ResearchOnline@JCU

JAMES COOK UNIVERSITY
AUSTRALIA

This file is part of the following work:

Evans, Edward Robert Jonathan (2022) ***Venom chemistry and ecology of Australian scorpions.*** **PhD Thesis, James Cook University.**

Access to this file is available from:
https://doi.org/10.25903/bfwf%2Db151

Copyright © 2022 Edward Robert Jonathan Evans.

The author has certified to JCU that they have made a reasonable effort to gain permission and acknowledge the owners of any third party copyright material included in this document. If you believe that this is not the case, please email researchonline@jcu.edu.au

Venom Chemistry and Ecology of Australian Scorpions

Edward Robert Jonathan Evans

BSc, MSc

Thesis submitted for the degree of

Doctor of Philosophy

2022

College of Public Health, Medical and Veterinary Sciences

James Cook University

Acknowledgements

Moving to the opposite side of the world to undertake my PhD research was a daunting task, but I am extremely thankful to the many friends, family and colleagues that made this adventure a thoroughly enjoyable and life-changing experience. First and foremost, I would like to extend my thanks to my wonderful supervisory panel, who could not have been more helpful and supportive throughout my candidature. They not only allowed me to direct my research questions at ideas and theories of particular interest to me, but also enabled me to answer those questions by providing excellent advice and technical support. I am incredibly grateful to Dr David Wilson, who showed endless patience and taught me how to perform many of the lab-based techniques that were new to me. Whenever I needed it, I could rely on David to be approachable to help troubleshoot problems or simply bounce ideas off, which ultimately made completing my project immeasurably easier. I also cannot thank Prof. Norelle Daly enough for her help teaching me how to analyse my data, and her always quick and decisive responses to questions that made it easy to forge a path forward. Dr Tobin Northfield first inspired me to undertake my PhD at JCU, following on from my short visit to Cairns in 2016 when undertaking my MSc in the UK. Tobin has always shown incredible enthusiasm for my work, and I have always valued his insights and interpretations which are often outside-the-box and encouraged me to follow lines of inquiry I would not otherwise have considered. Despite moving to the USA early on in my PhD, I greatly appreciate that Tobin has always made the effort to have virtual meetings with me when I've requested his help, even with the awkward time difference.

Several parts of my experiments were performed with collaborators beyond my supervisory panel, both within and external to JCU. I would like to thank Lachlan McIntyre for his support by calculating small molecule structures from NMR spectra, and Dr Steve Peigneur for performing voltage gated ion channel assays with my synthetic peptides. In addition, I would like to thank Dr Jeremy Potriquet and Dr Yide Wong for assisting in operating the 6600 mass spectrometer. Finally, Casey Schmidt and Dr Guangzu Zhao for teaching me how to synthesise peptides. I am additionally grateful to Alan Henderson who kindly allowed me to collect venom samples from his captive scorpion collection. Special thanks go to my close friends and colleagues in E4 building of the Australian Institute of Tropical Health and Medicine for the ongoing relationships that made every day in the lab enjoyable and educational.

When I first moved from the UK to Australia specifically to undertake my PhD, I anticipated it would be hard to adapt to only being able to catch up with friends and family at home once a year or so. However, I don't think anyone could have anticipated the global chaos that would ensue from the COVID-19 pandemic. Having been unable to return home for over two and a half years it has certainly added an extra challenge to overcome during my PhD that I could never have predicted, but my family's support from afar has been invaluable in getting me through. My parents, in particular, who nurtured my passion for wildlife throughout my life have been tremendously supportive, and our regular catchups allowed me to push through to the end.

Finally, I cannot thank the Northcote Trust enough for supporting me with a scholarship for the first three years of my study, paying both my tuition fees and a stipend. My PhD journey here at JCU was only realised thanks to the Northcote Trust, and it is difficult to articulate just how appreciative I am of their support. The remainder of my studies were funded by a JCU internal postgraduate scholarship and a CPHMVS writing-up grant, for which I am also extremely thankful to have been awarded.

Statement of the Contribution of Others

Nature of Assistance	Contribution	Co-Contributors
Intellectual support	Writing assistance	Dr David Wilson (JCU), Prof Norelle Daly (JCU), Dr Tobin Northfield (WSU)
	Data analysis assistance	Dr David Wilson (JCU), Prof Norelle Daly (JCU), Dr Tobin Northfield (WSU)
	Statistical support	Dr Tobin Northfield (WSU)
	Lab assistance	Dr David Wilson (JCU), Prof Norelle Daly (JCU), Casey Schmidt, Dr Guangzu Zhao (JCU), Dr Yide Wong (JCU), Dr Jeremy Potriquet (JCU/SCIEX)
	Editorial assistance	Dr David Wilson (JCU), Prof Norelle Daly (JCU), Dr Tobin Northfield (WSU)
	Small molecule characterisation	Lachlan McIntyre
Financial Support	Fees paid/waiver: *First 3 years* *After 3 years*	 Northcote Trust Scholarship JCU Postgraduate Scholarship
	Stipend *First 3 years* *3 years - completion*	 Northcote Trust Scholarship JCU Postgraduate Scholarship
	Write-up grant	CPHMVS Doctoral Completion Grant
	Research costs	Supervisors' grants and SSA fund
Data Collection	Ion channel assays	Dr Steve Peigneur (KU Leuven)

Abstract

Scorpion venom compositions have been subject to much scientific scrutiny over the years, but certain aspects of scorpion venom chemistry and how ecological interactions alter their evolution remain poorly described or understood. This is especially true of Australian scorpions, partly because Australian venom research has historically often prioritised the many more-toxic Australian animals, but also many Australian species belong to families that have not been thoroughly examined in general. Studies performed by toxinologists often focus on isolation/characterisation of peptide neurotoxins to understand specific activities, and many do not evaluate complete venom compositions or consider variation between samples. Conversely, experiments performed by evolutionary ecologists studying trophic interactions between scorpions and their prey/predators are frequently not complemented with high-resolution molecular analyses. By combining evolutionary/ecological theories with cutting edge molecular techniques, a much deeper level of knowledge can be obtained as to how venoms are shaped on both long- and short-timescales, which can have applications in a clinical, commercial, and research environment. This thesis contains experiments that were executed specifically to shed light on understudied areas of scorpion venom research, and fill knowledge gaps to help understand the diversity and function of scorpion venoms from both a molecular and ecological standpoint. Key knowledge gaps addressed include: i) venom optimisation in scorpions, ii) intraspecific variation in venom compositions, iii) the ‘sexual stings’ performed by male scorpions during courtship, iv) non-peptide small molecule constituents of scorpion venoms, and v) comparisons of chemical composition and use of ‘prevenom’ versus the full venom complement.

i) The use of venom has associated energetic and ecological costs, and there is evidence that venomous organisms balance these costs and optimise their venom use via different evolutionary and behavioural mechanisms associated with their ecology. Chapter 1 contains a literature review focussed on how scorpions can minimise the costs, whilst maximising the benefits, associated with venom use in different contexts.

ii) There is currently little knowledge of how scorpion venom composition changes over the course of a scorpion’s life span. The experiments performed in Chapter 2 show both ontogenetic and intersexual differences occur in marbled scorpion (*Lychas variatus*) venoms. By raising scorpions from birth to adulthood, ontogenetic shifts were observed both in the presence of specific molecules, and in the relative proportion of molecules in different molecular weight classes at the different life-stages. To the best of my knowledge, this is the first time ontogenetic variation has been demonstrated to occur in scorpion venoms using a proteomic approach. Furthermore, sex-

specific molecules were identified, and some were additionally associated with ontogeny, only appearing in the venom of adult males.

iii) 'Sexual stings' occur when male scorpions sting the female during courtship. Although sexual stings have been observed in the mating rituals of many species, the role of this unusual behaviour is still not understood. In Chapter 3, adult male-specific venom components were isolated and characterised from *L. variatus* venoms, to evaluate if males have venom components that may be specifically involved in sexual stinging. Three novel peptides were isolated: Lvar1, Lvar2, and Lvar3; which are two novel tetrapeptide-amides and a novel hexapeptide-amide unlike any known scorpion venom peptides, and structurally more similar to cell signalling molecules than neurotoxins. All three exhibited no activity on a suite of Na_V and K_V channels tested, nor caused toxicity in crickets or affected the behaviour of female scorpions in *in vivo* bioassays. The structural novelty and observed lack of toxicity suggests they are not typical toxins, and if they are involved in sexual stings they could have effects which are not directly observable (e.g., hormonal modulation of the female). However, future work is necessary to elucidate their activity and confirm their potential role in sexual stings.

iv) Little is known about the identity and function of specific non-peptide small molecules in scorpion venoms, despite their general presence in scorpion venoms being widely known. Chapter 4 contains an assessment of the non-peptide small molecule constituents of *Hormurus waigiensis* venom, and shows that adenosine, adenosine-monophosphate, glutamic acid, aspartic acid, and citric acid were all present. These molecules are all non-toxic, but may have auxiliary functions in the venom, for example improving the spread of toxins at the injection site. Although these results provide one of the most comprehensive assessments of small molecule constituents in a single scorpion species at present, it remains unknown how prevalent and diverse small molecules are in the venoms of other scorpion species.

v) There is strong evidence that scorpions vary their defensive behaviours in response to different perceived threats. In Chapter 5 threat perception and defensive response was assessed in desert scorpions (*Urodacus yaschenkoi*), with an extended chemical analysis of visually clear, opalescent, and milky venom secretions. The initial clear venom secretions of scorpions, termed 'prevenom', are chemically simplistic in some species. It has been suggested that prevenom may have evolved to control venom expenditure in low-threat encounters with predators, avoiding wasting a full complement of toxins when not required. Although prevenoms have been documented in a number of species, there have been few chemical analyses comparing it to venom, and past analyses have been performed in closely related buthid scorpions. Examination of defensive

behaviours in *U. yaschenkoi* showed that the scorpions increased their defences towards persistent attackers irrespective of attack rate, and more so to attackers that repeated attacks at a faster rate. Chemical analysis of individual venom samples produced showed that *U. yaschenkoi* prevenom is more chemically simplistic and contains fewer numbers of peptides >5000 Da than milky venom. This suggests that *U. yaschenkoi* prevenom will likely require less time and energy to replenish than main venom, providing evidence to support the idea that prevenom has evolved as a cost reducing mechanism in this species.

By considering the evolutionary and ecological processes that explain observed chemical differences in venom compositions, and supporting ecological and behavioural studies with venom composition analyses, the results presented in this thesis provide new evidence that builds on past studies, spanning multiple fields of research, and highlights new avenues for future investigations to be made.

Published Works Included in the Thesis

E**vans, E.R.J.**, Northfield, T.D., Daly, N.L., and Wilson, D.T. (2019). Venom costs and optimization in scorpions. *Frontiers in Ecology and Evolution* 7, 196.

Evans, E.R.J., Mcintyre, L., Northfield, T.D., Daly, N.L., and Wilson, D.T. (2020). Small molecules in the venom of the scorpion *Hormurus waigiensis*. *Biomedicines* 8, 259.

Published Works Completed During, But Not Included in the Thesis

Giribaldi, J., Haufe, Y., **Evans, E.R.J.**, Amar, M., Durner, A., Schmidt, C., Faucherre, A., Moha Ou Maati, H., Enjalbal, C., and Molgó, J. (2020). Backbone cyclization turns a venom peptide into a stable and equipotent ligand at both muscle and neuronal nicotinic receptors. *Journal of Medicinal Chemistry* 63, 12682-12692.

Giribaldi, J., Haufe, Y., **Evans, E.R.J.**, Wilson, D.T., Daly, N.L., Enjalbal, C., Nicke, A., and Dutertre, S. (2021). Synthesis, structural and pharmacological characterizations of CIC, a novel α-conotoxin with an extended N-terminal tail. *Marine Drugs* 19, 141.

Table of Contents

ACKNOWLEDGEMENTS .... **I**

STATEMENT OF THE CONTRIBUTION OF OTHERS .... **II**

ABSTRACT .... **III**

PUBLISHED WORKS INCLUDED IN THE THESIS .... **VI**

PUBLISHED WORKS COMPLETED DURING, BUT NOT INCLUDED IN THE THESIS .... **VI**

TABLE OF CONTENTS .... **VII**

LIST OF FIGURES AND TABLES .... **X**

INTRODUCTION **1**

Scorpions – An Overview 2
What are Scorpion Venoms? 2
The Components of Scorpion Venoms 3
Neglected Areas of Scorpion Venom Research 5
Scope of the Thesis 6
Bibliography 9

CHAPTER 1. VENOM COSTS AND OPTIMIZATION IN SCORPIONS **16**

1.1. Abstract 17
1.2. Introduction 18
1.3. The Costs of Venom Use in Scorpions 19
1.3.1. Direct 19
1.3.2. Indirect 19
1.4. The Evolution of Optimal Venom Use 20
1.5. Behavioural Mechanisms to Optimise Venom Use 22
1.5.1. The 'Decision' to Sting 22
1.5.2. 'Dry' Stings 23
1.5.3. Volume Injected 24
1.5.4. Composition Injected 24
1.6. Adaptive Plasticity 27
1.7. Conclusions and Future Directions 28
1.8. Bibliography 29

CHAPTER 2. ONTOGENETIC AND INTERSEXUAL DIFFERENCES IN THE VENOM COMPOSITION OF MARBLED SCORPIONS (*LYCHAS VARIATUS*) **34**

2.1. Abstract 34
2.2. Introduction 35
2.3. Materials and Methods 37
2.3.1. Scorpion Collection and Husbandry 37
2.3.2. Venom Extraction 37
2.3.3. Liquid Chromatography/Mass Spectrometry (LC/MS) 38
2.3.4. Data Analysis 38
2.4. Results 41
2.4.1. Overall Composition 41
2.4.2. Group Differences 44
2.4.3. Toxin Family Analysis 49
2.5. Discussion 51
2.6. Conclusions and Future Directions 55
2.7. Supplementary Materials 55
2.8. Bibliography 56

CHAPTER 3. LOVE STINGS? NOVEL SHORT AMIDATED PEPTIDES FROM THE VENOM OF ADULT MALE MARBLED SCORPIONS (*LYCHAS VARIATUS*) 65

3.1. Abstract 65
3.2. Introduction 66
3.3. Materials and Methods 68
3.3.1. Scorpion Collection and Husbandry 68
3.3.2. Venom Extraction 68
3.3.3. Liquid Chromatography/Mass Spectrometry (LC/MS) 69
3.3.4. Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) 69
3.3.5. Matrix-Assisted Laser Desorption Ionization Time-Of-Flight (MALDI-TOF) Mass Spectrometry 70
3.3.6. NMR Spectroscopy 70
3.3.7. Peptide Synthesis and Purification 70
3.3.8. Scorpion and Cricket Bioassay 71
3.3.9. Ion Channel Bioassay 73
3.4. Results 74
3.4.1. Isolation of Molecules of Interest 74
3.4.2. Characterisation of Molecules of Interest 78
3.4.3. Chemical Synthesis 83
3.4.4. Scorpion and Cricket Bioassay 83
3.4.5. Ion Channel Assay 85
3.5. Discussion 85
3.6. Conclusions and Future Directions 90
3.7. Supplementary Materials 91
3.8. Bibliography 99

CHAPTER 4. SMALL MOLECULES IN THE VENOM OF THE SCORPION *HORMURUS WAIGIENSIS* 106

4.1. Abstract 107
4.2. Introduction 108
4.3. Materials and Methods 110
4.3.1. Scorpion Collection 110
4.3.2. Venom Extraction and Purification 111
4.3.3. Liquid Chromatography/Mass Spectrometry (LC/MS) 111
4.3.4. Mass Spectrometry and NMR Analysis 112
4.4. Results 113
4.4.1. Venom Collection and Fractionation 113
4.4.2. NMR Analysis of RP-HPLC Fractions 113
4.4.3. High-Resolution Mass Spectrometry 114
4.4.4. Adenosine Quantitation by LC/MS 114
4.5. Discussion 120
4.5.1. Adenosine 120
4.5.2. AMP 121
4.5.3. Citric Acid/Citrate 122
4.5.4. Free Amino Acids 123
4.6. Conclusions and Future Directions 124
4.7. Bibliography 125

CHAPTER 5. YOU GET WHAT YOU DESERVE: EFFECT OF THREAT LEVEL AND REPETITIVE ATTACKS ON THE DEFENSIVE STINGING BEHAVIOURS OF THE DESERT SCORPION (*URODACUS YASCHENKOI*) 131

5.1. Abstract 131
5.2. Introduction 132
5.3. Methods 134
5.3.1. Scorpion Husbandry 134
5.3.2. Defensive Stinging Trial 134
5.3.3. Liquid Chromatography/Mass Spectrometry (LC/MS) 135
5.3.4. Data Processing/Analysis 136
5.4. Results 139

5.4.1. Defensive Investment *139*
5.4.2. Chemical Complexity *141*
5.4.3. Compositional Comparisons *144*
5.4.4. Toxin Families *146*
5.5. Discussion 147
5.6. Conclusions and Future Directions 151
5.7. Supplementary Materials 152
5.8. Bibliography 153

OVERALL SUMMARY AND FUTURE DIRECTIONS 157

Drivers of Venom Variation 157
Sexual Stinging 158
Non-Proteinaceous Small Molecules 159
Threat-Perception and Modulation of Venom Use 160
Final Words 161
Bibliography 162

List of Figures and Tables

Chapter 1. Venom Costs and Optimization in Scorpions

Figure 1. 1 Morphological comparison of four Australian scorpions 21
Figure 1. 2 From (A–D)–consecutive venom secretions collected from *Hormurus waigiensis* during one milking event using electrostimulation of the venom gland 26

Chapter 2. Ontogenetic and Intersexual Differences in the Venom Composition of Marbled Scorpions (*Lychas variatus*)

Figure 2. 1 Non-metric multidimensional scaling (NMDS) analysis comparing the composition of *Lychas variatus* venom across 84 samples containing 579 different venom components.................. 42
Figure 2. 2 Compositional comparison of male (blue) and female (pink) *Lychas variatus* venom 44
Figure 2. 3 Chemical complexity of *Lychas variatus* venom associated with different instars....... 46
Figure 2. 4 Boxplot showing the total number of different toxins identified in each sample for male and female *Lychas variatus* venom samples.. 47
Figure 2. 5 Venn diagram showing the shared presence of molecules across small (I2-3), medium (I4) and large (I5-6) *Lychas variatus* scorpions 48
Figure 2. 6 Relative proportion of the number of venom components in each *Lychas variatus* venom sample belonging to different molecular weight classes 50

Table 2. 1 Statistical outputs of PERMANOVAs testing the effect of instar and sex performed on distance matrices generated using Jaccard distributions from raw, unscaled data 43
Table 2. 2 Statistical outputs of MANOVAs performed testing the effect of instar and sex on the NMDS scores 43
Table 2. 3 Statistical output of linear mixed models testing the effect of instar and sex on the scores of NMDS1-3. 43

Supplementary Materials 2. 1 Stress plot of the NMDS ordination... 55

Chapter 3. Love stings? Novel Short Amidated Peptides from the Venom of Adult Male Marbled Scorpions (*Lychas variatus*)

Figure 3. 1 LC/MS trace of adult (I5) male and female (I5) *Lychas variatus* venom 75
Figure 3. 2 Reversed-phase high performance liquid chromatography (RP-HPLC) chromatogram of pooled adult male *Lychas variatus* venom 77
Figure 3. 3 MALDI-TOF MS and MS/MS spectra with calculated sequence of Lvar1...................... 80
Figure 3. 4 MALDI-TOF MS and MS/MS spectra with calculated sequence of Lvar2...................... 81
Figure 3. 5 MALDI-TOF MS and MS/MS spectra with calculated sequence of Lvar3...................... 82
Figure 3. 6 Results of *in vivo* bioassays performed with synthetic peptides and crude venom in **a)** female scorpions (*Lychas variatus*), and **b)** adult female crickets (*Acheta domesticus*)...................... 84

Table 3. 1 Mass (Da) of molecules present only in adult male *Lychas variatus* scorpion venoms, identified by LC/MS analysis 76
Table 3. 2 Observed ion ([M+H]+), theoretical monoisotopic mass, sequence, and name assigned to the three adult male specific peptides (Lvar1, Lvar2, and Lvar3)... 79

Supplementary Materials 3. 1 TOCSY 2D NMR spectrum of a HPLC fraction of crude adult male *Lychas variatus* venom containing Lvar1 ... 91
Supplementary Materials 3. 2 TOCSY 2D NMR spectrum of a HPLC fraction of crude adult male *Lychas variatus* venom containing Lvar2 ... 92
Supplementary Materials 3. 3 TOCSY 2D NMR spectrum of a HPLC fraction of crude adult male *Lychas variatus* venom containing Lvar3 ... 93
Supplementary Materials 3. 4 Reversed-phase high performance liquid chromatography (RP-HPLC) chromatogram showing coelution of synthetic and native Lvar1 ... 94
Supplementary Materials 3. 5 Reversed-phase high performance liquid chromatography (RP-HPLC) chromatogram showing coelution of synthetic and native Lvar3 ... 95
Supplementary Materials 3. 6 MALDI-TOF MS/MS fragmentation of synthetic Lvar1 ... 96
Supplementary Materials 3. 7 MALDI-TOF MS/MS fragmentation of synthetic Lvar2 ... 97
Supplementary Materials 3. 8 MALDI-TOF MS/MS fragmentation of synthetic Lvar3 ... 98

Chapter 4. Small Molecules in the Venom of the Scorpion *Hormurus waigiensis*

Figure 4. 1 Reversed-phase high performance liquid chromatography (RP-HPLC) chromatogram of pooled crude *Hormurus waigiensis* venom ... 115
Figure 4. 2 Chemical structure and 1D NMR spectrum of a fraction from *Hormurus waigiensis* venom containing aspartic acid and glutamic acid. ... 116
Figure 4. 3 Chemical structure and 1D NMR spectrum of a fraction from *Hormurus waigiensis* venom containing adenosine. ... 117
Figure 4. 4 Chemical structure and 1D NMR spectrum of a fraction from *Hormurus waigiensis* venom containing adenosine monophosphate. ... 118
Figure 4. 5 Chemical structure and 1D NMR spectrum of a fraction from *Hormurus waigiensis* venom containing citric acid. ... 119

Chapter 5. You Get What You Deserve: Effect of Threat Level and Repetitive Attacks on the Defensive Stinging Behaviours of the Desert Scorpion (*Urodacus yaschenkoi*)

Figure 5. 1 Mean defensive response of *Urodacus yaschenkoi* per simulated 'attack' in the sequence (n = 6) under low- and high-threat (attack frequency) treatments ... 140
Figure 5. 2 Box and whisker plot showing chemical complexity (total molecules) in each *Urodacus yaschenkoi* venom sample associated for each visual classification of sample ... 142
Figure 5. 3 Mean chemical complexity (total molecules) of *Urodacus yaschenkoi* venom samples collected in response to each simulated 'attack' in the sequence (n = 6) under low- and high-threat treatments ... 143
Figure 5. 4 Venn diagram of molecules shared between clear, opalescent, and milky *Urodacus yaschenkoi* venom samples ... 145
Figure 5. 5 Boxplot showing the proportion of molecules in different molecular weight classes associated with the visual classification of *Urodacus yaschenkoi* venom samples ... 146

Supplementary Materials 5. 1 Venn diagrams of the molecules shared between clear, opalescent, and milky *Urodacus yaschenkoi* venom samples ... 152

Introduction

Australia is a land famous for its many deadly animals. Some of the world's most venomous animals such as the taipan snake (*Oxyuranus microlepidotus*), funnel-web spider (*Atrax robustus*), cone snail (*Conus geographus*), blue-ringed octopus (*Hapalochlaena* spp.), box jellyfish (*Chironex fleckeri*/*Carukia barnesi*), and stonefish (*Synanceia horrida*) all call this country home (Wiener, 1957;Wiener, 1959;Broad et al., 1979;Currie, 1994;Fenner and Carney, 1999;Dutertre et al., 2014;Lago et al., 2015). Even highly venomous plants exist in Australian rainforests, such as gympie-gympie (*Dendrocnide moroides*), the stings of which cause excruciating and long-lasting pain (Gilding et al., 2020). There is, however, one clear exception to the Australian rule: scorpions. Australian scorpions are comparatively innocuous, and none cause deadly envenomation under normal circumstances. The most toxic species belong to the genus *Lychas*, with the majority of stings inducing localised pain and swelling (Isbister et al., 2003;Isbister et al., 2004). A combination of their relatively low toxicity, secretive lifestyle, and the small number of people living in rural Australia makes scorpion envenomation a relatively insignificant public health issue within the country. On the contrary, deadly species of scorpions exist in other regions of the world, and in some countries scorpion envenomation is a significant public health issue (Chippaux and Goyffon, 2008;Isbister and Bawaskar, 2014;Jean-Philippe et al., 2020;Lacerda et al., 2022).

As some scorpion species pose potential risk to human life, there is clear motivation to understand scorpion venom chemistry to improve treatment of sting victims. Additionally, there has been continual growth in the interest in venoms as sources of bioactive molecules which can be used as biochemical tools or developed into bioinsecticides or pharmaceuticals (Fox and Serrano, 2007;King, 2011;Klint et al., 2012;Windley et al., 2012;Ortiz et al., 2015;Herzig et al., 2020). Scorpion venoms are complex mixtures showing great diversity both between and within species, thereby making them excellent candidates for biodiscovery research (Ortiz et al., 2015), regardless of their risk to human life. Furthermore, scorpions use their venoms for both prey capture and defence and can therefore be models for studying various evolutionary and ecological processes (Polis et al., 1989;Gangur et al., 2018). Better understanding scorpion venom chemistry, and the underlying processes that drive compositional diversity, is therefore important for several lines of research, and can have applications in a clinical and commercial setting. Despite its importance, knowledge gaps remain in several aspects of scorpion venom research, such as the venom compositions of many neglected scorpion species, and fully understanding the ways that different evolutionary and ecological processes shape scorpion venom compositions. The investigations included within this thesis are focussed on aspects of scorpion venom research where ambiguity remains, aiming to gain

a greater overall understanding of scorpion venom compositions, and the ecological processes that shape them.

Scorpions – An Overview

Scorpions are ancient venomous taxa, having roamed the earth for over 400 million years (Laurie, 1900;Dunlop and Selden, 2009). They have a widespread distribution globally, being found on all continents except Antarctica. Currently more than 2500 species are recognised, with roughly half belonging to a single family: Buthidae (Rein, 2022). Buthidae is not only the largest family, but also contains every species that has the potential to cause dangerous envenomation in humans (Santos et al., 2016).

Scorpions have evolved venoms to assist in both capturing prey and defending against predators, which they deliver using their highly mobile metasoma and specialised stinging apparatus (Polis et al., 1989;van der Meijden and Kleinteich, 2017). As they use venom in both offensive and defensive contexts, scorpions experience evolutionary selective forces associated with both their prey and predators (Lima and Dill, 1990;Tian et al., 2008;Weinberger et al., 2009). These selective forces are, at least in part, responsible for driving the large interspecific (between species) and intraspecific (within species) variation observed in their venom compositions (Omran and McVean, 2000;Isbister et al., 2004;Abdel-Rahman et al., 2009;Ruiming et al., 2010;Sunagar et al., 2013;Miller et al., 2016;Ward et al., 2018;Olguín-Pérez et al., 2021). In addition to normal venom usage in prey-capture and defensive contexts, male scorpions frequently sting the female during courtship, and selection pressures associated with this poorly understood behaviour may also be responsible for driving diversity in venom compositions (Peretti, 2014;Olguín-Pérez et al., 2021).

What are Scorpion Venoms?

On a molecular level, scorpion venoms are complex mixtures containing many components that act in combination to induce toxicity (Inceoglu et al., 2003;Nascimento et al., 2006;Newton et al., 2007;de la Vega et al., 2010). These include salts, organic small molecules, and proteinaceous molecules ranging from small peptides to large and structurally complex enzymes (de la Vega et al., 2013;Cid-Uribe et al., 2020;Delgado-Prudencio et al., 2022). The venom mixture changes as it is continually released from the sting; the initial venom appears clear and contains a low peptide/protein concentration, and the later secretions become opaque-white and contain a high peptide/protein concentration (Yahel-Niv and Zlotkin, 1979;Inceoglu et al., 2003). Additionally, a

study on *Parabuthus leiosoma* found that there was large variation in the venom composition of consecutive stings (Sarhan et al., 2012).

Very few species have been subject to in-depth proteomic and transcriptomic studies that attempt to characterise the full set of venom components in a single species, and the vast majority of species remain entirely unstudied. To gain the most robust insights into whole venom composition/complexity, a combination of proteomic and transcriptomic/genomic approaches is required. High-throughput proteomics usually requires a sequence database to match peptide fragments against (Song et al., 2012;Cid-Uribe et al., 2020), and transcriptomic studies overlook non-peptide small molecule venom constituents, whilst additionally lacking information regarding any post-translational modifications present in the mature peptides (Delgado-Prudencio et al., 2019). A proteomic analysis of *Tityus serrulatus* revealed that a large proportion of the venom components are fragments of larger venom peptides/proteins, stressing the importance of combined approaches to understand the mechanisms that explain compositional diversity (Rates et al., 2008;de la Vega et al., 2013).

The Components of Scorpion Venoms

Peptides are the most abundant and diverse group of molecules in scorpion venoms, and are responsible for the majority of toxic effects (Possani et al., 2000;de la Vega et al., 2013;Cid-Uribe et al., 2020). Broadly speaking, scorpion venom peptides can be split into those that are structurally stabilised by disulfide bonds, and others that lack disulfide bonds (linear peptides) (Zeng et al., 2005;Sunagar et al., 2013;Almaaytah and Albalas, 2014;Cid-Uribe et al., 2020). Historically, the disulfide-bonded peptides have been the focus of research, as these include the most toxic molecules, and equally their high selectivity makes them of interest as biochemical tools (de la Vega and Possani, 2005;Dutertre and Lewis, 2010).

The best-known toxin family from scorpions is the CS-α/β peptides, characterised by their cysteine-stabilised α/β scaffold. CS-α/β peptides were traditionally split into the short chain toxins (<45 residues) and the long chain toxins (>55 residues), with short-chain toxins blocking K^+ channels and long chain toxins modulating Na^+ channels in different ways (de la Vega et al., 2013). Other disulfide bridge-containing peptides belong to structural families less commonly recorded in scorpions. Some adopt an inhibitory cystine knot (ICK motif), similar to those common in spider and cone snail venoms (Zhu et al., 2003;Daly and Craik, 2011). The scorpion ICK toxins, or calcines, act agonistically on intracellular calcium release channels (Ryanodine Receptors, RyR) (Fajloun et al., 2000;Gurrola et al., 2010;de la Vega et al., 2013). These molecules have the ability to cross cell

membranes without causing lysis, which makes them useful as a possible mechanism for intracellular drug delivery (Poillot et al., 2010;de la Vega et al., 2013). Another scorpion peptide group of great interest to researchers consists of chlorotoxin, originally described from *Leiurus quinquestriatus* (DeBin et al., 1993), and structurally related toxins which interact with Cl^- channels (Cid-Uribe et al., 2020). Chlorotoxin binds to tumour cells, and is able to cross the blood-brain barrier, which means it can be conjugated with a fluorescent tag to act as a 'tumour-paint' or can be used to target delivery of anti-tumour agents (Lyons et al., 2002;Veiseh et al., 2007;Sun et al., 2008;Huang et al., 2011;Dardevet et al., 2015). With advances in high-throughput proteomics and sequencing in the last decade, and as new species are studied, scorpion venom peptides with novel and exciting frameworks continue to be discovered. For example, the peptidergic scorpion toxin WaTx from *Urodacus manicatus* that activates transient receptor potential channel TRPA1 (King et al., 2019), and U_1-LITX-Lw1a from *Hormurus waigiensis* that has a disulfide-directed hairpin (DDH) motif which has shed light on the evolution of the abundant ICK motif (Smith et al., 2011;Smith et al., 2013b).

Far less is known about the scorpion venom peptides that lack disulfide bonds. These linear peptides are often relatively small, typically 13-56 amino acids in length, showing a diverse range of sequences and activities (Almaaytah and Albalas, 2014). Although researchers have historically focussed on disulfide-rich scorpion venom peptides, recent full-venom analyses demonstrate that the venom of different species can be dominated by the smaller peptides <3000 Da (de la Vega et al., 2013). The majority of linear scorpion peptides adopt a cationic amphipathic α-helical structure, and can be active on numerous biological targets that can lead to antimicrobial, anticancer, haemolytic, anti-inflammatory, immune-modulatory and bradykinin potentiating activities (Almaaytah and Albalas, 2014).

In addition to peptides, proteins and small molecules are also present in scorpion venoms. Scorpion venom proteins are typically enzymes, and can include hyaluronidases, phospholipases, metalloproteases and sphingomyelinases, transferases, and lyases (de la Vega et al., 2013;Delgado-Prudencio et al., 2022). Whilst not usually having a direct toxic effect, venom enzymes can have important roles within the venom such as helping spread toxins (Bordon et al., 2015) or be involved in post-translational modification (Delgado-Prudencio et al., 2019).

The organic small molecules in scorpion venoms have often been overlooked by researchers, and despite their presence frequently being reported in literature, very few have been characterised. Those presently documented in the venoms of different scorpion species belong to various classes of molecule, and include an alkaloid (Banerjee et al., 2018), two 1,4-benzoquinone

derivatives (Carcamo-Noriega et al., 2019), adenosine (Thien et al., 2017;Tran et al., 2017), citric acid (Fenton et al., 1995), spermidine (Arjunwadkar and Reddy, 1983) and 5-hydroxytryptamine (serotonin) (Adam and Weiss, 1959).

Neglected Areas of Scorpion Venom Research

Historically, most scorpion venom research was performed to better understand venom activity and therefore improve first aid or sting treatment of highly toxic species. For this reason, research was often biased towards members of the family Buthidae, which all dangerous species belong to (Santos et al., 2016). However, more recently there has been a shift in motivation to study venoms, and now a large portion of venom research is focussed on the biodiscovery of novel molecules with activities that can be harnessed as biochemical probes, pharmaceuticals, or bioinsecticides (King, 2011;Klint et al., 2012;Smith et al., 2013a). The growth of this field has brought fresh interest in the more obscure scorpion families, as it is known they can be sources of completely new peptide scaffolds, for example the disulfide-directed hairpin (DDH) scaffold of U_1-LITX-Lw1a (Smith et al., 2011;Smith et al., 2013b).

In addition, there has been past focus on the characterisation of the peptide venom constituents, as these are the most abundant/diverse molecules in the venom, are responsible for the main toxic effects, and their chemical properties make them favourable candidates for development as biochemical tools/pharmaceuticals. This has meant that there is a lag in understanding the other molecules in the venom, in particular the organic small molecules. Remarkably little is known about the identity, abundance, and function of small molecules in scorpion venoms, despite their presence being widely documented. This is surprising, as not only are small molecules known to play important functional roles in the venoms of other venomous organisms (McCormick and Meinwald, 1993;Aird, 2002;2005;Palma and Nakajima, 2005), there is also the potential for venom-based small molecule drug discovery (Wilson et al., 2017).

Although intraspecific variation in scorpion venoms is reported in numerous species, compositional differences have mainly been linked to geographic and sexual variation (Omran and McVean, 2000;Abdel-Rahman et al., 2009;De Sousa et al., 2010;Rodríguez-Ravelo et al., 2013;Estrada-Gómez et al., 2014;Miller et al., 2016;Uribe et al., 2017;Carcamo-Noriega et al., 2018;Ward et al., 2018). It is less clear if other factors, such as life-stage (ontogeny), affect scorpion venom compositions in similar ways to other venomous taxa (Herzig et al., 2004;Alape-Girón et al., 2008;Cipriani et al., 2017;Santana et al., 2017). One study on *Centruroides vittatus* identified

ontogenetic changes in toxin gene expression and venom toxicity (McElroy et al., 2017), but more work is required to understand if scorpion venoms show ontogenetic variation in a wider context.

Two curious aspects of venom use that appear to be to be unique to scorpions are 1) the existence of 'prevenom', and 2) the involvement of venom in courtship. Firstly, several species have been documented to produce clear 'prevenom' followed by milky venom (Yahel-Niv and Zlotkin, 1979;Gopalakrishnakone et al., 1995;Abdel-Rahman et al., 2009), but few chemical comparisons between them have been made (Yahel-Niv and Zlotkin, 1979;Inceoglu et al., 2003;Sarhan et al., 2012). The prevenom of *Parabuthus transvaalicus* and *Parabuthus leiosoma* was found to be more simplistic than milky venom (Inceoglu et al., 2003;Sarhan et al., 2012), and it has been suggested prevenom has evolved as a way to avoid wasting chemically complex venom when facing less threatening predators (Inceoglu et al., 2003;Nisani and Hayes, 2011;Nisani et al., 2012;Lira et al., 2017). However, most studies on scorpion venom composition fail to account for the differences between prevenom and venom. Moreover, the prevalence of prevenom in a taxonomically diverse range of species has not been assessed, nor have chemical comparisons been performed.

Furthermore, the 'sexual stings' performed by male scorpions during courtship are one of the most mysterious aspects of scorpion venoms. Despite its behavioural novelty piquing the interest of biologists, the role played by the sexual sting has still not been confirmed. Several speculated functions include directly subduing the female to prevent predation, to indirectly improve mating success via hormonal modulation, or acting as an honest indicator of fitness by the male (Inceoglu et al., 2003;Peretti, 2014;Olguín-Pérez et al., 2021). It was only recently shown that sexual stings involve venom transference in *Megacormus gertschi* (Olguín-Pérez et al., 2021), but whether males control the venom they inject into females, or if they possess molecules in their venom specifically involved with sexual stinging remains to be discovered.

Scope of the Thesis

Chapter 1 reviews the literature presently available on the mechanisms evolved by scorpions to optimise their venom use and outlines the ecological aspects of venom use in scorpions. Venom production/maintenance requires both energy and time, and consequently there are both metabolic costs associated with venom replenishment, and time post-expulsion when an animal may be left with reduced defences or ability to hunt (Hayes et al., 2002;McCue, 2006;Nisani et al., 2007). Organisms optimise their venom use by balancing these costs against the survival benefits venom provides, and past reviews have discussed the literature available in both snakes and spiders (Hayes

et al., 2002;Hayes, 2008;Morgenstern and King, 2013;Cooper et al., 2015). This chapter provides an exhaustive and up to date overview covering all aspects of venom optimisation in scorpions.

Chapter 2 contains an investigation into intraspecific (within-species) venom variation in marbled scorpions (*Lychas variatus*), specifically studying ontogenetic and interspecific differences. A past study identified changes in toxin gene expression and toxicity associated with ontogeny in *C. vittatus* (McElroy et al., 2017), but to the best of my knowledge ontogenetic venom variation has not previously been thoroughly examined in scorpions using a proteomic approach. As scorpions have slow growth rates, it can be difficult to study ontogenetic changes across their complete lifespan. Though some species take several years to reach maturity (Shorthouse and Marples, 1982), others are comparatively fast growing. Members of the genus *Lychas* have relatively fast growth rates, such as *Lychas tricarinatus* which reaches maturity in 220 days (Seiter and Stockmann, 2017), therefore *Lychas* spp. make excellent study species for experiments relating to ontogeny. I raised *L. variatus* from birth to adulthood and show that, like other scorpion species, *L. variatus* have sex-specific venom components. Additionally, ontogenetic shifts in venom composition were detected as both changes in the presence of specific molecules at the different life stages, but also changes in the relative proportions of molecules in different size classes. Furthermore, several molecules were identified that were unique to the venom of adult males, demonstrating that intraspecific variation in scorpion venoms can be associated to the interaction of multiple factors. To the best of my knowledge, this is the first evidence of sex-specific molecules in scorpion venoms that are additionally linked to a specific life stage.

Chapter 3 builds upon the findings of the previous chapter and includes the isolation and characterisation of three novel adult male-specific *L. variatus* venom components. Adult male-specific molecules were of particular interest, as in addition to using their venom in a conventional prey-capture and defensive context, the courtship of many species includes the male stinging the female (Olguín-Pérez et al., 2021). The experiments show the three adult male-specific molecules in *L. variatus* are structurally unlike typical scorpion venom components and display closer structural similarity to neuropeptides and hormones than neurotoxins. This chapter includes thorough discussion as to their potential role in the venom, focussing on their possible involvement in sexual stinging behaviours.

Chapter 4 tackles two areas of scorpion venom chemistry that are lacking data: non-buthid species and small molecule venom constituents. *Hormurus waigiensis* was selected for this study as relatively few investigations have been made into hormurid scorpion venoms, and none have previously focussed on small molecule venom constituents. By investigating the small molecules

present in *H. waigiensis* venoms, fresh insight is gained into how scorpion venoms function as complex mixtures of molecules working in combination to induce toxic effects. Five small molecules are isolated and characterised from the venom of *H. waigiensis*, which I believe may be the most currently identified from any single scorpion species. The five molecules are all non-toxic, and likely function within the venom as accessory molecules; for example, by acting to increase the effectiveness of other venom components.

