, 1987). This would suggest that venom proteins are produced, regulated and secreted in similar ways to salivary proteins and other secretory proteins (Fry, 2005). Thus, the phenotypic plasticity shown in the saliva composition of mice in response to dietary change (Da Costa *et al.,* 2008) provides a reasonably strong argument for the possibility that the same is true for snake venom. However, in this study the change in saliva composition was for the purpose of precipitating a foul-tasting compound, this is different to the function of venoms. Again, it is likely that there is not one single factor responsible for phenotypic plasticity of snake venom.

This is where I have identified a significant gap in the literature. Although we know that diet change happens alongside changes in venom composition, we don't know that one causes the other. A controlled experiment needs to be carried out to test the possible effects of a dietary shift on the composition of venom in snakes.

5 - Proposed Study

In order to contribute to the current gap in the literature, I carried out an experiment to determine the effect of dietary shifts on the composition snake venom. For a model species, this study used Eastern Saw-Scaled Vipers (*Echis carinatus sochureki*). This species was chosen due to its natural foraging behaviour and the availability of the species. *E. c. sochureki* are known to feed on both vertebrate and invertebrate prey in the wild and show high levels of venom specificity towards natural prey species as well as showing ontogenetic variation in venom composition (Barlow *et al.,* 2009). It seems logical that if snakes are able to show phenotypic plasticity of venom composition in response to dietary change, *E. c. sochureki* would be a logical candidate given the variation present in its natural diet. *E. c. sochureki* are also a medically significant species of snake which are responsible for many mortalities each year (Kularatne *et al.,* 2011; Warrell & Arnett, 1976; Weiss *et al.,* 1973).

Twenty juvenile *E. c. sochureki* were acquired by the university and kept in the venom room. A full record of diet from birth was kept so we know exactly what each snake has ever eaten. Upon arrival, snakes were fed on a diet of crickets for 3 months before a preliminary venom sample was taken. This gave a baseline sample for venom composition. 10 of the snakes (selected randomly) were then assigned to each condition. One condition remained on a diet of crickets while the other condition was weaned onto vertebrate prey in the form of neonatal mice. Additional venom samples were taken after 4 months and again after 8 months. After the third and final venom samples were taken, venom was analysed using Agilent Bioanalyzer on-chip technology and the protein composition of the venom was recorded. The final venom composition of each snake was compared to the baseline venom sample and 4-month venom sample to test for significant differences in venom composition.

Invertebrate fed snakes were fed more often than vertebrate feeders in order to control for the difference in mass between neonatal mice and crickets as well as accelerated growth rates in vertebrate feeders.

It was expected that the venom of vertebrate feeders would contain more haemotoxins at the end of the study and the invertebrate feeder's venom would contain more cytotoxic compounds. This result would be significant as it would influence the production of antivenom to include the venoms of snakes raised on different diets in order to produce more broadly effective antivenoms. The research also has implications for evolutionary biology as it could show that the loss or gain of venom proteins happens far more readily than first thought.

Chapter 2- Experimental Study

1 - Methodology-

1.1 – Ethics Statement

This research was screened in accordance with the College of Environmental Sciences and Engineering Research Ethics Review Procedure and no issues were identified.

1.2 - Experimental Conditions-

Twenty hatchling *Echis carinatus sochureki* were purchased, comprising of two clutches. One clutch contained fourteen snakes and the other contained six snakes. Snakes were each assigned a name comprising of the letters ECS (to represent *Echis carinatus sochureki*) followed by a number (table 4) All snakes were kept in a temperature-controlled room which maintained a temperature between 25°C and 30°C. All snakes were kept in appropriately sized plastic tubs with paper towel to function as substrate. All snakes were given a water bowl and a hide large enough for them to fit in. Any faeces and urates were removed, and water was replaced every other day. For the first six months of the experiment all the snakes were fed on invertebrate prey consisting of small brown crickets. After six months the snakes were split using a random number generator and half of each clutch was assigned to one of two groups (table 4). One group was maintained on an invertebrate diet of crickets while the other was weaned onto a vertebrate diet. Those placed on a vertebrate diet were first fed on the legs of neonate mice. After a few feeds they began to be fed appropriately sized infant mice. Meals were given weekly. In the instance that a snake refused food it would be assist fed, this was done by pinning the snake and inserting food into its mouth using forceps. Unfortunately, one snake (ECS18) died prior to conditions being implemented.

Table 4 – Using a random number generator, the snakes were split into two conditions. These conditions were labelled as vertebrate feeders and invertebrate feeders. The unhighlighted specimens were from the larger litter and those highlighted in green were from the second, smaller litter.

Invertebrate feeders-	**Vertebrate feeders-**
ECS01	ECS02
ECS05	ECS03
ECS06	ECS04
ECS08	ECS07
ECS10	ECS09
ECS13	ECS11
ECS14	ECS12
ECS15	ECS16
ECS17	ECS19
ECS20	

Throughout the experiment records were kept of what/when each snake ate (table 5) and how much each snake weighed (table 6) this record was used in order to check for extraneous variables.

Table 5- An example of the feeding record kept for the snakes after arriving at the university. The numbers represent the quantity of each food item eaten. The prefix A/F represents when a snake was assist fed. The prefix R represents when a snake refused food. The letter C signifies that the food item given was a brown cricket.

Feeding records for *Echis carinatus sochureki* post arrival at Bangor University						
Litter 1-	18/10/2019	19/10/2019	22/10/2019	23/10/2019	28/10/2019	29/10/2019
ECS01	1C		1C		1C	
ECS02	A/F 1C		1C		1C	
ECS03	1C		1C		1C	
ECS04	1C		1C		1C	
ECS05	1C		1C		1C	
ECS06	1C		1C		1C	
ECS07	1C		1C		R 1C	
ECS08	1C		1C		1C	
ECS09	1C		1C		1C	
ECS10	1C		1C		1C	
ECS11	1C		1C		1C	
ECS12	1C			1C	1C	
ECS13	1C		1C		R 1C	
ECS14	1C		1C		1C	
Litter 2-	18/10/2019	19/10/2019	22/10/2019	23/10/2019	28/10/2019	29/10/2019
ECS15	1C		1C		1C	
ECS16	1C		1C		1C	
ECS17	1C		1C		1C	
ECS18	R 1C	A/F 1C	R 1C	A/F 1C	R 1C	A/F 1C
ECS19	R 1C	A/F 1C	R 1C	A/F 1C	R 1C	A/F 1C
ECS20	R 1C	A/F 1C	R 1C	A/F 1C	R 1C	A/F 1C

Table 6- An example of weight records showing the weights of each snake before conditions were implemented (24/10/2019) and the weight of each snake after the end of the experiment at the final weight recording (06/07/2021). Boxes highlighted in red represent instances where the snake has died prior to the final weighing.

	Weight (g)	
	24/10/2019	**06/07/2021**
ECS01	4.7	
ECS02	4.2	11.7
ECS03	4.7	13.4
ECS04	4.7	14.9
ECS05	4.3	
ECS06	5.1	8
ECS07	4.6	19.2
ECS08	4.8	9.4
ECS09	4.7	18.4
ECS10	4.4	
ECS11	4.6	20.5
ECS12	4.7	14.9
ECS13	4.7	
ECS14	4.1	
ECS15	2.6	
ECS16	2.8	14.6
ECS17	2.9	6.8
ECS18	2.9	
ECS19	2.5	9.5
ECS20	2.6	

1.3 - Venom collection

Venom was collected from every living snake prior to conditions being implemented then again three months after conditions were implemented and a final time 12 months after conditions were implemented (table 5). Venom samples were collected by presenting the held snake with an Eppendorf tube with a layer of plastic film stretched over the top of it. After each snake had bitten and venom had been collected, the tube was closed, labelled and placed on ice. Venom samples were then stored at -80°C until used. There were two instances when no venom was given by the snake when extraction was attempted and another where the quantity of venom was too small (<4µl) to be analysed using the bioanalyzer. Additionally, a further 5 snakes died between the implementation of controls and the third venom extraction (table 7).

Table 7- The dates three dates at which venom samples were extracted for each snake. The instances when no venom was given are highlighted in yellow. Snakes which were deceased by each extraction are highlighted in red.