In Chapter 5, I return to the evolutionary and ecological theories discussed in Chapter 1, and assess how scorpions adapt their defensive behaviours in response to perceived threat to optimise venom use. In addition, I make the first chemical comparisons of prevenom and milky venom in *Urodacus yaschenkoi*. Experiments were performed on *U. yaschenkoi* as these large and robust scorpions produce relatively large volumes of venom, allowing for chemical analysis to be performed on individual sting samples. Scorpions can control their energetic investment in their defences via multiple mechanisms, such as by dry stinging, varying the volume of venom injected per sting, and controlling the composition of venom they deliver (prevenom versus venom). I show that in *U. yaschenkoi* both persistent attacks and more frequent attacks induce a greater defensive response per attack. I additionally show that *U. yaschenkoi* prevenom is more chemically simplistic and contains a lower relative proportion of larger peptides (>5000 Da) than main milky venom. The combined results of the behavioural study and chemical analysis of prevenom provide evidence to support the hypothesis that prevenom has evolved as a cost reducing mechanism in *U. yaschenkoi*.

The results presented in this thesis provide novel insights into the venom chemistry and ecology of Australian scorpion species. Whilst they contribute to filling some knowledge gaps in scorpion venom research, they also highlight that there is still much to learn about the ecological and evolutionary processes underlying the variability in venom composition and venom use.

Bibliography

Abdel-Rahman, M.A., Omran, M.a.A., Abdel-Nabi, I.M., Ueda, H., and Mcvean, A. (2009). Intraspecific variation in the Egyptian scorpion *Scorpio maurus palmatus* venom collected from different biotopes. *Toxicon* 53**,** 349-359.

Adam, K.R., and Weiss, C. (1959). Distribution of 5-hydroxytryptamine in scorpion venoms. *Nature* 183**,** 1398.

Aird, S.D. (2002). Ophidian envenomation strategies and the role of purines. *Toxicon* 40**,** 335-393.

Aird, S.D. (2005). Taxonomic distribution and quantitative analysis of free purine and pyrimidine nucleosides in snake venoms. *Comparative Biochemistry and Physiology B: Biochemistry and Molecular Biology* 140**,** 109-126.

Alape-Girón, A., Sanz, L., Escolano, J., Flores-Díaz, M., Madrigal, M., Sasa, M., and Calvete, J.J. (2008). Snake venomics of the lancehead pitviper *Bothrops asper*: geographic, individual, and ontogenetic variations. *Journal of Proteome Research* 7**,** 3556-3571.

Almaaytah, A., and Albalas, Q. (2014). Scorpion venom peptides with no disulfide bridges: a review. *Peptides* 51**,** 35-45.

Arjunwadkar, A.V., and Reddy, S.R.R. (1983). Spermidine in the venom of the scorpion, *Palamneus phipsoni*. *Toxicon* 21**,** 321-325.

Banerjee, S., Gnanamani, E., Lynch, S.R., ZuñIga, F.Z., JiméNez-Vargas, J.M., Possani, L.D., and Zare, R.N. (2018). An alkaloid from scorpion venom: chemical structure and synthesis. *Journal of Natural Products* 81**,** 1899-1904.

Bordon, K.C.F., Wiezel, G.A., Amorim, F.G., and Arantes, E.C. (2015). Arthropod venom hyaluronidases: biochemical properties and potential applications in medicine and biotechnology. *Journal of Venomous Animals and Toxins including Tropical Diseases* 21**,** 4-4.

Broad, A.J., Sutherland, S.K., and Coulter, A.R. (1979). The lethality in mice of dangerous Australian and other snake venom. *Toxicon* 17**,** 661-664.

Carcamo-Noriega, E.N., Olamendi-Portugal, T., Restano-Cassulini, R., Rowe, A., Uribe-Romero, S.J., Becerril, B., and Possani, L.D. (2018). Intraspecific variation of *Centruroides sculpturatus* scorpion venom from two regions of Arizona. *Archives of Biochemistry and Biophysics* 638**,** 52-57.

Carcamo-Noriega, E.N., Sathyamoorthi, S., Banerjee, S., Gnanamani, E., Mendoza-Trujillo, M., Mata-Espinosa, D., Hernández-Pando, R., Veytia-Bucheli, J.I., Possani, L.D., and Zare, R.N. (2019). 1, 4-Benzoquinone antimicrobial agents against *Staphylococcus aureus* and *Mycobacterium tuberculosis* derived from scorpion venom. *Proceedings of the National Academy of Sciences of the United States of America* 116**,** 12642-12647.

Chippaux, J.-P., and Goyffon, M. (2008). Epidemiology of scorpionism: a global appraisal. *Acta Tropica* 107**,** 71-79.

Cid-Uribe, J.I., Veytia-Bucheli, J.I., Romero-Gutierrez, T., Ortiz, E., and Possani, L.D. (2020). Scorpion venomics: a 2019 overview. *Expert Review of Proteomics* 17**,** 67-83.

Cipriani, V., Debono, J., Goldenberg, J., Jackson, T.N.W., Arbuckle, K., Dobson, J., Koludarov, I., Li, B., Hay, C., Dunstan, N., Allen, L., Hendrikx, I., Kwok, H.F., and Fry, B.G. (2017). Correlation between ontogenetic dietary shifts and venom variation in Australian brown snakes (*Pseudonaja*). *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 197**,** 53-60.

Cooper, A.M., Nelsen, D.R., and Hayes, W.K. (2015). The strategic use of venom by spiders. *Evolution of Venomous Animals and Their Toxins*, 1-18.

Currie, B. (1994). Clinical implications of research on the box-jellyfish *Chironex fleckeri*. *Toxicon* 32**,** 1305-1313.

Daly, N.L., and Craik, D.J. (2011). Bioactive cystine knot proteins. *Current Opinion in Chemical Biology* 15**,** 362-368.

Dardevet, L., Rani, D., Aziz, T.a.E., Bazin, I., Sabatier, J.-M., Fadl, M., Brambilla, E., and De Waard, M. (2015). Chlorotoxin: a helpful natural scorpion peptide to diagnose glioma and fight tumor invasion. *Toxins* 7**,** 1079-1101.

De La Vega, R.C.R., and Possani, L.D. (2005). Overview of scorpion toxins specific for Na+ channels and related peptides: biodiversity, structure–function relationships and evolution. *Toxicon* 46**,** 831-844.

De La Vega, R.C.R., Schwartz, E.F., and Possani, L.D. (2010). Mining on scorpion venom biodiversity. *Toxicon* 56**,** 1155-1161.

De La Vega, R.R., Vidal, N., and Possani, L.D. (2013). "Scorpion peptides," in *Handbook of Biologically Active Peptides*. Academic Press London), 423-429.

De Sousa, L., Borges, A., Vásquez-Suárez, A., Den Camp, H.J.O., Chadee-Burgos, R.I., Romero-Bellorín, M., Espinoza, J., De Sousa-Insana, L., and Pino-García, O. (2010). Differences in venom toxicity and antigenicity between females and males *Tityus nororientalis* (Buthidae) scorpions. *Journal of venom research* 1**,** 61.

Debin, J.A., Maggio, J.E., and Strichartz, G.R. (1993). Purification and characterization of chlorotoxin, a chloride channel ligand from the venom of the scorpion. *American Journal of Physiology-Cell Physiology* 264**,** C361-C369.

Delgado-Prudencio, G., Cid-Uribe, J.I., Morales, J.A., Possani, L.D., Ortiz, E., and Romero-Gutiérrez, T. (2022). The enzymatic core of scorpion venoms. *Toxins* 14**,** 248.

Delgado-Prudencio, G., Possani, L.D., Becerril, B., and Ortiz, E. (2019). The dual α-amidation system in scorpion venom glands. *Toxins* 11**,** 425.

Dunlop, J.A., and Selden, P.A. (2009). Calibrating the chelicerate clock: a paleontological reply to Jeyaprakash and Hoy. *Experimental and Applied Acarology* 48**,** 183.

Dutertre, S., Jin, A.-H., Alewood, P.F., and Lewis, R.J. (2014). Intraspecific variations in *Conus geographus* defence-evoked venom and estimation of the human lethal dose. *Toxicon* 91**,** 135-144.

Dutertre, S., and Lewis, R.J. (2010). Use of venom peptides to probe ion channel structure and function. *Journal of Biological Chemistry* 285**,** 13315-13320.

Estrada-Gómez, S., Cupitra, N.I., Arango, W.M., and Muñoz, L.J.V. (2014). Intraspecific variation of *Centruroides edwardsii* venom from two regions of Colombia. *Toxins* 6**,** 2082-2096.

Fajloun, Z., Kharrat, R., Chen, L., Lecomte, C., Di Luccio, E., Bichet, D., El Ayeb, M., Rochat, H., Allen, P., and Pessah, I.N. (2000). Chemical synthesis and characterization of maurocalcine, a scorpion toxin that activates Ca2+ release channel/ryanodine receptors. *FEBS Letters* 469**,** 179-185.

Fenner, P., and Carney, I. (1999). The Irukandji syndrome. A devastating syndrome caused by a north Australian jellyfish. *Australian Family Physician* 28**,** 1131-1137.

Fenton, A.W., West, P.R., Odell, G.V., Hudiburg, S.M., Ownby, C.L., Mills, J.N., Scroggins, B.T., and Shannon, S.B. (1995). Arthropod venom citrate inhibits phospholipase A2. *Toxicon* 33**,** 763-770.

Fox, J.W., and Serrano, S.M. (2007). Approaching the golden age of natural product pharmaceuticals from venom libraries: an overview of toxins and toxin-derivatives currently involved in therapeutic or diagnostic applications. *Current Pharmaceutical Design* 13**,** 2927-2934.

Gangur, A.N., Seymour, J.E., Liddell, M.J., Wilson, D., Smout, M.J., and Northfield, T.D. (2018). When is overkill optimal? Tritrophic interactions reveal new insights into venom evolution. *Theoretical Ecology* 11**,** 141-149.

Gilding, E.K., Jami, S., Deuis, J.R., Israel, M.R., Harvey, P.J., Poth, A.G., Rehm, F.B., Stow, J.L., Robinson, S.D., and Yap, K. (2020). Neurotoxic peptides from the venom of the giant Australian stinging tree. *Science Advances* 6**,** eabb8828.

Gopalakrishnakone, P., Cheah, J., and Gwee, M.C.E. (1995). Black scorpion (*Heterometrus longimanus*) as a laboratory animal: maintenance of a colony of scorpion for milking of venom for research, using a restraining device. *Laboratory Animals* 29**,** 456-458.

Gurrola, G.B., Capes, E.M., Zamudio, F.Z., Possani, L.D., and Valdivia, H.H. (2010). Imperatoxin A, a cell-penetrating peptide from scorpion venom, as a probe of Ca2+-release channels/ryanodine receptors. *Pharmaceuticals* 3**,** 1093-1107.

Hayes, W.K. (2008). "The snake venom-metering controversy: levels of analysis, assumptions, and evidence," in *The Biology of Rattlesnakes*.), 191-220.

Hayes, W.K., Herbert, S.S., Rehling, G.C., and Gennaro, J.F. (2002). "Factors that influence venom expenditure in viperids and other snake species during predatory and defensive contexts," in *Biology of the Vipers*.), 207-233.

Herzig, V., Cristofori-Armstrong, B., Israel, M.R., Nixon, S.A., Vetter, I., and King, G.F. (2020). Animal toxins—Nature's evolutionary-refined toolkit for basic research and drug discovery. *Biochemical Pharmacology* 181**,** 114096.

Herzig, V., Ward, R.J., and Dos Santos, W.F. (2004). Ontogenetic changes in *Phoneutria nigriventer* (Araneae, Ctenidae) spider venom. *Toxicon* 44**,** 635-640.

Huang, R., Ke, W., Han, L., Li, J., Liu, S., and Jiang, C. (2011). Targeted delivery of chlorotoxin-modified DNA-loaded nanoparticles to glioma via intravenous administration. *Biomaterials* 32**,** 2399-2406.

Inceoglu, B., Lango, J., Jing, J., Chen, L., Doymaz, F., Pessah, I.N., and Hammock, B.D. (2003). One scorpion, two venoms: prevenom of *Parabuthus transvaalicus* acts as an alternative type of venom with distinct mechanism of action. *Proceedings of the National Academy of Sciences of the United States of America* 100**,** 922-927.

Isbister, G.K., and Bawaskar, H.S. (2014). Scorpion envenomation. *New England Journal of Medicine* 371**,** 457-463.

Isbister, G.K., Volschenk, E.S., Balit, C.R., and Harvey, M.S. (2003). Australian scorpion stings: a prospective study of definite stings. *Toxicon* 41**,** 877-883.

Isbister, G.K., Volschenk, E.S., and Seymour, J.E. (2004). Scorpion stings in Australia: five definite stings and a review. *Internal Medicine Journal* 34**,** 427-430.

Jean-Philippe, C., Alfredo, C., Leslie, B., and Alejandro, A. (2020). Factors involved in the resilience of incidence and decrease of mortality from scorpion stings in Mexico. *Toxicon* 188**,** 65-75.

King, G.F. (2011). Venoms as a platform for human drugs: translating toxins into therapeutics. *Expert Opinion on Biological Therapy* 11**,** 1469-1484.

King, J.V.L., Emrick, J.J., Kelly, M.J., Herzig, V., King, G.F., Medzihradszky, K.F., and Julius, D. (2019). A cell-penetrating scorpion toxin enables mode-specific modulation of TRPA1 and pain. *Cell* 178**,** 1362-1374. e1316.

Klint, J.K., Senff, S., Rupasinghe, D.B., Er, S.Y., Herzig, V., Nicholson, G.M., and King, G.F. (2012). Spider-venom peptides that target voltage-gated sodium channels: pharmacological tools and potential therapeutic leads. *Toxicon* 60**,** 478-491.

Lacerda, A.B., Lorenz, C., De Azevedo, T.S., Cândido, D.M., Wen, F.H., Eloy, L.J., Bersusa, A.a.S., and Chiaravalloti Neto, F. (2022). Scorpion envenomation in the state of São Paulo, Brazil: Spatiotemporal analysis of a growing public health concern. *PLoS One* 17**,** e0266138.

Lago, J., Rodríguez, L.P., Blanco, L., Vieites, J.M., and Cabado, A.G. (2015). Tetrodotoxin, an extremely potent marine neurotoxin: distribution, toxicity, origin and therapeutical uses. *Marine Drugs* 13**,** 6384-6406.

Laurie, M. (1900). XIX.—On a Silurian Scorpion and some additional Eurypterid Remains from the Pentland Hills. *Earth and Environmental Science Transactions of The Royal Society of Edinburgh* 39**,** 575-590.

Lima, S.L., and Dill, L.M. (1990). Behavioral decisions made under the risk of predation: a review and prospectus. *Canadian Journal of Zoology* 68**,** 619-640.

Lira, A.F.A., Santos, A.B., Silva, N.A., and Martins, R.D. (2017). Threat level influences the use of venom in a scorpion species, *Tityus stigmurus* (Scorpiones, Buthidae). *Acta Ethologica* 20**,** 291-295.

Lyons, S.A., O'neal, J., and Sontheimer, H. (2002). Chlorotoxin, a scorpion-derived peptide, specifically binds to gliomas and tumors of neuroectodermal origin. *Glia* 39**,** 162-173.

Mccormick, K.D., and Meinwald, J. (1993). Neurotoxic acylpolyamines from spider venoms. *Journal of Chemical Ecology* 19**,** 2411-2451.

Mccue, M.D. (2006). Cost of producing venom in three North American pitviper species. *Copeia* 2006**,** 818-825.

Mcelroy, T., Mcreynolds, C.N., Gulledge, A., Knight, K.R., Smith, W.E., and Albrecht, E.A. (2017). Differential toxicity and venom gland gene expression in *Centruroides vittatus*. *PLoS One* 12**,** e0184695.

Miller, D.W., Jones, A.D., Goldston, J.S., Rowe, M.P., and Rowe, A.H. (2016). Sex differences in defensive behavior and venom of the striped bark scorpion *Centruroides vittatus* (Scorpiones: Buthidae). *Integrative and Comparative Biology* 56**,** 1022-1031.

Morgenstern, D., and King, G.F. (2013). The venom optimization hypothesis revisited. *Toxicon* 63**,** 120-128.

Nascimento, D.G., Rates, B., Santos, D.M., Verano-Braga, T., Barbosa-Silva, A., Dutra, A.A., Biondi, I., Martin-Eauclaire, M.F., De Lima, M.E., and Pimenta, A.M. (2006). Moving pieces in a taxonomic puzzle: venom 2D-LC/MS and data clustering analyses to infer phylogenetic relationships in some scorpions from the Buthidae family (Scorpiones). *Toxicon* 47**,** 628-639.

Newton, K.A., Clench, M.R., Deshmukh, R., Jeyaseelan, K., and Strong, P.N. (2007). Mass fingerprinting of toxic fractions from the venom of the Indian red scorpion, *Mesobuthus tamulus*: biotope-specific variation in the expression of venom peptides. *Rapid Communications in Mass Spectrometry* 21**,** 3467-3476.

Nisani, Z., Boskovic, D.S., Dunbar, S.G., Kelln, W., and Hayes, W.K. (2012). Investigating the chemical profile of regenerated scorpion (*Parabuthus transvaalicus*) venom in relation to metabolic cost and toxicity. *Toxicon* 60**,** 315-323.

Nisani, Z., Dunbar, S.G., and Hayes, W.K. (2007). Cost of venom regeneration in *Parabuthus transvaalicus* (Arachnida: Buthidae). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 147**,** 509-513.

Nisani, Z., and Hayes, W.K. (2011). Defensive stinging by *Parabuthus transvaalicus* scorpions: risk assessment and venom metering. *Animal Behaviour* 81**,** 627-633.

Olguín-Pérez, L., Francke, O.F., and Carbajal-Saucedo, A. (2021). Evidence of piercing and sexual differences in venom composition in a sexual stinging scorpion (Scorpiones: Euscorpiidae). *The Journal of Arachnology* 49**,** 98-107.

Omran, M.A., and Mcvean, A. (2000). Intraspecific variation in scorpion *Leiurus quinquestriatus* venom collected from Egypt (Sinai and Aswan deserts). *Journal of Toxicology: Toxin Reviews* 19**,** 247-264.

Ortiz, E., Gurrola, G.B., Schwartz, E.F., and Possani, L.D. (2015). Scorpion venom components as potential candidates for drug development. *Toxicon* 93**,** 125-135.

Palma, M.S., and Nakajima, T. (2005). A natural combinatorial chemistry strategy in acylpolyamine toxins from nephilinae orb-web spiders. *Toxin Reviews* 24**,** 209-234.

Peretti, A.V. (2014). "Chapter 2 - Sexual selection in Neotropical species: rules and exceptions," in *Sexual Selection,* eds. R.H. Macedo & G. Machado. (San Diego: Academic Press), 33-52.

Poillot, C., Dridi, K., Bichraoui, H., Pêcher, J., Alphonse, S., Douzi, B., Ronjat, M., Darbon, H., and De Waard, M. (2010). D-Maurocalcine, a pharmacologically inert efficient cell-penetrating peptide analogue. *Journal of Biological Chemistry* 285**,** 34168-34180.

Polis, G.A., Myers, C.A., and Holt, R.D. (1989). The ecology and evolution of intraguild predation: potential competitors that eat each other. *Annual review of ecology and systematics* 20**,** 297-330.

Possani, L.D., Merino, E., Corona, M., Bolivar, F., and Becerril, B. (2000). Peptides and genes coding for scorpion toxins that affect ion-channels. *Biochimie* 82**,** 861-868.

Rates, B., Ferraz, K.K.F., Borges, M.H., Richardson, M., De Lima, M.E., and Pimenta, A.M.C. (2008). *Tityus serrulatus* venom peptidomics: assessing venom peptide diversity. *Toxicon* 52**,** 611-618.

Rein, J.O. (2022). *The Scorpion Files. Trondheim: Norwegian University of Science and Technology* [Online]. Available: https://www.ntnu.no/ub/scorpion-files/ [Accessed 12/04/2022 2022].

Rodríguez-Ravelo, R., Coronas, F.I.V., Zamudio, F.Z., González-Morales, L., López, G.E., Urquiola, A.R., and Possani, L.D. (2013). The Cuban scorpion *Rhopalurus junceus* (Scorpiones, Buthidae): component variations in venom samples collected in different geographical areas. *Journal of Venomous Animals and Toxins Including Tropical Diseases* 19**,** 13.

Ruiming, Z., Yibao, M., Yawen, H., Zhiyong, D., Yingliang, W., Zhijian, C., and Wenxin, L. (2010). Comparative venom gland transcriptome analysis of the scorpion *Lychas mucronatus* reveals intraspecific toxic gene diversity and new venomous components. *BMC Genomics* 11**,** 452.

Santana, R.C., Perez, D., Dobson, J., Panagides, N., Raven, R.J., Nouwens, A., Jones, A., King, G.F., and Fry, B.G. (2017). Venom profiling of a population of the theraphosid spider *Phlogius crassipes* reveals continuous ontogenetic changes from juveniles through adulthood. *Toxins* 9**,** 116.

Santos, M.S.V., Silva, C.G.L., Neto, B.S., Júnior, C.R.P.G., Lopes, V.H.G., Júnior, A.G.T., Bezerra, D.A., Luna, J.V.C.P., Cordeiro, J.B., and Júnior, J.G. (2016). Clinical and epidemiological aspects of scorpionism in the world: a systematic review. *Wilderness & Environmental Medicine* 27**,** 504-518.

Sarhan, M.M., Fouda, M.M., Elbitar, A.M., Aly, H.A., and Sayed, A.B. (2012). Variation of protein profile among consecutive stings of the scorpion *Parabuthus leiosoma* (family: Buthidae) from Egypt, supports the venom-metering hypothesis in scorpions. *Al-Azhar Bulletin of Science* 23**,** 61-72.

Seiter, M., and Stockmann, M. (2017). The life history of the parthenogenetic scorpion *Lychas tricarinatus* (Simon, 1884) from Odisha province, India and supplementary notes on *Tityus trivittatus* Kraepelin, 1898 (Scorpiones, Buthidae). *Zoologischer Anzeiger* 270**,** 155-165.

Shorthouse, D.J., and Marples, T.G. (1982). The life stages and population dynamics of an arid zone scorpion *Urodacus yaschenkoi* (Birula 1903). *Australian Journal of Ecology* 7**,** 109-118.

Smith, J.J., Herzig, V., King, G.F., and Alewood, P.F. (2013a). The insecticidal potential of venom peptides. *Cellular and Molecular Life Sciences* 70**,** 3665-3693.

Smith, J.J., Hill, J.M., Little, M.J., Nicholson, G.M., King, G.F., and Alewood, P.F. (2011). Unique scorpion toxin with a putative ancestral fold provides insight into evolution of the inhibitor cystine knot motif. *Proceedings of the National Academy of Sciences* 108**,** 10478-10483.

Smith, J.J., Vetter, I., Lewis, R.J., Peigneur, S., Tytgat, J., Lam, A., Gallant, E.M., Beard, N.A., Alewood, P.F., and Dulhunty, A.F. (2013b). Multiple actions of ϕ-LITX-Lw1a on ryanodine receptors reveal a functional link between scorpion DDH and ICK toxins. *Proceedings of the National Academy of Sciences* 110**,** 8906-8911.

Song, J., Sun, R., Li, D., Tan, F., Li, X., Jiang, P., Huang, X., Lin, L., Deng, Z., and Zhang, Y. (2012). An improvement of shotgun proteomics analysis by adding next-generation sequencing transcriptome data in orange. *PLoS One* 7**,** e39494.

Sun, C., Fang, C., Stephen, Z., Veiseh, O., Hansen, S., Lee, D., Ellenbogen, R.G., Olson, J., and Zhang, M. (2008). Tumor-targeted drug delivery and MRI contrast enhancement by chlorotoxin-conjugated iron oxide nanoparticles.

Sunagar, K., Undheim, E., Chan, A., Koludarov, I., Muñoz-Gómez, S., Antunes, A., and Fry, B. (2013). Evolution stings: the origin and diversification of scorpion toxin peptide scaffolds. *Toxins* 5**,** 2456-2487.

Thien, T.V., Anh, H.N., Trang, N.T.T., Van Trung, P., Khoa, N.C., Osipov, A., Dubovskii, P., Ivanov, I., Arseniev, A., and Tsetlin, V. (2017). Low-molecular-weight compounds with anticoagulant activity from the scorpion *Heterometrus laoticus* venom. *Doklady Biochemistry and Biophysics* 476**,** 316-319.

Tian, C., Yuan, Y., and Zhu, S. (2008). Positively selected sites of scorpion depressant toxins: possible roles in toxin functional divergence. *Toxicon* 51**,** 555-562.

Tran, T., Hoang, A., Nguyen, T., Phung, T., Nguyen, K., Osipov, A., Ivanov, I., Tsetlin, V., and Utkin, Y. (2017). Anticoagulant activity of low-molecular weight compounds from *Heterometrus laoticus* scorpion venom. *Toxins* 9**,** 343.

Uribe, J.I.C., Vargas, J.M.J., Batista, C.V.F., Zuñiga, F.Z., and Possani, L.D. (2017). Comparative proteomic analysis of female and male venoms from the Mexican scorpion *Centruroides limpidus*: novel components found. *Toxicon* 125**,** 91-98.

Van Der Meijden, A., and Kleinteich, T. (2017). A biomechanical view on stinger diversity in scorpions. *Journal of Anatomy* 230**,** 497-509.

Veiseh, M., Gabikian, P., Bahrami, S.-B., Veiseh, O., Zhang, M., Hackman, R.C., Ravanpay, A.C., Stroud, M.R., Kusuma, Y., and Hansen, S.J. (2007). Tumor paint: a chlorotoxin: Cy5. 5 bioconjugate for intraoperative visualization of cancer foci. *Cancer Research* 67**,** 6882-6888.

Ward, M.J., Ellsworth, S.A., Hogan, M.P., Nystrom, G.S., Martinez, P., Budhdeo, A., Zelaya, R., Perez, A., Powell, B., and He, H. (2018). Female-biased population divergence in the venom of the Hentz striped scorpion (*Centruroides hentzi*). *Toxicon* 152**,** 137-149.

Weinberger, H., Moran, Y., Gordon, D., Turkov, M., Kahn, R., and Gurevitz, M. (2009). Positions under positive selection—Key for selectivity and potency of scorpion α-toxins. *Molecular Biology and Evolution* 27**,** 1025-1034.

Wiener, S. (1957). The Sydney funnel-web spider (*Atrax robustus*): I. Collection of venom and its toxicity in animals. *Medical Journal of Australia* 2**,** 377-383.

Wiener, S. (1959). Observations on the venom of the stone fish (*Synanceja trachynis*). *Medical Journal of Australia* 1**,** 620-627.

Wilson, D., Boyle, G., Mcintyre, L., Nolan, M., Parsons, P., Smith, J., Tribolet, L., Loukas, A., Liddell, M., and Rash, L. (2017). The aromatic head group of spider toxin polyamines influences toxicity to cancer cells. *Toxins* 9**,** 346.

Windley, M.J., Herzig, V., Dziemborowicz, S.A., Hardy, M.C., King, G.F., and Nicholson, G.M. (2012). Spider-venom peptides as bioinsecticides. *Toxins* 4**,** 191-227.

Yahel-Niv, A., and Zlotkin, E. (1979). Comparative studies on venom obtained from individual scorpions by natural stings. *Toxicon* 17**,** 435-446.

Zeng, X.C., Corzo, G., and Hahin, R. (2005). Scorpion venom peptides without disulfide bridges. *IUBMB Life* 57**,** 13-21.

Zhu, S., Darbon, H., Dyason, K., Verdonck, F., and Tytgat, J. (2003). Evolutionary origin of inhibitor cystine knot peptides. *The FASEB Journal* 17**,** 1765-1767.

Chapter 1. Venom Costs and Optimization in Scorpions

Frontiers in Ecology and Evolution **(2019), 7, 196**

doi: 10.3389/fevo.2019.00196

Edward R. J. Evans[1*], Tobin D. Northfield[2], Norelle L. Daly[1], David T. Wilson[1*]

[1]Center for Molecular Therapeutics, Australian Institute of Tropical Health and Medicine, James Cook University, Cairns, QLD, Australia

[2]Tree Fruit Research and Extension Center, Department of Entomology, Washington State University, Wenatchee, WA, USA

***Correspondence:**

Edward R. J. Evans edwardrobertjonathan.evans@my.jcu.edu.au

David T. Wilson david.wilson4@jcu.edu.au

Keywords: scorpion, venom, optimisation, evolution, plasticity, behaviour.

Author Contributions: The manuscript was written by EE and edited by TN, DW, and ND.

Acknowledgements: The Northcote Trust supports EE with a Northcote Graduate Scholarship.

1.1. Abstract

Scorpions use venoms as weapons to improve prey capture and predator defence, and these benefits must be balanced against costs associated with its use. Venom costs involve direct energetic costs associated with the production and storage of toxins, and indirect fitness costs arising from reduced venom availability. In order to reduce these costs, scorpions optimise their venom use via evolutionary responses, phenotypic plasticity, and behavioural mechanisms. Over long timescales, evolutionary adaptation to environments with different selection pressures appears to have contributed to interspecific variation in venom composition and stinger morphology. Furthermore, plastic responses may allow scorpions to modify and optimise their venom composition as pressures change. Optimal venom use can vary when facing each prey item and potential predator encountered, and therefore scorpions display a range of behaviours to optimise their venom use to the particular situation. These behaviours include varying sting rates, employing dry stings, and further altering the volume and composition of venom injected. Whilst these cost-reducing mechanisms are recognised in scorpions, relatively little is understood about the factors that influence them. Here, we review evidence of the costs associated with venom use in scorpions and discuss the mechanisms that have evolved to minimise them.

1.2. Introduction

Venomous organisms inject chemical cocktails into their predators and prey in order to disrupt normal biological functioning in their target (Fry et al., 2009;Casewell et al., 2013). These chemical weapons are often rich in proteins, peptides, and small molecules (Inceoglu et al., 2003;Escoubas et al., 2008;Calvete et al., 2009;Fry et al., 2009;Villar-Briones and Aird, 2018). Whilst venom provides survival benefits by aiding in prey capture and predator defence, the benefits come with costs. These costs are two-fold, involving direct energetic costs associated with production and storage of toxins (McCue, 2006;Nisani et al., 2007;Nisani et al., 2012), and further indirect costs associated with a reduced capacity to capture prey or defend when supplies are depleted. Whilst these costs have different types of impacts on venomous animals, the methods to reduce these costs can overlap. It has been proposed that due to costs associated with venom use, organisms will meter/optimise the volume of venom they inject in order to use their venom as economically as possible in different situations (Hayes et al., 2002;Wigger et al., 2002;Hayes, 2008;Morgenstern and King, 2013). Research into optimal venom use has primarily focussed on snakes (Hayes, 1995;Hayes et al., 2002;Young et al., 2002;Hayes, 2008), spiders (Wigger et al., 2002;Wullschleger and Nentwig, 2002;Hostettler and Nentwig, 2006;Nelsen et al., 2014;Cooper et al., 2015) and scorpions (Edmunds and Sibly, 2010;Nisani and Hayes, 2011;Lira et al., 2017), with the latter being the focus of this review.

Scorpions utilise venom to both capture prey and defend against predators, which can include organisms with very different physiologies and susceptibility to venom components (Gangur et al., 2017;van der Meijden et al., 2017). Over 400 million years of evolution (Dunlop and Selden, 2009) have led scorpions to develop a wide range of mechanisms that help minimise the costs of venom use. Successful prey capture and predator defence will ultimately affect a scorpion’s evolutionary fitness, and therefore selection on venom composition and concentration is generally influenced by both prey and/or predators (Tian et al., 2008;Weinberger et al., 2009;Gangur et al., 2018). As selection pressures vary between environments, so will optimal investment in venom (Gangur et al., 2018). Scorpions adapted to different ecological niches often show large differences in venom composition (de la Vega et al., 2010) and stinger morphology (van der Meijden et al., 2013;van der Meijden and Kleinteich, 2017), and this likely reflects responses to different selection pressures.

1.3. The Costs of Venom Use in Scorpions

1.3.1. Direct

Research on the energetic demands of venom use have focussed on costs of production, rather than maintenance, as it is difficult to measure the energy used in maintaining and storing toxins experimentally. Nisani et al. (2007) showed that after depleting the venom glands of scorpions (*Parabuthus transvaalicus*), metabolic rates increased by 39% during the first three days of regeneration. A later study (Nisani et al., 2012) found milked *P. transvaalicus* had on average a 21% higher metabolic rate than un-milked scorpions during the first eight days of regeneration, but in this second study the rate did not rise as high during the first three days (Nisani et al., 2007). Metabolic rates fluctuated throughout the experiment, and the authors suggested this likely reflected the asynchronous regeneration of toxins (Nisani et al., 2012). Whilst differences were observed between the studies, both identify a large increase in metabolic rate above baseline levels, indicating that in scorpions venom production is an important energetic expense (Nisani et al., 2007;Nisani et al., 2012). These studies also likely underestimate total energetic costs, as venom regeneration can take longer than eight days to complete (Carcamo-Noriega et al., 2019). Furthermore, scorpion venom varies between species in complexity, toxins utilised, and volume stored and injected (de la Vega et al., 2010;Sunagar et al., 2013;van der Meijden et al., 2015); all of which alter energy requirements. Energetic costs are also calculated against the baseline metabolic rate, which is likely to vary between species adapted to different ecological niches. Furthermore, as scorpions are ectotherms their metabolic rate will vary with environmental conditions, as Nime et al. (2013) showed scorpion activity is positively correlated with temperature.

1.3.2. Indirect

Indirect costs are associated with the ecological limitations arising from depleted venom supplies, such as increased predation risk or reduced ability to capture prey. Scorpions can store a limited volume of venom (van der Meijden et al., 2015), and regeneration of toxins can take at least two weeks to be complete (Carcamo-Noriega et al., 2019), reducing the ability to use venom for prey capture and predator defence until venom supplies are restored. Ecological costs of venom depletion cannot easily be quantified, as they will vary widely between species and environments with fluctuating selection pressures. Nonetheless, evaluation of behavioural changes that arise when venom stores are depleted may serve as evidence of ecological costs. Such a behavioural response has yet to be reported in scorpions, but some spiders with depleted venom supplies will adapt their hunting behaviour to target easily caught prey, as has been found in the wandering spider *Cupiennius salei* (Wullschleger and Nentwig, 2002). Scorpions and spiders often share many of their

natural predators and prey items, therefore the ecological costs of venom depletion in both organisms may bear some similarity. Comparable behaviours in scorpions might include a switch to smaller prey that can be successfully captured with only the pedipalps while venom is replenishing.

1.4. The Evolution of Optimal Venom Use

Compared with other venomous taxa, scorpions are unusual in that they possess two main weapons: their stinging apparatus and their pedipalps. Species vary in their relative investment in these two weapons depending on their ecological niche, leading to the great morphological diversity in scorpion stinging apparatus and pedipalps seen between species (Figure 1.1) (van der Meijden et al., 2013). Burrowing species, such as members of the family Scorpionidae, are often sit-and-wait predators (Hadley and Williams, 1968;Bub and Bowerman, 1979;Shachak and Brand, 1983;Shivashankar, 1994), and possess large pedipalps that can be used to dig, grab passing prey, and block predators from entering their burrow (van der Meijden et al., 2010). Large pedipalps are often accompanied by a small stinging apparatus. Small tail size may be due to a trade-off in investment between pedipalps and stinging apparatus (van der Meijden et al., 2013), or a reduced tail may simply improve mobility in the confines of a burrow. Vagrant scorpion species, such as many members of the family Buthidae, have no permanent residence and forage more actively (Hadley and Williams, 1968). This group of scorpions generally rely more heavily on their sting to capture prey and defend themselves, and have evolved powerful and highly mobile tails (Warburg, 1998;Coelho et al., 2017). Many of these scorpions have developed potent venom, and the family Buthidae contains all species with medically significant stings (Santos et al., 2016). Buthids often possess small pedipalps, suggesting an evolutionary trade-off between pedipalps and stinging apparatus may be present (van der Meijden et al., 2013), and/or small pedipalps may improve mobility and energetic efficiency while actively foraging. The evolution of potent venom in buthids may further have reduced the advantages that large pedipalps provide, making them energetically unfavourable.

Figure 1. 1 Morphological comparison of four Australian scorpions. A) *Australobuthus xerolimniorum* (Buthidae) B) *Lychas buchari* (Buthidae) C) *Hormurus waigiensis* (Hormuridae) D) *Urodacus* sp. (Urodacidae*). A. xerolimniorum* and *L. buchari* are more vagrant and active foragers, and have evolved relatively small chelicerae and thick powerful tails. *H. waigiensis* and *Urodacus* sp. are burrowing species, and have evolved larger chelicerae and smaller stinging apparatuses. Photographs by Edward Evans.

1.5. Behavioural Mechanisms to Optimise Venom Use

Optimal use of venom will vary between each interaction with prey and predators, influenced by the size and identity of the prey/predator. This has led scorpions to evolve a range of behavioural mechanisms allowing them to optimise their venom use when facing specific prey (Edmunds and Sibly, 2010) and under particular levels of threat (Nisani and Hayes, 2011).

1.5.1. The 'Decision' to Sting

To reduce the costs associated with unnecessary venom use, scorpions adapt their hunting strategies to the particular prey items targeted (Simone et al., 2018), and are less likely to use venom when capturing small or easily subdued prey (Rein, 1993;Edmunds and Sibly, 2010). Spiders appear to target the injection of their venom towards the thorax or head of prey items to maximise venom efficiency (Wigger et al., 2002;Carlson et al., 2014). However, to our knowledge it is not known if scorpions seek to apply stings to an optimal location, or if an optimal sting location even exists, as one study on *Bothriurus bonariensis* found that sting location did not affect the time taken to subdue prey (Simone et al., 2018).

Both the size and activity of prey items can influence a scorpion's choice to sting, as *Parabuthus liosoma*, *Parabuthus pallidus* and *Hadrurus spadix* sting larger and more active prey items more frequently (Rein, 1993;Edmunds and Sibly, 2010). Rein (1993) observed that the scorpions did not use their sting immediately when encountering prey, but would rather grab with their pedipalps and apply stings if the prey continued to struggle, presumably to minimise venom use whilst ensuring predation success. Ontogenetic changes in stinging behaviour can also be used to optimise venom use. Older *Paruroctonus boreus* and *Pandinus imperator* use their larger pedipalps to overpower prey and sting less often, avoiding using venom (Cushing and Matherne, 1980;Casper, 1985).

In addition to trade-offs between the use of venom and pedipalps for prey capture, there may be trade-offs in venom use and mobility when avoiding predation, as faster scorpions appear less likely to sting predators (Carlson et al., 2014;Miller et al., 2016). For example, female *Centuroides vittatus* scorpions that are heavier and less mobile, are more likely to sting a potential predator than males, which are more likely to sprint to safety (Carlson et al., 2014;Miller et al., 2016). Furthermore, within each sex, sprint speed decreases and sting rate increases with mass, indicating that higher rates of aggression are associated with reduced mobility (Carlson et al., 2014;Miller et al., 2016). Through fleeing, the males and smaller scorpions are not only able to avoid being eaten, but they also save their venom supply for future encounters and do not need to expend energy regenerating toxins, thus reducing ecological and energetic venom costs.

Unlike other scorpions, seven species of *Parabuthus* spray venom defensively, which may cause irritation to the sensitive tissues, such as eyes, of predators (Newlands, 1974;Nisani and Hayes, 2015). *P. transvaalicus* defensively spray when presented with both air-flow and touch stimuli simultaneously, suggesting the behaviour may be optimised towards high-threat scenarios where defensive tactics must be implemented before a predator gets close enough to sting (Nisani and Hayes, 2015). These scorpions are often attacked by predators such as grasshopper mice, which disarm scorpions by biting off their tails, and therefore place their face in close proximity to the telson (Nisani and Hayes, 2015). Compared with injection, sprayed venom has a higher risk of missing its target, likely increasing the costs of venom necessary to deter predators. However, these costs are likely offset by the advantage of deterring predators while the predator is still at a distance.