	Pre-condition extraction	Mid-condition extraction	Final extraction
ECS01	19/12/19	Deceased	Deceased
ECS02	26/11/19	17/3/20	27/10/20
ECS03	26/11/19	17/3/20	27/10/20
ECS04	26/11/19	No venom given	27/10/20
ECS05	26/11/19	Deceased	Deceased
ECS06	26/11/19	17/3/20	28/10/20
ECS07	26/11/19	17/3/20	27/10/20
ECS08	26/11/19	17/3/20	28/10/20
ECS09	26/11/19	17/3/20	27/10/20
ECS10	26/11/19	Deceased	Deceased
ECS11	26/11/19	17/3/20	27/10/20
ECS12	26/11/19	17/3/20	27/10/20
ECS13	19/12/19	17/3/20	28/10/20
ECS14	26/11/19	17/3/20	Deceased
ECS15	26/11/19	17/3/20	Deceased
ECS16	26/11/19	Very low venom yield	28/10/20
ECS17	26/11/19	17/3/20	28/10/20
ECS18	Deceased	Deceased	Deceased
ECS19	26/11/19	No venom given	28/10/20
ECS20	26/11/19	Deceased	Deceased

1.4 - Venom Composition Analysis

Venom was analysed using an Agilent 2100 bioanalyzer. The Agilent Protein 80 kit was used according to the makers protocol (Agilent Technologies, 2022). This gave me the size of each protein present (kDa) within the venom, the proteins relative concentration (ng/µl) and the proportion at which it occurred in each venom extraction (table 8).

Table 8- The first column shows the size of each protein present within the venom, the second shows the relative concentration of each protein within the venom and the third column shows the percentage of the whole venom that each protein represents. This specific example shows the composition of the venom of ECS02 that was extracted on 26/11/2019

Size kDa	Rel conc (ng/ul)	% Total
16.8	15.2	5
19.3	67.2	21.9
24.7	17.2	5.6
37.8	16.3	5.3
39.2	7	2.3
40.5	7	2.3
58.9	33.9	11.1
67.8	43.1	14.1
69.5	14.5	4.7
74.9	15.8	5.1
79.9	21.9	7.1
82.3	23.5	7.7
93.7	23.8	7.8

Due to the absence of an established data base, I was not able to accurately infer specific venom toxins or toxin families from the protein analysis. This analysis showed me the sizes of each protein present in the venom (kDa) and the proportion at which each protein occurs within the venom. In order to aid in further analysis and account for slight differences in the molecular weight of similar toxins from the same family, proteins were grouped into bins of molecular weight (table 9).

Table 9- As per the previous example (table 6), this venom composition is from the venom of ECS02 extracted on 26/11/2019. The left column shows the weight categories used to group the proteins present within the venom. The right column shows the percentage of the whole venom that each protein category represents.

Size (kDa)	% Total
0-10	0
10.1-20	26.9
20.1-30	5.6
30.1-40	7.6
40.1-50	2.3
50.1-60	11.1
60.1-70	18.8
70.1-80	12.2
80.1-90	7.7
90.1-100	7.8

1.5 - Data Analysis

As previously mentioned, the proteins present in the venoms were grouped by size as I did not have means to identify each protein present. The venom composition of venom from each snake from each sperate extraction was used to form a graph summarizing the venom composition for each venom extraction (figure 13). The mean percentage composition for each protein size category was calculated for both conditions at each extraction interval the standard deviation was also calculated (table 10). These means were used to form 6 further graphs in order the illustrate the average composition of venom for snakes from each condition at each of the three time intervals. While these graphs were useful to visualize any patterns and differences in venom composition, they do not show whether these patterns and differences are significant. Following Kolmogorov-Smirnoff tests of normality and Levene's tests of equality of variance, further statistical analysis was carried out. T-tests and Mann-Whitney U tests were completed to compare the average proportions of each protein-weight category at all three extraction intervals for both of the experimental conditions. A T-test was also carried out to determine whether the average weight of snakes in each condition varied significantly.

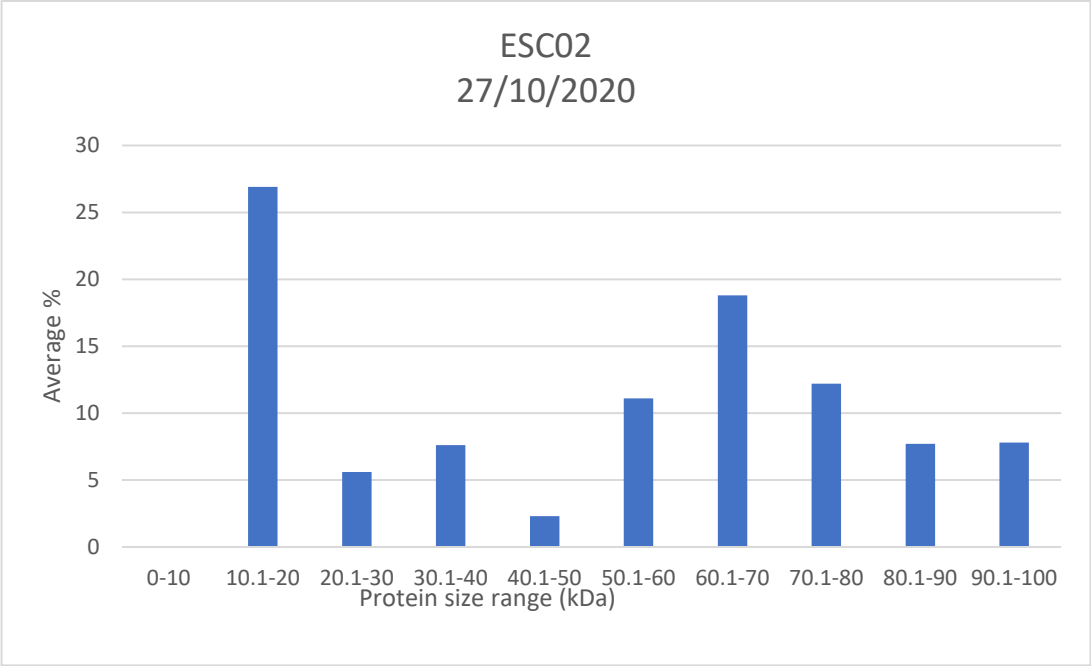

Figure 13. An example of one of the graphs created to visualise venom composition. This example shows the composition of the venom of ECS02 extracted on 27/10/2020. Each bar represents one size category (kDa) of proteins present in the venom and the percentage of the whole venom made up by that protein category. The chart shows that in this sample the most abundant protein size category is proteins between 20.1-30 kDa.

Table 10- An example of how average composition was calculated for each condition at each of the three venom extraction intervals. The example given shows the composition of venom for every vertebrate feeding snake extracted at the end of the experiment in October 2020. Also shown is the average composition of venom for vertebrate feeders after the final venom extraction alongside a measure of population standard deviation.

Vert feeders Oct 2020-												
	Total %											
Size range (kDa)	**ECS02-**	**ECS03-**	**ECS04-**	**ECS07-**	**ECS09-**	**ECS11-**	**ECS12-**	**ECS16-**	**ECS19-**		**Average (%)**	**Standard deviation:**
0-10	2.6	0	0		43.2	3.6	39.8	0	9.7		12.3625	17.10788835
10.1-20	24	6.4	0		14.8	6	0	30.4	47.6		16.15	15.71360875
20.1-30	38.5	66.9	19.9		11.4	35.1	0	25.2	0		24.625	20.91576857
30.1-40	0	0.7	0		5.3	0	0	0	25.3		3.9125	8.262934936
40.1-50	2.6	17.6	0		0	27	0	11.2	3		7.675	9.41219289
50.1-60	11.4	0.7	2.7		0	19.1	10.5	14.4	0		7.35	6.964014647
60.1-70	20.9	0	39.2		7.7	0	20.1	9.7	0		12.2	12.94275859
70.1-80	0	0	24.4		15.2	0	16.9	0	9.4		8.2375	9.065861446
80.1-90	0	7.7	13.8		2.4	9.2	10.7	7.5	5		7.0375	4.178198625
90.1-100	0	0	0		0	0	2	1.6	0		0.45	0.785811682

2 – Results

2.1 – Statistical Analysis

Tests of normality (Kolmogorov-Smirnov) and equality of variance (Levenes's test) were carried out on the data (tables 11 & 12). For all normally distributed data with equal variance, t-tests were then used and for data sets which were either not normally distributed, had a sample size which was too small to test for normality (n=<5) or showed inequality of variance, two-tailed Mann-Witney U tests were used to determine whether any changes in venom composition between conditions were significant (tables 13, 14 & 15). Statistical tests were carried out for each protein size group in order to determine whether the venoms varied significantly in their proportions of any individual protein group.

Table 11 – The Kolmogorov-Smirnov test statistics and p-values for each data set showing whether the data from each protein size bin for either condition group is normally distributed. cells highlighted in red show data sets where it cannot be concluded that data is distributed normally. Due to the low sample size for post-condition venom samples of invertebrate feed snakes (n=4), a test of normality could not be carried out and so it must be assumed that data is not normally distributed.

Protein size category (kDa)		Pre-condition vertebrate feeders	Pre-condition invertebrate feeders	Mid-condition vertebrate feeders	Mid-condition invertebrate feeders	Post-condition vertebrate feeders	Post-condition invertebrate feeders
0-10	K-S test stat	0.444	0.387	0.271	0.414	0.271	N/A sample size too small
	p-value	0.039	0.074	0.593	0.193	0.593	
	Normally distributed?	no	Yes	yes	Yes	yes	
10.1-20	K-S test stat	0.235	0.162	0.296	0.331	0.296	N/A sample size too small
	p-value	0.624	0.919	0.484	0.432	0.484	
	Normally distributed?	yes	Yes	yes	Yes	yes	
20.1-30	K-S test stat	0.192	0.185	0.328	0.194	0.328	N/A sample size too small
	p-value	0.836	0.825	0.358	0.946	0.358	
	Normally distributed?	yes	Yes	yes	Yes	yes	
30.1-40	K-S test stat	0.172	0.336	0.364	0.351	0.364	N/A sample size too small
	p-value	0.912	0.165	0.244	0.362	0.244	
	Normally distributed?	yes	Yes	yes	Yes	yes	
40.1-50	K-S test stat	0.347	0.237	0.516	0.282	0.516	N/A sample size too small
	p-value	0.179	0.549	0.03	0.634	0.03	
	Normally distributed?	yes	Yes	no	Yes	no	

50.1-60	K-S test stat	0.284	0.28	0.516	0.386	0.516	N/A sample size too small
	p-value	0.387	0.346	0.03	0.256	0.03	
	Normally distributed?	yes	Yes	no	Yes	no	
60.1-70	K-S test stat	0.328	0.36	0.32	0.385	0.32	N/A sample size too small
	p-value	0.232	0.114	0.388	0.263	0.388	
	Normally distributed?	yes	Yes	yes	Yes	yes	
70.1-80	K-S test stat	0.151	0.244	0.169	0.389	0.169	N/A sample size too small
	p-value	0.968	0.517	0.968	0.253	0.968	
	Normally distributed?	yes	Yes	yes	Yes	yes	
80.1-90	K-S test stat	0.261	0.295	0.253	0.369	0.253	N/A sample size too small
	p-value	0.495	0.287	0.674	0.308	0.674	
	Normally distributed?	yes	Yes	yes	Yes	yes	
90.1-100	K-S test stat	0.425	0.417	0.316	0.373	0.316	N/A sample size too small
	p-value	0.054	0.043	0.404	0.296	0.404	
	Normally distributed?	yes	No	yes	Yes	yes	

Table 12 – The result of the Levene's test of equal variance showing that in most normally distributed data sets, there was no significant difference between the variance shown within each data set, this is not the case for two normally distributed data sets. Equality of variance could not be tested for the post-condition data sets as the sample size for invertebrate feeders at this point was too low (n=4)

	Pre-condition			Mid-condition			Post-condition		
Protein size category (kDa)	f-ratio value	p-value	Sig diff?	f-ratio value	p-value	Sig diff?	f-ratio value	p-value	Sig diff?
0-10	0.024	0.879	no	8.348	0.015	yes	N/A	N/A	N/A
10.1-20	0.11	0.744	no	5.115	0.045	yes	N/A	N/A	N/A
20.1-30	1.769	0.201	no	0.032	0.861	no	N/A	N/A	N/A
30.1-40	0.638	0.435	no	0.107	0.75	no	N/A	N/A	N/A
40.1-50	1.162	0.296	no	8.309	0.015	yes	N/A	N/A	N/A
50.1-60	3.462	0.08	no	0.01	0.922	no	N/A	N/A	N/A
60.1-70	0.04	0.845	no	1.831	0.203	no	N/A	N/A	N/A
70.1-80	0.104	0.751	no	0.613	0.45	no	N/A	N/A	N/A
80.1-90	2.136	0.162	no	0.00047	0.983	no	N/A	N/A	N/A
90.1-100	0.047	0.831	no	1.727	0.216	no	N/A	N/A	N/A

Table 13- The p values of each Mann-Whitney U or t-test carried out in order to find any statistically significant differences in the composition of venom between invertebrate and vertebrate feeding snakes prior to conditions being implemented. The t-tests/Mann-Whitney U tests were carried out individually for each size category of proteins present within the venom. Sd= standard deviation, sig dif= significant difference.

size group (kDa)	**vert mean**	**vert sd**	**invert mean**	**invert sd**	**vert sample size**	**invert sample size**	**P/U value**	**</>P**	**Sig dif?**
0-10	0.911111111	1.652906	1.12	2.497919	9	10	42	p<0.05	no
10.1-20	23.32222222	14.82781	15.62	14.33867	9	10	0.2659	P>0.05	no
20.1-30	4.944444444	3.477902	5.3	5.908468	9	10	0.8768	P>0.05	no
30.1-40	7.177777778	4.201528	3.86	6.113461	9	10	0.1909	P>0.05	no
40.1-50	11.32222222	19.20288	10.33	10.97115	9	10	0.8901	P>0.05	no
50.1-60	5.711111111	9.375553	13.67	15.53506	9	10	0.2005	P>0.05	no
60.1-70	9.955555556	13.35133	9.83	13.88964	9	10	0.9842	P>0.05	no
70.1-80	26.44444444	19.40751	19.36	21.43013	9	10	0.4623	P>0.05	no
80.1-90	7.611111111	8.450305	18.27	28.952	9	10	0.3032	P>0.05	no
90.1-100	2.6	3.70705	2.64	4.600043	9	10	42	P<0.05	no

Table 14- The p values of each Mann-Whitney U or t-test carried out in order to find any statistically significant differences in the composition of venom between invertebrate and vertebrate feeding snakes 3 months after conditions being implemented. The t-tests/Mann-Whitney U tests were carried out individually for each size category of proteins present within the venom. Sd= standard deviation, sig dif= significant difference.

size group (kDa)	vert av	vert sd	invert av	invert sd	vert sample size	invert sample size	P/U value	</>P	Sig dif?
0-10	5.52857	5.51536	10.8167	16.2604	7	6	20	P<0.05	no
10.1-20	21.8286	24.4998	7.85	8.36894	7	6	16	P<0.05	no
20.1-30	13.6429	18.0748	27.1833	21.7107	7	6	0.2449	P>0.05	no
30.1-40	4.85714	8.88817	4.13333	5.88944	7	6	0.8684	P>0.05	no
40.1-50	2.25714	5.52885	13.5	14.0057	7	6	9	P<0.05	no
50.1-60	2.31429	5.66882	4.66667	5.78869	7	6	8	P<0.05	no
60.1-70	13.6714	17.254	6.65	11.0069	7	6	0.4103	P>0.05	no
70.1-80	20.8571	12.5301	11.3333	21.081	7	6	0.3345	P>0.05	no
80.1-90	10.2	11.6479	11.6479	11.4373	7	6	0.8259	P>0.05	no
90.1-100	4.84286	5.39467	7.8	14.388	7	6	0.6223	P>0.05	no