1.5.2. 'Dry' Stings

Scorpions can still avoid venom use when stinging in defensive encounters by employing 'dry' stings, where no venom is injected. Scorpions readily utilise dry stings in defensive situations (Nisani and Hayes, 2011;Lira et al., 2017;Rasko et al., 2018). The factors that determine whether venom is injected in a sting, however, are currently unclear. In *P. transvaalicus*, dry stinging behaviour is correlated with threat level, with the scorpions employing dry stings more frequently when the threat level is low (Nisani and Hayes, 2011). Additionally, this study suggests *P. transvaalicus*, when induced to sting multiple times in succession, use dry stings more frequently early in the stinging sequence and are more likely to inject venom as the threat persists (Nisani and Hayes, 2011). The combination of these results provides evidence that scorpions use dry stings in low threat situations to optimise their venom use. In contrast to the findings by Nisani and Hayes (2011) studies on dry stinging behaviour in other scorpion species have produced differing results. Lira et al. (2017) presented *Tityus stigmurus* with the same 'low-threat' and 'high-threat' stimuli described by Nisani and Hayes (2011), and found no correlation between dry sting rate and threat level. Furthermore, it has been shown that repeated simulated attacks against *Hadrurus arizonensis* lead to an increase in dry sting rate, despite venom remaining in the gland (Rasko et al., 2018). This latter result seems counter-intuitive, as scorpions might be expected to increase their defensive investment as a threat persists. Whilst the studies investigating the factors influencing dry stinging behaviour in scorpions are limited, the evidence supports the idea that at least some scorpion species utilise dry stings as a means to optimise their venom in defensive contexts (Nisani and Hayes, 2011), while others may not (Lira et al., 2017;Rasko et al., 2018). Further research should aim to identify whether interspecific differences are truly occurring, or if methodological differences between the studies are responsible for the observed differences. Furthermore, it is not currently known if scorpions utilise dry stings as a tactic to save venom when capturing prey, as spiders use

dry bites (Malli et al., 1998;Wigger et al., 2002). Spiders, however, have the ability to masticate their prey with their fangs, but the dry sting of a scorpion provides comparatively little aid in the incapacitation of prey. It is therefore unlikely that scorpions use dry stings to save venom when targeting prey.

1.5.3. Volume Injected

While scorpion stinging behaviour involves a dichotomy between dry stings versus stings with venom injected, scorpions also have the ability to vary the volume of venom they inject, both within each sting and through the application of multiple stings (Nisani and Hayes, 2011;van der Meijden et al., 2015). *P. transvaalicus* injected twice as much venom per single sting in high-threat situations compared with low-threat situations, indicating scorpions may use this tactic to vary their defensive investment in response to perceived threat level. The defensive sprays of *P. transvaalicus* display variable duration and flow rate suggesting the volume expelled could be controlled by contraction of the venom gland, but it is not currently known the volume sprayed is influenced by threat level (Nisani and Hayes, 2015).

The volume injected in single stings may be limited by morphological constraints, or the time that the aculeus is pierced into the target (van der Meijden et al., 2015). When scorpions are faced with repeated attacks from predators, they will continue to defensively sting as the attacks continue. Experiments into the defensive investment of scorpions in response to predation threat suggest that scorpions will repeatedly sting predators as the threat persists (Lira et al., 2017), but it is unclear whether the investment per attack increases or decreases with sting number (Nisani and Hayes, 2011;Rasko et al., 2018). Targeting prey, scorpions often hold on with their pedipalps and judiciously apply stings as the prey continues to struggle (Casper, 1985;Rein, 1993). The rate of stings increases with both prey size and activity (Edmunds and Sibly, 2010), suggesting that scorpions are being frugal with their venom, and only apply extra stings as necessary.

1.5.4. Composition Injected

In addition to reducing venom costs by metering the volume of venom they inject, scorpions are able to alter the composition of venom injected into their target and avoid unnecessarily injecting costly venom components. Scorpion venom is heterogenous and changes in composition as it is expelled from the aculeus (Figure 1.2). As the venom is secreted from the aculeus tip, the initial expulsion is a clear liquid, followed by an opalescent liquid, and finally turns milky coloured and viscous (Yahel-Niv and Zlotkin, 1979). These different secretions also vary in toxicity (Yahel-Niv and Zlotkin, 1979). Inceoglu et al. (2003) found that the initial clear secretion in *P. transvaalicus* constituted around 5% of the total venom volume within the gland, and they termed this

'prevenom'. Prevenom and main milky venom have distinct compositions and modes of action in both invertebrate and vertebrate targets (Inceoglu et al., 2003). The different mode of actions of prevenom and main venom may act together to induce greater toxicity, in a similar way to the toxin cabals employed by cone snails which target different pathways simultaneously (Olivera et al., 1999;Inceoglu et al., 2003). *P. transvaalicus* prevenom was found to contain six-times less peptide and protein concentration compared to the main venom, but a sixteen-fold higher potassium (K^+) salt concentration (Inceoglu et al., 2003). The authors suggested this extremely high K^+ concentration causes large and rapid depolarisation of nerves in the target, causing quick paralysis in insects and pain in vertebrates (Inceoglu et al., 2003). Prevenom not only contains a much lower peptide/protein concentration, but a comparatively simplistic composition (Inceoglu et al., 2003). It is therefore expected that prevenom is metabolically cheaper to produce than the main venom, as K^+ salt likely requires less energy to be replenished than peptides requiring production and folding (Inceoglu et al., 2003). The relative costs of prevenom versus main venom have not been calculated experimentally, but in *P. transvaalicus* prevenom components appear to regenerate quickly and at little metabolic cost compared with other toxins (Nisani et al., 2012). Prevenom may therefore have evolved as a mechanism to avoid injecting larger volumes of peptide rich mixtures, thereby minimising both metabolic and ecological costs of depletion, although this connection is difficult to test experimentally (Nisani et al., 2012).

By using prevenom first, scorpions can save their main peptide-rich venom for high-threat situations or when initially low-threat situations escalate. This hypothesis is supported by evidence that the composition of venom (prevenom vs main venom) scorpions inject is context dependent. In low-threat situations, scorpions are able to avoid injecting their metabolically 'expensive' mixtures of toxins, by injecting only prevenom (Nisani and Hayes, 2011). *P. transvaalicus* inject their main venom more frequently in high-threat situations, and in later stings when induced to sting repeatedly at both low and high threat levels (Nisani and Hayes, 2011). Furthermore, in low-threat situations, *T. stigmurus* injected prevenom in all trials, but when faced with the high-threat treatments most of the scorpions injected their main milky venom secretion (Lira et al., 2017). The use of prevenom in low threat situations not only minimises metabolic costs but also reduces ecological costs, as prevenom appears to regenerate faster than the main venom components (Nisani et al., 2012).

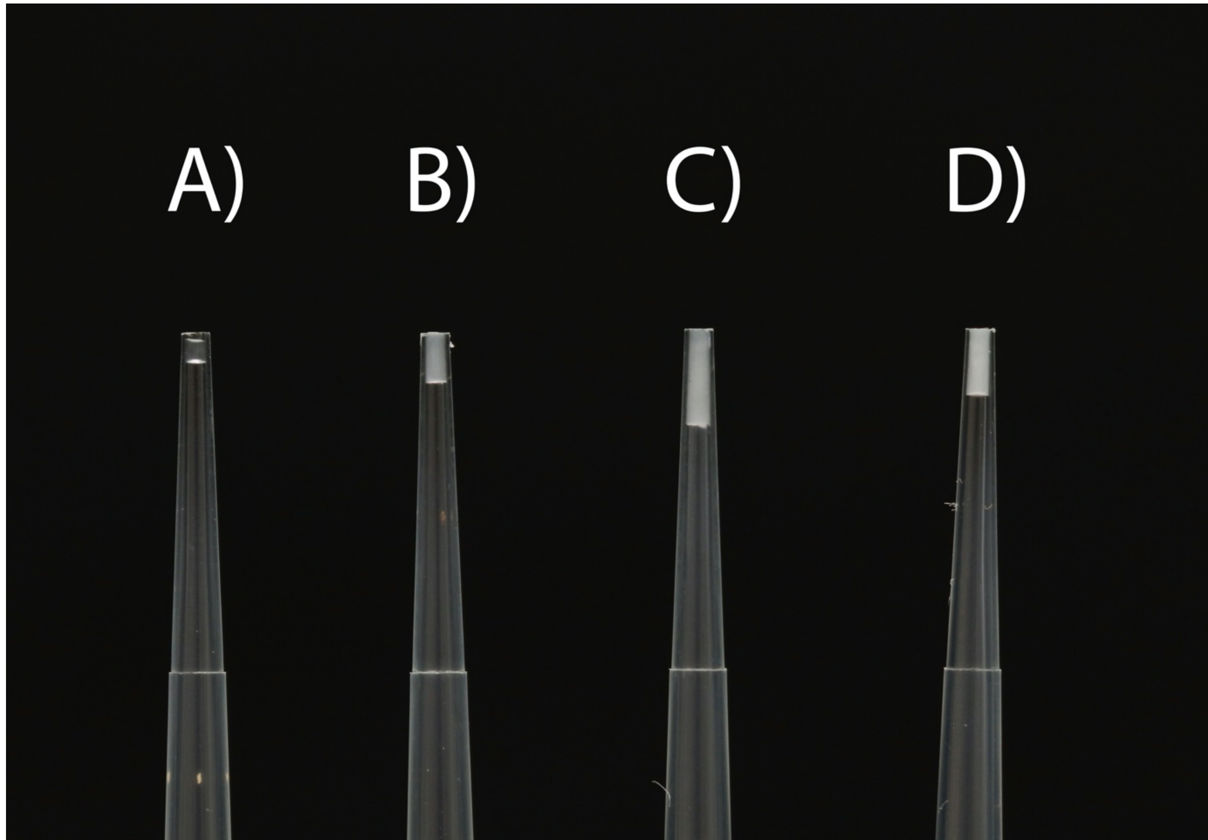


Figure 1. 2 From (A–D)–consecutive venom secretions collected from *Hormurus waigiensis* during one milking event using electrostimulation of the venom gland. (A) The initial secretion is relatively clear, containing the compositionally simple prevenom. (B) The secretion changes from clear to milky (C,D). The later venom secretions are comparatively opaque and white, representing the peptide/protein rich mixture. Photograph by Edward Evans.

1.6. Adaptive Plasticity

Recent evidence suggests scorpions can modify their venom composition in response to predator exposure (Gangur et al., 2017). Repeated periodical encounters with a surrogate vertebrate predator (a taxidermied mouse) over a six week period led *Hormurus waigiensis* to appear to produce a higher relative abundance of some vertebrate specific toxins used in defensive situations, and a lower relative abundance of certain toxins specific to their invertebrate prey (Gangur et al., 2017). This study provided the first evidence for adaptive plasticity in venom compositions, and suggested it has evolved as a mechanism to allow for the optimisation of venom use (Gangur et al., 2017). Modification of venom composition in response to environmental pressures could allow scorpions to further optimise venom use in different environments. In environments with few predators, scorpions may not require large quantities of defensive toxins, but as predator abundance increases so does the need to defend themselves. Therefore, ability to plastically change venom composition can allow scorpions to prioritise their resources and minimise the costs of venom use. It is currently unclear what environmental cues (e.g. olfactory) led to the plastic response observed by (Gangur et al., 2017). Furthermore, it is unclear if the response was targeted specifically at the presence of the mouse, or if it was a uniform response to increased predation pressure.

Unlike the response from simulated top-down predation pressure, Gangur et al. (2017) did not identify changes in venom composition in response to a scavenging versus predacious diet, where venom is not required for prey capture. This may be due to the unpredictable nature of scavenging, and the potential need to kill prey in the future, regardless of current carrion availability. Alternatively, venom may need to be maintained for its defensive function. In contrast, it may also be that experimental conditions do not represent the bottom-up pressures experienced in the wild, as crickets may be more easily subdued that natural prey items. *H. waigiensis* is a burrowing species that has evolved large pedipalps and a small stinging apparatus, and further studies should evaluate whether more active species that rely more heavily on their sting to capture prey respond differently to a changing diet.

1.7. Conclusions and Future Directions

Scorpions experience direct costs associated with the production and storage of toxins, and indirect costs associated with impaired ecological function when their venom is depleted. Optimal venom use minimises these costs, maximising the survival benefit venom provides. On the broadest scales, optimal venom investment has contributed to the divergence of stinger morphology and venom compositions between species adapted to different environments (Tian et al., 2008;Sunagar et al., 2013;van der Meijden et al., 2013). Optimal venom use can be influenced by factors such as prey/predator identity, and scorpions therefore utilise a suite of behavioural tactics to minimise waste. These include varying sting frequency, employing dry stings, and further controlling the volume and composition of venom injected (Nisani and Hayes, 2011). Scorpions may also plastically adapt their venom composition (Gangur et al., 2017), allowing them to optimise venom use as selection pressures change. Whilst the presence of these mechanisms and behaviours are well documented, the factors influencing them are poorly understood. Current knowledge of venom optimisation has generally relied upon correlative research, where the selective forces driving the correlations are inferred, rather than directly measured. There is evidence of each, venom costs, benefits for prey capture and predator defence, and behavioural and trait phenotypes that appear to reduce these costs and maximise benefits. However, there is little direct evidence tying changes in phenotypes to changes in costs or benefits to describe a mechanistic link. Controlled selection experiments or phylogenetic studies that consider species interactions can help describe links between selection and evolutionary response in arms races (Pimentel, 1968;Kursar et al., 2009;Toju et al., 2011;Betts et al., 2018), and may help better describe how observed venom optimisation mechanisms have evolved. Future work is needed to investigate whether observed changes are due to adaptive responses or physiological limitations, the extent that these mechanisms are influenced by the environment, and how widespread they are across different scorpion species.

1.8. Bibliography

Betts, A., Gray, C., Zelek, M., Maclean, R.C., and King, K.C. (2018). High parasite diversity accelerates host adaptation and diversification. *Science* 360**,** 907-911.

Bub, K., and Bowerman, R.F. (1979). Prey capture by the scorpion *Hadrurus arizonensis* Ewing (Scorpiones: Vaejovidae). *Journal of Arachnology***,** 243-253.

Calvete, J.J., Sanz, L., Angulo, Y., Lomonte, B., and Gutiérrez, J.M. (2009). Venoms, venomics, antivenomics. *FEBS Letters* 583**,** 1736-1743.

Carcamo-Noriega, E.N., Possani, L.D., and Ortiz, E. (2019). Venom content and toxicity regeneration after venom gland depletion by electrostimulation in the scorpion *Centruroides limpidus*. *Toxicon* 157**,** 87-92.

Carlson, B.E., Mcginley, S., and Rowe, M.P. (2014). Meek males and fighting females: sexually-dimorphic antipredator behavior and locomotor performance is explained by morphology in bark scorpions (*Centruroides vittatus*). *PLoS One* 9**,** e97648.

Casewell, N.R., Wüster, W., Vonk, F.J., Harrison, R.A., and Fry, B.G. (2013). Complex cocktails: the evolutionary novelty of venoms. *Trends in Ecology & Evolution* 28**,** 219-229.

Casper, G.S. (1985). Prey capture and stinging behavior in the emperor scorpion, *Pandinus imperator* (Koch)(Scorpiones, Scorpionidae). *Journal of Arachnology***,** 277-283.

Coelho, P., Kaliontzopoulou, A., Rasko, M., and Meijden, A. (2017). A 'striking' relationship: scorpion defensive behaviour and its relation to morphology and performance. *Functional Ecology* 31**,** 1390-1404.

Cooper, A.M., Nelsen, D.R., and Hayes, W.K. (2015). The strategic use of venom by spiders. *Evolution of Venomous Animals and Their Toxins***,** 1-18.

Cushing, B.S., and Matherne, A. (1980). Stinger utilization and predation in the scorpion *Paruroctonus boreus*. *The Great Basin Naturalist***,** 193-195.

De La Vega, R.C.R., Schwartz, E.F., and Possani, L.D. (2010). Mining on scorpion venom biodiversity. *Toxicon* 56**,** 1155-1161.

Dunlop, J.A., and Selden, P.A. (2009). Calibrating the chelicerate clock: a paleontological reply to Jeyaprakash and Hoy. *Experimental and Applied Acarology* 48**,** 183.

Edmunds, M.C., and Sibly, R.M. (2010). Optimal sting use in the feeding behavior of the scorpion *Hadrurus spadix*. *Journal of Arachnology* 38**,** 123-125.

Escoubas, P., Quinton, L., and Nicholson, G.M. (2008). Venomics: unravelling the complexity of animal venoms with mass spectrometry. *Journal of Mass Spectrometry* 43**,** 279-295.

Fry, B.G., Roelants, K., Champagne, D.E., Scheib, H., Tyndall, J.D., King, G.F., Nevalainen, T.J., Norman, J.A., Lewis, R.J., and Norton, R.S. (2009). The toxicogenomic multiverse: convergent recruitment of proteins into animal venoms. *Annual Review of Genomics and Human Genetics* 10**,** 483-511.

Gangur, A.N., Seymour, J.E., Liddell, M.J., Wilson, D., Smout, M.J., and Northfield, T.D. (2018). When is overkill optimal? Tritrophic interactions reveal new insights into venom evolution. *Theoretical Ecology* 11**,** 141-149.

Gangur, A.N., Smout, M., Liddell, M.J., Seymour, J.E., Wilson, D., and Northfield, T.D. (2017). Changes in predator exposure, but not in diet, induce phenotypic plasticity in scorpion venom. *Proceedings of the Royal Society of London. Series B: Biological Sciences* 284**,** 20171364.

Hadley, N.F., and Williams, S.C. (1968). Surface activities of some North American scorpions in relation to feeding. *Ecology* 49**,** 726-734.

Hayes, W.K. (1995). Venom metering by juvenile prairie rattlesnakes, *Crotalus v. viridis*: effects of prey size and experience. *Animal Behaviour* 50**,** 33-40.

Hayes, W.K. (2008). "The snake venom-metering controversy: levels of analysis, assumptions, and evidence," in *The Biology of Rattlesnakes*.), 191-220.

Hayes, W.K., Herbert, S.S., Rehling, G.C., and Gennaro, J.F. (2002). "Factors that influence venom expenditure in viperids and other snake species during predatory and defensive contexts," in *Biology of the Vipers*.), 207-233.

Hostettler, S., and Nentwig, W. (2006). Olfactory information saves venom during prey-capture of the hunting spider *Cupiennius salei* (Araneae: Ctenidae). *Functional Ecology* 20**,** 369-375.

Inceoglu, B., Lango, J., Jing, J., Chen, L., Doymaz, F., Pessah, I.N., and Hammock, B.D. (2003). One scorpion, two venoms: prevenom of *Parabuthus transvaalicus* acts as an alternative type of venom with distinct mechanism of action. *Proceedings of the National Academy of Sciences of the United States of America* 100**,** 922-927.

Kursar, T.A., Dexter, K.G., Lokvam, J., Pennington, R.T., Richardson, J.E., Weber, M.G., Murakami, E.T., Drake, C., Mcgregor, R., and Coley, P.D. (2009). The evolution of antiherbivore defenses and their contribution to species coexistence in the tropical tree genus *Inga*. *Proceedings of the National Academy of Sciences of the United States of America* 106**,** 18073-18078.

Lira, A.F.A., Santos, A.B., Silva, N.A., and Martins, R.D. (2017). Threat level influences the use of venom in a scorpion species, *Tityus stigmurus* (Scorpiones, Buthidae). *Acta Ethologica* 20**,** 291-295.

Malli, H., Imboden, H., and Kuhn-Nentwig, L. (1998). Quantifying the venom dose of the spider *Cupiennius salei* using monoclonal antibodies. *Toxicon* 36**,** 1959-1969.

Mccue, M.D. (2006). Cost of producing venom in three North American pitviper species. *Copeia* 2006**,** 818-825.

Miller, D.W., Jones, A.D., Goldston, J.S., Rowe, M.P., and Rowe, A.H. (2016). Sex differences in defensive behavior and venom of the striped bark scorpion *Centruroides vittatus* (Scorpiones: Buthidae). *Integrative and Comparative Biology* 56**,** 1022-1031.

Morgenstern, D., and King, G.F. (2013). The venom optimization hypothesis revisited. *Toxicon* 63**,** 120-128.

Nelsen, D.R., Kelln, W., and Hayes, W.K. (2014). Poke but don't pinch: risk assessment and venom metering in the western black widow spider, *Latrodectus hesperus*. *Animal Behaviour* 89**,** 107-114.

Newlands, G. (1974). The venom-squirting ability of *Parabuthus* scorpions (Arachnida: Buthidae). *South African Journal of Medical Sciences* 39**,** 175-178.

Nime, M.F., Casanoves, F., Vrech, D.E., and Mattoni, C.I. (2013). Relationship between environmental variables and surface activity of scorpions in the Arid Chaco ecoregion of Argentina. *Invertebrate Biology* 132**,** 145-155.

Nisani, Z., Boskovic, D.S., Dunbar, S.G., Kelln, W., and Hayes, W.K. (2012). Investigating the chemical profile of regenerated scorpion (*Parabuthus transvaalicus*) venom in relation to metabolic cost and toxicity. *Toxicon* 60**,** 315-323.

Nisani, Z., Dunbar, S.G., and Hayes, W.K. (2007). Cost of venom regeneration in *Parabuthus transvaalicus* (Arachnida: Buthidae). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 147**,** 509-513.

Nisani, Z., and Hayes, W.K. (2011). Defensive stinging by *Parabuthus transvaalicus* scorpions: risk assessment and venom metering. *Animal Behaviour* 81**,** 627-633.

Nisani, Z., and Hayes, W.K. (2015). Venom-spraying behavior of the scorpion *Parabuthus transvaalicus* (Arachnida: Buthidae). *Behavioural Processes* 115**,** 46-52.

Olivera, B.M., Walker, C., Cartier, G.E., Hooper, D., Santos, A.D., Schoenfeld, R., Shetty, R., Watkins, M., Bandyopadhyay, P., and Hillyard, D.R. (1999). Speciation of cone snails and interspecific hyperdivergence of their venom peptides: potential evolutionary significance of introns. *Annals of the New York Academy of Sciences* 870**,** 223-237.

Pimentel, D. (1968). Population regulation and genetic feedback. *Science* 159**,** 1432-1437.

Rasko, M., Coelho, P., Simone, Y., and Van Der Meijden, A. (2018). How to attack a scorpion: venom metering during a repeated attack. *Animal Behaviour* 145**,** 125-129.

Rein, J.O. (1993). Sting use in two species of *Parabuthus scorpions* (Buthidae). *Journal of Arachnology***,** 60-63.

Santos, M.S.V., Silva, C.G.L., Neto, B.S., Júnior, C.R.P.G., Lopes, V.H.G., Júnior, A.G.T., Bezerra, D.A., Luna, J.V.C.P., Cordeiro, J.B., and Júnior, J.G. (2016). Clinical and epidemiological aspects of scorpionism in the world: a systematic review. *Wilderness & Environmental Medicine* 27**,** 504-518.

Shachak, M., and Brand, S. (1983). The relationship between sit and wait foraging strategy and dispersal in the desert scorpion, *Scorpio maurus palmatus*. *Oecologia* 60**,** 371-377.

Shivashankar, T. (1994). Advanced sub social behaviour in the scorpion *Heterometrus fulvipes* Brunner (Arachnida). *Journal of Biosciences* 19**,** 81-90.

Simone, Y., Garcia, L.F., Lacava, M., Van Der Meijden, A., and Viera, C. (2018). Predatory versatility in females of the scorpion *Bothriurus bonariensis* (Scorpiones: Bothriuridae): overcoming prey with different defensive mechanisms. *Journal of Insect Behavior***,** 1-14.

Sunagar, K., Undheim, E., Chan, A., Koludarov, I., Muñoz-Gómez, S., Antunes, A., and Fry, B. (2013). Evolution stings: the origin and diversification of scorpion toxin peptide scaffolds. *Toxins* 5**,** 2456-2487.

Tian, C., Yuan, Y., and Zhu, S. (2008). Positively selected sites of scorpion depressant toxins: possible roles in toxin functional divergence. *Toxicon* 51**,** 555-562.

Toju, H., Abe, H., Ueno, S., Miyazawa, Y., Taniguchi, F., Sota, T., and Yahara, T. (2011). Climatic gradients of arms race coevolution. *American Naturalist* 177**,** 562-573.

Van Der Meijden, A., Coelho, P., and Rasko, M. (2015). Variability in venom volume, flow rate and duration in defensive stings of five scorpion species. *Toxicon* 100**,** 60-66.

Van Der Meijden, A., Coelho, P.L., Sousa, P., and Herrel, A. (2013). Choose your weapon: defensive behavior is associated with morphology and performance in scorpions. *PLoS one* 8**,** e78955.

Van Der Meijden, A., Herrel, A., and Summers, A. (2010). Comparison of chela size and pincer force in scorpions; getting a first grip. *Journal of Zoology* 280**,** 319-325.

Van Der Meijden, A., and Kleinteich, T. (2017). A biomechanical view on stinger diversity in scorpions. *Journal of Anatomy* 230**,** 497-509.

Van Der Meijden, A., Koch, B., Van Der Valk, T., Vargas-Muñoz, L.J., and Estrada-Gómez, S. (2017). Target-specificity in scorpions; comparing lethality of scorpion venoms across arthropods and vertebrates. *Toxins* 9**,** 312.

Villar-Briones, A., and Aird, S. (2018). Organic and peptidyl constituents of snake venoms: the picture is vastly more complex than we imagined. *Toxins* 10**,** 392.

Warburg, M. (1998). Qualitative and quantitative analysis of intra-and interspecific behavioural patterns among scorpions. *Journal of Ethology* 16**,** 115-121.

Weinberger, H., Moran, Y., Gordon, D., Turkov, M., Kahn, R., and Gurevitz, M. (2009). Positions under positive selection—Key for selectivity and potency of scorpion α-toxins. *Molecular Biology and Evolution* 27**,** 1025-1034.

Wigger, E., Kuhn-Nentwig, L., and Nentwig, W. (2002). The venom optimisation hypothesis: a spider injects large venom quantities only into difficult prey types. *Toxicon* 40**,** 749-752.

Wullschleger, B., and Nentwig, W. (2002). Influence of venom availability on a spider's prey-choice behaviour. *Functional Ecology* 16**,** 802-807.

Yahel-Niv, A., and Zlotkin, E. (1979). Comparative studies on venom obtained from individual scorpions by natural stings. *Toxicon* 17**,** 435-446.

Young, B.A., Lee, C.E., and Daley, K.M. (2002). Do snakes meter venom? *BioScience* 52**,** 1121-1126.

Lychas variatus adult female – Edward Evans

Chapter 2. Ontogenetic and Intersexual Differences in the Venom Composition of Marbled Scorpions (*Lychas variatus*)

2.1. Abstract

The chemical composition of venom varies not only between different species, but also within species. Many factors are known to act in combination to cause within-species (intraspecific) variation in venom compositions, including sex, locality, life-stage, seasonality, and environmental conditions such as temperature. Despite being a relatively well-studied venomous taxon, and some species being dangerously toxic to humans, relatively little is known regarding how scorpion venoms can vary within species, and the factors that drive variation. Scorpions display large regional and sexual differences in their venom compositions, but the influence of other factors have not been thoroughly investigated. In this study I examined intraspecific variation in marbled scorpion *Lychas variatus* venoms, and found they display both ontogenetic shifts and intersexual differences in composition. Of 579 molecule masses identified, 18 were unique to the venom of young (second-third instar) scorpions, 4 to medium (fourth instar), and 26 to adult (fifth-sixth instar) scorpions. Interestingly, 65 were uniquely shared between the small and medium scorpions, and 113 between the medium and large, suggesting a transitional change in the venom composition associated with size. Differences were also observed in the relative proportion of molecules in each sample belonging to different molecular weight classes. Per sample, fourth instar venoms contained the highest number of small toxins (500-2500 Da) and the lowest number of large toxins (5000-9000 Da). Additionally, sex-specific venom components were identified. 16 molecules appeared only in male venoms, whilst 115 were unique to female venoms. Furthermore, male venoms contained lower relative proportions of molecules in the 2500-5000 Da range. The findings of this study highlight the need to better understand drivers of intraspecific venom variation in scorpions, as it could provide new avenues for biodiscovery of unstudied molecules. Furthermore, ontogenetic and intersexual differences in their venoms will affect overall activity, and therefore in some species age/sex may be important to consider when treating sting victims or when selecting appropriate animals for antivenom production.

2.2. Introduction

The toxins produced by venomous organisms show great structural and functional diversity across the natural world (Fry et al., 2009;Casewell et al., 2013;Herzig et al., 2020a). Venoms are usually mixtures of toxins, displaying large compositional diversity between species (interspecific), and between members of the same species (intraspecific)(Casewell et al., 2020;Yu et al., 2020). Intraspecific variation in venom composition can be very large and is often associated with the ecology of the animals (Andrade and Abe, 1999;Barlow et al., 2009;Cipriani et al., 2017;Casewell et al., 2020;Herzig et al., 2020b).

As venoms have predominantly evolved for prey capture and predator defence, they can be under strong selective pressure, driving diversification between populations experiencing different prey/predators (Duda and Palumbi, 1999;Tian et al., 2008;Sunagar et al., 2016;Gangur et al., 2018;Yu et al., 2020). Additionally, the production and maintenance of venoms has energetic costs, and balancing of these costs against the survival benefits provided by venom in different environments can further drive diversification (Wigger et al., 2002;Morgenstern and King, 2013;Evans et al., 2019). Understanding the drivers of intraspecific venom variation is important, as compositional differences can directly impact the activity of the venom and possibly affect the symptoms experienced by envenomated patients (de Haro et al., 2002;Oliveira et al., 2013) or affect industrial processes that utilise venoms as a resource material, such as antivenom production (Fry et al., 2003;Senji Laxme et al., 2021). For example, the presence of the most human-toxic component of the Sydney funnel web (*Atrax robustus*) venom, δ-ACTX-Ar1a, is associated with the maturation of males (Herzig et al., 2020b), which may lead to differences in the severity of envenomation associated with spiders of different sex/age. Additionally, if antivenoms are produced using animals from a specific population, they may be ineffective when treating envenomation by an animal from another population with a different venom composition (Gillissen et al., 1994;Fry et al., 2003;Senji Laxme et al., 2021). It can therefore be instrumental within both clinical and industrial settings to fully understand how venom can vary between members of the same species.

Intraspecific variation in venoms is caused by a complicated network of biotic and abiotic factors, and venoms are seen to vary with geography (Winter et al., 2010;Cologna et al., 2013;Chang et al., 2015), sex (Rash et al., 2000;Furtado et al., 2006;Herzig et al., 2008), life-stage (ontogeny) (Andrade and Abe, 1999;Deslippe and Guo, 2000;Escoubas et al., 2002;McElroy et al., 2017;Santana et al., 2017;Avella et al., 2022), seasonality (Keegan et al., 1960;Junior et al., 2010), individuality (Duran et al., 2020), stage of venom regeneration (Pimenta et al., 2003;Nisani et al., 2012), and environmental conditions such as temperature (O'Hara et al., 2018;Sachkova et al., 2020).

Compositional variation can involve differences in the relative abundance of molecules, or differences in the presence/absence of specific toxins, both of which can impact the activity/toxicity of the venom as a whole (Oliveira et al., 2013). In certain regions of the world, highly toxic scorpion species cause many human deaths each year (Chippaux and Goyffon, 2008), and gaining a greater understanding of how scorpion venoms vary between individuals could provide insights that help optimise first aid, or selection of specific individuals for antivenom production. In addition, scorpion venoms are of interest to researchers for the biodiscovery of novel biochemical tools or pharmaceuticals, and intraspecific variability may provide insights into previously missed molecular candidates (King, 2011;Sunagar et al., 2014;Fry et al., 2015;Ortiz et al., 2015;Santana et al., 2017).

Scorpion venoms show large compositional differences between populations and the sexes. Venom divergence between scorpion populations may partly be due to genetic drift, but also local adaptation in different environments (Omran and McVean, 2000;Newton et al., 2007;Rodríguez-Ravelo et al., 2013;Estrada-Gómez et al., 2014;Carcamo-Noriega et al., 2018). Sex-related differences in scorpion venoms appear to be common across species, including in complexity (Abdel-Rahman et al., 2009), the relative abundance of toxins (Schwartz et al., 2008;Rodríguez-Ravelo et al., 2015), and the presence of sex-specific toxins (Yamaji et al., 2004;Özkan et al., 2011;Uribe et al., 2017). Both regional and sexual variation in scorpion venoms has been demonstrated to affect toxicity (De Sousa et al., 2010;Oliveira et al., 2013), and therefore these factors may be important to consider in a clinical setting or when selecting animals for antivenom production, as is known for snakes (Fry et al., 2003;Casewell et al., 2013).

The impact of other factors on intraspecific variability in scorpion venoms have been less well studied, but it has been reported that the venom toxicity and toxin gene expression in striped bark scorpions (*Centruroides vittatus*) varies across the different life-stages (McElroy et al., 2017). Ontogenetic shifts are observed in the venoms of other well-studied venomous taxa such as spiders (de Andrade et al., 1999;Escoubas et al., 2002;Herzig et al., 2004;Herzig, 2010;Santana et al., 2017) and snakes (Madrigal et al., 2012;García-Osorio et al., 2020), but have not been widely examined in scorpions. Ontogenetic changes in venoms are often associated with the ecology of the animals, arising for example, from a shift in diet as the animal grows (Cipriani et al., 2017), or a change in exposure to predators at a particular life stage (Herzig et al., 2020b). A study by Gangur et al. (2017) demonstrated that the venom composition of *Hormurus waigiensis* scorpions showed plasticity in response to exposure to a taxidermied mouse, providing strong evidence that scorpion venoms are shaped by their predators. As geography and sex have large impacts on scorpion venom composition, other factors likely also affect their predator/prey interactions and may contribute to venom variability. Sexual dimorphism in scorpions may be linked to sexual differences in

hunting/defensive strategy (Miller et al., 2016), particularly when there are sexual size differences that lead to different prey types being targeted. Indeed, *Pandinus imperator* (Scorpionidae) and *Paruroctonus boreus* (Vaejovidae) show ontogenetic shifts in hunting strategy, with smaller scorpions stinging prey items more frequently (Cushing and Matherne, 1980;Casper, 1985), and such differences could lead to compositional changes in the venom under different optimisation mechanisms (Evans et al., 2019).

In this study I tested whether marbled scorpions (*Lychas variatus*) display ontogenetic shifts in their venom composition, and whether sexual differences are present. I raised captive born *L. variatus* to adulthood, collected venom samples at different life stages, and performed liquid chromatography/mass spectrometry (LC/MS) analyses to identify compositional differences between the samples.

2.3. Materials and Methods

2.3.1. Scorpion Collection and Husbandry

Wild female scorpions were collected from a single rainforest population in the Cairns region (Queensland, Australia), and gave birth to captive-born young. Adult scorpions were housed individually in round plastic food containers (11 cm diameter: 8.5 cm height) with 3-5cm depth substrate (coco-fibre/sand mixture). They were stored at 25 °C in a temperature-controlled room with no light source. Captive-born scorpions were separated from the mother once they had moulted to second instar (I2) and housed in small, round plastic containers (6 cm diameter: 3 cm height). Once they had moulted to the fourth instar (I4), they were moved into the same sized containers as adults. Male *L. variatus* attained their adult size at fifth instar (I5), whilst some females additionally moulted to sixth instar (I6). I was uncertain if females were sexually mature at I5, but for the analysis I considered that fifth and sixth instar (I5-6) females were adult. A cricket was offered to the scorpions once a week, and uneaten food items were removed after 1-3 days to minimise stress. Females that had recently given birth and still had young on their backs were not provided with food items at this stage, as the introduction of a cricket caused great disturbance to the mother and offspring.

2.3.2. Venom Extraction

Scorpions were restrained onto a sponge using parafilm, and a square-wave stimulator (Arthur H. T. Thomas Co. Scientific Apparatus, Philadelphia, PA, USA) was used to electrostimulate the venom glands and extract venom into a pipette tip. Young scorpions (I2-3) were stimulated using

15V DC current, 0.5 ms pulse duration, 1 pulse/sec frequency, and the voltage was raised to 20 V for older scorpions (I4-6). Individual scorpion venom samples were suspended in 10 µL of Type 1 water (T1W) and stored at -20 °C prior to further analysis. Not all scorpions were milked at successive instars, as repeated electrostimulation of the venom gland can reduce the survivorship of scorpions (Czaykowsky, 2020). A total of 84 venom samples were collected, belonging to 35 individual scorpions. The number of samples representing each instar was as follows: I2 =17, I3 = 15, I4 = 23, I5 = 21, I6 = 8.

2.3.3. Liquid Chromatography/Mass Spectrometry (LC/MS)

Individual scorpion venoms were analysed by LC/MS using a Shimadzu LCMS-2020 mass spectrometer combined with a Shimadzu Prominence HPLC system (Shimadzu, Kyoto, Japan). As the quantity of venom produced by each scorpion depended on its size, crude samples (venom in 10 µL T1W) were diluted based on the scorpion's instar and made up to 10 µL with T1W prior to LC/MS analysis. As I2 and I3 scorpions produced very small quantities of venom, the approximately 10 µL crude samples were not diluted. For I4 scorpions 5 µL of sample was combined with 5 µL T1W for injection, and for I5-6 scorpions 3 µL of the stored sample was combined with 7 µL T1W.

Each 10 µL preparation was injected onto a reversed-phase high performance liquid chromatography (RP-HPLC) column (Phenomenex Aeris 3.6 µm PEPTIDE XB-C_{18} 100 Å, 2.1 x 150 mm; Phenomenex, Torrence, CA, USA) by a Shimadzu SIL-20ACHT autosampler, with the column oven at 30 °C. A 1% gradient of solvent B (90% acetonitrile [ACN; OPTIMA LCMS grade, Thermo Fisher Scientific, Scoresby, VIC, Australia]/0.09% formic acid [FA; Sigma-Aldrich, St. Louis, MO, USA]/H_2O) against solvent A (solvent A: 0.1% FA) was run (0-60 % solvent B) for 60 min at a flow rate of 0.250 mL/min using Shimadzu LC-20AD pumps. UV absorbance was monitored at 214 nm and 280 nm by a Shimadzu SPD-20A detector. Mass spectra were collected in positive ion mode with the following settings: scan range of *m/z* 250-2000, detector voltage of 1.15 kV, nebulizing gas flow of 1.5 L/min, drying gas flow of 3.0 L/min. Shimadzu LabSolutions v5.96 software (Shimadzu, Japan) was used to collect and analyse data. Mass lists for each sample were created by manually scanning through each peak and using the Shimadzu multi-charge ion analysis tool, and all mass lists were combined into a dataset containing the presence/absence of all toxin masses observed.

2.3.4. Data Analysis

All subsequent analysis was carried out in R version 4.0.5 (R Core Team, 2021), and data visualisation performed with the ggplot2 package (Wickham, 2016). The presence/absence dataset of all molecules recorded across the samples was subset by removing molecules present in less than

four samples and below 500 Da in mass. This threshold was selected as molecules present in less than four samples were either rare within the venoms or present below the detectable threshold in other samples. Additionally, molecules under 500 Da were removed as these often coeluted early and were difficult to accurately record across samples.

Overall chemical composition was analysed using non-metric multidimensional scaling (NMDS) ordinations based on Jaccard dissimilarity matrices, to visualise the effect of instar and sex. The best NMDS ordination was selected based on the optimal stress value, when adding an additional dimension (K) failed to reduce the stress by 0.05. To conduct statistical inference tests that align with NMDS visualisations, I performed permutational multivariate analysis of variance (PERMANOVA) with sex and instar as fixed effects, and the Jaccard distance matrix created from the raw (unscaled) data as response variables using the vegan package (Oksanen et al., 2020). As 19 samples were from scorpions that deceased before they attained a size where their sex could be determined, the combined effects of sex and instar were tested on a subset dataset with unsexed scorpions removed. The significance of each term was evaluated sequentially in the order instar, sex, instar x sex. The unsexed scorpions were mostly individuals that died at a small size, hence many of the removed samples belonged to small scorpions. I therefore also tested the effect of instar alone on a distance matrix created using the full dataset that included these scorpions for which sex could not be determined. Using NMDS score outputs rather than raw data allowed evaluation of a more simplified matrix, allowing evaluation of sex and instar effects with a multivariate analysis of variance (MANOVA). The effect of instar and sex were tested together on scorpions that had their sex determined, but again as some samples belonged to unsexed scorpions for which sex identification data was missing, the effect of instar was additionally tested separately on the full set of samples. Univariate linear mixed models (LMMs) were then performed to test the effect of instar and sex on each NMDS score using the lme4 R package (Bates et al., 2015). As some of the samples belonged to the same scorpion (some scorpions were only milked once, whilst others were milked up to five times at different instars), the identity of the scorpions was included as a random factor in the model (as a random intercept) to account for variation in venom composition between individuals.

The presence/absence of specific molecules associated with sex and ontogeny was calculated and visualised using Venn diagrams produced using the VennDiagram R package (Chen and Boutros, 2011). The effect of instar and sex on chemical complexity (total molecules per sample) was evaluated using LMMs that incorporated the scorpion identity as a random factor. Due to the large number of unsexed samples belonging to small scorpions, and no male scorpions moulting to

sixth instar, the effect of instar alone was again tested on the complete set of samples. Post-hoc Tukey tests were performed to make pairwise comparisons between specific instars.