Table 15- The U values of each Mann-Whitney U test carried out in order to find any statistically significant differences in the composition of venom between invertebrate and vertebrate feeding snakes 11 months after conditions were implemented. The tests were carried out individually for each size category of proteins present within the venom. Sd= standard deviation, sig dif= significant difference

size group (kDa)	vert av	vert sd	invert av	invert sd	vert sample size	invert sample size	U value	</>P	Sig dif?
0-10	12.3625	17.10789	4.5875	5.4914	8	4	16.5	p<0.05	no
10.1-20	16.15	15.71361	22.0375	16.26781	8	4	13	p<0.05	no
20.1-30	24.625	20.91577	10.775	7.106115	8	4	16	p<0.05	no
30.1-40	3.9125	8.262935	4.8375	7.54763	8	4	17	p<0.05	no
40.1-50	7.675	9.412193	5.3375	2.957485	8	4	13	p<0.05	no
50.1-60	7.35	6.964015	4.025	5.062917	8	4	11	p<0.05	no
60.1-70	12.2	12.94276	20.525	15.28891	8	4	12.5	p<0.05	no
70.1-80	8.2375	9.065861	11.775	17.03029	8	4	10.5	p<0.05	no
80.1-90	7.0375	4.178199	15.0375	11.633	8	4	11.5	p<0.05	no
90.1-100	0.45	0.785812	1.125	1.948557	8	4	10	p<0.05	no

2.2 – Comparisons of Average Venom Compositions

Initial analysis showed that the average venom composition of the snake's prior to conditions being implemented is very similar between the two groups (figures 14 and 15). The bar graphs generated, aid in visualising the average venom composition of snakes in each condition and see that they are very similar. In fact, proteins sized between 0-10 kDa make up 1% of average venom composition in both groups and proteins in the size range of 90.1-100 kDa make up 3% of total average venom composition in both groups. Both groups have proteins between 70.1-80 kDa in size as their most abundant average size category (22% of total average venom composition in invert feeders and 26% in vertebrate feeders). Both groups also showed similar average proportions of proteins between 40.1-50 kDa and 60.1-70 kDa (11% and 11% for invert feeders, 11% and 10% for vertebrate feeders respectively). Other size categories vary more between groups but not greatly, for example proteins between 30.1-40 kDa make up 4% of total

average venom composition in invert feeders and 7% in vertebrate feeders. Both conditions also show very high standard deviation meaning that variation between each induvial snake was high in relation to the small sample sizes. The statistical analysis above shows that the venoms of each group did not vary significantly in the composition of their venoms (table 13).

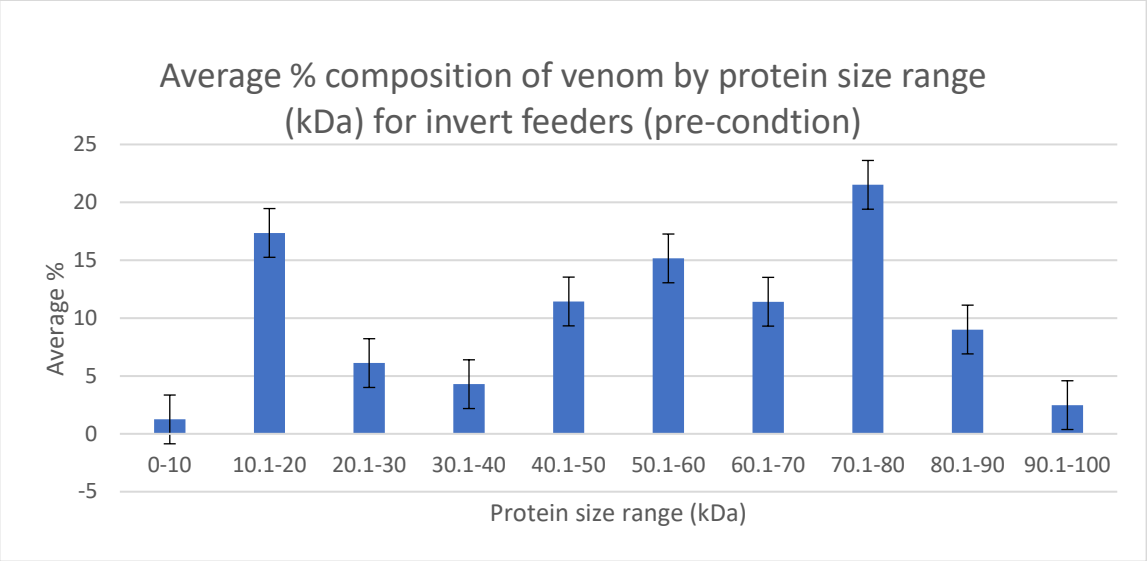

Figure 14. The average venom composition of invertebrate feeders prior to the implementation of conditions. The categories represent size groupings of proteins present in the venom (kDa) each bar shows the percentage of the total venom composition that each size category comprises. Error bars showing the standard deviation within each size category are also included.

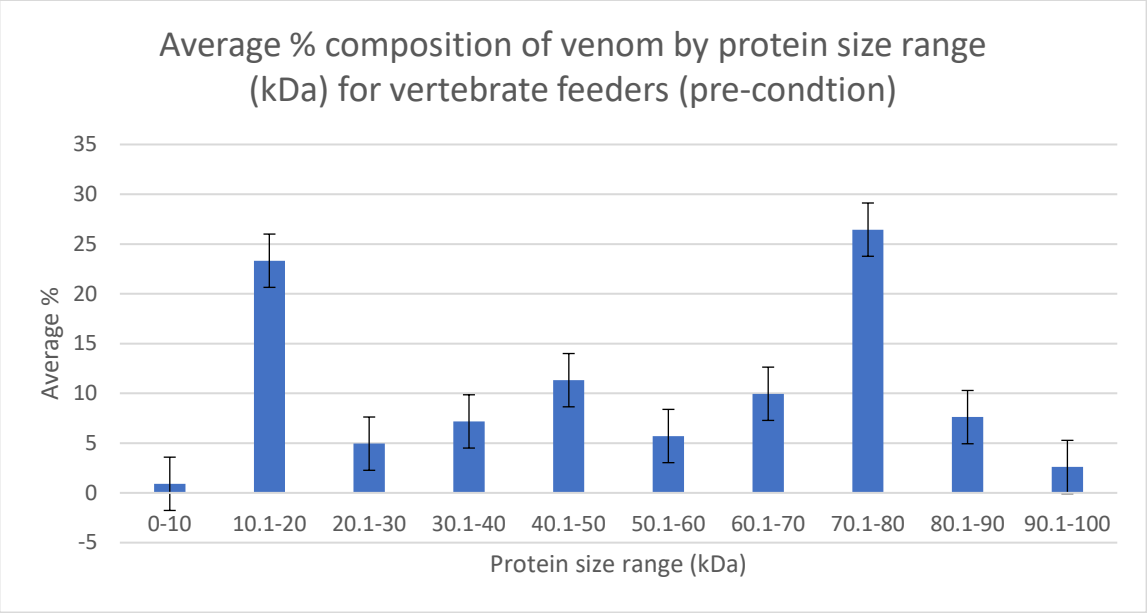

Figure 14. The average venom composition of vertebrate feeders prior to the implementation of conditions. The categories represent size groupings of proteins present in the venom (kDa) each bar shows the percentage of the total venom composition that each size category comprises. Error bars showing the standard deviation within each size category are also included.

After three months of conditions being implemented the composition of venom had changed between the two groups. In vertebrate feeders the size group which made up the greatest percentage of average total venom composition was proteins between 10.1-20 kDa (22% of total venom composition) while in invert feeders, the most common average size category was proteins between 20.1-30 kDa (27% of total venom composition). The average venom of vertebrate feeders had four size categories which each made up less than 5% of total venom composition (30.1-40, 40.1-50, 50.1-60 and 90.1-100 kDa) while the average venom of invert feeders had only two size categories which made up less than 5% of total venom composition (30.1-40 and 50.1-60 kDa). Thus, it can be said that the average composition of invertebrate feeding snakes shows a more equal distribution of protein sizes present and so is less skewed towards any single size category. The size category which was least prevalent in the average venom composition of vertebrate feeders was proteins between 40.1-50 kDa (2%) while in the average venom composition of invert feeders, this category made up 14% of total venom composition and the least abundant category was proteins between 30.1-40 kDa (4%), a size category which made up 5% of average venom composition in vertebrate feeders. The error bars show that standard deviation within each sample is very high meaning that there is a lot of variation in the venom composition of individual snakes within each condition in relation to the small sample sizes. All differences in average venom composition between each group described above are not statistically significant (table 14).