Ontogenetic differences were further characterised in the relative proportion of molecules belonging to different toxin families, by binning molecules into mass ranges based loosely on those of typical scorpion venom toxin families. The selected ranges were small molecules/small peptides (<2500 Da), medium peptides (2500-5000 Da), large peptides (5000-9000 Da), and very large peptides/proteins (>9000 Da). These were selected as <2500 Da would largely represent small molecules and non-disulfide-bridged peptides (NDBPs)(Zeng et al., 2005;Almaaytah and Albalas, 2014), 2500-5000 Da would largely represent short-chain toxins acting on K^+, Cl^- and Ca^{2+} channels (Possani et al., 2000;de la Vega et al., 2003;de la Vega and Possani, 2004;Cid-Uribe et al., 2020), 5000-9000 Da would include long-chain toxins targeting Na^+ channels (Gordon et al., 1998;de la Vega and Possani, 2005), and >9000 Da would contain all other larger peptides/proteins (Delgado-Prudencio et al., 2022). For each sample, the relative proportion of molecules belonging to each size class was calculated, and generalised linear mixed models (GLMMs) were used to assess differences between samples associated instar and sex using the glmmTMB R package (Brooks et al., 2017). A combination of Gaussian, beta, and gamma distributions were tested for each molecular size class, and the distribution that best fit the data was selected (measured by the lowest AIC value). Post-hoc pairwise Tukey comparisons were then performed to evaluate differences between specific instars and sex using the multcomp package in R (Hothorn et al., 2008).

2.4. Results

2.4.1. Overall Composition

A total of 1418 different molecules were recorded across all samples based on the manually created mass lists, and these data were reduced by removing molecules present in less than four different samples and under 500 Da in size. With these conditions, 579 molecules were identified in the venom of *L. variatus*. An NMDS ordination revealed that the chemical composition of these samples appeared to be associated with instar, as colouring the samples by instar showed clustering along the NMDS1 axis (Figure 2.1). Clustering was not visibly associated with sex. The final model (K = 3) had a stress value of 0.1295 (Supplementary Materials 2.1).

A PERMANOVA on the Jaccard distance matrix describing raw, unscaled data identified that differences in overall venom composition (i.e., presence/absence of all compounds) were significantly associated with instar, sex, and the interaction between the two (instar $p = 0.001$, sex $p = 0.002$, instar × sex $p = 0.002$)(Table 2.1). MANOVAs performed to test the effect of instar and sex on the output scores of NMDS dimensions 1 through 3 for each scorpion also showed that instar, sex, and their interaction variable described significant variation in the overall venom composition of the samples (instar $p < 0.0001$; sex $p = 0.0268$; instar × sex $p = 0.0018$)(Table 2.2). A MANOVA was additionally performed on the full dataset showing a significant effect of instar on NMDS1-3 (instar $p < 0.0001$)(Table 2.2).

In addition to multivariate analysis, univariate LMMs were used to test the effect of instar and sex on the scores of NMDS1-3. Instar significantly described the variation in NMDS1 (Table 2.3). Instar and the interaction between sex and instar significantly described differences in NMDS3 (Table 2.3).

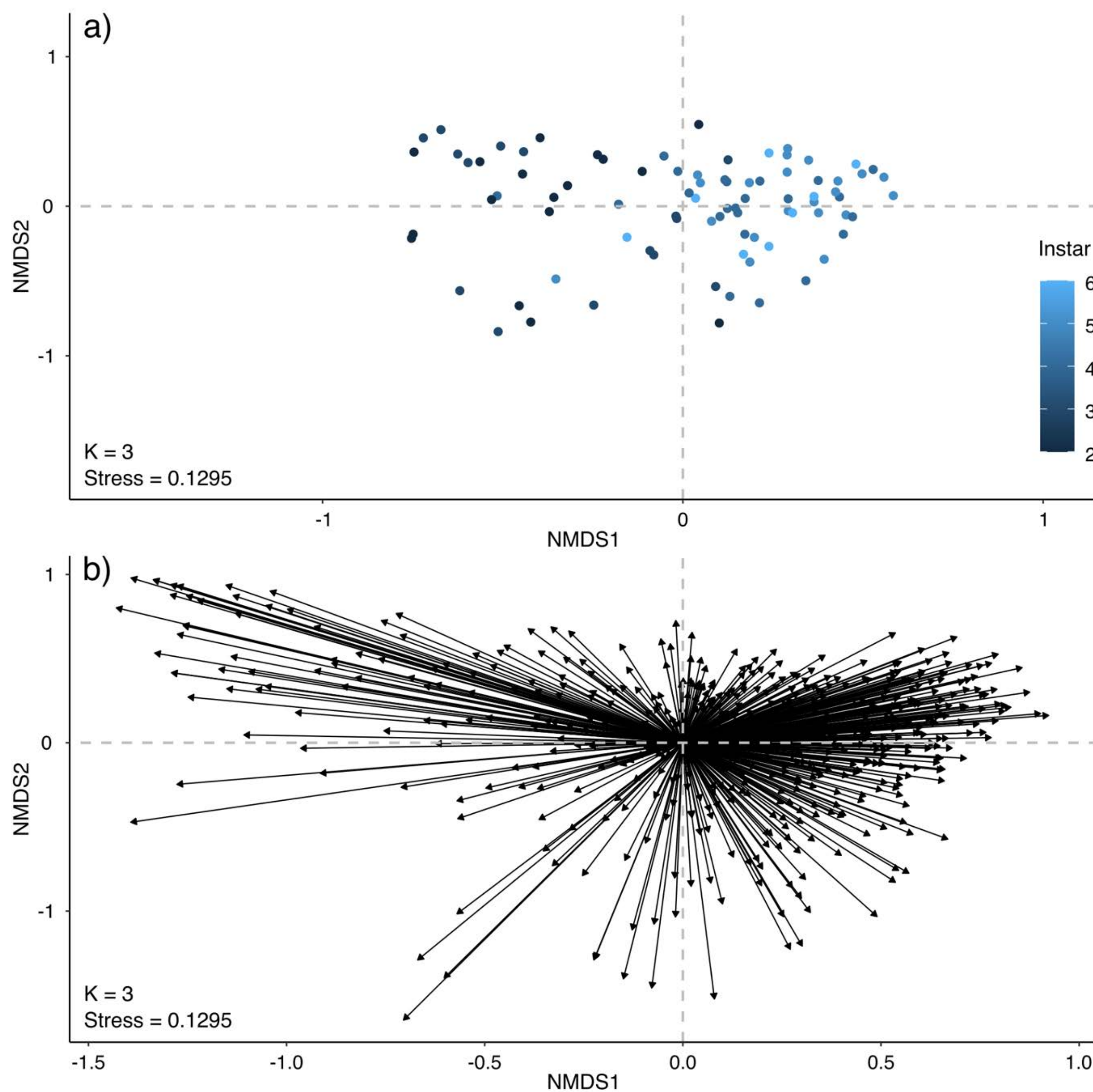


Figure 2. 1 Non-metric multidimensional scaling (NMDS) analysis comparing the composition of *Lychas variatus* venom across 84 samples containing 579 different venom components. **a)** The values of NMDS1 and NMDS2 plotted for each sample, showing clustering across different instars. The samples are coloured by instar (I2-6, light blue-dark blue) **b)** NMDS1 and NMDS2 values of the 579 molecules plotted. For simplicity, the labels for the molecules associated with each arrow are not presented. Preliminary analysis did not reveal any pattern in the grouping of molecules.

Table 2. 1 Statistical outputs of PERMANOVAs testing the effect of instar and sex performed on distance matrices generated using Jaccard distributions from raw, unscaled data. The subset dataset had 19 unsexed scorpion samples removed, but these samples were included in the full dataset to test the effect of instar.

Dataset	Variable	DF	Sum of Sqs	R^2	*F* value	*p* (>*F*)
Subset (unsexed removed)	Instar	4	2.4329	0.1549	2.8959	0.001
	Sex	1	0.4453	0.0284	2.1202	0.002
	Instar × Sex	3	1.0633	0.0677	1.6875	0.002
	Residual	56	11.7617	0.7490		
	Total	64	15.7032	1.0000		
Full (unsexed included)	Instar	4	3.0356	0.1476	3.4207	0.001
	Residual	79	17.5267	0.8524		
	Total	83	20.5623	1.0000		

Table 2. 2 Statistical outputs of MANOVAs performed testing the effect of instar and sex on the NMDS scores. The subset dataset had 19 unsexed scorpion samples removed, but they were included in the full dataset to test the effect of instar alone.

Dataset	Variable	DF_1	Pillai	Approx *F* value	Num DF	Den DF	*p* (>*F*)
Subset (unsexed removed)	Instar	4	1.0612	7.6625	12	168	<0.0001
	Sex	1	0.1553	3.3083	3	54	0.0268
	Instar × Sex	3	0.4275	3.1018	9	168	0.0018
	Residuals	56					
Full (unsexed included)	Instar	4	0.9135	8.6469	12	237	<0.0001
	Residuals	79					

Table 2. 3 Statistical output of linear mixed models testing the effect of instar and sex on the scores of NMDS1-3.

Type III tests of fixed effects		NMDS1			NMDS2			NMDS3		
	DF_1	Den DF	*F* value	*p* (>*F*)	Den DF	*F* value	*p* (>*F*)	Den DF	*F* value	*p* (>*F*)
Instar	4	48.9820	16.1357	<0.0001	46.56706	0.2470	0.9100	46.3046	18.7676	<0.0001
Sex	1	27.0919	0.4145	0.5251	22.359	0.3774	0.5452	24.2184	1.2232	0.2800
Instar x Sex	3	47.2589	1.0006	0.4009	44.39907	1.9723	0.1319	44.3712	9.8546	<0.0001

2.4.2. Group Differences

2.4.2.1. Sex

To evaluate how chemical composition is associated with age and sex, the presence/absence of specific molecules in each group was compared. A total number of 464 molecules were identified from the venoms of males, and 563 from female venoms. 16 molecules were only observed in the male venoms, whilst 115 were unique to females (Figure 2.2). Of the 16 that were male-specific, 12 were additionally specific to adults (I5). No molecules were specific to I6 females, and only 5 were specific to I5/6 female samples. The adult male-specific venom components are of particular interest, as adult males of many scorpion species use their venom to sting the females during courtship.

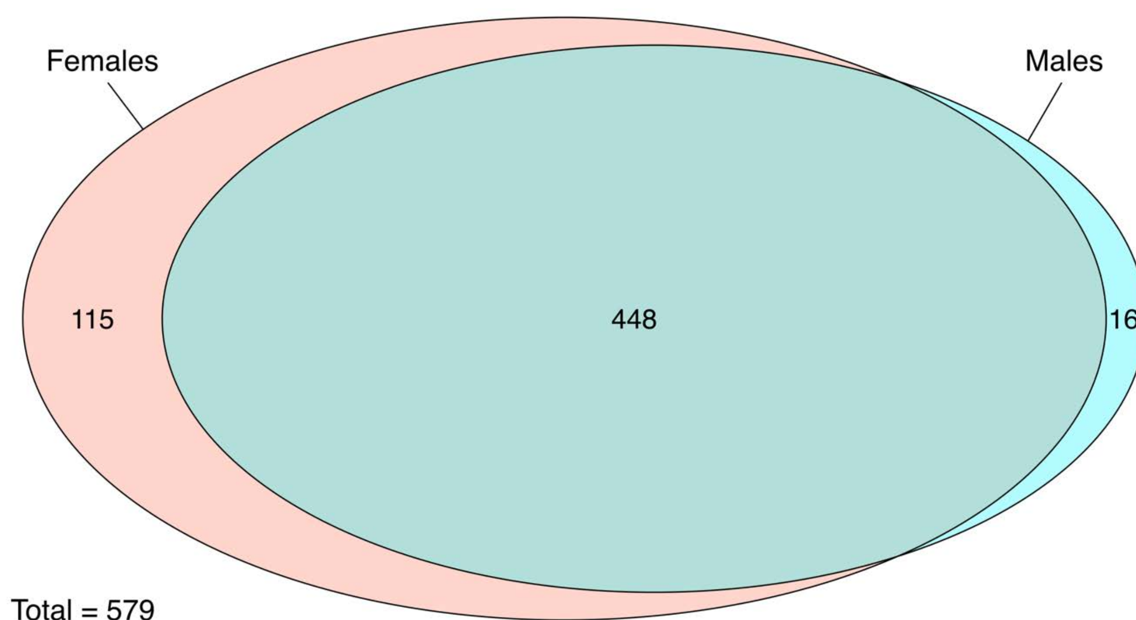


Figure 2. 2 Compositional comparison of male (blue) and female (pink) *Lychas variatus* venom. Of the total 579 compounds analysed, 563 were observed in the females (16 male specific components) and 464 were observed in male venoms (115 female specific components). The majority of molecules (448) were not sex-specific.

2.4.2.2. Instar

The total number of toxins per sample (chemical complexity) increased with instar (Figure 2.3a), suggesting that young scorpions may have more simplistic venom than older scorpions. It may also be an artefact of small scorpions producing small volumes of venom, and therefore some molecules may have been present below the level of detection of the LC/MS system. A LMM incorporating the scorpion's identity for each sample as a random factor showed differences in the mean venom components detected were significantly described by instar ($F_{4,50.124}$ = 5.0978, p = 0.0016), but not sex ($F_{1,29.888}$ = 0.1052, p = 0.7480); however, the instar by sex interaction did describe significant differences in the mean ($F_{3,48.637}$ = 2.8102, p = 0.049). The significant interaction of instar and sex may be due to there only being one male I2 sample, and/or the presence of only female I6 samples, but adult (I5) male samples also contained on average fewer molecules than I5 female samples (Figure 2.4). However, testing each instar separately, there was no significant effect of sex detected on the mean total number of molecules per sample. As unsexed samples were not incorporated in this model, a LMM was used to test the effect of instar alone which also showed that the mean number of molecules per sample was significantly associated with instar ($F_{4,75.498}$ = 14.621, p = 6.901e-09). Pairwise Tukey tests on the output of this model showed that I2 samples on average contained significantly fewer molecules than I4 ($p < 0.001$), I5 ($p < 0.001$) and I6 ($p < 0.001$). Likewise, I3 samples contained fewer than I4 ($p < 0.001$), I5 (p = 0.0033) and I6 (p = 0.0.1953)(Figure 2.3a).

Interestingly, the total diversity of the toxins observed at each instar across the different samples was highest in the fourth instar (Figure 2.3b). This could in part be due to I4 being represented by the most samples tested (23), but this would not explain the lower toxin diversity observed for I5 scorpions (21 tested). The large toxin diversity recorded across the I4 samples suggests there might be a transition in venom composition from young scorpions (I2-3) to sub-adult/adult scorpions (I5-6), with medium scorpions possessing a mixture of the two. Ontogenetic differences were observed across 242 of the 579 molecules studied; 18 were specific to small (I2-3) scorpions, 4 were only observed in medium (I4) scorpions, whilst 26 were unique to large (I5-6) scorpions. Although the number of molecules specific to each size group was relatively low, a large number of molecules were shared between the size groups supporting the idea of a transitional change. Small and medium scorpions shared 65 molecules that were absent in large individuals, whilst medium and large scorpions shared 113 molecules that were absent in small individuals. A comparatively low number (16) were shared between small and large scorpions that were not observed in medium scorpions. The large similarity of small/medium and medium/large compared

with small/large supports the idea of a transitional change in venom composition as the scorpions grow larger (Figure 2.5).

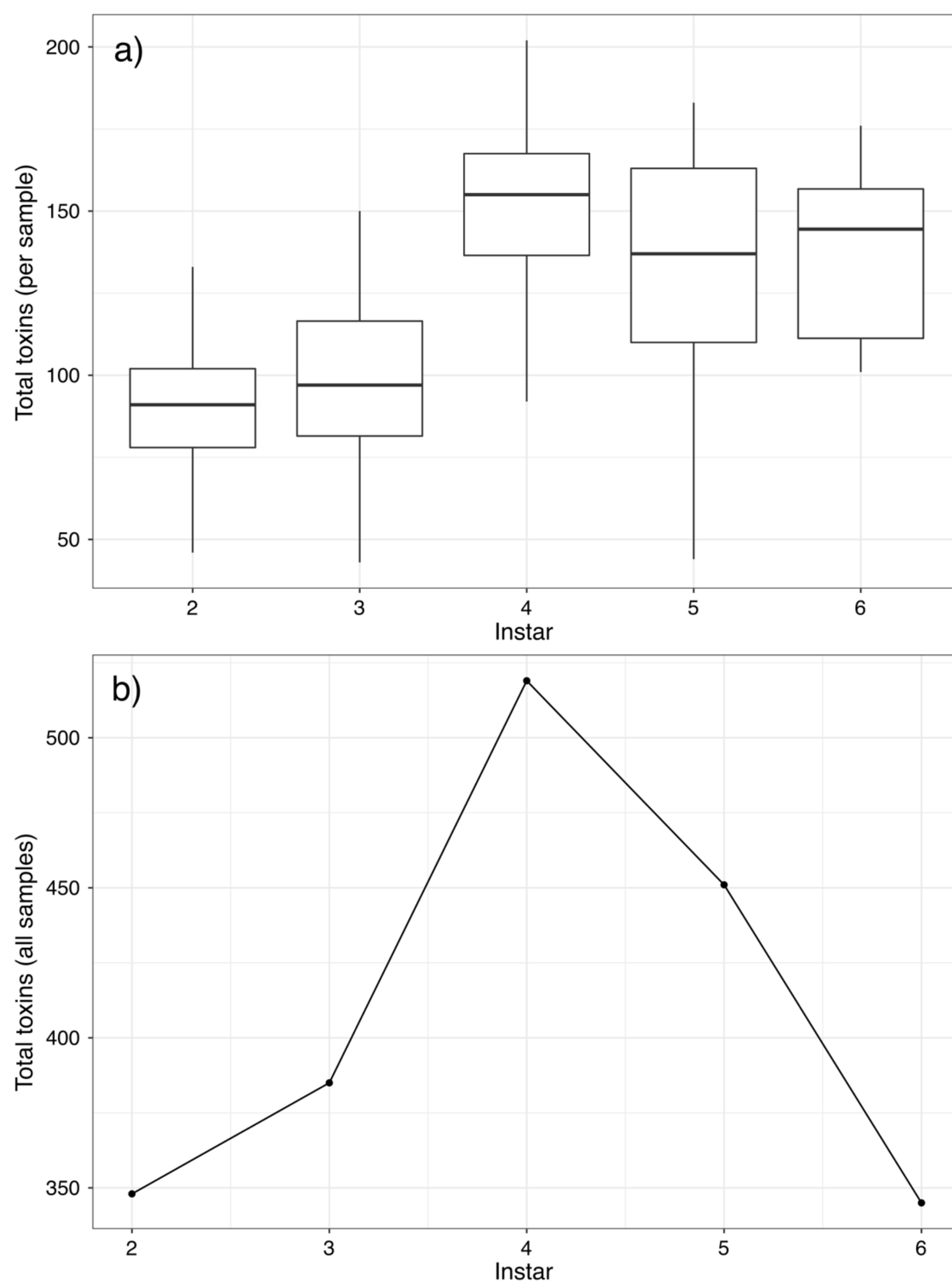


Figure 2. 3 Chemical complexity of *Lychas variatus* venom associated with different instars. **a)** Boxplot showing the total number of toxins identified in each sample at each instar, irrespective of molecule identity. The I4, I5 and I6 samples contained significantly higher numbers of molecules than I2 scorpions. **b)** The total number of different toxins identified across all venom samples at each instar. The greatest toxin diversity was observed in medium sized (I4) scorpion venoms. The total number of different molecules observed at each instar were: I2 = 348, I3 = 385, I4 = 519, I5 = 451, I6 = 345.

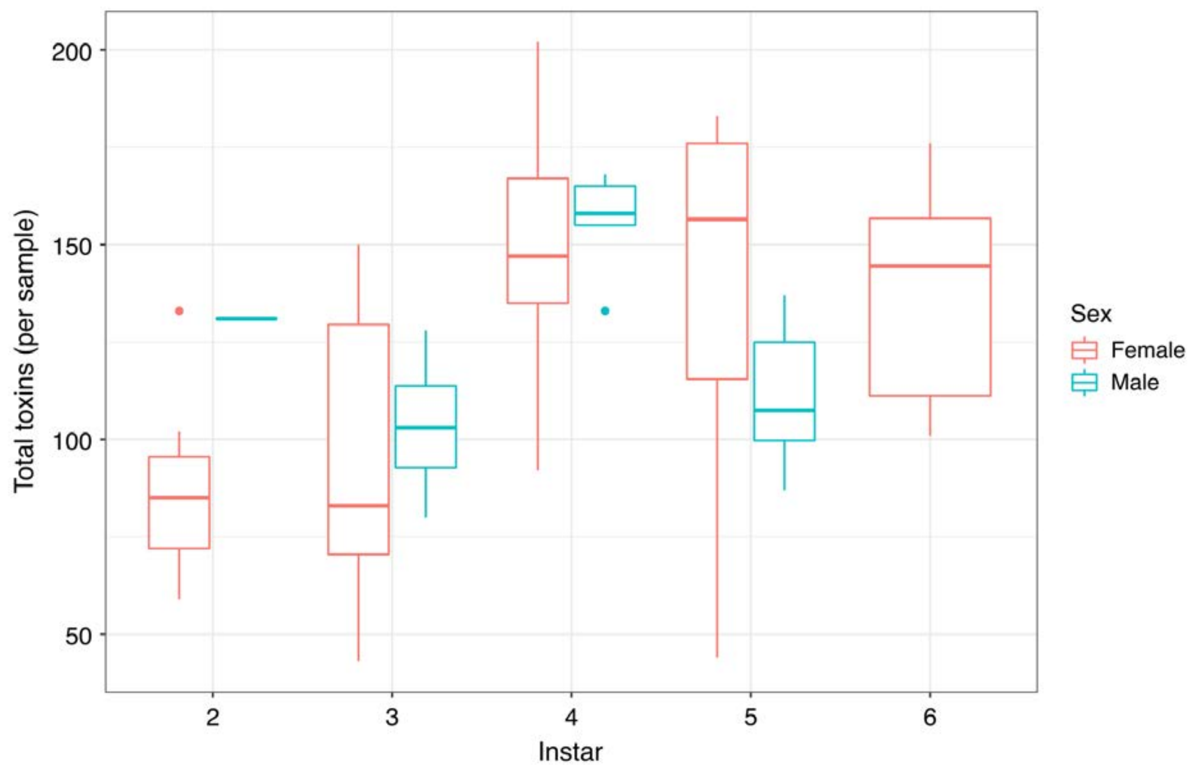


Figure 2. 4 Boxplot showing the total number of different toxins identified in each sample for male and female *Lychas variatus* venom samples. Samples belonging to scorpions that had not had their sex determined are not included.

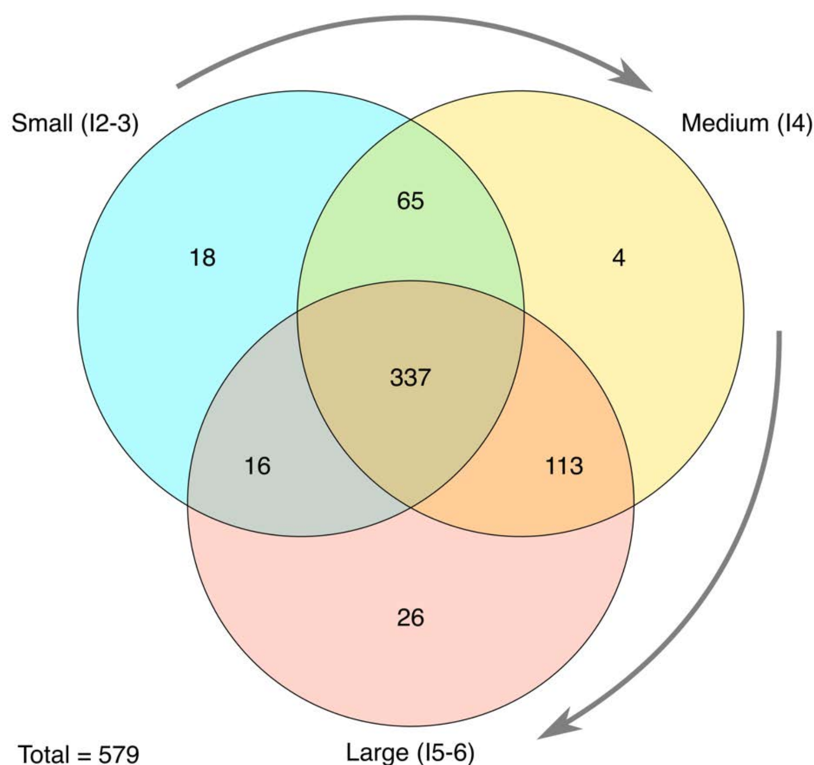


Figure 2. 5 Venn diagram showing the shared presence of molecules across small (I2-3), medium (I4) and large (I5-6) *Lychas variatus* scorpions. 337 molecules were shared across all size groups. 18 molecules were unique to small scorpions, 4 to medium scorpions, and 26 to large scorpions. Small and medium scorpions shared 65 molecules which were not observed in adult venoms, whilst medium and large scorpions shared 113 molecules which were not observed in small scorpions. The large number of molecules shared between small/medium and medium/large suggests a transitional change in venom composition from small to large scorpions. Equally, only a relatively small number (16) of molecules were shared between small and large scorpions that were not found in the medium sized venoms.

2.4.3. Toxin Family Analysis

Having shown that venom composition changes across the life stages, it was of interest to determine whether the proportion of molecules belonging to different toxin families within the venom also changed. A general linear mixed model showed instar significantly altered the mean ratio of molecules sized 500-2500 Da (χ^2(df = 4) = 14.983, p = 0.0047), but no difference was associated with sex. Post-hoc pairwise Tukey tests identified the means of I4 samples were significantly different to I5 samples (z = -3.726, p = 0.0018) and the comparison between I4 and I6 was close to the significance threshold (z = -2.679, p = 0.0548). As there were no significant differences between the sexes detected, the effect of instar alone was tested on the full set of samples. This reaffirmed that differences in the relative proportion of molecules 500-2500 Da were associated with instar (χ^2(df = 4) = 15.471, p = 0.0031), and pairwise comparisons identified significant differences between I4 and I5 (z = -3.879, $p < 0.001$), and I4 and I6 (z = -2.752, p = 0.0456) samples. These results indicate the venoms of I4 scorpions contained a significantly higher proportion of small molecules/peptides (500-2500 Da) than I5 and I6 scorpions (Figure 2.6a).

The relative proportion of medium peptides (2500-5000 Da) between the samples was also tested using a general linear mixed model, and both instar (χ^2(df = 4) = 12.6192, p = 0.0133) and sex (χ^2(df = 4) = 4.0453, p = 0.0443) described significant variation in the model. Pairwise Tukey tests identified that the mean proportion of medium peptides in I3 (z = 2.987, p = 0.0230), I4 (z = 3.394, p = 0.0061), I5 (z = 2.770, p = 0.0432), and I6 (z = 3.093, p = 0.0166) were all significantly higher than I2 samples. I2 scorpion venoms therefore contained a significantly lower proportion of molecules 2500-5000 Da than the other instars. Additionally, male venoms overall contained significantly lower proportions of molecules in this molecular weight range than female venoms (z = -2.043, p = 0.0411). Interestingly however, when including unsexed samples, the differences in the mean were no longer significant between the instars, presumably because some second instar scorpions that had high numbers of medium peptides did not survive long enough to have their sex determined, or due to additional variability in sex that is left unexplained from not including the "sex" parameter. However, further research would be needed to evaluate this further.

The proportion of large peptides (5000-9000 Da) in the samples was best described using a generalized linear mixed model with a gamma distribution and log link function. Differences were associated with instar (χ^2(df = 4) = 22.1289, p = 0.0002), but did not occur between the sexes. Pairwise comparisons identified significant differences between I2 and I4 samples (z = -3.796, p = 0.00132), and between I4 and I5 samples (z = 4.733, $p < 0.001$). This shows that the venoms of the I4 scorpions contained a significantly lower proportion of large peptides (5000-9000 Da) than I2 and I5

scorpions (Figure 2.6c), irrespective of sex. This was supported by another generalised linear mixed model with a gamma distribution on the full set of samples. Again, differences in the proportion of molecules 5000-9000 Da was described by instar (χ^2(df = 4) = 26.237, $p < 0.0001$), and post-hoc comparisons showed significant differences between I2 and I4 ($z = -4.266$, $p < 0.001$), and between I4 and I5 ($z = 5.189$, $p < 0.001$).

Differences in the proportion of very large peptides/proteins (>9000 Da) was not associated with instar or sex when tested with a general linear mixed model. For modelling these very large molecules, a Gaussian distribution was selected as some samples contained zero molecules in this size class, therefore a beta or gamma distribution could not be used.

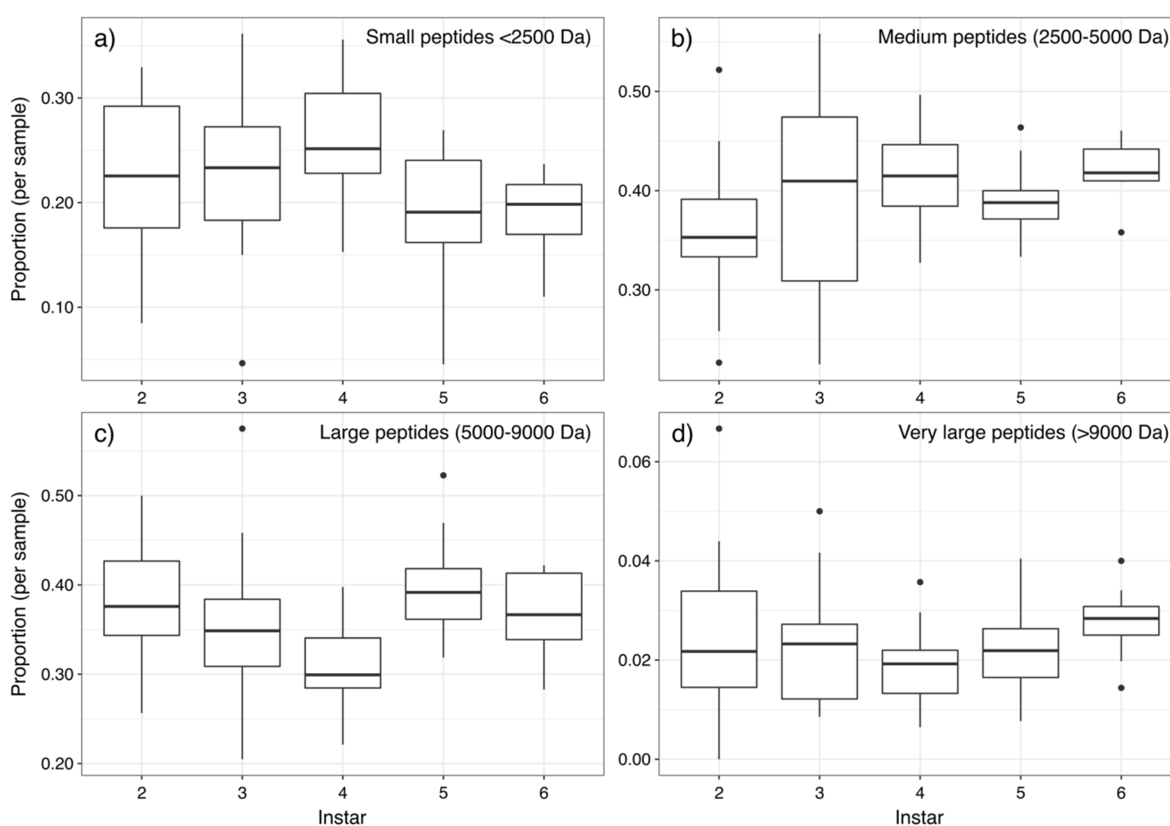


Figure 2. 6 Relative proportion of the number of venom components in each *Lychas variatus* venom sample belonging to different molecular weight classes: Small molecules/peptides (500-2500 Da), medium peptides (2500-5000 Da), large peptides (5000-9000 Da), and very large peptides/proteins (>9000 Da). A total of 84 samples are included, including samples belonging to unsexed scorpions.

2.5. Discussion

The results of this study show that the venom composition of marbled scorpions (*L. variatus*) display changes associated with ontogeny and sex. The presence/absence of molecules within the venom was closely associated with instar, with small scorpions and large scorpions possessing different molecules in their venoms. The 65 molecules shared between small (I2-3) and medium (I4) scorpions that were absent in large scorpions (I5-6), and the 115 shared between medium and large that were absent in small scorpions, is evidence of a transitional change in venom composition as the scorpions grow. It was also identified that differences in the venom of male and female scorpions could also be associated with ontogeny. Of the 16 male-specific molecules observed, 12 were unique to adult (I5) scorpions. To my knowledge, these data provide the first evidence of intersexual differences in scorpion venoms linked to ontogeny and exemplifies how intraspecific drivers of venom variation can be complex, showing chemical differences may be associated with a combination of factors.

Ontogenetic shifts in venoms are widely reported in venomous taxa, particularly in well-studied groups such as snakes and spiders. As venoms are used to capture prey or defend against potential predators, changes in venom composition at different life stages may be linked with changes in diet (Mackessy, 1988;Daltry et al., 1996;Andrade and Abe, 1999;Gibbs et al., 2011;Chang et al., 2015;Cipriani et al., 2017), or changes in exposure to predators (Gangur et al., 2017;Herzig et al., 2020b). For example, the juveniles of many brown snake species (*Pseudonaja* spp.) shift from predominantly targeting reptiles to incorporating mammals in their diet as they grow, and this corresponds to a shift in their venom composition and activity (Shine, 1977;Jackson et al., 2016;Cipriani et al., 2017). Alternatively, defensive use of venom may explain ontogenetic changes in composition. A stark example of this is observed in adult male Sydney funnel-web spiders (*Atrax robustus*), where, upon maturing, males leave their web and roam in search of a female. This change in behaviour exposes them to a higher risk of predation, and has been suggested that this leads to the expression of δ-ACTX-Ar1a within their venom thereby improving their defences (Herzig et al., 2020b). It is unclear if dietary-change or predator exposure drive the observed ontogenetic shift in *L. variatus* venom, but as scorpions use their venom heavily in both prey-capture and defensive contexts, it is possible that both may be partly responsible. Ontogenetic changes in venom toxicity and toxin gene expression have been previously documented in *C. vittatus*, but no data has been gathered for other scorpion species.

Like most scorpion species, *L. variatus* is a generalist predator that will consume a diverse range of invertebrate prey items in the wild (Polis and McCormick, 1986). This includes members of

the Lepidoptera, Orthoptera, Hemiptera, Blattodea, Hymenoptera, Dermaptera, and Araneae (personal observation). In this study, *L. variatus* were raised solely on a diet of *A. domesticus* of varying sizes, as it has been shown in *T. serrulatus* that the captive diet of scorpions can influence venom composition (Pucca et al., 2014). Different sized scorpions are known to adopt different prey-capture and defensive strategies (Cushing and Matherne, 1980;Casper, 1985;Polis et al., 1989;McReynolds, 2012;Miller et al., 2016;Lira et al., 2020), and therefore selection may act to favour different venom compositions for different sized scorpions. If *L. variatus* are also employing different hunting/defensive strategies as they grow, this may explain the observed ontogenetic shifts in their venom composition.

In addition to the observed ontogenetic differences in the presence/absence of specific molecules in *L. variatus* venom, the venoms of small scorpions appeared to be more simplistic. Fewer molecules per sample were identified in small (I2 and I3) scorpions than medium-large (I4-6) scorpions (Figure 2.3a). As scorpion venoms are complex mixtures containing many peptides, they are energetically costly to produce (Nisani et al., 2007), and limited resource availability may explain the lower chemical complexity observed in the small scorpions. For example, it has been documented in some snakes that venom composition can increase in complexity as they grow (Alape-Girón et al., 2008). I2 and I3 scorpions produced extremely small volumes of venom, and the fewer molecules observed may additionally be attributed to some molecules being present below the detectability threshold of the Shimadzu LC/MS system. To confirm if the venom of young scorpions is truly more simplistic, future work is required using pooled venom samples or using a more sensitive instrument.

By collating molecules into molecular weight classes that approximately correspond to known scorpion toxin families, ontogenetic differences in relative numbers of different types of molecules in the venom were observed. Overall, molecules in the size ranges 2500-5000 Da and 5000-9000 Da made up the greatest number of the venom components per sample, and only a small number were >9000 Da (Figure 2.6). I4 venoms contained the highest relative numbers of small molecules/peptides (500-2500 Da), significantly higher than the I5 and I6 scorpions. Correspondingly, I4 venoms contained the lowest relative numbers of large peptides (5000-9000 Da), significantly lower than I2 and I5 venoms. The relative number of medium peptides (2500-5000 Da) appeared to steadily increase from I2-I6 (Figure 2.6b), and pairwise comparisons on a subset of samples that did not include unsexed scorpions showed I2 samples contained significantly lower numbers than the other instars. Although the difference between the instars is relatively small, the relative number of molecules in each molecular weight class was associated with ontogeny and demonstrates that the venom undergoes broad-scale changes as the scorpions grow.

The greatest overall toxin diversity was observed across the I4 samples (Figure 2.3b). Whilst this appears to be associated with I4 samples containing a mixture of venom components present in both smaller and larger scorpions (Figure 2.5), it is also important to consider sampling effects. I4 samples were the most numerous (I2 =17, I3 = 15, I4 = 23, I5 = 21, I6 = 8), and the different number of replicates between the instars could also partially explain differences in the overall toxin diversity observed.

Many scorpion species are reported to display intersexual differences in their venom compositions (Yamaji et al., 2004;Schwartz et al., 2008;Abdel-Rahman et al., 2009;Özkan et al., 2011;Rodríguez-Ravelo et al., 2015;Uribe et al., 2017), and I also identified sex-specific venom components in *L. variatus*. Female venoms contained 115 molecules that were not observed in any of the male samples, whilst 16 molecules were unique to male samples. Additionally, male venoms contained significantly lower relative numbers of components in the 2500-5000 Da range. Sexual differences may arise if males and females are consistently using their venom in different ways, for example targeting different prey items or encountering different predators, as toxin genes will experience different selective forces between the sexes (Ward et al., 2018). Many scorpion species, including *L. variatus*, are sexually dimorphic in body size (McLean et al., 2018). Size differences can affect motility, and it has been demonstrated in some scorpions than males and females adopt different defensive strategies (Carlson et al., 2014;Miller et al., 2016). Male and female *L. variatus* may also adopt different prey-capture/defensive strategies associated with their different morphologies, and selection may drive the observed differences in their venom compositions.

Intersexual differences in scorpion venoms may also be associated with sexual stinging behaviours. The courtship of many scorpion species involves the male stinging the female, and although it has recently been shown that venom is injected in sexual stings, it remains unknown if males inject a specific set of molecules or their complete venom mixture (Olguín-Pérez et al., 2021). The underlying mechanisms of this unconventional use of venom are poorly understood, and it is not presently known if it is an attempt by the male to reduce the risk of predation by the female, an indicator of fitness, or perhaps increasing mating success by another mechanism (Inceoglu et al., 2003;Jiao and Zhu, 2010;Peretti, 2014;Olguín-Pérez et al., 2021). Regardless of the sexual sting's function, the use of venom in this context is unique to adult males, therefore selection may lead to differences between males and females, potentially also at this specific life-stage. Of the 16 male-specific components identified, 12 were additionally unique to adult males, suggesting their identity is worth investigating as candidates to be involved in this behaviour. Whilst 12 of the 16 male-specific molecules were additionally unique to adults (I5), only 5 of the 115 female-specific molecules were unique to I5-6 female scorpions. The comparatively large number of adult male

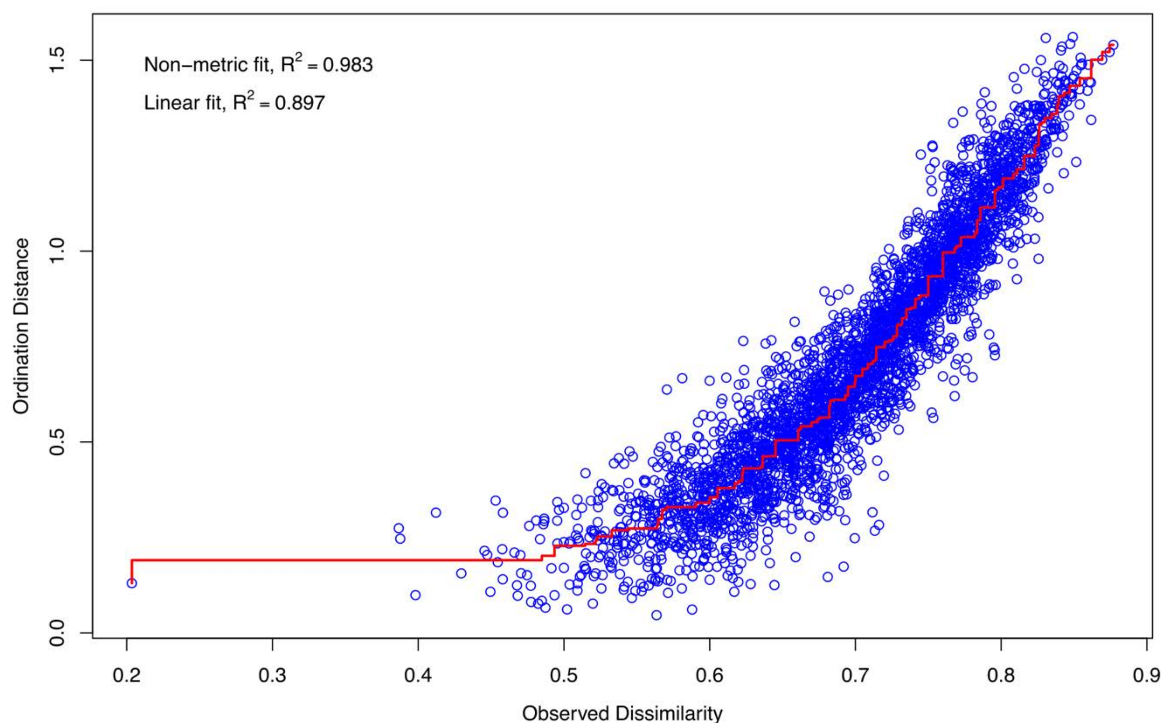
specific molecules suggests that they may fulfil a specific function in the venom at this life stage. Furthermore, of the 12 adult male specific molecules, 6 had masses below 800 Da. This is smaller than most currently known scorpion venom peptides and further suggests they may not be typical toxins. The breeding biology of *L. variatus* has not been studied, therefore it is currently unknown if the courtship of this species involves a sexual sting, but sexual stinging behaviours have been observed in *Lychas spinatus besti* which is currently considered a junior synonym of *L. variatus* (Koch, 1977;Kovařík, 1997;Newton, 2008;Kovařík, 2019). To identify if these adult male specific molecules might be involved in sexual stinging, future work is necessary to isolate, characterise, and test the activity of these venom components.