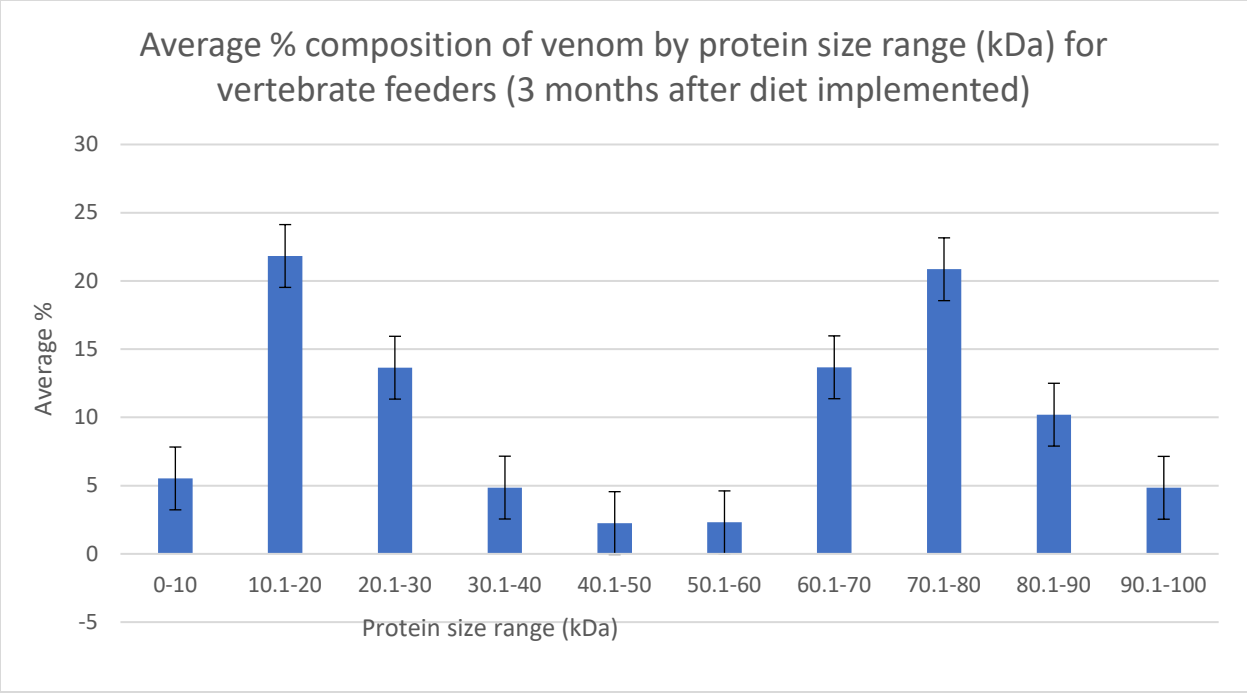

Figure 15. The average venom composition of vertebrate feeders 3 months after the implementation of conditions. The categories represent size groupings of proteins present in the venom (kDa) each bar shows the percentage of the total venom composition that each size category comprises. Error bars showing the standard deviation within each size category are also included.

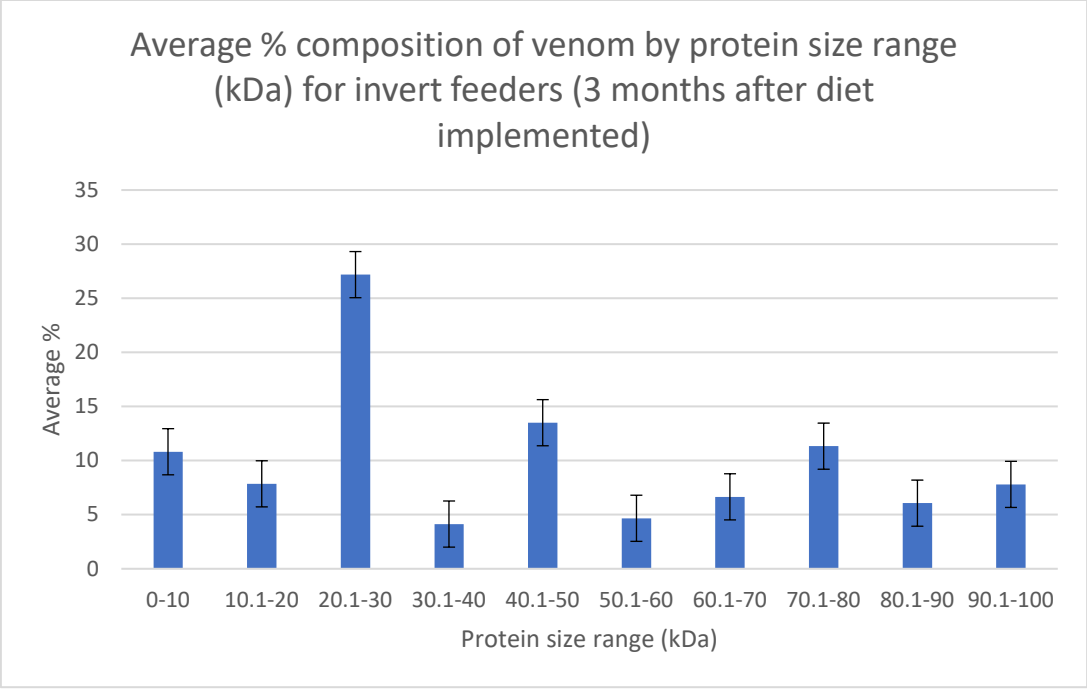

Figure 16. The average venom composition of invertebrate feeders 3 months after the implementation of conditions. The categories represent size groupings of proteins present in the venom (kDa), each bar shows the percentage of the total venom composition that each size category comprises. Error bars showing the standard deviation within each size category are also included.

The final venom extraction shows much the same pattern, it seems like there is a clear difference in the average composition of venoms between vertebrate feeders and invertebrate feeders when you look at figures 17 and 18. The most prevent protein size category in the average venom composition of vertebrate feeders was proteins between 20.1-30 kDa (25% of total average venom composition) compared to proteins between 10.1-20 kDa (22% of total average venom composition) in invert feeders. The average venom composition of vertebrate feeders contained only a negligible presence of proteins between 90.1-100 kDa (<0.5% of total average venom composition) while the average venom composition of invert feeders showed a slightly higher proportion of proteins between 90.1-100 kDa (1.1% of total average venom composition). The average venom composition of invertebrate feeders at this point resembles that of vertebrate feeders three months after conditions were implemented with a far less equal spread of protein size categories. 58% of the average venom composition of invert feeders at this point is made up of only three protein size categories (10.1-20, 60.1-70 and 80.1-90 kDa), similarly 48% of the average venom composition of vertebrate feeders was made up of proteins of these size categories three months after conditions were implemented. This relationship is explored further below. The error bars show that standard deviation within each sample is very high meaning that there is a lot of variation in the venom composition of individual snakes within each condition in relation to the small sample sizes. All differences in average venom composition between each group described above are not statistically significant (table 15).

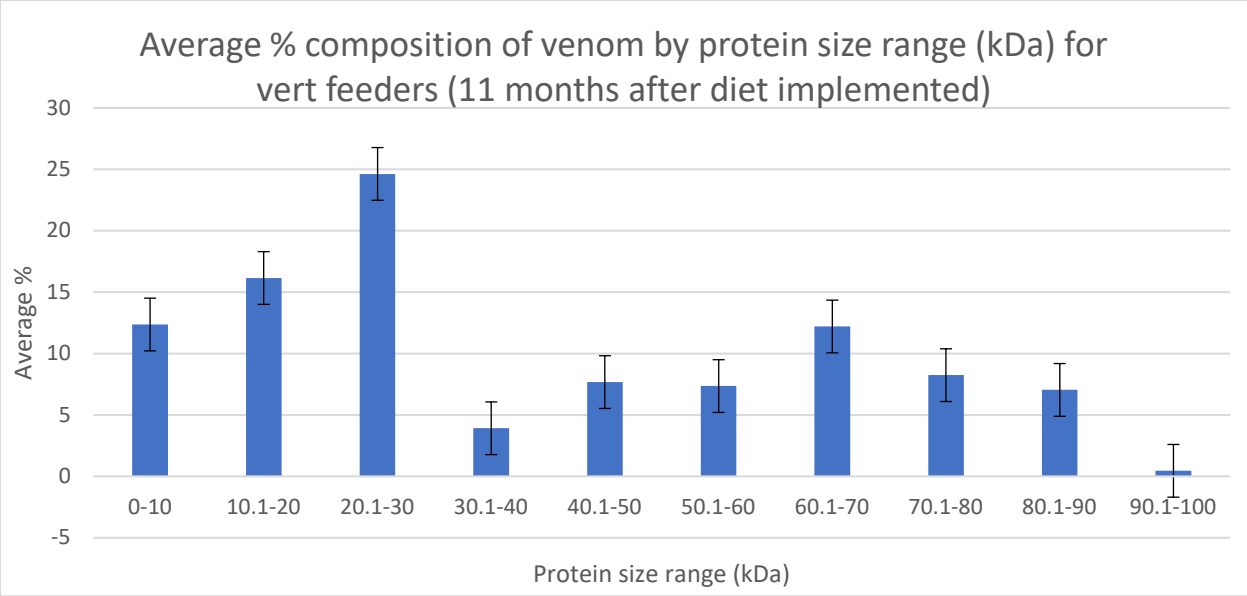

Figure 17. The average venom composition of invertebrate feeders 11 months after the implementation of conditions. The categories represent size groupings of proteins present in the venom (kDa), each bar shows the percentage of the total venom composition that each size category comprises. Error bars showing the standard deviation within each size category are also included.

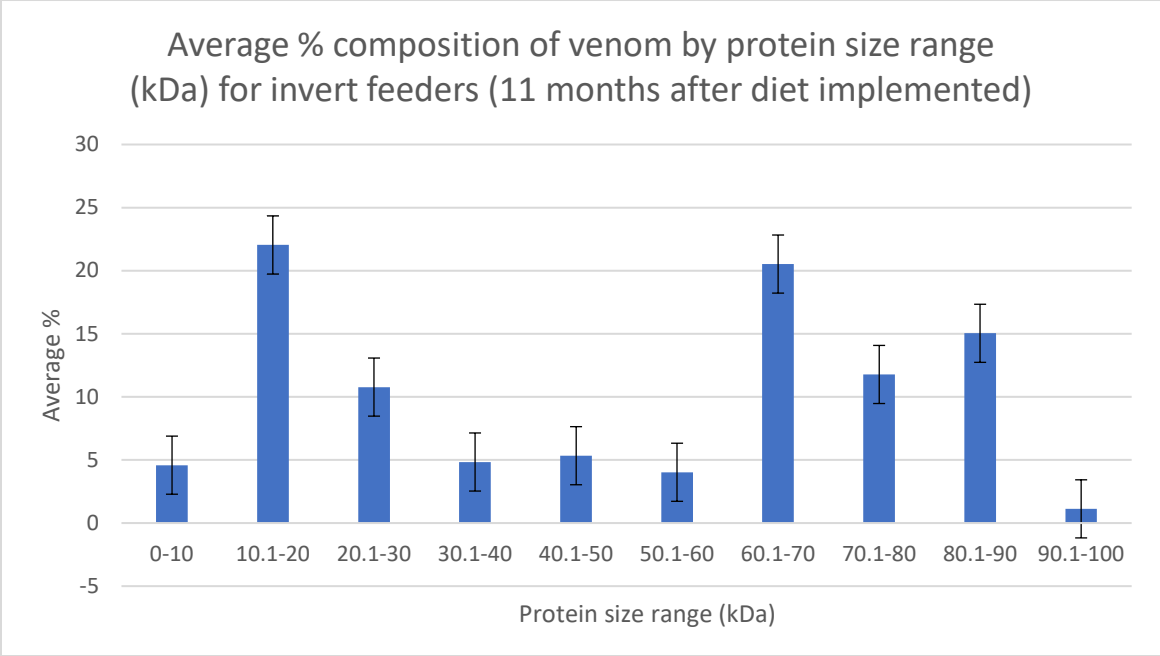

Figure 18. The average venom composition of invertebrate feeders 11 months after the implementation of conditions. The categories represent size groupings of proteins present in the venom (kDa), each bar shows the percentage of the total venom composition that each size category comprises. Error bars showing the standard deviation within each size category are also included.

As previously mentioned, the average venom composition of invertebrate feeders 11 months after conditions were implemented showed a similar pattern to that of vertebrate feeders after only 3 months. The most abundant protein size category in the average venom composition of both groups at this point was 10.1-20 kDa (22% for both groups) followed by 60.1-70 kDa (21% in invert feeders and 14% in vertebrate feeders) and 70.1-80 kDa (12% in invert feeders and 21% in vertebrate feeders). Both groups also showed a less equal spread of protein sizes within their average venom compositions with both groups having five protein size categories which each made up less than 10% of the total venom composition, four of which each made up less than 5% of total average venom composition. Additionally, both groups showed very small proportions of the largest and smallest protein categories within their average venom composition; 0-10 kDa and 90.1- 100 kDa (5% and 1% for invert feeders and 6% and 5% for vertebrate feeder respectively). These similarities can be visualized in figure 19 below.

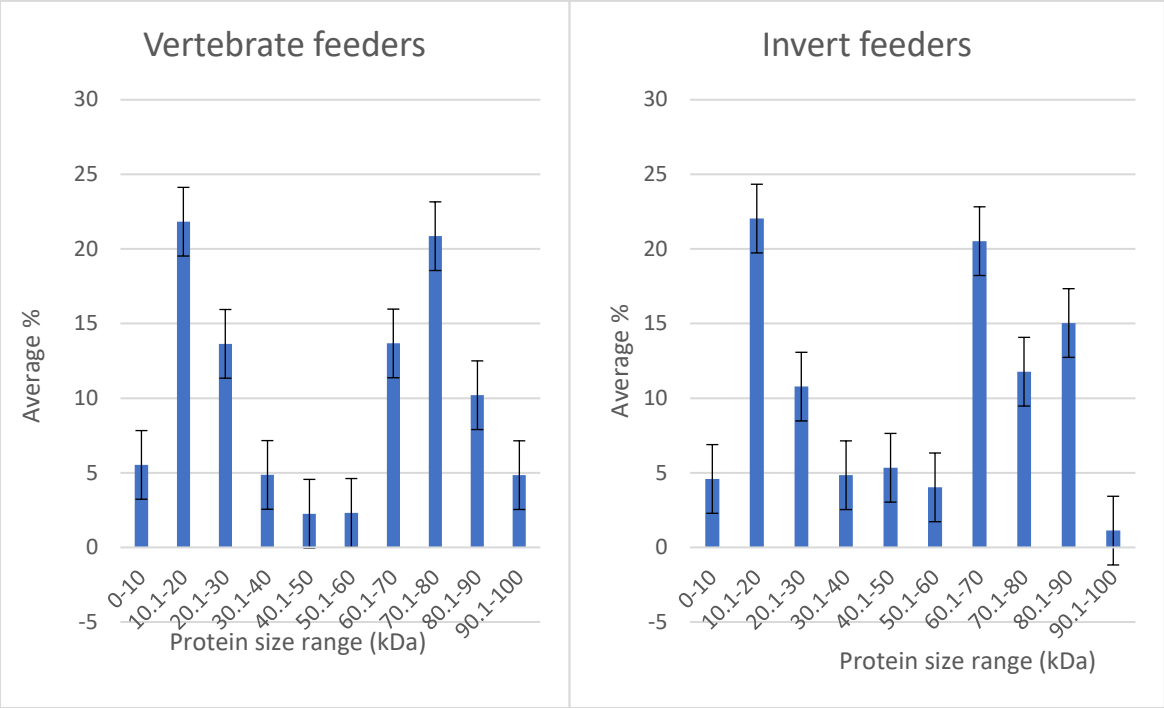

Figure 19: The average venom composition of vertebrate feeders 3 months after the implementation of conditions (left) and invertebrate feeders 11 months after conditions were implemented (right). Each bar shows the percentage of the total venom composition that each size category comprises. Error bars showing the standard deviation within each size category are also included.