There is currently only a small body of research studying intraspecific variation in scorpion venoms, despite its potential importance in clinics, industry, and research. Although *L. variatus* is not considered dangerous to humans, with stings usually causing localised pain (Isbister et al., 2004), ontogenetic and intersexual differences may equally occur in other scorpion species that cause significant envenomation. Intersexual differences in overall venom toxicity have been documented in *Tityus nororientalis* and *C. vittatus* (De Sousa et al., 2010;Miller et al., 2016), and venom toxicity has been linked to ontogeny in *C. vittatus* (McElroy et al., 2017). The results of these studies suggest that the sex and age of a scorpion could therefore affect the severity of sting victims and may be an important consideration when treating envenomated patients. Additionally, intraspecific variability in snake venoms is known to impact the effectiveness of antivenoms (Kalita et al., 2018;Sousa et al., 2018;Tan et al., 2020), and this will likely translate to the effectiveness of scorpion antivenoms. Gaining a better understanding of the drivers of scorpion venom variation could therefore be useful when selecting the most appropriate animals/venoms for use in antivenom production. Furthermore, scorpion venoms provide a large resource of bioactive molecules which are of interest in biodiscovery research (Ortiz et al., 2015), and the results of this study indicate that incorporating animals of different age and sex may greatly increase the total number of molecules available for screening.

2.6. Conclusions and Future Directions

The venoms of *L. variatus* display ontogenetic and sexual differences in their venom composition, but the underlying explanation for these differences remains elusive. Without knowing the structure/activity of the toxins specifically associated with the observed differences, it is difficult to conclude why these differences occur. For example, in spiders it has been suggested the expression of toxins with known activities at different life-stages may be linked with their ecology (de Andrade et al., 1999;Herzig et al., 2004;Herzig et al., 2020b). Similar selection pressures may drive variation in *L. variatus* venoms but understanding changes in specific toxins/venom activity would be necessary to draw conclusions as to why the observed compositional differences occur. It may therefore be beneficial to target future research towards scorpion species which have well-studied venom compositions, or highly toxic species with specific toxins of interest.

2.7. Supplementary Materials



Supplementary Materials 2. 1 Stress plot of the NMDS ordination. The number of dimensions (K) = 3.

2.8. Bibliography

Abdel-Rahman, M.A., Omran, M.a.A., Abdel-Nabi, I.M., Ueda, H., and Mcvean, A. (2009). Intraspecific variation in the Egyptian scorpion *Scorpio maurus palmatus* venom collected from different biotopes. *Toxicon* 53**,** 349-359.

Alape-Girón, A., Sanz, L., Escolano, J., Flores-Díaz, M., Madrigal, M., Sasa, M., and Calvete, J.J. (2008). Snake venomics of the lancehead pitviper *Bothrops asper*: geographic, individual, and ontogenetic variations. *Journal of Proteome Research* 7**,** 3556-3571.

Almaaytah, A., and Albalas, Q. (2014). Scorpion venom peptides with no disulfide bridges: a review. *Peptides* 51**,** 35-45.

Andrade, D.V., and Abe, A.S. (1999). Relationship of venom ontogeny and diet in *Bothrops*. *Herpetologica***,** 200-204.

Avella, I., Calvete, J.J., Sanz, L., Wüster, W., Licata, F., Quesada-Bernat, S., Rodríguez, Y., and Martínez-Freiría, F. (2022). Interpopulational variation and ontogenetic shift in the venom composition of Lataste's viper (*Vipera latastei*, Boscá 1878) from northern Portugal. *Journal of Proteomics* 263**,** 104613.

Barlow, A., Pook, C.E., Harrison, R.A., and Wüster, W. (2009). Coevolution of diet and prey-specific venom activity supports the role of selection in snake venom evolution. *Proceedings of the Royal Society B: Biological Sciences* 276**,** 2443-2449.

Bates, D., Mächler, M., Bolker, B., and Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software* 67**,** 1-48.

Brooks, M.E., Kristensen, K., Van Benthem, K.J., Magnusson, A., Berg, C.W., Nielsen, A., Skaug, H.J., Machler, M., and Bolker, B.M. (2017). glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. *The R Journal* 9**,** 378-400.

Carcamo-Noriega, E.N., Olamendi-Portugal, T., Restano-Cassulini, R., Rowe, A., Uribe-Romero, S.J., Becerril, B., and Possani, L.D. (2018). Intraspecific variation of *Centruroides sculpturatus* scorpion venom from two regions of Arizona. *Archives of Biochemistry and Biophysics* 638**,** 52-57.

Carlson, B.E., Mcginley, S., and Rowe, M.P. (2014). Meek males and fighting females: sexually-dimorphic antipredator behavior and locomotor performance is explained by morphology in bark scorpions (*Centruroides vittatus*). *PLoS One* 9**,** e97648.

Casewell, N.R., Jackson, T.N.W., Laustsen, A.H., and Sunagar, K. (2020). Causes and consequences of snake venom variation. *Trends in Pharmacological Sciences* 41**,** 570-581.

Casewell, N.R., Wüster, W., Vonk, F.J., Harrison, R.A., and Fry, B.G. (2013). Complex cocktails: the evolutionary novelty of venoms. *Trends in Ecology & Evolution* 28**,** 219-229.

Casper, G.S. (1985). Prey capture and stinging behavior in the emperor scorpion, *Pandinus imperator* (Koch)(Scorpiones, Scorpionidae). *Journal of Arachnology***,** 277-283.

Chang, D., Olenzek, A.M., and Duda Jr, T.F. (2015). Effects of geographical heterogeneity in species interactions on the evolution of venom genes. *Proceedings of the Royal Society B: Biological Sciences* 282**,** 20141984.

Chen, H., and Boutros, P.C. (2011). VennDiagram: a package for the generation of highly-customizable Venn and Euler diagrams in R. *BMC Bioinformatics* 12**,** 35.

Chippaux, J.-P., and Goyffon, M. (2008). Epidemiology of scorpionism: a global appraisal. *Acta Tropica* 107**,** 71-79.

Cid-Uribe, J.I., Veytia-Bucheli, J.I., Romero-Gutierrez, T., Ortiz, E., and Possani, L.D. (2020). Scorpion venomics: a 2019 overview. *Expert Review of Proteomics* 17**,** 67-83.

Cipriani, V., Debono, J., Goldenberg, J., Jackson, T.N.W., Arbuckle, K., Dobson, J., Koludarov, I., Li, B., Hay, C., Dunstan, N., Allen, L., Hendrikx, I., Kwok, H.F., and Fry, B.G. (2017). Correlation between ontogenetic dietary shifts and venom variation in Australian brown snakes (*Pseudonaja*). *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 197**,** 53-60.

Cologna, C.T., Dos Santos Cardoso, J., Jourdan, E., Degueldre, M., Upert, G., Gilles, N., Uetanabaro, A.P.T., Neto, E.M.C., Thonart, P., and De Pauw, E. (2013). Peptidomic comparison and characterization of the major components of the venom of the giant ant *Dinoponera quadriceps* collected in four different areas of Brazil. *Journal of Proteomics* 94**,** 413-422.

Cushing, B.S., and Matherne, A. (1980). Stinger utilization and predation in the scorpion *Paruroctonus boreus*. *The Great Basin Naturalist***,** 193-195.

Czaykowsky, C. (2020). *Preliminary investigation of the effects of sex and electrical venom extraction on venom composition in the southern devil scorpion, Vaejovis carolinianus.* Senior Research

Projects, Southern Adventist University.

Daltry, J.C., Wüster, W., and Thorpe, R.S. (1996). Diet and snake venom evolution. *Nature* 379**,** 537-540.

De Andrade, R.M.G., De Oliveira, K.C., Giusti, A.L., Da Silva, W.D., and Tambourgi, D.V. (1999). Ontogenetic development of *Loxosceles intermedia* spider venom. *Toxicon* 37**,** 627-632.

De Haro, L., Robbe-Vincent, A., Saliou, B., Valli, M., Bon, C., and Choumet, V. (2002). Unusual neurotoxic envenomations by *Vipera aspis aspis* snakes in France. *Human & Experimental Toxicology* 21**,** 137-145.

De La Vega, R.C.R., and Possani, L.D. (2004). Current views on scorpion toxins specific for K+-channels. *Toxicon* 43**,** 865-875.

De La Vega, R.C.R., and Possani, L.D. (2005). Overview of scorpion toxins specific for Na+ channels and related peptides: biodiversity, structure–function relationships and evolution. *Toxicon* 46**,** 831-844.

De La Vega, R.C.R.G., Merino, E., Becerril, B., and Possani, L.D. (2003). Novel interactions between K+ channels and scorpion toxins. *Trends in Pharmacological Sciences* 24**,** 222-227.

De Sousa, L., Borges, A., Vásquez-Suárez, A., Den Camp, H.J.O., Chadee-Burgos, R.I., Romero-Bellorín, M., Espinoza, J., De Sousa-Insana, L., and Pino-García, O. (2010). Differences in venom toxicity and antigenicity between females and males *Tityus nororientalis* (Buthidae) scorpions. *Journal of venom research* 1**,** 61.

Delgado-Prudencio, G., Cid-Uribe, J.I., Morales, J.A., Possani, L.D., Ortiz, E., and Romero-Gutiérrez, T. (2022). The enzymatic core of scorpion venoms. *Toxins* 14**,** 248.

Deslippe, R.J., and Guo, Y.-J. (2000). Venom alkaloids of fire ants in relation to worker size and age. *Toxicon* 38**,** 223-232.

Duda, T.F., and Palumbi, S.R. (1999). Molecular genetics of ecological diversification: duplication and rapid evolution of toxin genes of the venomous gastropod *Conus*. *Proceedings of the National Academy of Sciences* 96**,** 6820-6823.

Duran, L.H., Rymer, T.L., and Wilson, D.T. (2020). Variation in venom composition in the Australian funnel-web spiders *Hadronyche valida*. *Toxicon: X* 8**,** 100063.

Escoubas, P., Corzo, G., Whiteley, B.J., Célérier, M.L., and Nakajima, T. (2002). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and high-performance liquid chromatography study of quantitative and qualitative variation in tarantula spider venoms. *Rapid Communications in Mass Spectrometry* 16**,** 403-413.

Estrada-Gómez, S., Cupitra, N.I., Arango, W.M., and Muñoz, L.J.V. (2014). Intraspecific variation of *Centruroides edwardsii* venom from two regions of Colombia. *Toxins* 6**,** 2082-2096.

Evans, E.R.J., Northfield, T.D., Daly, N.L., and Wilson, D.T. (2019). Venom costs and optimization in scorpions. *Frontiers in Ecology and Evolution* 7**,** 196.

Fry, B.G., Koludarov, I., Jackson, T.N., Holford, M., Terrat, Y., Casewell, N.R., Undheim, E.A., Vetter, I., Ali, S.A., and Low, D.H. (2015). Seeing the woods for the trees: understanding venom evolution as a guide for biodiscovery.

Fry, B.G., Roelants, K., Champagne, D.E., Scheib, H., Tyndall, J.D., King, G.F., Nevalainen, T.J., Norman, J.A., Lewis, R.J., and Norton, R.S. (2009). The toxicogenomic multiverse: convergent recruitment of proteins into animal venoms. *Annual Review of Genomics and Human Genetics* 10**,** 483-511.

Fry, B.G., Winkel, K.D., Wickramaratna, J.C., Hodgson, W.C., and Wüster, W. (2003). Effectiveness of snake antivenom: species and regional venom variation and its clinical impact. *Journal of Toxicology: Toxin Reviews* 22**,** 23-34.

Furtado, M.F.D., Travaglia-Cardoso, S.R., and Rocha, M.M.T.D. (2006). Sexual dimorphism in venom of *Bothrops jararaca* (Serpentes: Viperidae). *Toxicon* 48**,** 401-410.

Gangur, A.N., Seymour, J.E., Liddell, M.J., Wilson, D., Smout, M.J., and Northfield, T.D. (2018). When is overkill optimal? Tritrophic interactions reveal new insights into venom evolution. *Theoretical Ecology* 11**,** 141-149.

Gangur, A.N., Smout, M., Liddell, M.J., Seymour, J.E., Wilson, D., and Northfield, T.D. (2017). Changes in predator exposure, but not in diet, induce phenotypic plasticity in scorpion venom. *Proceedings of the Royal Society of London. Series B: Biological Sciences* 284**,** 20171364.

García-Osorio, B., Lomonte, B., Bénard-Valle, M., López De León, J., Román-Domínguez, L., Mejía-Domínguez, N.R., Lara-Hernández, F., Alagón, A., and Neri-Castro, E. (2020). Ontogenetic changes in the venom of *Metlapilcoatlus nummifer*, the mexican jumping viper. *Toxicon* 184**,** 204-214.

Gibbs, H.L., Sanz, L., Chiucchi, J.E., Farrell, T.M., and Calvete, J.J. (2011). Proteomic analysis of ontogenetic and diet-related changes in venom composition of juvenile and adult Dusky Pigmy rattlesnakes (*Sistrurus miliarius barbouri*). *Journal of Proteomics* 74**,** 2169-2179.

Gillissen, A., Theakston, R.D.G., Barth, J., May, B., Krieg, M., and Warrell, D.A. (1994). Neurotoxicity, haemostatic disturbances and haemolytic anaemia after a bite by a Tunisian saw-scaled or carpet viper (*Echis* 'pyramidum'-complex): Failure of antivenom treatment. *Toxicon* 32**,** 937-944.

Gordon, D., Savarin, P., Gurevitz, M., and Zinn-Justin, S. (1998). Functional anatomy of scorpion toxins affecting sodium channels. *Journal of Toxicology: Toxin Reviews* 17**,** 131-159.

Herzig, V. (2010). Ontogenesis, gender, and molting influence the venom yield in the spider *Coremiocnemis tropix* (Araneae, Theraphosidae). *Journal of Venom Research* 1**,** 76.

Herzig, V., Cristofori-Armstrong, B., Israel, M.R., Nixon, S.A., Vetter, I., and King, G.F. (2020a). Animal toxins—Nature's evolutionary-refined toolkit for basic research and drug discovery. *Biochemical Pharmacology* 181**,** 114096.

Herzig, V., Khalife, A.A., Chong, Y., Isbister, G.K., Currie, B.J., Churchill, T.B., Horner, S., Escoubas, P., Nicholson, G.M., and Hodgson, W.C. (2008). Intersexual variations in Northern (*Missulena pruinosa*) and Eastern (*M. bradleyi*) mouse spider venom. *Toxicon* 51**,** 1167-1177.

Herzig, V., Sunagar, K., Wilson, D.T., Pineda, S.S., Israel, M.R., Dutertre, S., Mcfarland, B.S., Undheim, E.A., Hodgson, W.C., and Alewood, P.F. (2020b). Australian funnel-web spiders evolved human-lethal δ-hexatoxins for defense against vertebrate predators. *Proceedings of the National Academy of Sciences* 117**,** 24920-24928.

Herzig, V., Ward, R.J., and Dos Santos, W.F. (2004). Ontogenetic changes in *Phoneutria nigriventer* (Araneae, Ctenidae) spider venom. *Toxicon* 44**,** 635-640.

Hothorn, T., Bretz, F., and Westfall, P. (2008). Simultaneous inference in general parametric models. *Biometrical Journal: Journal of Mathematical Methods in Biosciences* 50**,** 346-363.

Inceoglu, B., Lango, J., Jing, J., Chen, L., Doymaz, F., Pessah, I.N., and Hammock, B.D. (2003). One scorpion, two venoms: prevenom of *Parabuthus transvaalicus* acts as an alternative type of venom with distinct mechanism of action. *Proceedings of the National Academy of Sciences of the United States of America* 100**,** 922-927.

Isbister, G.K., Volschenk, E.S., and Seymour, J.E. (2004). Scorpion stings in Australia: five definite stings and a review. *Internal Medicine Journal* 34**,** 427-430.

Jackson, T.N.W., Koludarov, I., Ali, S.A., Dobson, J., Zdenek, C.N., Dashevsky, D., Op Den Brouw, B., Masci, P.P., Nouwens, A., and Josh, P. (2016). Rapid radiations and the race to redundancy: An investigation of the evolution of Australian elapid snake venoms. *Toxins* 8**,** 309.

Jiao, G.B., and Zhu, M.S. (2010). Courtship and mating of *Scorpiops luridus* Zhu Lourenço & Qi, 2005 (Scorpiones: Euscorpiidae) from Xizang province, China. *Journal of Venomous Animals and Toxins including Tropical Diseases* 16**,** 155-165.

Junior, R.S.F., Sciani, J.M., Marques-Porto, R., Junior, A.L., Orsi, R.D.O., Barraviera, B., and Pimenta, D.C. (2010). Africanized honey bee (*Apis mellifera*) venom profiling: Seasonal variation of melittin and phospholipase A2 levels. *Toxicon* 56**,** 355-362.

Kalita, B., Mackessy, S.P., and Mukherjee, A.K. (2018). Proteomic analysis reveals geographic variation in venom composition of Russell's viper in the Indian subcontinent: implications for clinical manifestations post-envenomation and antivenom treatment. *Expert Review of Proteomics* 15**,** 837-849.

Keegan, H.L., Hedeen, R.A., and Whittemore Jr, F.W. (1960). Seasonal variation in venom of black widow spiders. *The American Journal of Tropical Medicine and Hygiene* 9**,** 477-479.

King, G.F. (2011). Venoms as a platform for human drugs: translating toxins into therapeutics. *Expert Opinion on Biological Therapy* 11**,** 1469-1484.

Koch, L.E. (1977). *The taxonomy, geographic distribution and evolutionary radiation of Australo-Papuan scorpions.* Western Australian Museum.

Kovařík, F. (1997). Revision of the genera *Lychas* and *Hemilychas*, with descriptions of six new species (Scorpiones: Buthidae). *Acta Societatis Zoologicae Bohemicae* 61**,** 311-371.

Kovařík, F. (2019). Taxonomic reassessment of the genera *Lychas*, *Mesobuthus*, and *Olivierus*, with descriptions of four new genera (Scorpiones: Buthidae). *Euscorpius* 2019**,** 1-27.

Lira, A.F.A., Almeida, F.M.F., and Albuquerque, C.M.R. (2020). Reaction under the risk of predation: effects of age and sexual plasticity on defensive behavior in scorpion *Tityus pusillus* (Scorpiones: Buthidae). *Journal of Ethology* 38**,** 13-19.

Mackessy, S.P. (1988). Venom ontogeny in the Pacific rattlesnakes *Crotalus viridis helleri* and *C. v. oreganus*. *Copeia* 1988**,** 92-101.

Madrigal, M., Sanz, L., Flores-Díaz, M., Sasa, M., Núñez, V., Alape-Girón, A., and Calvete, J.J. (2012). Snake venomics across genus *Lachesis*. Ontogenetic changes in the venom composition of *Lachesis stenophrys* and comparative proteomics of the venoms of adult *Lachesis melanocephala* and *Lachesis acrochorda*. *Journal of Proteomics* 77**,** 280-297.

Mcelroy, T., Mcreynolds, C.N., Gulledge, A., Knight, K.R., Smith, W.E., and Albrecht, E.A. (2017). Differential toxicity and venom gland gene expression in *Centruroides vittatus*. *PLoS One* 12**,** e0184695.

Mclean, C.J., Garwood, R.J., and Brassey, C.A. (2018). Sexual dimorphism in the arachnid orders. *PeerJ* 6**,** e5751.

Mcreynolds, C.N. (2012). Ontogenetic shifts in microhabitat use, foraging and temporal activity for the striped bark scorpion *Centruroides vittatus* (Scorpiones: Buthidae). *Euscorpius* 2012**,** 1-19.

Miller, D.W., Jones, A.D., Goldston, J.S., Rowe, M.P., and Rowe, A.H. (2016). Sex differences in defensive behavior and venom of the striped bark scorpion *Centruroides vittatus* (Scorpiones: Buthidae). *Integrative and Comparative Biology* 56**,** 1022-1031.

Morgenstern, D., and King, G.F. (2013). The venom optimization hypothesis revisited. *Toxicon* 63**,** 120-128.

Newton, K.A., Clench, M.R., Deshmukh, R., Jeyaseelan, K., and Strong, P.N. (2007). Mass fingerprinting of toxic fractions from the venom of the Indian red scorpion, *Mesobuthus tamulus*: biotope-specific variation in the expression of venom peptides. *Rapid Communications in Mass Spectrometry* 21**,** 3467-3476.

Newton, M. (2008). "A guide to keeping Australian scorpions in captivity: with notes on general biology and identification". Newton Publishing, Adelaide, S. Aust).

Nisani, Z., Boskovic, D.S., Dunbar, S.G., Kelln, W., and Hayes, W.K. (2012). Investigating the chemical profile of regenerated scorpion (*Parabuthus transvaalicus*) venom in relation to metabolic cost and toxicity. *Toxicon* 60**,** 315-323.

Nisani, Z., Dunbar, S.G., and Hayes, W.K. (2007). Cost of venom regeneration in *Parabuthus transvaalicus* (Arachnida: Buthidae). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 147**,** 509-513.

O'hara, E.P., Caldwell, G.S., and Bythell, J. (2018). Equistatin and equinatoxin gene expression is influenced by environmental temperature in the sea anemone *Actinia equina*. *Toxicon* 153**,** 12-16.

Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., Mcglinn, D., Minchin, P.R., O'hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., and Wagner, H. (2020). "vegan: Community Ecology Package". 2.5-7 ed.).

Olguín-Pérez, L., Francke, O.F., and Carbajal-Saucedo, A. (2021). Evidence of piercing and sexual differences in venom composition in a sexual stinging scorpion (Scorpiones: Euscorpiidae). *The Journal of Arachnology* 49**,** 98-107.

Oliveira, F.N., Mortari, M.R., Carneiro, F.P., Guerrero-Vargas, J.A., Santos, D.M., Pimenta, A.M., and Schwartz, E.F. (2013). Another record of significant regional variation in toxicity of *Tityus serrulatus* venom in Brazil: a step towards understanding the possible role of sodium channel modulators. *Toxicon* 73**,** 33-46.

Omran, M.A., and Mcvean, A. (2000). Intraspecific variation in scorpion *Leiurus quinquestriatus* venom collected from Egypt (Sinai and Aswan deserts). *Journal of Toxicology: Toxin Reviews* 19**,** 247-264.

Ortiz, E., Gurrola, G.B., Schwartz, E.F., and Possani, L.D. (2015). Scorpion venom components as potential candidates for drug development. *Toxicon* 93**,** 125-135.

Özkan, Ö., Çiftçi, G., and Karaer, Z. (2011). Electrophoretical comparison of proteins of *Mesobuthus eupeus* and *Mesobuthus gibbosus* scorpion venoms. *Kafkas Univ Vet Fak Derg* 17**,** S153-S158.

Peretti, A.V. (2014). "Chapter 2 - Sexual selection in Neotropical species: rules and exceptions," in *Sexual Selection,* eds. R.H. Macedo & G. Machado. (San Diego: Academic Press), 33-52.

Pimenta, A.M., Almeida, F.D.M., De Lima, M.E., Martin-Eauclaire, M.F., and Bougis, P.E. (2003). Individual variability in *Tityus serrulatus* (Scorpiones, Buthidae) venom elicited by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Communications in Mass Spectrometry* 17**,** 413-418.

Polis, G.A., and Mccormick, S.J. (1986). Scorpions, spiders and solpugids: predation and competition among distantly related taxa. *Oecologia* 71**,** 111-116.

Polis, G.A., Myers, C.A., and Holt, R.D. (1989). The ecology and evolution of intraguild predation: potential competitors that eat each other. *Annual review of ecology and systematics* 20**,** 297-330.

Possani, L.D., Merino, E., Corona, M., Bolivar, F., and Becerril, B. (2000). Peptides and genes coding for scorpion toxins that affect ion-channels. *Biochimie* 82**,** 861-868.

Pucca, M.B., Amorim, F.G., Cerni, F.A., Bordon, K.D.C.F., Cardoso, I.A., Anjolette, F.a.P., and Arantes, E.C. (2014). Influence of post-starvation extraction time and prey-specific diet in *Tityus serrulatus* scorpion venom composition and hyaluronidase activity. *Toxicon* 90**,** 326-336.

R Core Team (2021). "R: A language and environment for statistical computing". (Vienna, Austria: R Foundation for Statistical Computing).

Rash, L.D., King, R.G., and Hodgson, W.C. (2000). Sex differences in the pharmacological activity of venom from the white-tailed spider (*Lampona cylindrata*). *Toxicon* 38**,** 1111-1127.

Rodríguez-Ravelo, R., Batista, C.V.F., Coronas, F.I.V., Zamudio, F.Z., Hernández-Orihuela, L., Espinosa-López, G., Ruiz-Urquiola, A., and Possani, L.D. (2015). Comparative proteomic analysis of male and female venoms from the Cuban scorpion *Rhopalurus junceus*. *Toxicon* 107**,** 327-334.

Rodríguez-Ravelo, R., Coronas, F.I.V., Zamudio, F.Z., González-Morales, L., López, G.E., Urquiola, A.R., and Possani, L.D. (2013). The Cuban scorpion *Rhopalurus junceus* (Scorpiones, Buthidae): component variations in venom samples collected in different geographical areas. *Journal of Venomous Animals and Toxins Including Tropical Diseases* 19**,** 13.

Sachkova, M.Y., Macrander, J., Surm, J.M., Aharoni, R., Menard-Harvey, S.S., Klock, A., Leach, W.B., Reitzel, A.M., and Moran, Y. (2020). Some like it hot: population-specific adaptations in venom production to abiotic stressors in a widely distributed cnidarian. *BMC Biology* 18**,** 121.

Santana, R.C., Perez, D., Dobson, J., Panagides, N., Raven, R.J., Nouwens, A., Jones, A., King, G.F., and Fry, B.G. (2017). Venom profiling of a population of the theraphosid spider *Phlogius crassipes* reveals continuous ontogenetic changes from juveniles through adulthood. *Toxins* 9**,** 116.

Schwartz, E.F., Camargos, T.S., Zamudio, F.Z., Silva, L.P., Bloch Jr, C., Caixeta, F., Schwartz, C.A., and Possani, L.D. (2008). Mass spectrometry analysis, amino acid sequence and biological activity of venom components from the Brazilian scorpion *Opisthacanthus cayaporum*. *Toxicon* 51**,** 1499-1508.

Senji Laxme, R.R., Attarde, S., Khochare, S., Suranse, V., Martin, G., Casewell, N.R., Whitaker, R., and Sunagar, K. (2021). Biogeographical venom variation in the Indian spectacled cobra (*Naja naja*) underscores the pressing need for pan-India efficacious snakebite therapy. *PLoS Neglected Tropical Diseases* 15**,** e0009150.

Shine, R. (1977). Habitats, diets, and sympatry in snakes: a study from Australia. *Canadian Journal of Zoology* 55**,** 1118-1128.

Sousa, L.F., Zdenek, C.N., Dobson, J.S., Op Den Brouw, B., Coimbra, F.C.P., Gillett, A., Del-Rei, T.H.M., Chalkidis, H.D.M., Sant'anna, S., Teixeira-Da-Rocha, M.M., Grego, K., Travaglia Cardoso, S.R., Moura Da Silva, A.M., and Fry, B.G. (2018). Coagulotoxicity of *Bothrops* (lancehead pit-vipers) venoms from Brazil: differential biochemistry and antivenom efficacy resulting from prey-driven venom variation. *Toxins* 10**,** 411.

Sunagar, K., Morgenstern, D., Reitzel, A.M., and Moran, Y. (2016). Ecological venomics: How genomics, transcriptomics and proteomics can shed new light on the ecology and evolution of venom. *Journal of Proteomics* 135**,** 62-72.

Sunagar, K., Undheim, E.a.B., Scheib, H., Gren, E.C.K., Cochran, C., Person, C.E., Koludarov, I., Kelln, W., Hayes, W.K., King, G.F., Antunes, A., and Fry, B.G. (2014). Intraspecific venom variation in the medically significant Southern Pacific Rattlesnake (*Crotalus oreganus helleri*): Biodiscovery, clinical and evolutionary implications. *Journal of Proteomics* 99**,** 68-83.

Tan, K.Y., Ng, T.S., Bourges, A., Ismail, A.K., Maharani, T., Khomvilai, S., Sitprija, V., Tan, N.H., and Tan, C.H. (2020). Geographical variations in king cobra (*Ophiophagus hannah*) venom from Thailand, Malaysia, Indonesia and China: On venom lethality, antivenom immunoreactivity and in vivo neutralization. *Acta Tropica* 203**,** 105311.

Tian, C., Yuan, Y., and Zhu, S. (2008). Positively selected sites of scorpion depressant toxins: possible roles in toxin functional divergence. *Toxicon* 51**,** 555-562.

Uribe, J.I.C., Vargas, J.M.J., Batista, C.V.F., Zuñiga, F.Z., and Possani, L.D. (2017). Comparative proteomic analysis of female and male venoms from the Mexican scorpion *Centruroides limpidus*: novel components found. *Toxicon* 125**,** 91-98.

Ward, M.J., Ellsworth, S.A., Hogan, M.P., Nystrom, G.S., Martinez, P., Budhdeo, A., Zelaya, R., Perez, A., Powell, B., and He, H. (2018). Female-biased population divergence in the venom of the Hentz striped scorpion (*Centruroides hentzi*). *Toxicon* 152**,** 137-149.

Wickham, H. (2016). *ggplot2: elegant graphics for data analysis.* springer.

Wigger, E., Kuhn-Nentwig, L., and Nentwig, W. (2002). The venom optimisation hypothesis: a spider injects large venom quantities only into difficult prey types. *Toxicon* 40**,** 749-752.

Winter, K.L., Isbister, G.K., Mcgowan, S., Konstantakopoulos, N., Seymour, J.E., and Hodgson, W.C. (2010). A pharmacological and biochemical examination of the geographical variation of *Chironex fleckeri* venom. *Toxicology letters* 192**,** 419-424.

Yamaji, N., Dai, L., Sugase, K., Andriantsiferana, M., Nakajima, T., and Iwashita, T. (2004). Solution structure of IsTX: A male scorpion toxin from *Opisthacanthus madagascariensis* (Ischnuridae). *European journal of biochemistry* 271**,** 3855-3864.

Yu, C., Yu, H., and Li, P. (2020). Highlights of animal venom research on the geographical variations of toxin components, toxicities and envenomation therapy. *International Journal of Biological Macromolecules* 165**,** 2994-3006.

Zeng, X.C., Corzo, G., and Hahin, R. (2005). Scorpion venom peptides without disulfide bridges. *IUBMB Life* 57**,** 13-21.

Lychas variatus adult female (left) and adult male (right) – Edward Evans

Chapter 3. Love stings? Novel Short Amidated Peptides from the Venom of Adult Male Marbled Scorpions (*Lychas variatus*)

3.1. Abstract

Sexual stinging occurs where adult male scorpions of many species use their venom to sting the female during courtship, and currently the underlying molecular mechanisms are unknown. I investigated if adult male marbled scorpions (*Lychas variatus*) have additional venom components that could be attributed to sexual stinging using a combination of LC/MS, RP-HPLC, MALDI-TOF and NMR techniques. I identified two novel tetrapeptide-amides Lvar1 (HLPH-NH_2) and Lvar2 (HLPR-NH_2), and a hexapeptide-amide Lvar3 (VFPRPY-NH_2) which were specific to the venom of adult males. These peptides were absent in the venom of females and immature males. Their small size and lack of disulfide bonds indicates these molecules are unlike typical scorpion toxins and may have a different function within the venom. I found these molecules are not toxic in domestic crickets, and do not affect the behaviour of female scorpions (*L. variatus*). In addition, they were not active on a collection of Na_V and K_V channels tested. The combination of their structural novelty, specificity to adult male venoms, and apparent lack of toxicity towards insect prey supports the idea that these molecules are not typical venom toxins and are strong candidates to be involved in sexual stinging, suggesting future studies are needed to determine their role in mating.

3.2. Introduction

Venoms have evolved in most incidences to incapacitate potential prey or to defend against potential predators (Schendel et al., 2019), for example by causing pain or paralysis. There are, however, unusual cases where venom is used in a less-typical way; for example against competitors (Arbuckle, 2017;Nekaris et al., 2020), or as a part of courtship. 'Sexual stinging' behaviours have been documented in at least 20 scorpion species belonging to six families (Olguín-Pérez et al., 2021), and consist of the male carefully stinging a female usually before initiating the courtship dance, when a pair of scorpions lock pedipalps and the male attempts to manoeuvre the female on top of a spermatophore he has deposited.

Despite its importance in mating, the function of sexual stinging remains unclear. It has been suggested that sexual stings may act as a mechanism to stimulate the female (Inceoglu et al., 2003), subdue her and reduce the predation risk towards the male (Jiao and Zhu, 2010), or perhaps improve reproductive success by other mechanisms such as influencing mate choice (Olguín-Pérez et al., 2021). It was recently shown that sexual stings involve the transfer of venom, rather than being a purely ritualistic behaviour (Olguín-Pérez et al., 2021); however, it remains unknown if a specific set of molecules are injected rather than all the toxins. For example, as scorpions can control expulsion of their venom from the gland (Nisani and Hayes, 2011), males could inject chemically simplistic 'prevenom', thereby reducing the exposure of the female to the main toxic components of the venom (Inceoglu et al., 2003). Scorpions possess highly complex venoms which can contain hundreds of different molecules (Batista et al., 2006), but it is unknown if there are specific components associated with sexual stinging behaviour.

Scorpions display complicated courtship behaviours which have been well-described in a number of species (Tallarovic et al., 2000;Briceño and Bonilla, 2009;Jiao and Zhu, 2010;Toscano-Gadea, 2010). Courtship typically can be broken down into three stages: initiation, the courtship dance or "*promenade a deux*", and insemination (Zizzari et al., 2014;Olguín-Pérez et al., 2021). A range of behaviours are involved with each stage, such as vibrating their bodies, gripping pedipalps, clubbing with their metasoma, massaging the chelicera, rubbing with their legs, and sexual stinging (Alexander, 1959;Peretti and Carrera, 2005). Sexual stings, although observed in several species, do not typically feature in the courtship of all scorpion species (Peretti and Carrera, 2005;Chantall-Rocha and Japyassú, 2017), but have been observed to occur as frequently as 14 times in a single *Hadrurus arizonensis* mating event (Tallarovic et al., 2000), and in *Bothriurus buecherli* each sting can last 35.2 min (Toscano-Gadea, 2010), indicating at least for some species it is a very important part of courtship (Polis, 1990;Peretti, 2014;Olivero et al., 2019;Olguín-Pérez et al., 2021). Interspecific

variation is reported in the sting frequency, sting duration, and location of the sting (Tallarovic et al., 2000;Toscano-Gadea, 2010;Olguín-Pérez et al., 2021), implying that the underlying mechanism may not be uniform across all species.

Identifying if venom components have a role in mating is difficult because male and female venom may differ for several reasons. Intersexual differences in venoms have been documented in a diverse range of venomous animals (Herzig et al., 2008;Binford et al., 2016;Lopes-Ferreira et al., 2016;Sarhan et al., 2017;Nystrom et al., 2019), and could be attributed to a wide range of biological or ecological factors such as differential hunting strategies or predator exposure between the sexes (Miller et al., 2016;Herzig et al., 2020). Scorpions display intersexual differences in both the relative abundance of venom constituents, and the presence of different molecules within their venom (Abdel-Rahman et al., 2009;De Sousa et al., 2010;Özkan et al., 2011;Rodríguez-Ravelo et al., 2015;Uribe et al., 2017), and such differences have been reported to impact the toxicity of male and female venoms (De Sousa et al., 2010;Miller et al., 2016). Many scorpion species display sexual dimorphism (Carrera et al., 2009;McLean et al., 2018), and it is likely that this could lead to compositional differences in venom if it affects their hunting strategy or ability to escape predation. For example, females often have larger/stockier abdomens as they have greater reproductive investment due to egg production, whilst males are frequently more slender and often exhibit longer tails (McLean et al., 2018). Such morphological differences can affect the motility of scorpions, and may influence the hunting/defensive strategies employed by the sexes (Carlson et al., 2014;Miller et al., 2016;González-Gómez et al., 2019). When male and female scorpions are consistently employing different hunting and defensive strategies, and/or are consistently exposed to different prey and predators, over time their venoms may have diverged due to optimisation under different pressures (Wigger et al., 2002;Morgenstern and King, 2013;Evans et al., 2019). In addition to this, it is unknown if intersexual differences in scorpion venom composition could be attributed to sexual stinging, and if males have unique molecules to solely serve a function associated with this behaviour.

Adult male scorpions differ from both subadult males and females in that they can use their venom for sexual stinging, in addition to prey capture and defence. Therefore, in this study I investigated adult-male specific molecules present in the venom of marbled scorpions (*Lychas variatus*), to identify any that may be involved in sexual stinging. The breeding biology of *L. variatus* is not documented, including whether sexual stinging occurs in this species. However, sexual stinging has previously been reported in *Lychas spinatus besti* (Newton, 2008), which is currently considered synonymous with *L. variatus* (Koch, 1977;Kovařík, 1997;2019). I isolated and characterised three novel peptides (Lvar1, Lvar2, and Lvar3) which were only observed in the venom of adult male *L.*

variatus, are much smaller than most known scorpion venom toxins, and are structurally unlike other small scorpion peptides. Synthetic versions of the peptides were produced for bioactivity testing in female scorpions, in crickets, and across a suite of ion channels.

3.3. Materials and Methods

3.3.1. Scorpion Collection and Husbandry

Wild scorpions were collected from a single rainforest population in the Cairns region (Queensland, Australia), and additionally wild-caught females gave birth to captive born young. Adult scorpions were housed individually in round plastic food containers (11 cm diameter: 8.5 cm height) with 3-5cm depth substrate (coco-fibre/sand mixture). They were stored at 25 °C in a temperature-controlled room with no light source. Captive born scorpions were separated from the mother once they had moulted to second instar (I2) and housed in small, round plastic containers (6 cm diameter: 3 cm height). Once they had moulted to the fourth instar (I4), they were moved into the same sized containers as adults. A cricket was offered to the scorpions once a week, and uneaten food items were removed after 1-3 days to minimise stress. Females that had recently given birth and still had young on their backs were not provided with food items at this stage, as I experienced the introduction of a cricket caused great disturbance to the mother and offspring.

3.3.2. Venom Extraction

Scorpions were restrained onto a sponge using parafilm, and a square-wave stimulator (Arthur H. T. Thomas Co. Scientific Apparatus, Philadelphia, PA, USA) was used to electrostimulate the venom glands and extract venom into a pipette tip. Young scorpions (I2-3) were stimulated using 15V DC current, 0.5 ms pulse duration, 1 pulse/sec frequency, and the voltage was raised to 20 V for older scorpions (I4-6). Individual scorpion venom samples were suspended in 10 µL of Type 1 water (T1W) and stored at -20 °C prior to further analysis.

An additional 20 wild-caught adult male (I5) scorpions were milked and pooled together in 20 µL T1W. Venom was extracted from these scorpions a total of four times with intervals of 3-5 weeks to allow for recovery and venom replenishment. These four samples were further pooled together to produce one concentrated sample containing venom from a total of 80 milking events. This concentrated sample was used for molecule isolation and characterisation.

3.3.3. Liquid Chromatography/Mass Spectrometry (LC/MS)

To identify molecules of potential interest, individual scorpion venoms were analysed by liquid chromatography/mass spectrometry (LC/MS) using a Shimadzu LCMS-2020 mass spectrometer combined with a Shimadzu Prominence HPLC system (Shimadzu, Kyoto, Japan). As the quantity of venom produced by each scorpion depended on its size, crude samples (venom in 10 µL T1W) were diluted based on the scorpion's instar and made up to 10 µL with T1W prior to LC/MS analysis. As I2 and I3 scorpions produced very small quantities of venom, the approximately 10 µL crude samples were not diluted. For I4 scorpions 5 µL of sample was combined with 5 µL T1W for injection, and for I5-6 scorpions 3 µL of the stored sample was combined with 7 µL T1W.

Each 10 µL preparation was injected onto a reversed-phase high performance liquid chromatography (RP-HPLC) column (Phenomenex Aeris 3.6 µm PEPTIDE XB-C_{18} 100 Å, 2.1 x 150 mm; Phenomenex, Torrence, CA, USA) by a Shimadzu SIL-20ACHT autosampler, with the column oven at 30 °C. A 1% gradient of solvent B (90% acetonitrile [ACN; OPTIMA LCMS grade, Thermo Fisher Scientific, Scoresby, VIC, Australia]/0.09% formic acid [FA; Sigma-Aldrich, St. Louis, MO, USA]/water) against solvent A (solvent A: 0.1% FA) was run (0-60 % solvent B) for 60 min at a flow rate of 0.250 mL/min using Shimadzu LC-20AD pumps. UV absorbance at 214 nm and 280 nm was recorded by a Shimadzu SPD-20A detector. Mass spectra were collected in positive ion mode with the following settings: scan range of *m/z* 250-2000, detector voltage of 1.15 kV, nebulizing gas flow of 1.5 L/min, drying gas flow of 3.0 L/min. Shimadzu LabSolutions v5.96 software (Shimadzu, Japan) was used to collect and analyse data. Mass lists for each sample were created by manually scanning through each peak and using the multi-charge ion analysis tool. These mass lists were compared to identify molecules of interest that were specific to adult male venoms.

3.3.4. Reversed-Phase High Performance Liquid Chromatography (RP-HPLC)

Pooled adult male venom was fractionated using reversed-phase high performance liquid chromatography (RP-HPLC) to isolate the molecules of interest identified from the LC/MS results. The sample was line-loaded onto a semi-preparative C_{18} column (Phenomenex Jupiter 250 × 10 mm, 10 µm, 100 Å) and a 0.5% gradient of solvent B (90% acetonitrile/0.05% TFA) in solvent A (0.05% TFA) was run at a flow rate of 3 mL/min for 120 min. A total of 384 fractions were collected. Three molecules were selected for further analysis as they had low molecular weights, were present in reasonably high quantities, and eluted in relatively pure RP-HPLC fractions.

3.3.5. Matrix-Assisted Laser Desorption Ionization Time-Of-Flight (MALDI-TOF) Mass Spectrometry

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was performed on RP-HPLC fractions using a SCIEX TOF/TOF 5800 MALDI (SCIEX, Framingham, MA, USA) to identify fractions containing the molecules of interest. Fractions were spotted onto a 384-well stainless-steel target plate using 0.5 µL of sample and 0.5 µL of α–cyano-4-hydroxycinnamic acid (CHCA; Sigma-Aldrich, St. Louis, MO, USA) matrix, and calibration was undertaken using Calibration Mix Solution 2 (SCIEX, Framingham, MA, USA). Each spot was analysed using reflector positive mode (scan range *m/z* 450-600 and *m/z* 600-4500, averaged over 2000 laser shots) to identify the fractions containing the molecules of interest. Tandem-MS (MS/MS) was performed in 1 kV positive ion mode, both with/without collision-induced dissociation (CID). Spectra were averaged over 2000 shots. The fragmentation pattern was used to manually *de novo* sequence the molecules and elucidate their structure. Fractions predominantly containing the molecules of interest were pooled prior to further analysis.

3.3.6. NMR Spectroscopy

The fractions of interest were analysed using a Bruker Avance III 600MHz NMR spectrometer (Bruker, Billerica, MA, USA) equipped with a cryoprobe. Lyophilised venom fractions were dissolved in 90% H_20/10% D_2O (Cambridge Isotope Laboratories, Woburn, MA, USA). Two-dimensional ^1H-^1H TOCSY, ^1H-^1H NOESY, ^1H-^{15}N HSQC, and ^1H-^{13}C HSQC spectra were collected using standard Bruker pulse sequences. All experiments were run at 290K with an interscan delay of 1 s and spectra were referenced to external 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS; Cambridge Isotope Laboratories). Mixing times of 80 ms (TOCSY) and 200-250 ms (NOESY) were used. Spectra were analysed in Topspin v3.6.1 (Bruker, Billerica, MA, USA) and CcpNMR v3 (Skinner et al., 2016). The spectra were studied for characteristic peak shifts and NMRseq techniques were used (Wilson and Daly, 2018) to confirm the sequences of the molecules calculated from the MS/MS data.

3.3.7. Peptide Synthesis and Purification

The three peptides identified and named Lvar1, Lvar2 and Lvar3 were synthesised using solid-phase fluorenylmethyloxycarbonyl (Fmoc) chemistry-based peptide synthesis using Rink amide (Auspep) resin on a 0.1 and 0.2 mmole scale. The amino acids (Novabiochem) were activated by mixing for 15 min with O-(1H-6-chlorobenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU, Iris Biotech GmbH) and N,N-diisopropylethylamine (DIPEA, Auspep) in dimethylformamide (DMF, Auspep). Deprotection of the N-terminal was achieved by adding 20% piperidine (ChemSupply) in DMF for 5 min, draining, and then repeating for 5 min in the same solution. Lvar1 and Lvar3 were assembled manually, whilst Lvar2 was assembled with the aid of a

Protein Technologies PS3 synthesiser (Gyros Protein Technologies, Sweden). A double coupling method was used in the assemblage of Lvar3 peptide. The peptides were cleaved from the resin by swirling the resin for 2 hours in a mixture of 95% trifluoroacetic acid (TFA, Auspep), 2.5% triisopropylsilane (TIPS, Sigma-Aldrich) and water. The TFA was then evaporated off using nitrogen gas, and the cleaved peptides were precipitated using ice-cold diethylether, dissolved into 50% acetonitrile/water/0.05% TFA, and then lyophilised using a ScanSpeed 40 speed vacuum concentrator (LaboGene, Lillerød, Denmark). The lyophilised peptides were then dissolved into water and purified using RP-HPLC with a C_{18} preparative column (Phenomenex Jupiter 250 × 21.2 mm, 10 µm, 300 Å) with the buffers described in section 2.4. A 1% gradient of solvent B (0-60%) was used, and fractions were collected manually based on 214nm absorbance. MALDI-TOF mass spectrometry and LC/MS was then used to analyse the fractions to identify masses and identify which fractions should be pooled using the relevant methods described in section 2.4. 40 µL of the final pooled peptide samples were then analysed by RP-HPLC equipped with an analytical column (Agilent Eclipse Plus C_{18}, 100 x 4.6 mm, 3.5 µm) to test purity. To confirm the synthetic peptides had the same retention times as their native counterparts, coelutions were performed using HPLC by injecting each synthetic peptide separately and in combination with the native peptides at a proportion of ~2:1. Each synthetic peptide was then subject to MALDI-TOF MS/MS following the methods described in section 2.5 to ensure they showed the same fragmentation pattern as their native counterparts.

3.3.8. Scorpion and Cricket Bioassay

The activities of the three peptides were tested in wild-caught adult female *L. variatus* scorpions and adult female domestic brown crickets (*Acheta domesticus*) purchased commercially (PETstock, Australia). To determine a biologically relevant quantity of each peptide for injection, the amount of Lvar1, Lvar2, and Lvar3 in the venom of male scorpions was estimated using LC/MS and comparing the areas (214nm absorbance) of the peaks containing each of the three peptides. Serial dilutions of the synthetic peptides and eight male venoms were run using the LC/MS methods described in section 2.3. The 214 nm peak areas of the serial dilutions of the synthetics were used to produce standard curves, and the areas of peaks containing each peptide within the male venoms were compared against the standard curve to estimate the quantity of each present. The maximum value estimated to be in the male venom was then doubled to select a high, but biologically relevant dose for injection.

Each synthetic peptide was dissolved separately into Ringer's solution (112mM NaCl, 2mM KCl, 2mM CaCl2 and 10mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (Life

Technologies Corporation, Grand Island, NY, USA) (Nisani et al., 2012)) so that a 1 μL injection of the final preparations contained 0.451 μg of Lvar1, 0.761 μg Lvar2, and 0.437 μg Lvar3 respectively. Ringer's solution was used as a negative control in both crickets and female scorpions. Crude *Urodacus yaschenkoi* venom was used as a positive control in female scorpions. The toxicity of crude adult male *L. variatus* venom was tested in female scorpions by drawing freshly collected venom directly into the needle of a syringe pre-loaded with insect saline immediately prior to injection of 1 μL. A positive control was also performed in crickets using a pooled sample containing 20 adult male *L. variatus* venoms suspended in 50 μL T1W. 1 μL of this mixture was drawn into the needle of a syringe preloaded with insect saline immediately before injection into the cricket. Each peptide was tested in eight female scorpions and eight crickets. Ten female scorpions each were injected with *U. yaschenkoi* venom and insect saline. Crude male *L. variatus* venom was tested in ten female scorpions. Eight crickets each were injected with insect saline and *L. variatus* venom.

1 μL injections were made using a 100 μL SGE syringe (Trajan Scientific and Medical, Vic, Australia) and a hand microapplicator (Burkard Scientific, UK). Scorpions were restrained on a petri dish using parafilm and injected deep into the mesosoma, by entering the needle at the posterior end and inserting it towards the cephalothorax. Crickets were carefully held and injected into the ventral side of the pronotum. Following injection, scorpions were placed into a large food container (24 cm length: 21.5 cm width: 10.5 cm height) and their behaviour monitored at 1 min, 3 min, and 5 min time points. At these points they were touched repeatedly with forceps to elicit a defensive or flight response, otherwise they remained motionless. Scorpion behaviour after injection was classified as normal (unhindered flight response or rapid defensive behaviour), slow (slow-paced flight response, delayed defensive response, or coordination impeded by spasms), or paralysed (uncontrollable spasming or movement fully impeded). Crickets were placed into round plastic containers (6 cm diameter; 3 cm height) post-injection and their righting response was tested at the same time points by flipping the container and observing the ability of the cricket to right itself. If crickets were unable to right themselves after 10 sec they were considered immobilised, whereas those unable to after 30 sec were considered paralysed. Crickets able to quickly right themselves were deemed unaffected.

A permutation-based chi-squared test was used to test whether injection of the three compounds and the negative control (insect saline) had any effect on scorpion or cricket behaviour within 5 min post-injection in R version 4.0.5 (R Core Team, 2021). This nonparametric approach used Monte Carlo simulation with 10,000 permutations to calculate *p* values, because the expected number of crickets paralysed was low for any of the treatments evaluated, invalidating the chi square approximation in the Pearson chi squared test that relies on large sample sizes.

3.3.9. Ion Channel Bioassay

Ion channel assays were performed by Steve Peigneur using synthetic Lvar1, Lvar2 and Lvar3 following protocols described in detail previously (Peigneur et al., 2019a;Peigneur et al., 2019b). For the expression of K_V channels (rK_V1.1, rK_V1.2, hK_V1.3, rK_V1.4, rK_V1.5, rK_V1.6, rK_V2.1, hK_V10.1, hERG, and Shaker IR) and Na_V channels (rNa_V1.2, rNa_V1.4, hNa_V1.5, mNa_V1.6, hNa_V1.7, hNa_V1.8, BgNa_V1, VdNa_V1) in *Xenopus laevis* oocytes, the linearised plasmids were transcribed using the T7 or SP6 mMESSAGE-mMACHINE transcription kit (Ambion). In total, 50 nL of cRNA (1 ng/nL) was injected into oocytes, which were incubated in ND96 solution containing 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, and 5 mM HEPES (pH = 7.4), supplemented with 50 mg/L gentamycin sulfate. Recordings were performed using a Geneclamp 500 amplifier (Molecular Devices) controlled by a pClamp data acquisition system (Axon Instruments); bath solution was ND96. Voltage and current electrodes were filled with 3 M KCl. Resistances of both electrodes were kept between 0.7 and 1.5 MΩ. Elicited currents were sampled at 1 kHz and filtered at 0.5 kHz (for potassium currents) or sampled at 20 kHz and filtered at 2 kHz (for sodium currents) using a four-pole low-pass Bessel filter. Leak subtraction was performed using a-P/4 protocol. Currents were evoked by a 100 ms (Na_V) or 500 ms (K_V) depolarization to the voltage corresponding to the maximal activation of the channels in control conditions from a holding potential of −90 mV. Data were sampled at 500 Hz and filtered at 200 Hz. Peak current amplitude was measured prior to and following the incubation of the peptide. All data were obtained in at least three independent experiments.

3.4. Results

3.4.1. Isolation of Molecules of Interest

A total of 84 samples (6 adult males: 78 female/juvenile scorpions) were subjected to LC/MS analysis to identify peaks and molecules only present in adult male scorpions. For illustration, LC/MS analysis of two I5 sibling scorpions (one male, one female) from the same clutch and raised in captivity under identical conditions display compositional differences indicated by the presence/absence of specific peaks (Figure 3.1). By comparing mass lists generated for each sample, a total of 12 molecules were found to be unique to adult males (Table 3.1). Molecules observed in less than three samples were not considered, as those appearing in just one or two samples may have been erroneously identified, or present below detectable levels in most samples.

RP-HPLC was then performed on a pooled sample of adult male venom (Figure 3.2). The LC/MS and RP-HPLC data were used in combination to select three molecules for further analysis as they i) had low molecular mass ii) were present in sufficiently high quantities and iii) eluted in relatively clean RP-HPLC peaks (Figure 3.2). The selected molecules of interest had masses of 501.3 Da, 520.3 Da, and 776.4 Da, and were present in at least five out of six adult male venoms tested (Table 3.1) but not detected in the 78 female/juvenile samples tested. RP-HPLC fractions containing the molecules of interest were pooled.

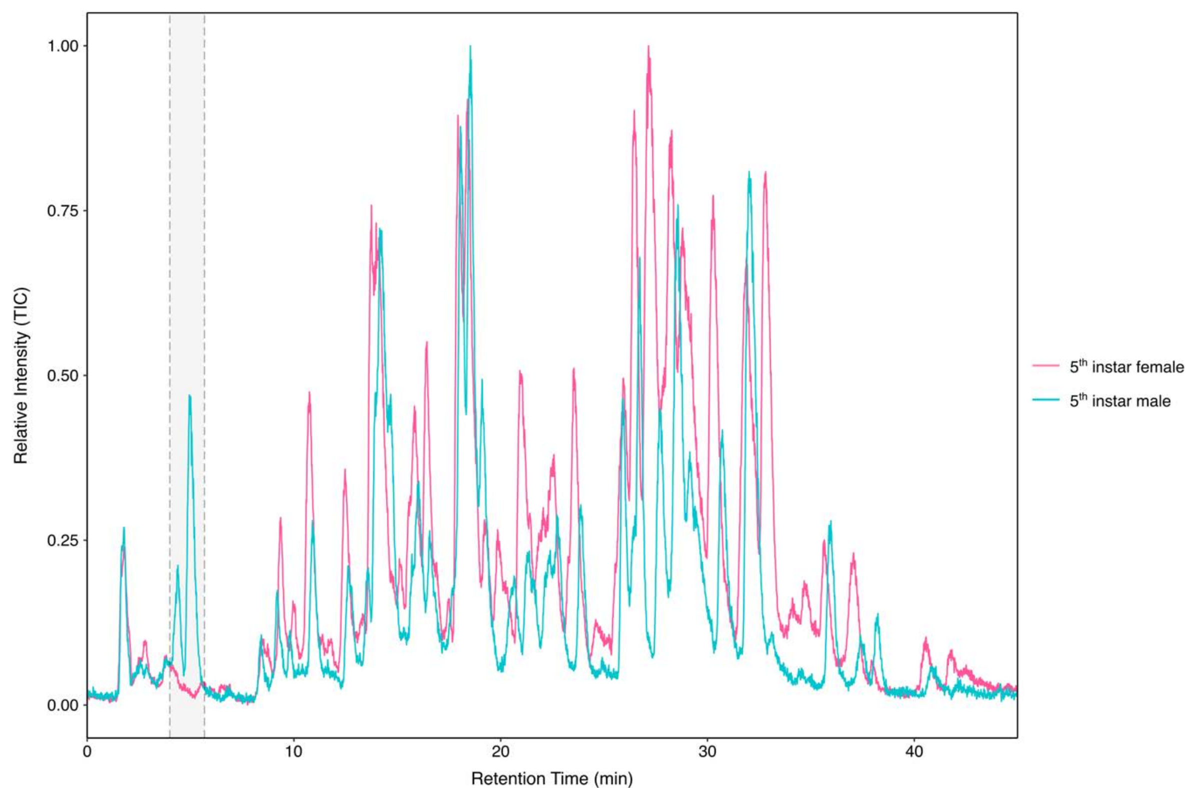


Figure 3. 1 LC/MS trace of adult (I5) male and female (I5) *Lychas variatus* venom (Phenomenex Aeris 3.6 µm PEPTIDE XB-C18 100 Å column; flowrate 0.25mL/min; solvent A H_2O/0.1% FA, solvent B 90% ACN/H_2O/0.09% FA; 0–45% solvent B in 45min; scan range *m/z* 250-2000, detector voltage 1.15 kV, nebulizing gas flow 1.5 L/min, drying gas flow 3.0 L/min). The two scorpions were siblings from the same clutch, raised in captivity under identical conditions. Male venom is represented by the blue trace and female venom in pink. Clear differences between the traces are present in the shaded area from approximately 4-5.5 min.

Table 3. 1 Mass (Da) of molecules present only in adult male *Lychas variatus* scorpion venoms, identified by LC/MS analysis. The total number of samples they were identified within is shown. 82 samples were tested (adult males n = 6, other scorpions n = 76) and only molecules observed in at least four samples included.

Reconstructed mass (Da)	Presence in samples (n=6)
501.3	6
520.3	5
621.4	6
640.0	6
776.4	6
790.4	5
3853.6	4
4024.1	4
4390.2	5
4400.2	6
5278.9	4
5986.5	4

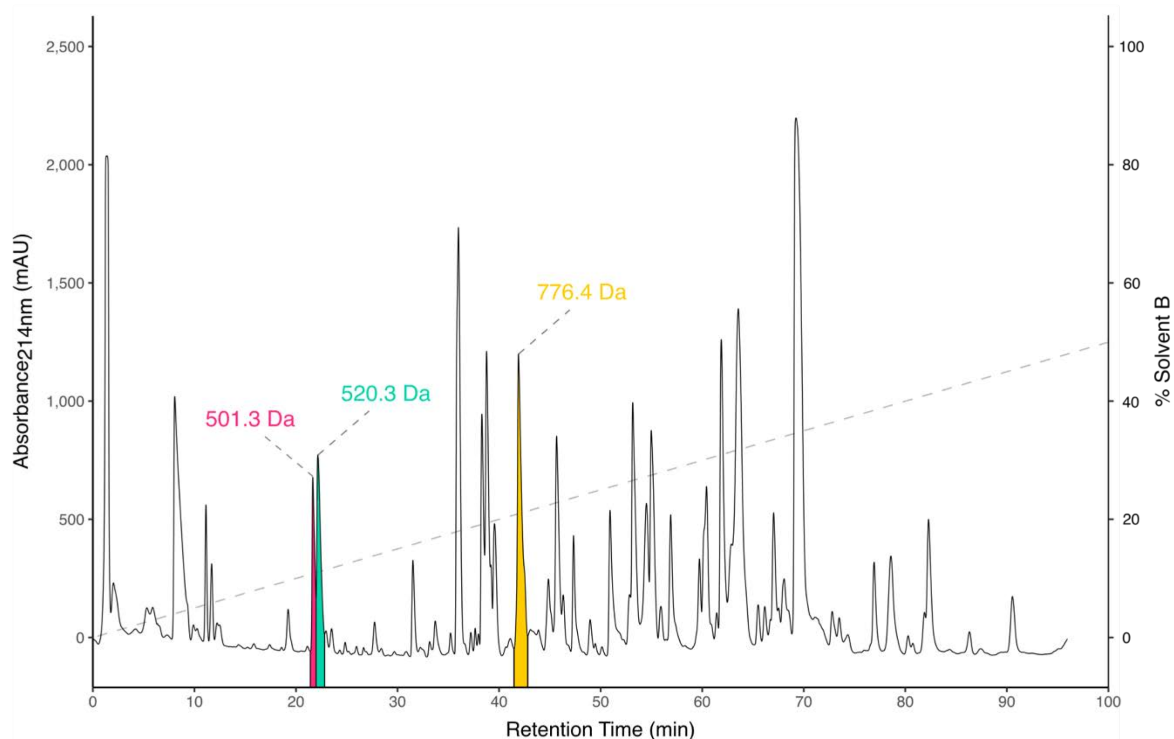


Figure 3. 2 Reversed-phase high performance liquid chromatography (RP-HPLC) chromatogram of pooled adult male *Lychas variatus* venom (Phenomenex Jupiter® C_{18} column; 250 × 10 mm, 10 µm, 100 Å; 3mL/min flowrate; solvent A H_2O/0.05% TFA, solvent B 90% ACN/H_2O/0.045% TFA; 0.5% gradient solvent B (0-60% B; 120 min), absorbance at 214 nm). Peaks containing molecules of interest are highlighted in pink (501.3 Da), blue (520.3 Da) and yellow (776.4 Da).

3.4.2. Characterisation of Molecules of Interest

MS/MS fragmentation in combination with 2D-NMR was used to characterise the primary structure of each molecule by manual *de novo* sequencing and NMRseq techniques (Wilson and Daly, 2018). The sequence determined, and name assigned to the three molecules (501.3 Da = Lvar1, 520.3 Da = Lvar2, and 776.4 Da = Lvar3) is summarised in Table 3.2. The RP-HPLC peak containing Lvar1 at 21.650 min made up 1.6% of the total peak area of the chromatogram (Figure 3.2). MALDI-TOF MS analysis on the corresponding fraction identified the $[M + H]^+$ of Lvar1 as 502.2604 *m/z* (Figure 3.3). MS/MS fragmentation on this precursor mass showed a fragmentation pattern corresponding to the sequence HLPH-NH_2 (Figure 3.3). NOESY and TOCSY NMR spectra obtained for this fraction contained peaks with chemical shifts that provided support for the sequence determined; however, the inter-residue peaks were not strong enough to elucidate the sequence from NMR data alone (Supplementary Materials 3.1). The Lvar2 RP-HPLC peak at 22.156 min, accounted for 2.67% of the total peak area of the chromatogram (Figure 3.2). MALDI-TOF MS analysis on these fractions identified the $[M + H]^+$ of Lvar2 as 521.3096 *m/z* (Figure 3.4). MS/MS fragmentation of this mass showed a fragmentation pattern corresponding the sequence HLPR-NH_2 (Figure 3.4). TOCSY NMR spectra supported this elucidated sequence (Supplementary Materials 3.2). The inter-residue peaks in the NOESY spectra were not sufficiently strong to determine the primary sequence of the peptide without the MS/MS fragmentation pattern. The MS spectra for Lvar2 showed a different isotope distribution for the $[M + H]^+$ ion compared to the other two peptides and suggests another molecule one mass unit larger than 521.3096 *m/z* may also be present (Figure 3.4), possibly corresponding to a C-terminal acid form of Lvar2. The Lvar3 RP-HPLC peak at 41.907 min accounted for 4.77% of the total peak area of the chromatogram (Figure 3.2). The $[M + H]^+$ of Lvar3 was found to be 777.3743 by MALDI-TOF MS (Figure 3.5), and MS/MS fragmentation of this precursor ion showed a fragmentation pattern for the sequence VFPRPY-NH_2 (Figure 3.5). The b_1 ion was only just detectable in the spectra displayed in Figure 3.5 but was more easily observed when collision-induced dissociation (CID) was used. Spectra collected with CID were less clear overall, and therefore not presented. 2D NMR experiments confirmed the sequence VFPRPY-NH_2. Peaks were observed in the TOCSY and NOESY 2D NMR spectra supported this sequence, with some sequential interaction between the amino acids evident through the NOESY inter-residue peaks. The assigned TOCSY spectrum is presented without the NOESY spectrum for ease of visualisation (Supplementary Materials 3.3).

Table 3. 2 Observed ion ([M+H]+), theoretical monoisotopic mass, sequence, and name assigned to the three adult male specific peptides (Lvar1, Lvar2, and Lvar3) calculated using 2D NMR and MALDI-TOF MS/MS analysis.

Observed mass $[M+H]^+$	Theoretical monoisotopic mass (Da)	Peptide sequence	Name
502.2604	501.270	HLPH-NH_2	Lvar1
521.3096	520.312	HLPR-NH_2	Lvar2
777.3743	776.422	VFPRPY-NH_2	Lvar3

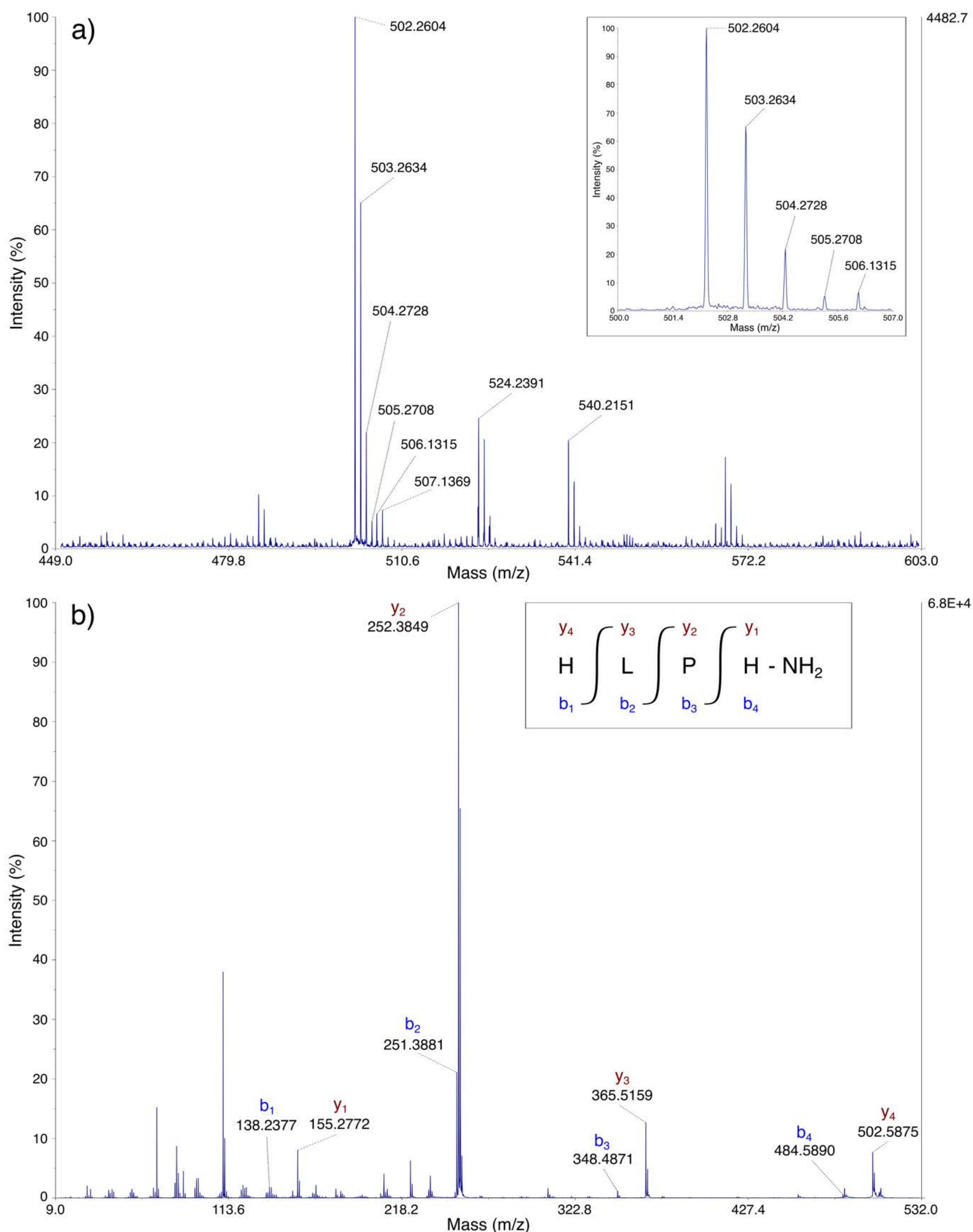


Figure 3. 3 MALDI-TOF MS and MS/MS spectra with calculated sequence of Lvar1. **a)** MALDI-TOF MS spectrum of native Lvar1 $[M+H]^+$ 502.2604 *m/z*. The $[M+Na]^+$ (524.2391 *m/z*) and $[M+K]^+$ (540.2151 *m/z*) adducts are also visible within the spectrum. The top-right inset is an expanded view of the spectrum between 500.0-507.0 *m/z*. **b)** MALDI-TOF MS/MS fragmentation on a RP-HPLC fraction containing native Lvar1 with a precursor $[M+H]^+$ 502.26 *m/z*. The fragment ions and elucidated sequence (HLPH-NH_2) are displayed in the top-right panel.

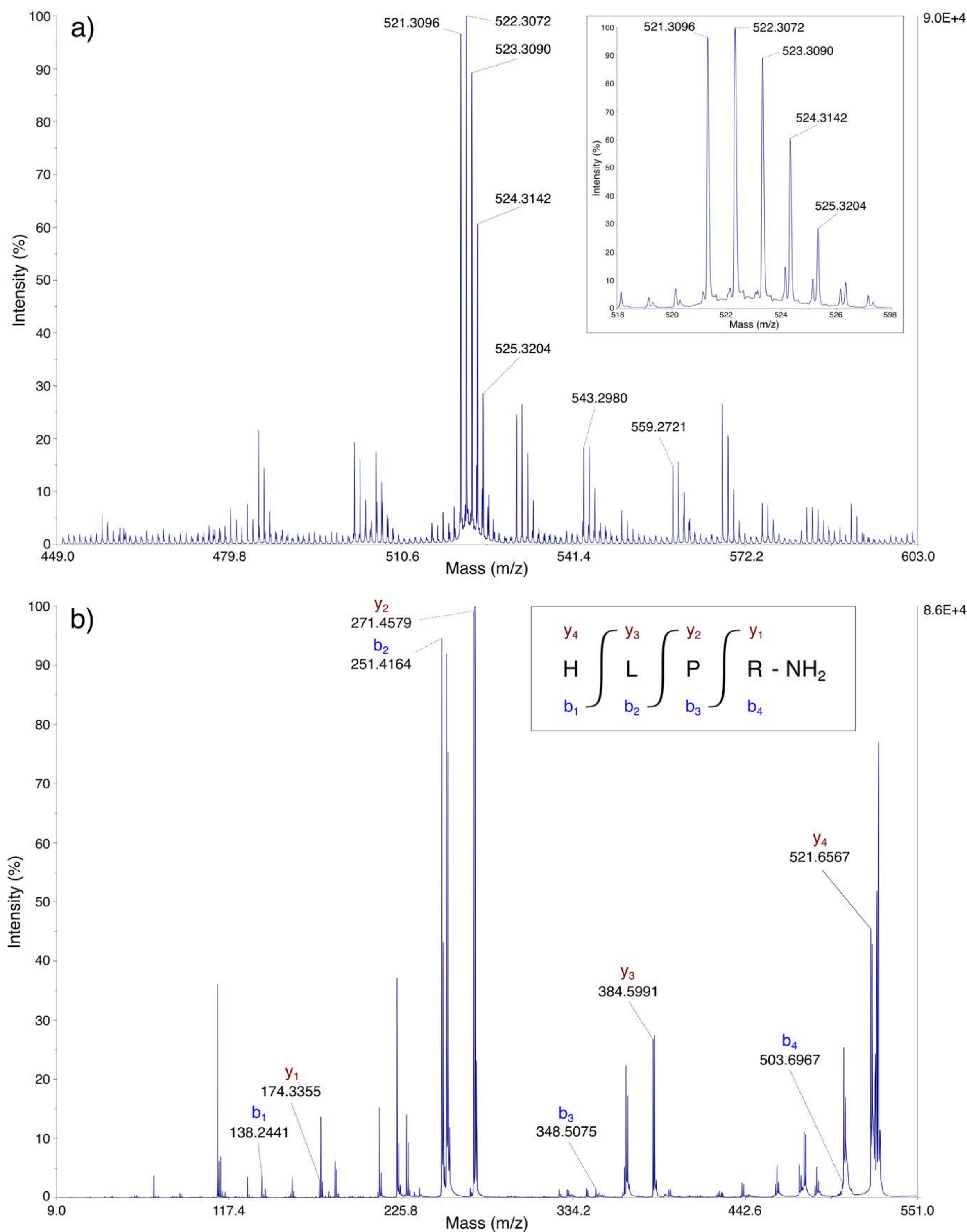


Figure 3. 4 MALDI-TOF MS and MS/MS spectra with calculated sequence of Lvar2. **a)** MALDI-TOF MS spectrum of native Lvar2 $[M+H]^+$ 521.3096 *m/z*. The $[M+Na]^+$ (543. 3142 *m/z*) and $[M+K]^+$ (559.2721 *m/z*) adducts are also visible within the spectrum. The top-right inset is an expanded view of the spectrum between 518.0-598.0 *m/z*. **b)** MALDI-TOF MS/MS fragmentation on a RP-HPLC fraction containing native Lvar2 with a precursor $[M+H]^+$ 521.309 *m/z*. The fragment ions and elucidated sequence (HLPR-NH_2) are displayed in the top-right panel.

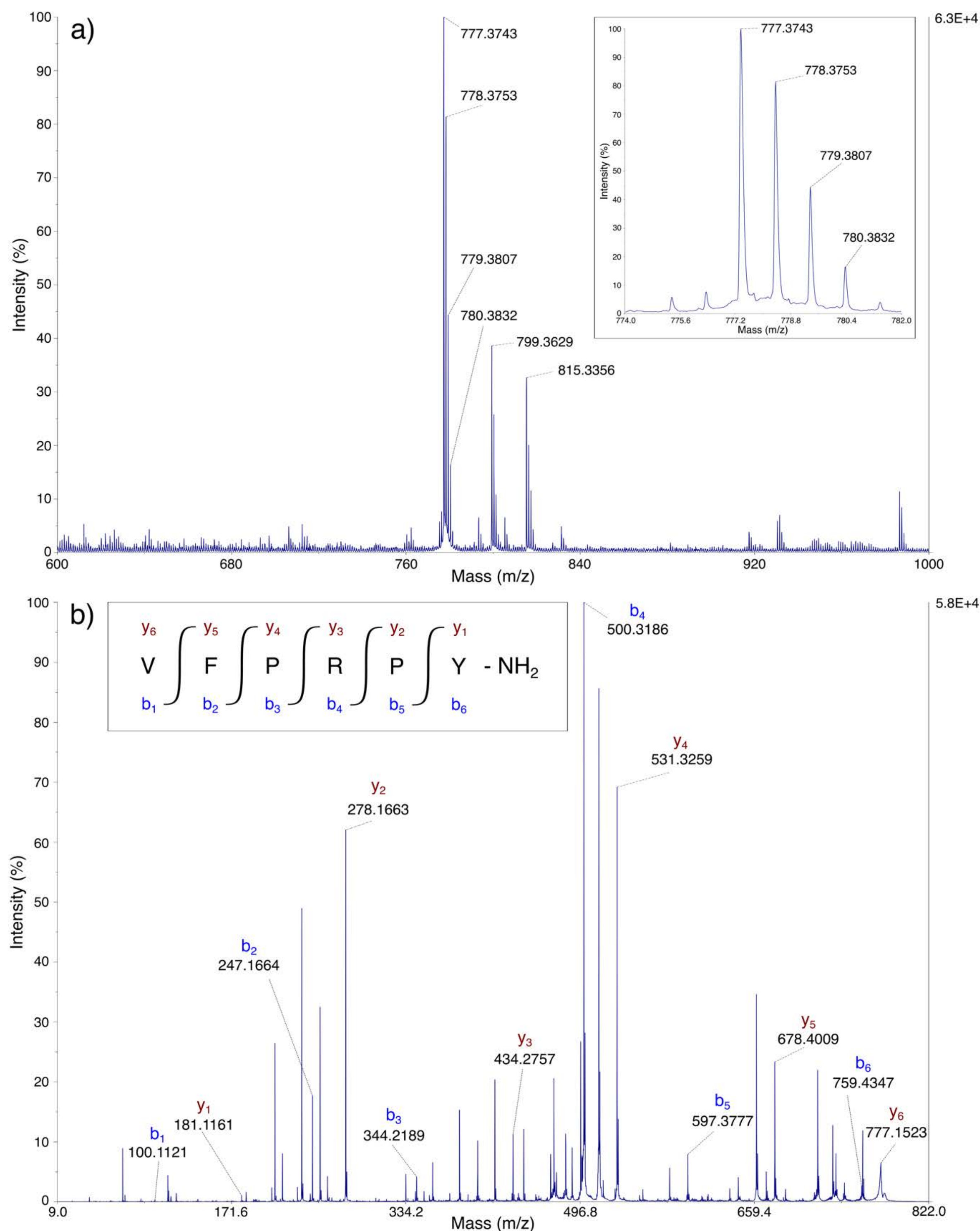


Figure 3. 5 MALDI-TOF MS and MS/MS spectra with calculated sequence of Lvar3. **a)** MALDI-TOF MS spectrum of native Lvar3 $[M+H]^+$ 777.3743 *m/z*. The $[M+Na]^+$ (799.3629 *m/z*) and $[M+K]^+$ (815.3356 *m/z*) adducts are also visible within the spectrum. The top-right inset is an expanded view of the spectrum between 774.0-782.0 *m/z*. **b)** MALDI-TOF MS/MS fragmentation on a RP-HPLC fraction containing native Lvar3 with a precursor $[M+H]^+$ 777.364 *m/z*. The fragment ions and elucidated sequence (VFPRPY-NH_2) are displayed in the top-left panel.

3.4.3. Chemical Synthesis

Synthetic versions of Lvar1, Lvar2, and Lvar3 were synthesised using Fmoc-based solid-phase peptide synthesis. Synthetic peptides were required for activity testing as *L. variatus* is a small scorpion and collection of sufficient native material is not feasible. Synthetic Lvar1, and Lvar3 coeluted with the native toxins using analytical RP-HPLC indicating the synthetic molecule was the same as the native (Supplementary Materials 3.4-3.5). Coelution of Lvar2 was not possible due to instability and degradation of the native material. MS/MS performed on the three synthetic molecules showed the same MS/MS fragmentation pattern as the counterpart native molecules (Supplementary Materials 3.6-3.8).

3.4.4. Scorpion and Cricket Bioassay

Injection of 1 μL containing 0.451 μg Lvar1, 0.761 μg Lvar2, and 0.437 μg Lvar3 had no clearly discernible toxic or behavioural effects on adult female scorpions (χ^2 = 4.4785, df = NA, p = 0.2730) and crickets (χ^2 = 6.4, df = NA, p = 0.2291) during 5 min post-injection (Figure 3.6). Of the eight female scorpions injected with Lvar1, none displayed symptoms following injection except one female appeared slower than normal at 3 min following injection. As her behaviour appeared normal at the 1 min and 5 min time points, it is unlikely that this was associated with Lvar1 activity. Additionally, all eight crickets injected with Lvar1 were unaffected. None of the eight female scorpions injected with Lvar2 showed signs of abnormal behaviour at 1 min, 3 min, nor 5 min following injection. Two out of the eight crickets became paralysed in one of their back legs following injection of Lvar2, but this was witnessed in past bioassay work, appearing to be a side-effect of the injection method in crickets, perhaps caused by damaging a nerve with the needle. Two out of the eight female scorpions injected with Lvar3 appeared slow at the 1 min, 3 min and 5 min timepoints following injection, however the other six appeared unaffected following the injection. Given the low number of scorpions affected, it is more likely that their behaviour was impacted by damage caused by the needle during injection rather than Lvar3 activity. It was noted that one of these scorpions was difficult to catch and restrain, and therefore exhaustion likely explained her slow behaviour post injection. In a positive control using crude *U. yaschenkoi* venom, seven out of ten female *L. variatus* displayed either spasming or complete paralysis, whilst three appeared unaffected. Crude male *L. variatus* was also tested in ten adult females. Following injection, one became paralysed, two appeared to be slow, whilst seven displayed no symptoms. Male *L. variatus* venom caused paralysis in all eight crickets injected. A negative control of ringer's solution had no effect on ten female *L. variatus* injected, nor eight crickets (Figure 3.6).