Vertebrate feeding snakes showed a shift from venoms showing an average composition with a higher proportion of larger proteins to an average composition with a higher proportion of smaller proteins (figure 20) over time. The size group which made up the greatest percentage of average total venom composition in vertebrates prior to conditions being implemented was

proteins between 70.1-80 kDa (26% of total average venom composition). This shifted to proteins between 20.1-30 kDa (25% of total average venom composition) 11 months after condition had been implemented. However, the second most prevalent protein category in the average venom composition of vertebrate feeding snakes prior to conditions being implemented was proteins between 10.1-20 kDa (23% of total average venom composition). In the average venom composition of vertebrate feeding snakes 11 months after the implementation of conditions, the second most prevalent protein size category was also proteins between 10.1-20 kDa (16% of total average venom composition).

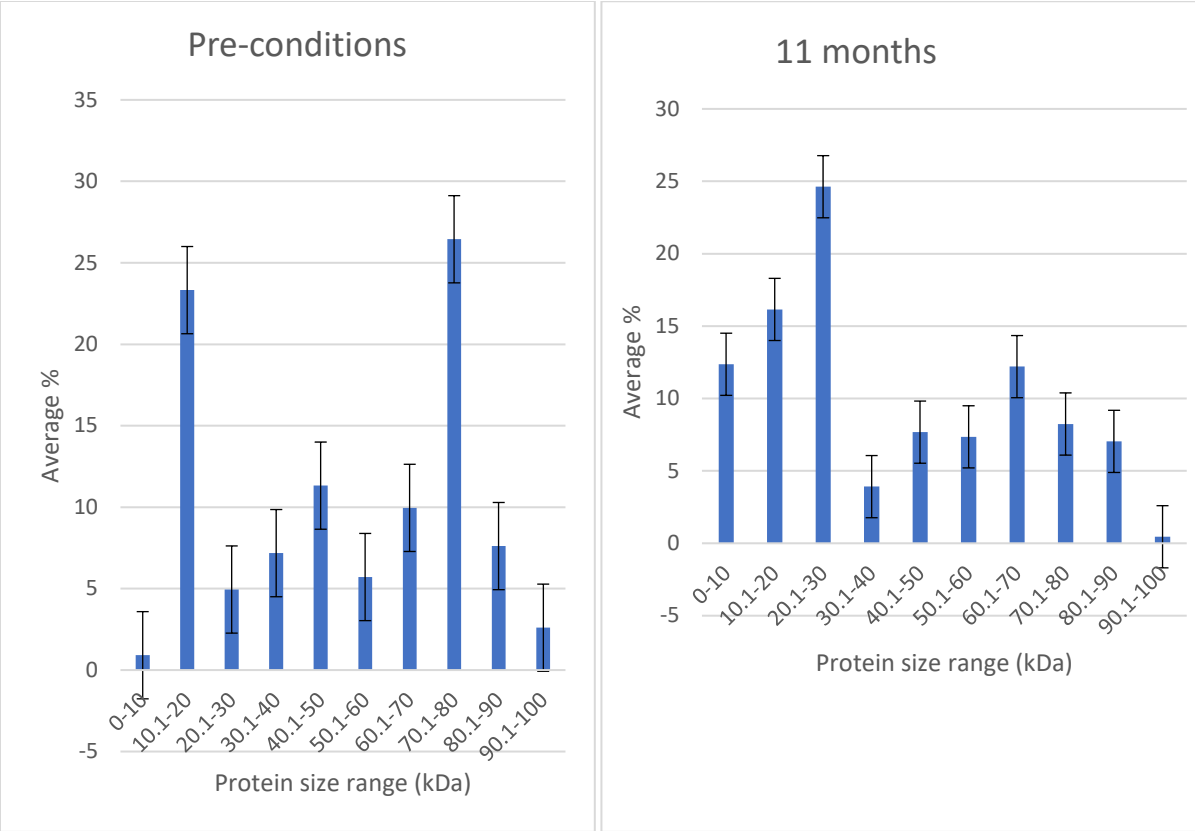

Figure 20: The average venom composition of vertebrate feeders prior to the implementation of conditions (left) and vertebrate feeders 11 months after conditions were implemented (right). Each bar shows the percentage of the total venom composition that each size category comprises. Error bars showing the standard deviation within each size category are also included.

The same pattern was not shown by invert feeders, which showed no clear shift towards smaller or larger proteins after 11 months of conditions being implemented (figure 21). The size group which made up the greatest percentage of average total venom composition in invertebrates prior to conditions being implemented was proteins between 70.1-80 kDa (22% of total average venom composition). This shifted to proteins between 10.1-20 kDa (22% of total average venom composition) 11 months after condition had been implemented. However, the second most

prevalent protein categories in the average venom compositions of vertebrate feeding snakes prior to conditions being implemented and 11 months after conditions being implemented were proteins between 10.1-20 kDa and protein between 60.1-70 kDa respectively (17% and 21% of total average venom composition respectively). This shows no clear shift from small proteins to larger proteins or vice versa for invertebrate feeding snakes.

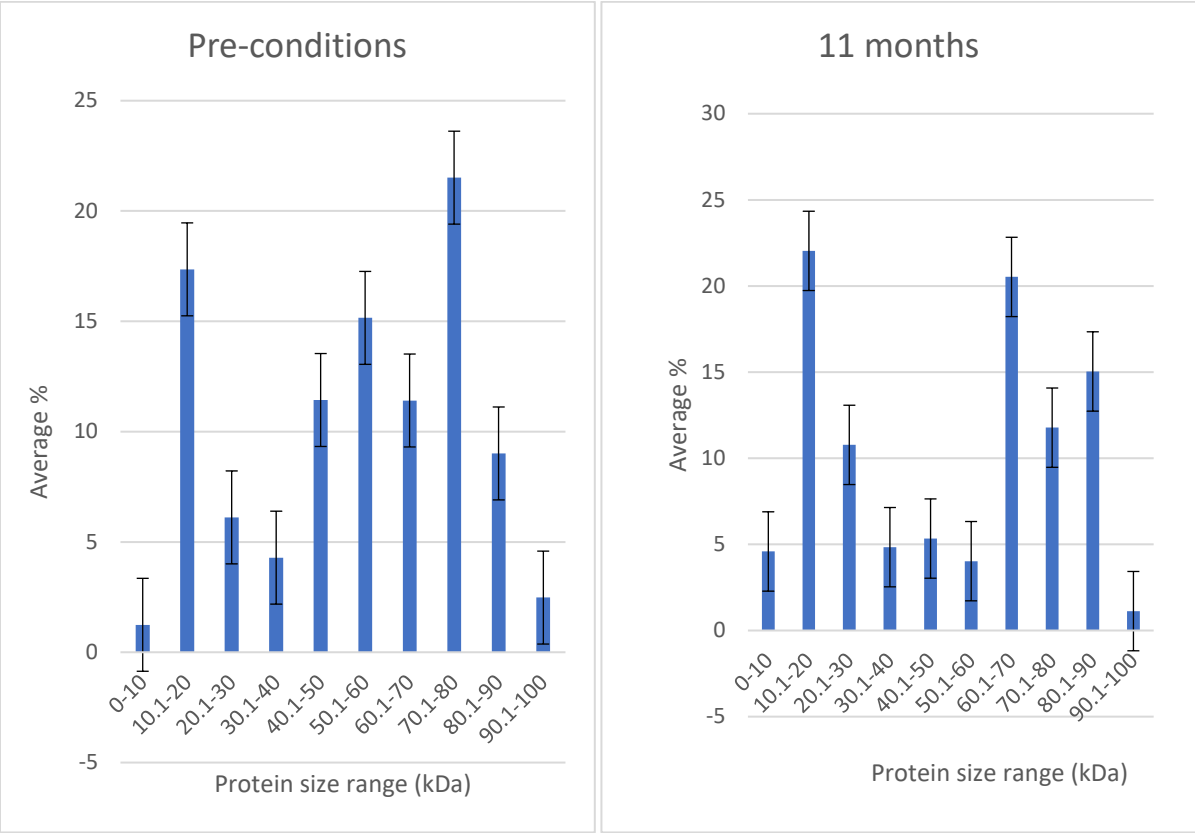

Figure 21: The average venom composition of invertebrate feeders prior to the implementation of conditions (left) and invertebrate feeders 11 months after conditions were implemented (right). Each bar shows the percentage of the total venom composition that each size category comprises. Error bars showing the standard deviation within each size category are also included.