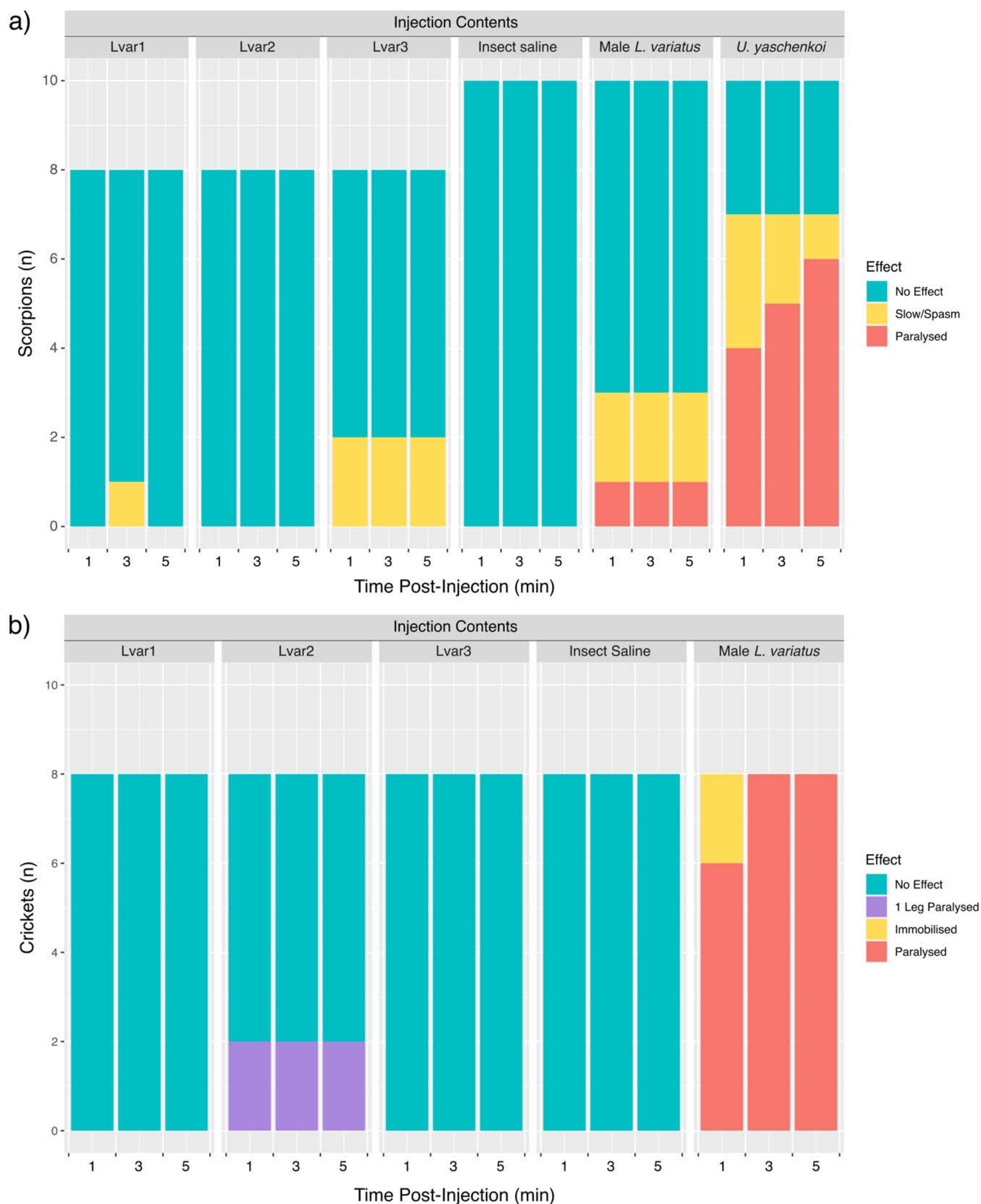


Figure 3. 6 Results of *in vivo* bioassays performed with synthetic peptides and crude venom in **a)** female scorpions (*Lychas variatus*), and **b)** adult female crickets (*Acheta domesticus*). Lvar1, Lvar2, Lvar3, and crude adult male *L. variatus* venom were tested in both crickets and female scorpions. Female scorpions were additionally injected with crude *Urodacus yaschenkoi* venom as a positive control due to the observed lack of toxicity of male *L. variatus* venom.

3.4.5. Ion Channel Assay

2 µg of synthetic Lvar1, Lvar2 and Lvar3 showed no activity against the suite of ion channels tested in patch-clamp assays. K_V subtypes included rK_V1.1, rK_V1.2, hK_V1.3, rK_V1.4, rK_V1.5, rK_V1.6, rK_V2.1, hK_V10.1, hERG, and Shaker IR. Na_V subtypes included rNa_V1.2, rNa_V1.4, hNa_V1.5, mNa_V1.6, hNa_V1.7, hNa_V1.8, BgNa_V1, VdNa_V1.

3.5. Discussion

To identify if male venoms contained molecules that may be specifically involved in sexual stinging, I examined *L. variatus* venoms for molecules that were only present in adult males, and that did not appear to be structurally related to typical venom peptides that are known to vary between the sexes. With these criteria, Lvar1, Lvar2, and Lvar3 were selected as potential candidates. The molecules were isolated and characterised, and found to be two tetrapeptide-amides (Lvar1 and Lvar2) and a hexapeptide-amide (Lvar3) structurally unlike previously characterised scorpion venom peptides. These molecules were found to be non-toxic towards crickets, and not to be active upon a suite of Na_v and K_v channel subtypes. The combination of their structural novelty, apparent lack of toxicity in crickets, and presence only within adult male venoms, makes them strong candidates to be involved in sexual stinging.

The function of sexual stinging in scorpions remains an open debate, and at present the underlying chemical mechanisms are unknown. It has been suggested sexual stings could serve to supress predation of the male during courtship (Inceoglu et al., 2003;Lira et al., 2018), though females can appear to be non-aggressive during and in response to sexual stings (Jiao and Zhu, 2010;Olivero et al., 2019), and sexual cannibalism may not be particularly prevalent in scorpions (Peretti et al., 2008). Sexual stings could serve an anti-predation function, but they may alternatively improve reproductive success by another mechanism, for example by increasing the receptiveness of the female by chemical signalling or immunostimulation (Olguín-Pérez et al., 2021). Venom transference from male to female could improve mating success via multiple mechanisms, for example by providing antimicrobial protection from specific venom peptides or by modulating reproductive hormones (Olguín-Pérez et al., 2021). Chemical signals are known to affect female receptiveness during the courtship of scorpions (Peretti, 1997;Olivero et al., 2015), and if venom transference is occurring in sexual stings they could act as another avenue of chemical communication. Additionally, as sexual stings are performed prior to mating, they could serve as honest indicators of male fitness (Olguín-Pérez et al., 2021). Interestingly, the duration of sexual stings in *Bothriurius bonariensis* was found to be positively correlated to the body condition of the

female, suggesting sexual stinging could be associated with mate choice (Olivero et al., 2019). If sexual stinging impacts mate choice, selection acting upon genes coding for venom peptides involved in this behaviour could contribute to the large intersexual venom variation observed across different scorpion species (Peretti, 2014;Sentenská et al., 2017;Ward et al., 2018;Olguín-Pérez et al., 2021), and may explain the presence of Lvar1, Lvar2, and Lvar3 only in adult males.

Lvar1, Lvar2 and Lvar3 do not appear to be structurally similar to typical scorpion venom toxins. Scorpion venoms contain a mixture of structurally diverse molecules, many of which are disulfide-bonded peptides that act upon ion channels (Possani et al., 2000;Zhijian et al., 2006;Cid-Uribe et al., 2020), and whilst non-disulfide-bonded peptides (NDBPs) are also present (Zeng et al., 2005;de la Vega et al., 2010;Valdez-Velázquez et al., 2013;Almaaytah and Albalas, 2014;Harrison et al., 2014) there are few examples of NDBPs as small as Lvar1, Lvar2, and Lvar3. Small NDBPs often have similar properties to neuropeptides – a diverse group of small proteinaceous molecules secreted by neurons and involved in cell signalling (Hökfelt et al., 2000;Burbach, 2011). An example is FMRFamide (Phe-Met-Arg-Phe-NH_2), a tetrapeptide-amide like Lvar1 and Lvar2, which is a member of closely related and highly conserved family of neuropeptides present in different phyla involved in feeding and reproduction (Price and Greenberg, 1977;Walker et al., 2009;Quillet et al., 2016). Cell-signalling molecules such as neuropeptides, neuropeptide-like molecules, and hormones are important venom constituents in a taxonomically diverse range of animals, including cone snails (Cruz et al., 1987;Robinson et al., 2017), snakes (Stewart et al., 1971), varanid lizards (Fry et al., 2010), wasps (Konno et al., 2016;Arvidson et al., 2019;Nihei et al., 2021), spiders (McCowan and Garb, 2014), centipedes (Undheim et al., 2015), and are also present in the defensive secretions of frogs (Montecucchi et al., 1984;Gozzini et al., 1985;Bowie and Tyler, 2006). Neuropeptide-like molecules have also been characterised from scorpion venoms, but presently there are few examples. At the time of writing, a search of the UniProt database (https://www.uniprot.org/) shows 29 scorpion venom peptides ten amino acids in length or less, four of which are under seven amino acids long, displaying both structural and functional diversity.

The known scorpion venom peptides under ten amino acids in length exhibit a diverse range of activities. The smallest known scorpion peptides, and closest in size to Lvar1 and Lvar2, includes two tetrapeptides found in *Pandanus imperator* venom named tetrapandins (Shalabi et al., 2004). Tetrapandin 1 (LWSG) and tetrapandin 2 (LWKT) are known to interact with store-operated channels to mediate store-operated Ca^{2+} entry (Shalabi et al., 2004). A tetrapeptide with a similar sequence to tetrapandin-2 called tetrascorpin-1, AaTs-1, was also recently characterised from *Androctonus australis* venom (Aissaoui-Zid et al., 2021). AaTs-1 (IWKS) was shown to enhance expression of tumor suppressor protein p53 and the G-coupled formyl-peptide receptor FPRL-1, likely leading to

inhibition of store-operated Ca^{2+} entry (Aissaoui-Zid et al., 2021). Whilst the AaTs-1 and the tetrapandins appear to be structurally and functionally related, a different tetrapeptide [des-Arg^1]-proctolin (YLPT) from *Tityus serrulatus* venom has close sequence similarity to the insect neuropeptide proctolin (RYLPT) (Duzzi et al., 2016). [des-Arg^1]-proctolin inhibits both human neprilysin and neprilysin-like enzymes in cockroaches, suggesting a possible role in both defensive and predatory stings. The difference in the activities of these known tetrapeptides show that, although Lvar1 and Lvar2 are of equal size, their activity is unlikely to be correlated as their sequences are unrelated. Other activities of these small peptides include a pentapeptide KEILG from *Tityus serrulatus* venom that inhibits thimet oligopeptidase (EP24.15) activity (Carvalho et al., 2013), whilst peptides 7.1 (RLRSKGKK), 7.2 (RLRSKG) and 8 (KIWRS) from *Tityus* serrulatus were found to modulate macrophage responses to increase IL-6 production and inhibit angiotensin-converting enzyme (Pucca et al., 2016). Additionally, *Bm*K-YA (YGGYMNPA-NH_2) described from *Buthus martensii* shows sequence similarity to enkephalin, and interacts with opioid G-coupled protein receptors (Zhang et al., 2012). This functional diversity demonstrates that, without observing sequence similarity of Lvar1, Lvar2, and Lvar3 with other known small peptides, it is not possible to predict their activity or mechanism of action. A search of the NeuroPep database (http://isyslab.info/NeuroPep) showed that Lvar1, Lvar2, and Lvar3 do not share close sequence similarity to currently known neuropeptides. Additionally, many closely related neuropeptides share C-terminal motifs; for example, the RFamides present in cone snail and wasp venoms end in a C-terminal RF (Maillo et al., 2002;Robinson et al., 2015;Konno et al., 2016). The three novel peptides reported here, however, all differ at the C-terminal. Lvar1 (HLPH-NH_2) and Lvar2 (HLPR-NH_2) are identical except for their C-terminal residue, whilst Lvar3 (VFPRPY-NH_2) is overall different.

Lvar1, Lvar2, and Lvar3 showed no activity against a suite of sodium and potassium voltage-gated ion channels, which are common targets for conventional scorpion disulfide-bonded peptides (Cid-Uribe et al., 2020). This lack of activity is not surprising, given the large structural difference between the novel peptides and conventional scorpion neurotoxins. Additionally, the peptides appeared to be non-toxic towards crickets in *in vivo* assays, with no clear response observed after intrathoracic injection. This suggests that the molecules may not be present in the venom to aid in the incapacitation of insect prey; however, this cannot be ruled out until the mode of action of these molecules is established.

As the novel male-specific molecules show close resemblance to cell-signalling molecules rather than neurotoxins, and additionally appear to be non-toxic in crickets, this supports the hypothesis they could be involved in sexual stinging. I therefore tested these molecules in adult female scorpions, but there was no significant or discernible effect on their behaviour post-injection.

This suggests that if these molecules are indeed involved in sexual stinging, they either have an effect which is not directly observable, or they do not alter behaviours outside of the context of courtship. The method used to test female behaviour might not capture changes that are closely tied to receptiveness in courtship. For example, if these molecules were making a female more receptive to a male's advances and making her movements appear slow, it may only be observed in the presence of a male or during the act of courtship. The females' behaviour post-injection was tested by touching the scorpions with forceps (as they otherwise remained motionless), and this likely to be interpreted as a threat by the female (in addition to being restrained for injection). Behavioural responses under these conditions may therefore not reflect those in response to a sexual sting in the absence of threat. It is also possible that the method of injection was not effective to elicit a response. The location of sexual stings appears to be important, with some species always stinging in the joint of the pedipalp (Olguín-Pérez et al., 2021), while others frequently inject into the mesosoma (Lira et al., 2018). As I injected the molecules deep into the abdomen of the females, this may not have been the most appropriate location for the molecules to have an effect. As *L. variatus* are small scorpions, deep injection was required to reduce the risk of the leaking haemolymph washing out the injected peptides when the needle was withdrawn. The expulsion of haemolymph suggests that injection via needle is likely more invasive than a sexual sting, as it was recorded that no haemolymph was emitted during or after the sexual stings of *Bothriurus buecherli*, despite insertion of the aculeus into the female appearing intense (Toscano-Gadea, 2010). Pilot controls with a shallow injection of crude *U. yaschenkoi* venom showed little to no effect, indicating that the venom had been washed out when the needle was withdrawn. With a deeper injection, the effectiveness of the positive control was 70% (Figure 3.6). Although measures were taken to reduce the amount of haemolymph leaking, such as loosely restraining the scorpions with parafilm and withdrawing the needle slowly, it is possible that leaking haemolymph may still have washed some of the peptides out, which could explain the lack of observable effect. Interestingly, whilst injection of crude *U. yaschenkoi* venom caused toxicity in the females, the majority of scorpions injected with crude male *L. variatus* venom appeared unaffected, suggesting male venom may not have toxic effects in the females, but future work is required to test this. Unfortunately, it is near impossible to test the effect of crude male venom and the isolated molecules under conditions that would accurately mimic those during courtship in a natural setting. Understanding if these molecules are truly involved in sexual stinging would likely require knowledge of their molecular targets and activities.

Although it has recently been demonstrated that sexual stings can involve venom injection (Olguín-Pérez et al., 2021), it is not known if there is controlled transference of a specific set of

molecules. Despite *P. transvaalicus* not typically using a sexual sting (Olivero et al., 2019), Inceoglu et al. (2003) first suggested the low toxicity, but paralytic, prevenom of *P. transvaalicus* could be injected in sexual stings to avoid causing toxic effects in the female. Data collected from the sexually stinging species *Euscorpius alpha* also suggests there may be some association between sexual stings and prevenom, with large differences observed in the venom gland morphology of males and females, including the types and proportions of secretory cells present (Sentenská et al., 2017). Almost 90% of the secretory cells in male venom glands were type A cells containing no protein granules, compared to less than 25% type A cells in female glands (Sentenská et al., 2017). The authors suggest, but have not confirmed, that type A secretory cells may produce prevenom, following the assumption that prevenom of *E. alpha* likely contains a lower protein concentration akin to *P. transvaalicus* prevenom (Inceoglu et al., 2003;Sentenská et al., 2017). Type A cells were also only distally located in female *E. alpha* venom glands, possibly explaining why prevenom appears first when venom is expelled from the aculeus (Sentenská et al., 2017). As Lvar1, Lvar2 and Lvar3 were characterised from venom collected via electrostimulation, the peptides may be present in either or both prevenom/main venom. Future examination of the presence of these molecules in both natural defensive stings, and within prevenom versus milky venom may shed more light on their role in sexual stinging, and additionally on the role of prevenom in sexual stings.

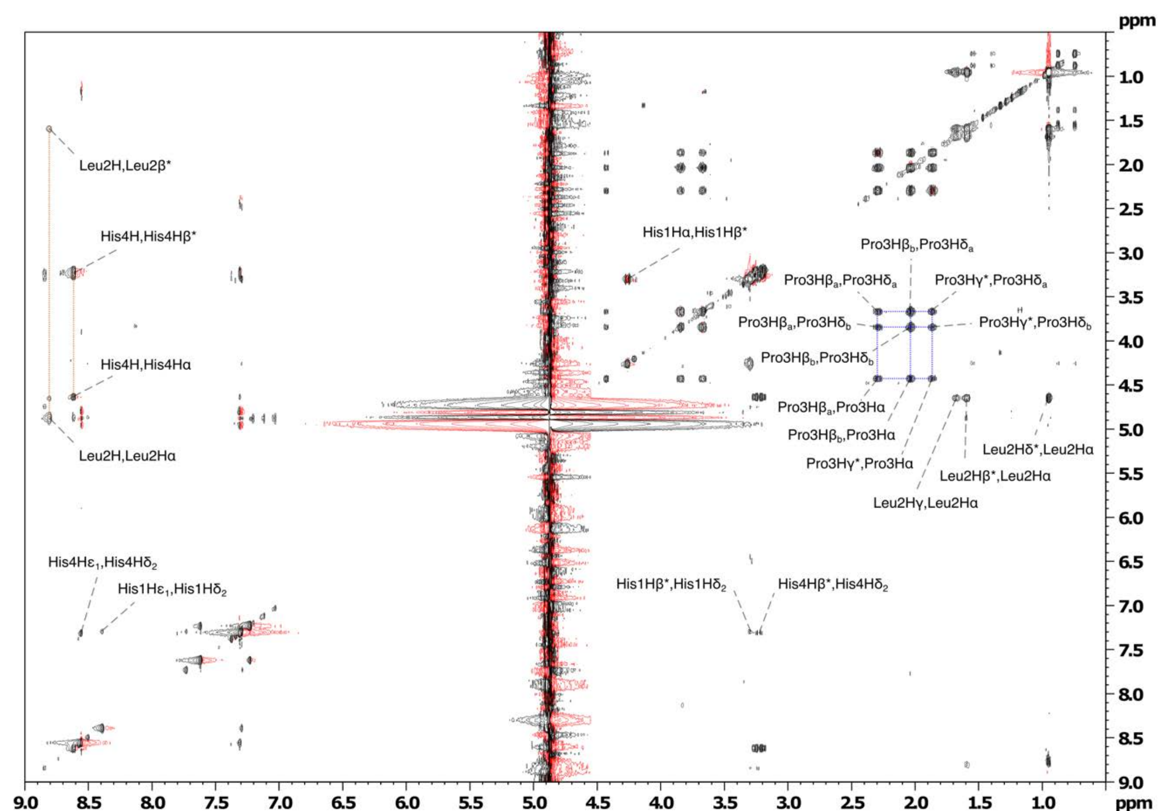
An alternative explanation for the appearance of these molecules in the venom of adult males is that they may be hormones involved in maturation that have either made their way into the venom naturally, or have leaked into the venom expelled during milking because of cell damage. Preliminary LC/MS analysis on the haemolymph of both an adult male and female *L. variatus* and did not reveal the presence of these peptides at a detectable level, but this was not sufficiently in-depth to confirm their absence.

3.6. Conclusions and Future Directions

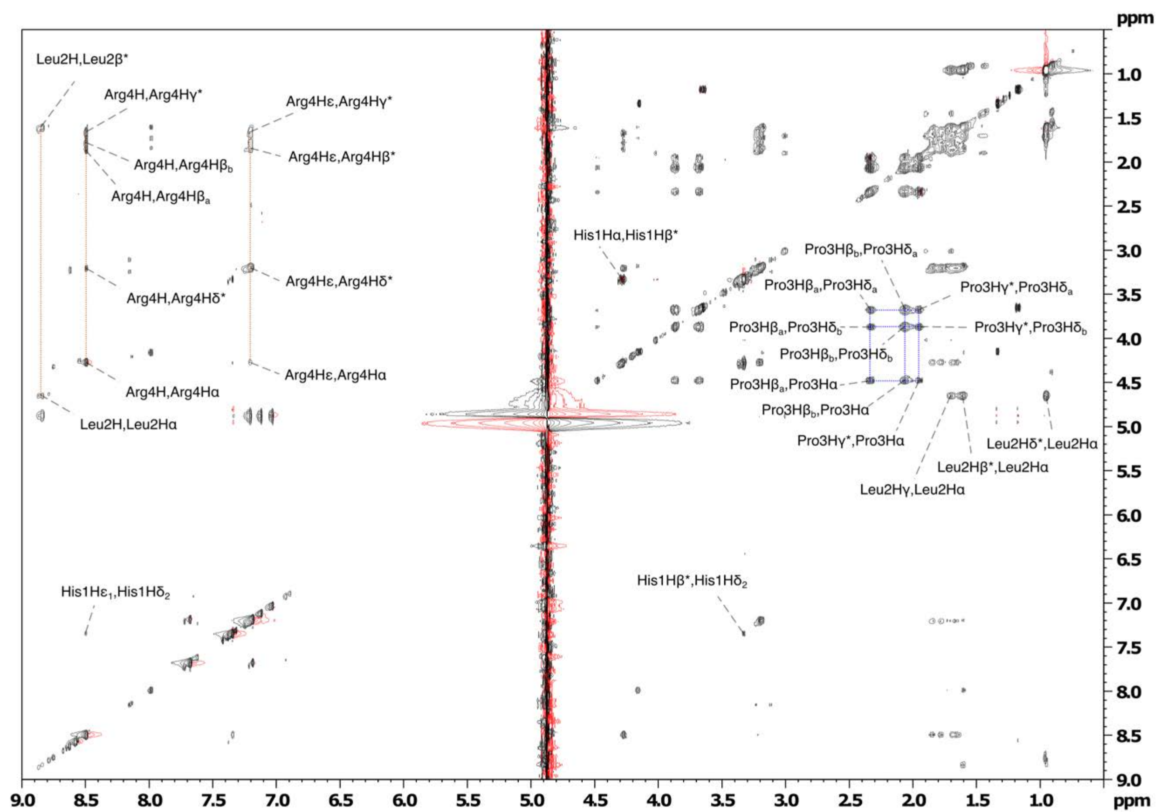
Three novel peptides were isolated from *L. variatus* venom, that appeared only in the venom of adult males. Their very small size and unique sequences sets them apart from known scorpion venom peptides, and they are structurally more like cell-signalling molecules than typical scorpion toxins. These results show that at the concentration tested these molecules are not toxic to crickets, did not affect the behaviour of female *L. variatus*, and had no activity on a suite of Na_V and K_v channels at the concentration tested. The synthesis of these results suggests the molecules may serve a function in sexual stinging, but at present their involvement in this behaviour cannot be confirmed.

If Lvar1, Lvar2, and Lvar3 are involved in sexual stinging, the observed lack of effect on female behaviour suggests they could serve an immunomodulatory function, act as an indicator of fitness, or modulate hormones associated with female receptiveness or reproduction. Future work could test this by investigating the effect of Lvar1, Lvar2, and Lvar3 on the endocrine system of female scorpions, by monitoring hormone levels in the haemolymph before and alter injection of venom peptides. Additionally, identifying the molecular targets of these peptides will ultimately help understand if their activity translates to a role in sexual stinging. In a wider context, screening the venoms of other scorpion species (particularly those documented to sexually sting) for other structurally related peptides, and adult male-specific components, could help improve our understanding of sexual stinging behaviours in scorpions.

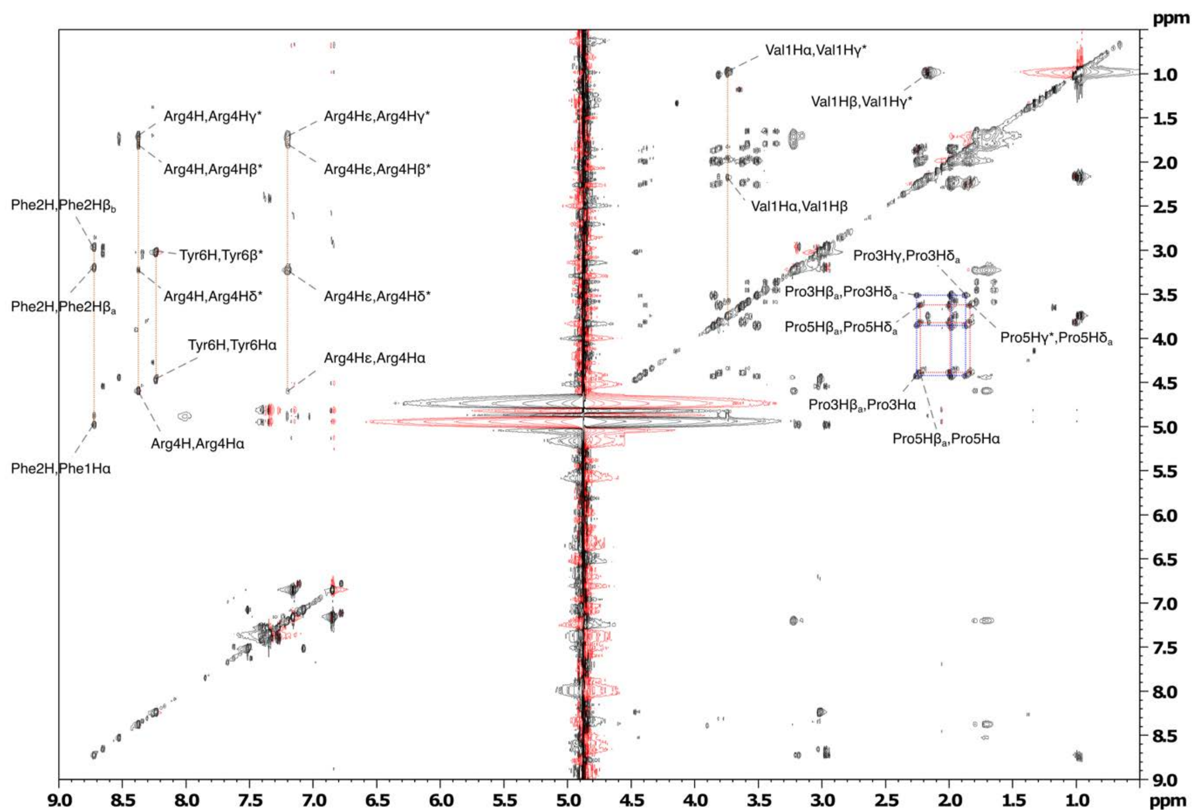
3.7. Supplementary Materials



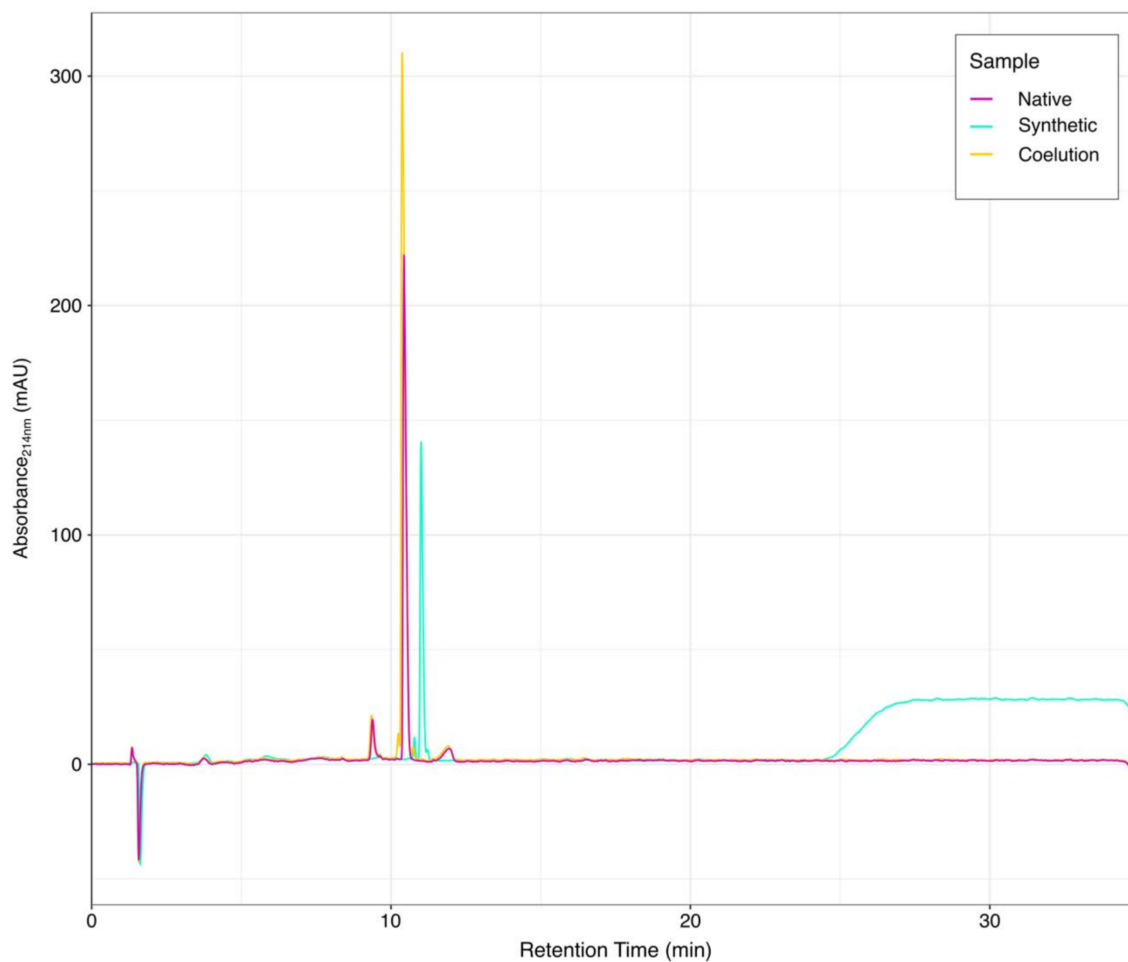
Supplementary Materials 3. 1 TOCSY 2D NMR spectrum of a HPLC fraction of crude adult male *Lychas variatus* venom containing Lvar1. Vertical orange lines represent peak interactions in the amide region (top-left), and blue lines represent interactions between the protons in the proline (top-right).



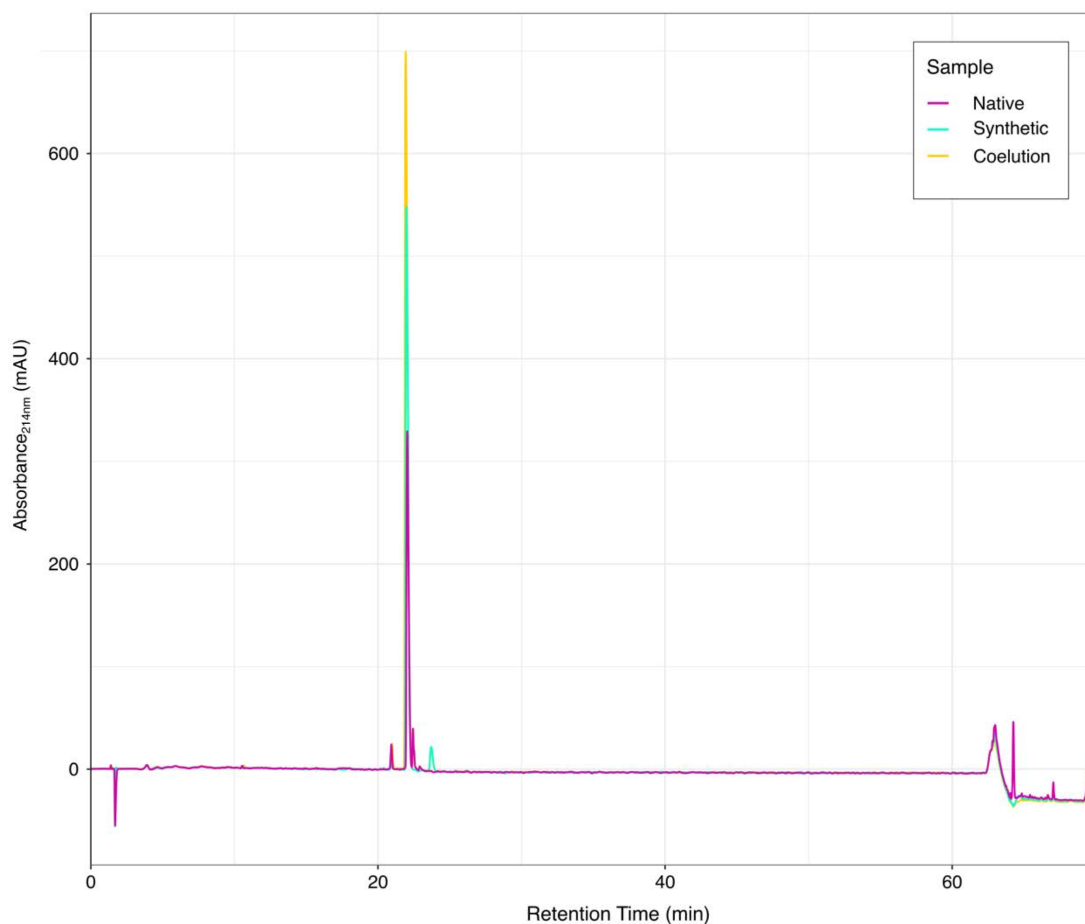
Supplementary Materials 3. 2 TOCSY 2D NMR spectrum of a HPLC fraction of crude adult male *Lychas variatus* venom containing Lvar2. Vertical orange lines represent peak interactions in the amide region (top-left), and blue lines represent interactions between the protons in the proline (top-right).



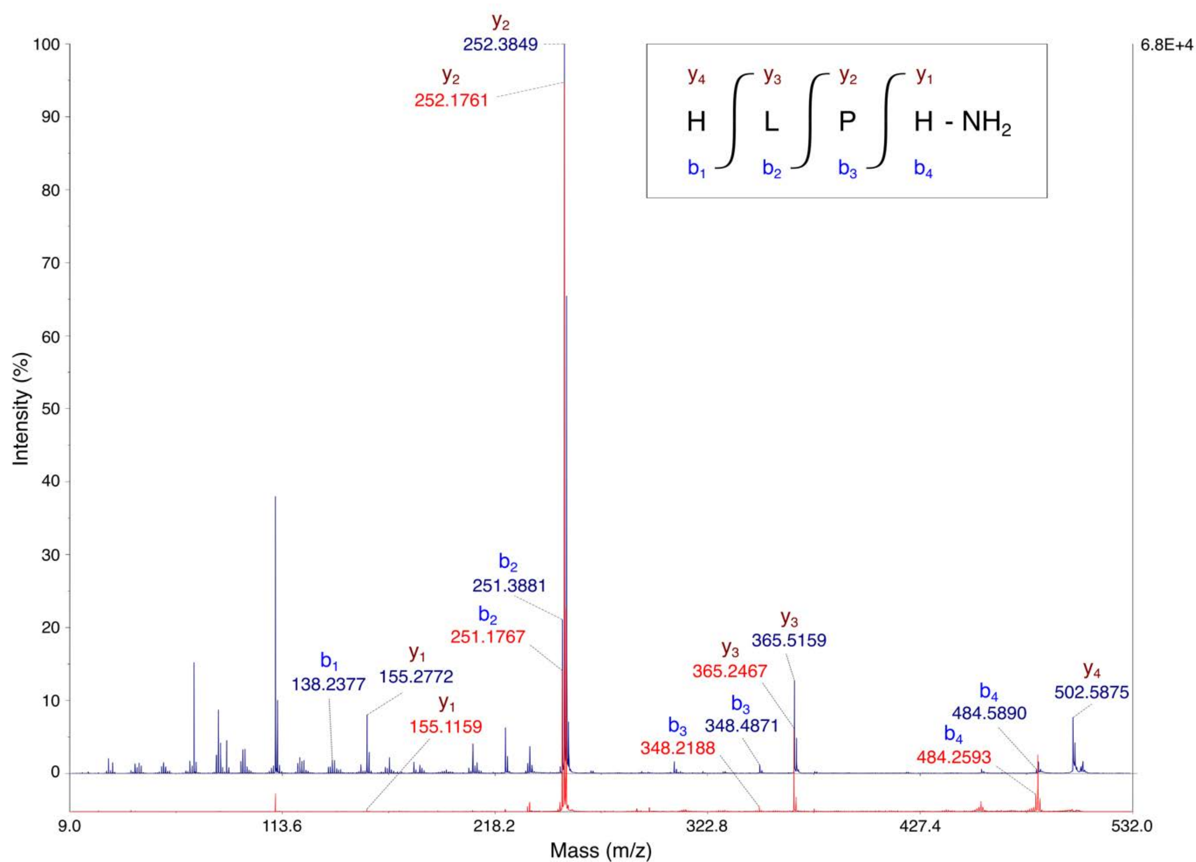
Supplementary Materials 3. 3 TOCSY 2D NMR spectrum of a HPLC fraction of crude adult male *Lychas variatus* venom containing Lvar3. Vertical orange lines represent peak interactions in the amide region (top-left), and for valine 1 (top-right). Blue and red lines represent interactions between the protons in proline 3 and proline 5 respectively (top-right). Not all proline peaks are labelled for simplicity.



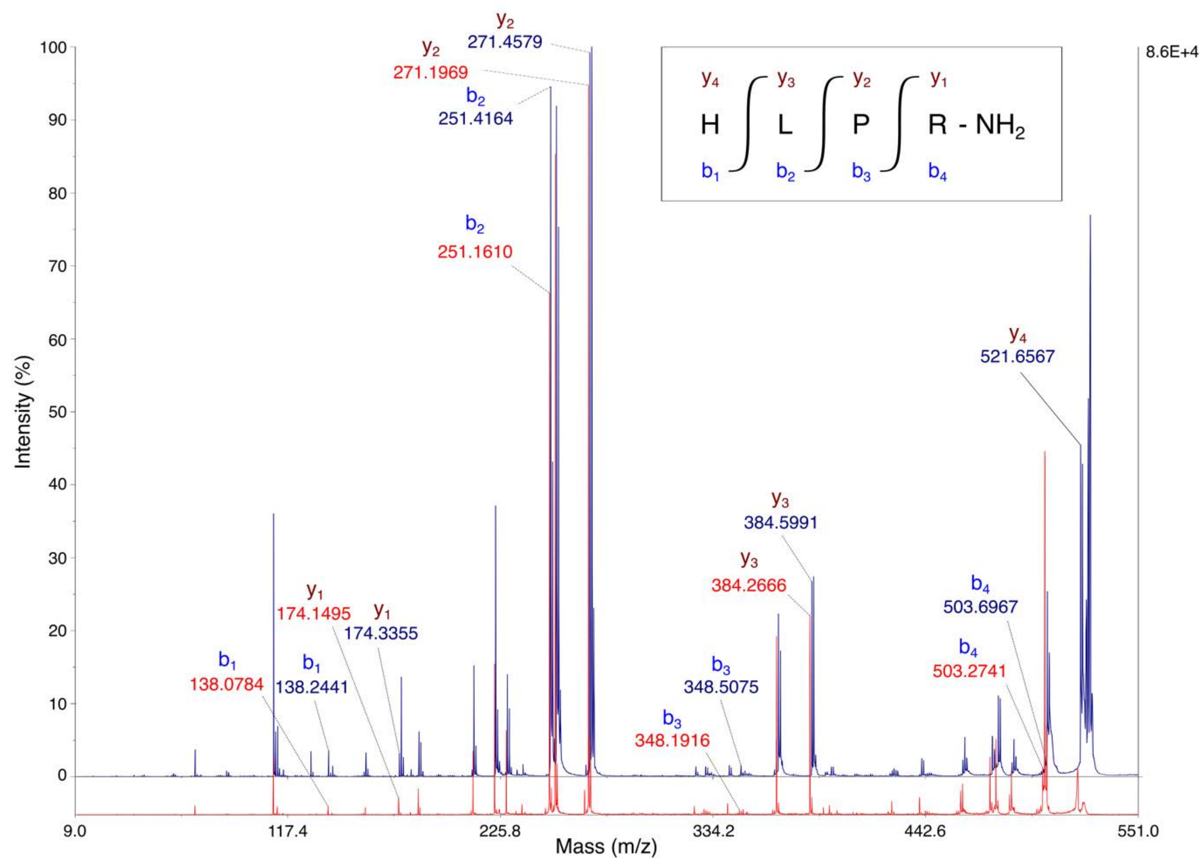
Supplementary Materials 3. 4 Reversed-phase high performance liquid chromatography (RP-HPLC) chromatogram showing coelution of synthetic and native Lvar1 (Agilent Eclipse Plus C_{18}, 100 x 4.6 mm, 3.5 µm; 1mL/min flowrate; solvent A H_2O/0.05% TFA, solvent B 90% ACN/H_2O/0.045% TFA; gradient solvent B (0-8% B 0-8 min; 8-14% B 8-32 min; 14-0% B 32-35 min), absorbance at 214 nm). Native Lvar1 is plotted in pink, synthetic in blue, and the coelution in yellow.