When comparing the average venom compositions across time for each condition, the patterns observed are overshadowed by the size of the error bars present, indicating that differences between the venom compositions of each snake within the conditions outweigh any patterns observed.

However, there was a significant difference in the average weight (g) of vertebrate feeding snakes (mean=15.233, SD=3.38) and the average weight (g) of invertebrate feeding snakes (mean=8.067, SD=1.062); $t(10)=-3.512$, $p=0.0056$ at the end of the experiment. Conversely, there was no significant difference in the average weight (g) of vertebrate feeding snakes (mean=4.167, SD=0.827) and the average weight (g) of invertebrate feeding snakes (mean=4.02, SD=0.906); $t(17)=-0.367$, $p=0.7182$ prior to conditions being implemented.

3 - Discussion

I must accept my null hypothesis that snakes raised on a vertebrate diet will show no significant difference in venom composition when compared to snakes raised on an invertebrate diet. However, it is possible that this is partially due to the small sample size. It is possible that the limited sample size resulted in individual differences in snake venom composition within experimental conditions undermining any differences shown between experimental conditions. However, this can not be assumed and it is also important to consider that the results I achieved are representative. *E. c. sochureki* may not show any phenotypic plasticity of venom composition in response to dietary change.

The graphs generated do show some interesting trends at first glance. However, none of the differences observed are statistically significant. For example, when examining figure 19 there appear to be similarities between average venom composition of the invertebrate feeders 11 months after conditions were implemented and that of vertebrate feeders only 3 months after conditions were implemented. This, coupled with the significant difference in the average weight of the snakes in each condition after the end of the experiment suggests that those being fed on invertebrates may have been developing at a far slower rate than those fed on vertebrates.

Both conditions showed a less equal spread of protein size categories within their average venom composition 11 months after conditions were implemented. This may be indicative of an ontogenetic shift towards a more prey-specific venom composition. Venom composition may shift from one which favours a more generalized diet to a more specific diet, as small younger snakes may be dependent on a broader prey composition. When snakes get older and thus larger, they often feed on larger prey items and so have to feed less often. Smaller, younger snakes will need to eat smaller meals on a more regular basis may have to take advantage of any prey item which they encounter, thus making them rely on a more generalized diet while older, larger snakes will be able to specify more and feed exclusively on larger vertebrate prey.

The difference in the physiological condition (determined by the mean weight of each group) of the snakes at the end of the conditions may come as a surprise, seeing as the macronutrient profiles of invertebrates and vertebrates are generally comparable on a gram-to-gram basis (Tennie *et al.,* 2014). However, the neonatal mice being fed to the vertebrate feeders were larger than the crickets that were being fed to the invertebrate feeders. We attempted to off-set this by feeding the invertebrate feeders on a more regular basis, but it was not possible to ensure that all snakes consumed the same mass of food on a weekly basis without resorting to regular stressful assist feeding which could have resulted in more mortality or injury.

It is perhaps fair to infer from this, that a difference in venom composition in relation diet is possible if only due to a poorer quality diet resulting in slower development of venom. This would make sense as venoms are often very metabolically costly compounds to create (McCue, 2006; Nystrom *et al.,* 2022; Pintor *et al.,*2010). It is known that diet can affect the rate of venom production (Chacón *et al.,* 2012; Tobassum *et al.,* 2018) so it would not be unreasonable to suggest that perhaps the rate of ontogenetic development of snake venom may be somewhat influenced by the quality of the snake's diet.

However, it is also important to consider that the reason no significant difference in venom composition in response to dietary change was recorded may be because there is no difference to record. While it is clear that venom composition does change alongside changes in diet, as explored in section 4.3.3 of the literature review, it is very feasible that this type of shift in venom composition is not the result of a change in diet but rather a change that would occur regardless of a dietary change. Of course, a shift towards a venom composition with a higher efficiency at immobilising prey items of a certain type in conjunction with other changes that cause the prey type in question to become more available to the snake will have survival benefits. The patterns observed could thus be described by considering that the development of a new venom phenotype is likely to be influenced by the physiological condition of the snake. Therefore, a delayed development of venom phenotype showed by invertebrate feeding snakes in comparison to vertebrate feeding snakes would be solely due to the poorer condition of those on an invertebrate diet. As previously mentioned, venom production is very metabolically expensive. If the venom phenotype expressed by older snakes contains more metabolically expensive proteins or simply a greater quantity of venom proteins, this shift may be delayed if the snake in question is in worse condition and has less 'expendable energy' to spare.

Alternatively, a change in body size may be the trigger for the change in venom phenotype. If this is the case, those in a worse physiological condition will grow at a slower rate and thus will develop the new venom phenotype at a later date. As diet is clearly linked to physiological condition, this would imply a link between diet and plasticity on venom phenotype, if not the type of plasticity tested for in the above experiment. The significant difference in the average weight of snakes from each condition and the similarities between the average venom composition of vertebrate feeders after 3 months and invertebrate feeders after 11 month supports the idea that diet impacts on physiological condition and potentially, in turn, on venom composition.

From my study I believe that it is fair to infer that a cricket only diet is not a high-quality diet for *E. c. sochureki.* The mortality rate for those raised on just crickets was notably higher (6 out of the original 10 having died before the final milking) than that of those raised on vertebrates after the conditions were implemented (1 out of the original 9 having died before the final milking).

I believe that further research in this area has the potential to reveal some very interesting trends between diet composition and phenotypic plasticity of venom composition. The patterns shown in my results and discussed above, show some interesting, if statistically insignificant changes in venom composition, potentially in response to dietary change. With a more robust experimental design and a larger sample size these potential responses may well be shown to be significant.

I maintain that controlled feeding experiments are still an effective way to investigate phenotypic plasticity in response to dietary change. However, the results gained show that any further research here needs to utilise a larger sample size in order to account for the variation of venom composition shown between individual snakes being reared on the same diet. Additionally, a diet consisting entirely of crickets is not suitable for this species, thus any further controlled feeding experiments carried out on the species studied here should either rear snakes to a healthy size on a vertebrate or mixed diet prior to implementing any dietary changes or rear

all snakes on a mixture of both vertebrate and invertebrate prey with the separate conditions changing the ratio of prey items present within the diets of snakes rather than a complete separation. Furthermore, sex must be removed as a potential extraneous variable.

3.1 - Conclusion

To conclude, despite the failure of this study to find any statistically significant evidence that *E.c.sochureki* show phenotypic plasticity in regards to venom composition in response to dietary change, the results were still interesting and further research in this field may yet find a direct link between phenotypic plasticity of venom composition and dietary composition. If the patterns shown in my results are amplified by further research, it may be concluded that snakes show a delayed development of venom composition when feeding on a diet dominated by invertebrates in comparison to a diet dominated by vertebrates. Further controlled feeding experiments of this type must utilise larger sample sizes in order to account for differences in venom composition between individual snakes.

Venom composition is a greatly plastic trait and is affected by an enormous range of variables, it seems logical that diet must play a part in the composition of venoms on not only an intra-species level but on an intra-specific level. This is an exciting and fast developing field which promises to show great progress in our understanding of what drives phenotypic plasticity of venom composition in snakes. I, for one, look forward to seeing what comes next.

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