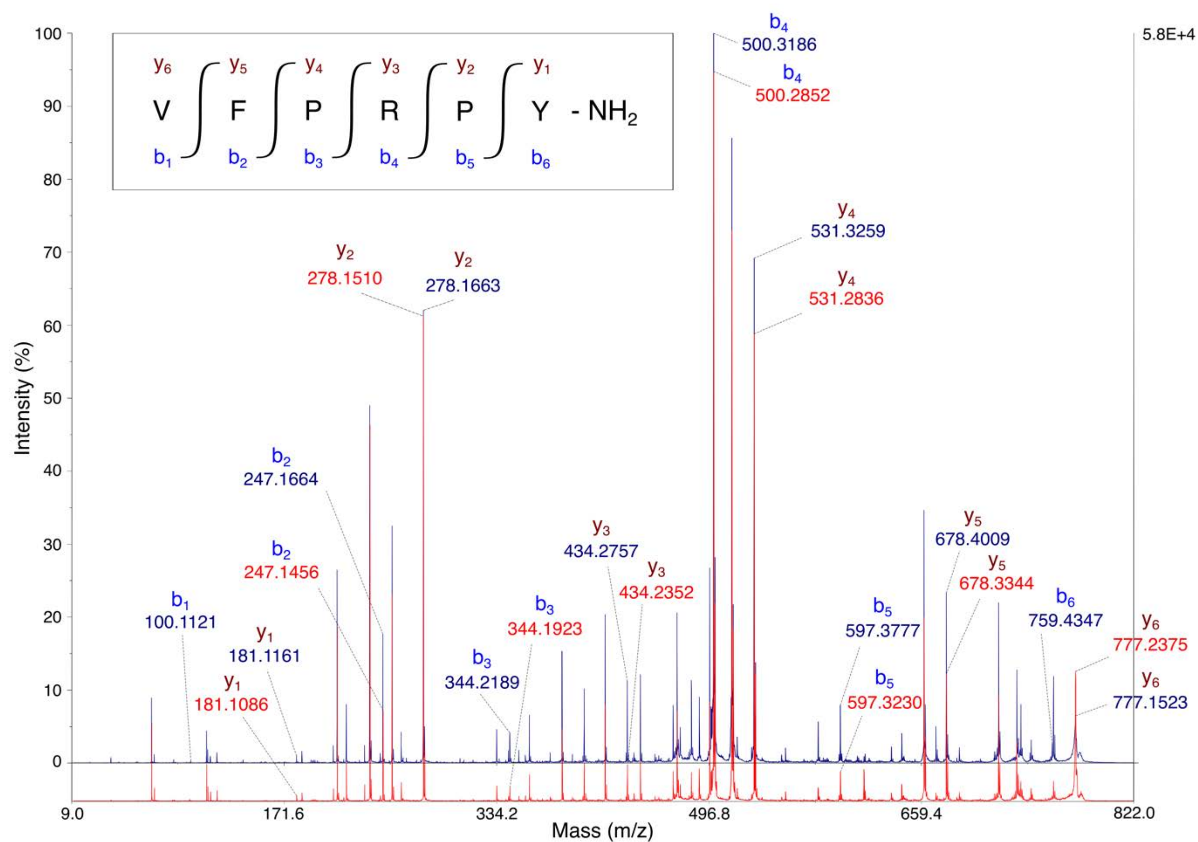
Supplementary Materials 3. 5 Reversed-phase high performance liquid chromatography (RP-HPLC) chromatogram showing coelution of synthetic and native Lvar3 (Agilent Eclipse Plus C_{18}, 100 x 4.6 mm, 3.5 µm; 1mL/min flowrate; solvent A H_2O/0.05% TFA, solvent B 90% ACN/H_2O/0.045% TFA; gradient solvent B (0-20% B 0-20 min; 20-30% B 20-60 min; 30-90% B 60-62 min; 90% B 62-67 min; 90-0% B 67-70 min), absorbance at 214 nm). Native Lvar3 is plotted in pink, synthetic in blue, and the coelution in yellow.



Supplementary Materials 3. 6 MALDI-TOF MS/MS fragmentation of synthetic Lvar1 (red) and a RP-HPLC fraction containing native Lvar1 (blue) with a precursor $[M+H]^+$ 502.39 *m/z* and $[M+H]^+$ 502.26 *m/z* respectively. The fragment ions and elucidated sequence (HLPH-NH_2) are displayed in the top-right panel.



Supplementary Materials 3. 7 MALDI-TOF MS/MS fragmentation of synthetic Lvar2 (red) and a RP-HPLC fraction containing native Lvar2 (blue) with a precursor $[M+H]^+$ 521.43 *m/z* and $[M+H]^+$ 521.309 *m/z* respectively. The fragment ions and elucidated sequence (HLPR-NH_2) are displayed in the top-right panel.



Supplementary Materials 3. 8 MALDI-TOF MS/MS fragmentation of synthetic Lvar3 (red) and a RP-HPLC fraction containing native Lvar3 (blue) with a precursor $[M+H]^+$ 777.569 *m/z* and $[M+H]^+$ 777.364 *m/z* respectively. The fragment ions and elucidated sequence (HLPH-NH_2) are displayed in the top-left panel.

3.8. Bibliography

Abdel-Rahman, M.A., Omran, M.a.A., Abdel-Nabi, I.M., Ueda, H., and Mcvean, A. (2009). Intraspecific variation in the Egyptian scorpion *Scorpio maurus palmatus* venom collected from different biotopes. *Toxicon* 53**,** 349-359.

Aissaoui-Zid, D., Saada, M.-C., Moslah, W., Potier-Cartereau, M., Lemettre, A., Othman, H., Gaysinski, M., Abdelkafi-Koubaa, Z., Souid, S., and Marrakchi, N. (2021). AaTs-1: a tetrapeptide from *Androctonus australis* scorpion venom, inhibiting U87 glioblastoma cells proliferation by p53 and FPRL-1 up-regulations. *Molecules* 26**,** 7610.

Alexander, A.J. (Year). "Courtship and mating in the buthid scorpions", in: *Proceedings of the Zoological Society of London*: Wiley Online Library), 145-169.

Almaaytah, A., and Albalas, Q. (2014). Scorpion venom peptides with no disulfide bridges: a review. *Peptides* 51**,** 35-45.

Arbuckle, K. (2017). Evolutionary context of venom in animals. *Evolution of Venomous Animals and their Toxins* 24**,** 3-31.

Arvidson, R., Kaiser, M., Lee, S.S., Urenda, J.-P., Dail, C., Mohammed, H., Nolan, C., Pan, S., Stajich, J.E., and Libersat, F. (2019). Parasitoid jewel wasp mounts multipronged neurochemical attack to hijack a host brain. *Molecular & Cellular Proteomics* 18**,** 99-114.

Batista, C.V., D'suze, G., Gómez-Lagunas, F., Zamudio, F.Z., Encarnación, S., Sevcik, C., and Possani, L.D. (2006). Proteomic analysis of *Tityus discrepans* scorpion venom and amino acid sequence of novel toxins. *Proteomics* 6**,** 3718-3727.

Binford, G.J., Gillespie, R.G., and Maddison, W.P. (2016). Sexual dimorphism in venom chemistry in *Tetragnatha* spiders is not easily explained by adult niche differences. *Toxicon* 114**,** 45-52.

Bowie, J.H., and Tyler, M.J. (2006). "Host defense peptides from Australian amphibians: caerulein and other neuropeptides," in *Handbook of Biologically Active Peptides*. Elsevier), 283-289.

Briceño, R.D., and Bonilla, F. (2009). Substrate vibrations in the scorpion *Centruroides margaritatus* (Scorpiones: Buthidae) during courtship. *Revista de Biología Tropical* 57**,** 267-274.

Burbach, J.P.H. (2011). "What are neuropeptides?," in *Neuropeptides*. Springer), 1-36.

Carlson, B.E., Mcginley, S., and Rowe, M.P. (2014). Meek males and fighting females: sexually-dimorphic antipredator behavior and locomotor performance is explained by morphology in bark scorpions (*Centruroides vittatus*). *PLoS One* 9**,** e97648.

Carrera, P.C., Mattoni, C.I., and Peretti, A.V. (2009). Chelicerae as male grasping organs in scorpions: sexual dimorphism and associated behaviour. *Zoology* 112**,** 332-350.

Carvalho, D.C., Duzzi, B., Kuniyoshi, A.K., Fioramonte, M., Gozzo, F.C., Melo, R.L., Tambourgi, D.V., Rioli, V., and Portaro, F.C. (2013). Insights into scorpion venom peptides: Alternative processing of β-KTx propeptide from *Tityus serrulatus* venom results in a new naturally occurring thimet oligopeptidase inhibitor. *Peptides* 40**,** 30-33.

Chantall-Rocha, S., and Japyassú, H.F. (2017). Diffuse resistance courtship in the scorpion *Rhopalurus rochai* (Scorpiones: Buthidae). *Behavioural Processes* 135**,** 45-55.

Cid-Uribe, J.I., Veytia-Bucheli, J.I., Romero-Gutierrez, T., Ortiz, E., and Possani, L.D. (2020). Scorpion venomics: a 2019 overview. *Expert Review of Proteomics* 17**,** 67-83.

Cruz, L.J., De Santos, V., Zafaralla, G.C., Ramilo, C.A., Zeikus, R., Gray, W.R., and Olivera, B.M. (1987). Invertebrate vasopressin/oxytocin homologs. Characterization of peptides from *Conus geographus* and *Conus straitus* venoms. *Journal of Biological Chemistry* 262**,** 15821-15824.

De La Vega, R.C.R., Schwartz, E.F., and Possani, L.D. (2010). Mining on scorpion venom biodiversity. *Toxicon* 56**,** 1155-1161.

De Sousa, L., Borges, A., Vásquez-Suárez, A., Den Camp, H.J.O., Chadee-Burgos, R.I., Romero-Bellorín, M., Espinoza, J., De Sousa-Insana, L., and Pino-García, O. (2010). Differences in venom toxicity and antigenicity between females and males *Tityus nororientalis* (Buthidae) scorpions. *Journal of venom research* 1**,** 61.

Duzzi, B., Cajado-Carvalho, D., Kuniyoshi, A.K., Kodama, R.T., Gozzo, F.C., Fioramonte, M., Tambourgi, D.V., Portaro, F.V., and Rioli, V. (2016). [des-Arg1]-Proctolin: A novel NEP-like enzyme inhibitor identified in *Tityus serrulatus* venom. *Peptides* 80**,** 18-24.

Evans, E.R.J., Northfield, T.D., Daly, N.L., and Wilson, D.T. (2019). Venom costs and optimization in scorpions. *Frontiers in Ecology and Evolution* 7**,** 196.

Fry, B.G., Winter, K., Norman, J.A., Roelants, K., Nabuurs, R.J., Van Osch, M.J., Teeuwisse, W.M., Van Der Weerd, L., Mcnaughtan, J.E., Kwok, H.F., Scheib, H., Greisman, L., Kochva, E., Miller, L.J., Gao, F., Karas, J., Scanlon, D., Lin, F., Kuruppu, S., Shaw, C., Wong, L., and Hodgson, W.C. (2010). Functional and structural diversification of the Anguimorpha lizard venom system. *Mol Cell Proteomics* 9**,** 2369-2390.

González-Gómez, J.C., Valenzuela-Rojas, J.C., García, L.F., Franco Pérez, L.M., Guevara, G., Buitrago, S., Cubillos, A., and Van Der Meijden, A. (2019). Sexual dimorphism in the biomechanical and toxicological performance in prey incapacitation of two morphologically distinct scorpion species (*Chactas* sp. and *Centruroides* sp.). *Biological Journal of the Linnean Society* 129**,** 190-198.

Gozzini, L., Montecucchi, P.C., Erspamer, V., and Melchiorri, P. (1985). Tryptophyllins from extracts of *Phyllomedusa rhodei* skin: new tetra-, penta-and heptapeptides. Further studies. *International Journal of Peptide and Protein Research* 25**,** 323-329.

Harrison, P.L., Abdel-Rahman, M.A., Miller, K., and Strong, P.N. (2014). Antimicrobial peptides from scorpion venoms. *Toxicon* 88**,** 115-137.

Herzig, V., Khalife, A.A., Chong, Y., Isbister, G.K., Currie, B.J., Churchill, T.B., Horner, S., Escoubas, P., Nicholson, G.M., and Hodgson, W.C. (2008). Intersexual variations in northern (*Missulena pruinosa*) and eastern (*M. bradleyi*) mouse spider venom. *Toxicon* 51**,** 1167-1177.

Herzig, V., Sunagar, K., Wilson, D.T., Pineda, S.S., Israel, M.R., Dutertre, S., Mcfarland, B.S., Undheim, E.A., Hodgson, W.C., and Alewood, P.F. (2020). Australian funnel-web spiders evolved human-lethal δ-hexatoxins for defense against vertebrate predators. *Proceedings of the National Academy of Sciences* 117**,** 24920-24928.

Hökfelt, T., Broberger, C., Xu, Z.-Q.D., Sergeyev, V., Ubink, R., and Diez, M. (2000). Neuropeptides—an overview. *Neuropharmacology* 39**,** 1337-1356.

Inceoglu, B., Lango, J., Jing, J., Chen, L., Doymaz, F., Pessah, I.N., and Hammock, B.D. (2003). One scorpion, two venoms: prevenom of *Parabuthus transvaalicus* acts as an alternative type of venom with distinct mechanism of action. *Proceedings of the National Academy of Sciences of the United States of America* 100**,** 922-927.

Jiao, G.B., and Zhu, M.S. (2010). Courtship and mating of *Scorpiops luridus* Zhu Lourenço & Qi, 2005 (Scorpiones: Euscorpiidae) from Xizang province, China. *Journal of Venomous Animals and Toxins including Tropical Diseases* 16**,** 155-165.

Koch, L.E. (1977). *The taxonomy, geographic distribution and evolutionary radiation of Australo-Papuan scorpions.* Western Australian Museum.

Konno, K., Kazuma, K., and Nihei, K.-I. (2016). Peptide toxins in solitary wasp venoms. *Toxins* 8**,** 114.

Kovařík, F. (1997). Revision of the genera *Lychas* and *Hemilychas*, with descriptions of six new species (Scorpiones: Buthidae). *Acta Societatis Zoologicae Bohemicae* 61**,** 311-371.

Kovařík, F. (2019). Taxonomic reassessment of the genera *Lychas*, *Mesobuthus*, and *Olivierus*, with descriptions of four new genera (Scorpiones: Buthidae). *Euscorpius* 2019**,** 1-27.

Lira, A.F.A., Pordeus, L.M., Rego, F.N.a.A., Iannuzzi, K., and Albuquerque, C.M.R. (2018). Sexual dimorphism and reproductive behavior in the Brazilian scorpion *Tityus pusillus* (Scorpiones, Buthidae). *Invertebrate Biology* 137**,** 221-230.

Lopes-Ferreira, M., Sosa-Rosales, I., Bruni, F.M., Ramos, A.D., Portaro, F.C.V., Conceição, K., and Lima, C. (2016). Analysis of the intersexual variation in *Thalassophryne maculosa* fish venoms. *Toxicon* 115**,** 70-80.

Maillo, M., Aguilar, M.B., Lopéz-Vera, E., Craig, A.G., Bulaj, G., Olivera, B.M., and Heimer De La Cotera, E.P. (2002). Conorfamide, a *Conus* venom peptide belonging to the RFamide family of neuropeptides. *Toxicon* 40**,** 401-407.

Mccowan, C., and Garb, J.E. (2014). Recruitment and diversification of an ecdysozoan family of neuropeptide hormones for black widow spider venom expression. *Gene* 536**,** 366-375.

Mclean, C.J., Garwood, R.J., and Brassey, C.A. (2018). Sexual dimorphism in the arachnid orders. *PeerJ* 6**,** e5751.

Miller, D.W., Jones, A.D., Goldston, J.S., Rowe, M.P., and Rowe, A.H. (2016). Sex differences in defensive behavior and venom of the striped bark scorpion *Centruroides vittatus* (Scorpiones: Buthidae). *Integrative and Comparative Biology* 56**,** 1022-1031.

Montecucchi, P.C., Gozzini, L., Erspamer, V., and Melchiorri, P. (1984). Primary structure of tryptophan-containing peptides from skin extracts of *Phyllomedusa rhodei* (tryptophyllins). *International Journal of Peptide and Protein Research* 23**,** 276-281.

Morgenstern, D., and King, G.F. (2013). The venom optimization hypothesis revisited. *Toxicon* 63**,** 120-128.

Nekaris, K.a.I., Campera, M., Nijman, V., Birot, H., Rode-Margono, E.J., Fry, B.G., Weldon, A., Wirdateti, W., and Imron, M.A. (2020). Slow lorises use venom as a weapon in intraspecific competition. *Current Biology* 30**,** R1252-R1253.

Newton, M. (2008). "A guide to keeping Australian scorpions in captivity: with notes on general biology and identification". Newton Publishing, Adelaide, S. Aust).

Nihei, K.-I., Peigneur, S., Tytgat, J., Lange, A.B., and Konno, K. (2021). Isolation and characterization of FMRFamide-like peptides in the venoms of solitary sphecid wasps. *Peptides* 142**,** 170575.

Nisani, Z., Boskovic, D.S., Dunbar, S.G., Kelln, W., and Hayes, W.K. (2012). Investigating the chemical profile of regenerated scorpion (*Parabuthus transvaalicus*) venom in relation to metabolic cost and toxicity. *Toxicon* 60**,** 315-323.

Nisani, Z., and Hayes, W.K. (2011). Defensive stinging by *Parabuthus transvaalicus* scorpions: risk assessment and venom metering. *Animal Behaviour* 81**,** 627-633.

Nystrom, G.S., Ward, M.J., Ellsworth, S.A., and Rokyta, D.R. (2019). Sex-based venom variation in the eastern bark centipede (*Hemiscolopendra marginata*). *Toxicon* 169**,** 45-58.

Olguín-Pérez, L., Francke, O.F., and Carbajal-Saucedo, A. (2021). Evidence of piercing and sexual differences in venom composition in a sexual stinging scorpion (Scorpiones: Euscorpiidae). *The Journal of Arachnology* 49**,** 98-107.

Olivero, P.A., González, A., Mattoni, C.I., and Peretti, A.V. (2015). Chemical caressess: geographical variation of male sexual signals in a Neotropical scorpion. *Behaviour* 152**,** 1745-1763.

Olivero, P.A., Vrech, D.E., Oviedo-Diego, M.A., Mattoni, C.I., and Peretti, A.V. (2019). Courtship performance as function of body condition in an 'ancient' form of sperm transfer. *Animal Biology* 69**,** 33-46.

Özkan, Ö., Çiftçi, G., and Karaer, Z. (2011). Electrophoretical comparison of proteins of *Mesobuthus eupeus* and *Mesobuthus gibbosus* scorpion venoms. *Kafkas Univ Vet Fak Derg* 17**,** S153-S158.

Peigneur, S., Cheneval, O., Maiti, M., Leipold, E., Heinemann, S.H., Lescrinier, E., Herdewijn, P., De Lima, M.E., Craik, D.J., and Schroeder, C.I. (2019a). Where cone snails and spiders meet: design of small cyclic sodium-channel inhibitors. *The FASEB Journal* 33**,** 3693-3703.

Peigneur, S., Devi, P., Seldeslachts, A., Ravichandran, S., Quinton, L., and Tytgat, J. (2019b). Structure-function elucidation of a new α-conotoxin, MilIA, from *Conus milneedwardsi*. *Marine drugs* 17**,** 535.

Peretti, A.V. (1997). Relación de las glándulas caudales de machos de escorpiones Bothriuridae con el comportamiento sexual (Scorpiones). *Revue Arachnologique* 12**,** 31-41.

Peretti, A.V. (2014). "Chapter 2 - Sexual selection in Neotropical species: rules and exceptions," in *Sexual Selection,* eds. R.H. Macedo & G. Machado. (San Diego: Academic Press), 33-52.

Peretti, A.V., Acosta, L.E., and Benton, T.G. (2008). Sexual cannibalism in scorpions: fact or fiction? *Biological Journal of the Linnean Society* 68**,** 485-496.

Peretti, A.V., and Carrera, P. (2005). Female control of mating sequences in the mountain scorpion *Zabius fuscus*: males do not use coercion as a response to unreceptive females. *Animal Behaviour* 69**,** 453-462.

Polis, G.A. (1990). Life history. *The biology of scorpions*.

Possani, L.D., Merino, E., Corona, M., Bolivar, F., and Becerril, B. (2000). Peptides and genes coding for scorpion toxins that affect ion-channels. *Biochimie* 82**,** 861-868.

Price, D.A., and Greenberg, M.J. (1977). Structure of a molluscan cardioexcitatory neuropeptide. *Science* 197**,** 670-671.

Pucca, M.B., Cerni, F.A., Pinheiro-Junior, E.L., Zoccal, K.F., Bordon, K.D.C.F., Amorim, F.G., Peigneur, S., Vriens, K., Thevissen, K., Cammue, B.P.A., Júnior, R.B.M., Arruda, E., Faccioli, L.H., Tytgat, J., and Arantes, E.C. (2016). Non-disulfide-bridged peptides from *Tityus serrulatus* venom: Evidence for proline-free ACE-inhibitors. *Peptides* 82**,** 44-51.

Quillet, R., Ayachi, S., Bihel, F., Elhabazi, K., Ilien, B., and Simonin, F. (2016). RF-amide neuropeptides and their receptors in mammals: Pharmacological properties, drug development and main physiological functions. *Pharmacology & Therapeutics* 160**,** 84-132.

R Core Team (2021). "R: A language and environment for statistical computing". (Vienna, Austria: R Foundation for Statistical Computing).

Robinson, S.D., Li, Q., Bandyopadhyay, P.K., Gajewiak, J., Yandell, M., Papenfuss, A.T., Purcell, A.W., Norton, R.S., and Safavi-Hemami, H. (2017). Hormone-like peptides in the venoms of marine cone snails. *General and Comparative Endocrinology* 244**,** 11-18.

Robinson, S.D., Safavi-Hemami, H., Raghuraman, S., Imperial, J.S., Papenfuss, A.T., Teichert, R.W., Purcell, A.W., Olivera, B.M., and Norton, R.S. (2015). Discovery by proteogenomics and characterization of an RF-amide neuropeptide from cone snail venom. *Journal of Proteomics* 114**,** 38-47.

Rodríguez-Ravelo, R., Batista, C.V.F., Coronas, F.I.V., Zamudio, F.Z., Hernández-Orihuela, L., Espinosa-López, G., Ruiz-Urquiola, A., and Possani, L.D. (2015). Comparative proteomic analysis of

male and female venoms from the Cuban scorpion *Rhopalurus junceus*. *Toxicon* 107**,** 327-334.

Sarhan, M., Mostafa, A., Elbehiry, S.E., El Reheem, A., Abd, M., and Saber, S.A. (2017). Intersexual variation in tail length, venom composition, toxicity, and anticancer activity of *Cerastes cerastes* (Viperidae). *The Egyptian Journal of Hospital Medicine* 66**,** 81-90.

Schendel, V., Rash, L.D., Jenner, R.A., and Undheim, E.A. (2019). The diversity of venom: the importance of behavior and venom system morphology in understanding its ecology and evolution. *Toxins* 11**,** 666.

Sentenská, L., Graber, F., Richard, M., and Kropf, C. (2017). Sexual dimorphism in venom gland morphology in a sexually stinging scorpion. *Biological journal of the Linnean Society* 122**,** 429-443.

Shalabi, A., Zamudio, F., Wu, X., Scaloni, A., Possani, L.D., and Villereal, M.L. (2004). Tetrapandins, a new class of scorpion toxins that specifically inhibit store-operated calcium entry in human embryonic kidney-293 cells. *Journal of Biological Chemistry* 279**,** 1040-1049.

Skinner, S.P., Fogh, R.H., Boucher, W., Ragan, T.J., Mureddu, L.G., and Vuister, G.W. (2016). CcpNmr AnalysisAssign: a flexible platform for integrated NMR analysis. *Journal of Biomolecular NMR* 66**,** 111-124.

Stewart, J.M., Ferreira, S.H., and Greene, L.J. (1971). Bradykinin potentiating peptide PCA-Lys-Trp-Ala-Pro: an inhibitor of the pulmonary inactivation of Bradykinin and conversion of angiotensin I to II. *Biochemical Pharmacology* 20**,** 1557-1567.

Tallarovic, S.K., Melville, J.M., and Brownell, P.H. (2000). Courtship and mating in the giant hairy desert scorpion, *Hadrurus arizonensis* (Scorpionida, Iuridae). *Journal of insect behavior* 13**,** 827-838.

Toscano-Gadea, C.A. (2010). Sexual behavior of *Bothriurus buecherli* (Scorpiones: Bothriuridae) and comparison with the *B. prospicuus* group. *The Journal of Arachnology* 38**,** 360-363.

Undheim, E.a.B., Grimm, L.L., Low, C.-F., Morgenstern, D., Herzig, V., Zobel-Thropp, P., Pineda, S.S., Habib, R., Dziemborowicz, S., and Fry, B.G. (2015). Weaponization of a hormone: convergent recruitment of hyperglycemic hormone into the venom of arthropod predators. *Structure* 23**,** 1283-1292.

Uribe, J.I.C., Vargas, J.M.J., Batista, C.V.F., Zuñiga, F.Z., and Possani, L.D. (2017). Comparative proteomic analysis of female and male venoms from the Mexican scorpion *Centruroides limpidus*: novel components found. *Toxicon* 125**,** 91-98.

Valdez-Velázquez, L.L., Quintero-Hernández, V., Romero-Gutiérrez, M.T., Coronas, F.I., and Possani, L.D. (2013). Mass fingerprinting of the venom and transcriptome of venom gland of scorpion *Centruroides tecomanus*. *PLoS One* 8**,** e66486.

Walker, R.J., Papaioannou, S., and Holden-Dye, L. (2009). A review of FMRFamide-and RFamide-like peptides in Metazoa. *Invertebrate Neuroscience* 9**,** 111-153.

Ward, M.J., Ellsworth, S.A., Hogan, M.P., Nystrom, G.S., Martinez, P., Budhdeo, A., Zelaya, R., Perez, A., Powell, B., and He, H. (2018). Female-biased population divergence in the venom of the Hentz striped scorpion (*Centruroides hentzi*). *Toxicon* 152**,** 137-149.

Wigger, E., Kuhn-Nentwig, L., and Nentwig, W. (2002). The venom optimisation hypothesis: a spider injects large venom quantities only into difficult prey types. *Toxicon* 40**,** 749-752.

Wilson, D., and Daly, N. (2018). Nuclear magnetic resonance seq (NMRseq): a new approach to peptide sequence tags. *Toxins* 10**,** 437.

Zeng, X.C., Corzo, G., and Hahin, R. (2005). Scorpion venom peptides without disulfide bridges. *IUBMB life* 57**,** 13-21.

Zhang, Y., Xu, J., Wang, Z., Zhang, X., Liang, X., and Civelli, O. (2012). Bm K-YA, an enkephalin-like peptide in scorpion venom. *PLoS One* 7**,** e40417.

Zhijian, C., Feng, L., Yingliang, W., Xin, M., and Wenxin, L. (2006). Genetic mechanisms of scorpion venom peptide diversification. *Toxicon* 47**,** 348-355.

Zizzari, Z.V., Smolders, I., and Koene, J.M. (2014). Alternative delivery of male accessory gland products. *Frontiers in Zoology* 11**,** 1-8.

Hormurus waigiensis adult female – Edward Evans

Chapter 4. Small Molecules in the Venom of the Scorpion *Hormurus waigiensis*

***Biomedicines* (2020), 8, 259**

doi: 10.3390/biomedicines8080259

Edward R. J. Evans[1], Lachlan McIntyre[2], Tobin D. Northfield[3], Norelle L. Daly[1] and David T. Wilson[1,*]

[1]Centre for Molecular Therapeutics, AITHM, James Cook University, Cairns, QLD 4878, Australia;

[2]Independent Researcher, P.O. Box 78, Bamaga

[3]Department of Entomology, Tree Fruit Research and Extension Center, Washington State University, Wenatchee, WA 98801, USA

***Correspondence:**

David T. Wilson david.wilson4@jcu.edu.au; Tel.: +61-7-4232-1707

Keywords: venom; scorpion; adenosine; purine; nucleoside; nucleotide; citric acid; glutamic acid; aspartic acid

Author Contributions: E.R.J.E, D.T.W., and N.L.D. designed the study. E.R.J.E. and D.T.W. carried out the experiments. E.R.J.E., D.T.W., and L.M. analysed the results. E.R.J.E., D.T.W., and N.L.D. wrote the paper. T.D.N. edited the paper. All authors analysed the results and approved the final version of the manuscript.

Acknowledgments: This work was supported in part by an Australian Research Council Linkage Infrastructure, Equipment and Facilities (LIEF) grant (#LE160100218). The authors are grateful to Jeremy Potriquet and Yide Wong for assistance with the SCIEX 6600 TripleTOF mass spectrometry and Alan Henderson (Minibeast Wildlife, QLD) for providing scorpion specimens.

4.1. Abstract

Despite scorpion stings posing a significant public health issue in particular regions of the world, certain aspects of scorpion venom chemistry remain poorly described. Although there has been extensive research into the identity and activity of scorpion venom peptides, non-peptide small molecules present in the venom have received comparatively little attention. Small molecules can have important functions within venoms; for example, in some spider species the main toxic components of the venom are acylpolyamines. Other molecules can have auxiliary effects that facilitate envenomation, such as purines with hypotensive properties utilised by snakes. In this study, we investigated some non-peptide small molecule constituents of *Hormurus waigiensis* venom using LC/MS, reversed-phase HPLC, and NMR spectroscopy. We identified adenosine, adenosine monophosphate (AMP), and citric acid within the venom, with low quantities of the amino acids glutamic acid and aspartic acid also being present. Purine nucleosides such as adenosine play important auxiliary functions in snake venoms when injected alongside other venom toxins, and they may have a similar role within *H. waigiensis* venom. Further research on these and other small molecules in scorpion venoms may elucidate their roles in prey capture and predator defence, and gaining a greater understanding of how scorpion venom components act in combination could allow for the development of improved first aid.

4.2. Introduction

Scorpion envenomation poses a significant public health issue in certain areas of the world, particularly in northern Saharan Africa, South and East Africa, the Near- and Middle-East, southern India, Mexico, Brazil, and within the Amazonian basin (Chippaux and Goyffon, 2008). Although the effects of scorpion venom can be severe when injected by a scorpion, individual venom components can have a wide range of positive applications in medicine when administered in a controlled way (Ahmadi et al., 2020). Past research into scorpion venoms has primarily focused on peptide constituents, as these molecules display the greatest diversity within the venom, are often responsible for the greatest toxic effects, and have the largest potential for development of therapeutics or bioinsecticides (Possani et al., 1999;King, 2011;Smith et al., 2013a;Ortiz et al., 2015). However, scorpions possess complex venom containing a mixture of proteins, peptides, small molecules, and salts (Inceoglu et al., 2003). Whilst non-peptide small molecules are often reported to be present within scorpion venom, and despite being known to play important roles in the venom of different taxa (Aird, 2002;Daly and Wilson, 2018;Villar-Briones and Aird, 2018), the identities and functions of small molecules in scorpion venom remain poorly described. Identifying the small molecules present in scorpion venoms may allow a greater understanding of how scorpion venoms function, have evolved, and may help improve treatment of stings. Additionally, certain venom-derived small molecules may have applications as therapeutics (Wilson et al., 2017). At present, only a very small number of non-peptide small molecules (<1 kDa) have been confidently characterised from scorpion venoms, including an alkaloid described from *Megacormus gertschi* (Banerjee et al., 2018), two 1,4-benzoquinone derivatives from *Diplocentrus melici* (Carcamo-Noriega et al., 2019), adenosine from *Heterometrus laoticus* (Thien et al., 2017;Tran et al., 2017), and citric acid from *Centruroides sculpturatus* (Fenton et al., 1995). Other published material identified the presence of spermidine in the venom of *Palamneus phipsoni* (*Heterometrus phipsoni*) (Arjunwadkar and Reddy, 1983;Francke, 2019), and 5-hydroxytryptamine (serotonin) in *Leiurus quinquestriatus* and *Buthotus minax* (*Hottentotta minax*) (Adam and Weiss, 1959;Francke, 2019); however, no definitive data were collected, and therefore re-examination of these venoms with modern analytical techniques would help to confirm their presence. Furthermore, free amino acids, nucleotides, lipids, amines, heterocyclic compounds, and inorganic salts are reportedly present in scorpion venoms (Dai et al., 2002;Luna-Ramírez et al., 2015;Ortiz et al., 2015), but their specific molecular compositions and functions remain generally unknown.

Venomous organisms commonly possess low-molecular weight non-peptide molecules within their venom (Daly and Wilson, 2018;Villar-Briones and Aird, 2018). The identities and functions of the low-molecular weight non-peptide molecules found in different organisms may

provide insights into those that may be found in scorpions. Spiders are one of the most closely related groups of venomous organisms to scorpions, and are also one of the most widely studied groups of venomous animals. Different spider species possess a large range of small molecules within their venom, including acylpolyamines and polyamines (Skinner et al., 1990;McCormick and Meinwald, 1993;Itagaki et al., 1997;Hisada et al., 1998;Palma and Nakajima, 2005;Wilson et al., 2017); alkaloids (Cesar et al., 2005;Marques et al., 2005;Saidemberg et al., 2009); nucleosides, nucleotides, and analogues (Chan et al., 1975;Savel-Niemann, 1989;Horni et al., 2001;Rodrigues et al., 2004;Taggi et al., 2004;Schroeder et al., 2008); and free-amino acids (Schanbacher et al., 1973;Savel-Niemann, 1989), alongside other molecules that have been subject to less intensive study (Escoubas et al., 2000;Vassilevski et al., 2009;Kuhn-Nentwig et al., 2011). Given that scorpions use their venom against similar predators and prey to spiders, the small molecules they possess may be similar to those found in spiders.

Small molecules can act directly as toxins within venom, have facilitatory roles that increase the overall toxicity or effectiveness of the venom, or alternatively have functional roles linked to the maintenance of toxins within the venom gland (Yoshioka et al., 1992;Odell et al., 1999;Aird, 2002;Gomes et al., 2011). For example, polyamines in the venom of spiders in the genus *Nephila* are sufficiently toxic towards insects to directly aid in the incapacitation of prey (Yoshioka et al., 1992). Alternatively, nigriventrine from the Brazilian armed spider (*Phoneutria nigriventer*) can induce convulsions in mammals, and therefore help the spider to defend against predators (Gomes et al., 2011). Whilst such molecules directly contribute to venom toxicity when targeting prey or potential predators, some small-molecule venom constituents do not induce a toxic effect when injected into a target organism, but can still play an important role within venom by acting in conjunction with toxins to facilitate envenomation or incapacitation of the target (Aird, 2002;2005). For example, purine nucleosides such as adenosine, guanosine, and inosine are present within a wide range of elapid and viperine snake venoms, and whilst these molecules generally have very low toxicity and are naturally present within the target organism, injection of high concentrations alongside other toxins can improve the overall effectiveness of the venom by facilitating envenomation and incapacitation (Aird, 2002;2005). Aird (2002) explains that snake venom has three primary modes of action against prey—immobilisation by hypotension, immobilisation by paralysis, and digestion—and that the suite of small molecules injected have key roles in all three functions. As the body of research investigating the small molecule constituents of snake venoms is expanding, there is growing understanding that non-toxic small molecules can play important auxiliary roles within venoms, and future research should aim to elucidate the complex interactions that occur during envenomation (Villar-Briones and Aird, 2018). In addition to these functions as toxins or facilitators

of toxins, small molecules can have functions within the venom gland associated with maintenance and production of toxins. For example, citrate, which can inhibit the action of venom proteins within the gland, may be in the venom to prevent self-harm (Francis et al., 1992;Fenton et al., 1995;Odell et al., 1999). As scorpion venoms contain a suite of uncharacterised small molecules, it is possible that some may induce toxicity directly, have auxiliary effects that increase overall venom toxicity, or have functions associated with the production and storage of toxins.

In this study, we investigated some of the small molecule compositions of Australian rainforest scorpion (*Hormurus waigiensis*) venom using reversed-phase high-performance liquid chromatography (RP-HPLC), liquid chromatography/mass spectrometry (LC/MS), and nuclear magnetic resonance (NMR) spectroscopy. *H. waigiensis* is a burrowing scorpion that is widely distributed across Southeast Asia, the Pacific, New Guinea, and Australia (Koch, 1977). Within Australia, it is found in tropical and sub-tropical forests along the eastern coast from northern New South Wales to Cape York, with reports of populations also present in the Northern Territory and Western Australia (Monod and Volschenk, 2004). Stings from this species are considered mild, with the venom causing moderate pain and swelling (Isbister et al., 2004). Presently just one toxin, ϕ-liotoxin-Lw1a (ϕ-LITX-Lw1a), has been characterised from *H. waigiensis* (Smith et al., 2011;Smith et al., 2013b). The toxin ϕ-LITX-Lw1a was the first scorpion toxin reported to adopt a disulfide-directed β-hairpin (DDH) structure and may provide a missing link explaining how the three-disulfide inhibitor cystine knot (ICK) motif has evolved in scorpions (Smith et al., 2011;Smith et al., 2013b). The remaining constituents of *H. waigiensis* venom have not been characterised. Scorpion venom research has historically been skewed towards medically significant species, all of which belong to the family Buthidae (Santos et al., 2016). *H. waigiensis* is a member of the family Hormuridae, which has been subject to far fewer investigations by toxinologists. Given the structural novelty of ϕ-LITX-Lw1a, *H. waigiensis* venom may contain other molecules with significant structural differences to those present in more thoroughly studied Buthid venoms.

4.3. Materials and Methods

4.3.1. Scorpion Collection

Scorpions were collected from rainforest sites in the vicinity of Kuranda (QLD, Australia), and kept on the premises of Minibeast Wildlife, Kuranda. The scorpions were housed in plastic food containers with substrate and a piece of bark, and kept at ambient temperature. Prior to milking, individuals had been maintained in captivity for less than one year.

4.3.2. Venom Extraction and Purification

Venom samples were collected from *H. waigiensis* using a square-wave stimulator (Arthur H. T. Thomas Co. Scientific Apparatus, Philadelphia, PA, USA) to electrostimulate the venom gland at 25V DC, 0.5 ms pulse duration, at a frequency of 1 pulse/sec. The samples were pooled into 20 µL of MilliQ water and stored at −20 °C. Pooled samples collected on two separate dates were further pooled prior to analysis. A further five scorpions (two male and three female) were milked and the samples were stored separately in 30 µL of MilliQ water for LC/MS analysis.

Crude pooled venom was fractionated by reversed-phase HPLC (RP-HPLC) using a Phenomenex Jupiter® C_{18} column (250 × 10 mm, 10 µm, 100 Å; Phenomenex, Torrence, CA, USA). Fractionation of the venom components was achieved using a linear gradient of two mobile phases: H_2O/0.05% trifluoroacetic acid (TFA; Auspep, Tullamarine, VIC, Australia) (solvent A) and 90% acetonitrile (ACN; Sigma-Aldrich, St. Louis, MO, USA)/H_2O/0.045% TFA (solvent B). Separation used a gradient of 0–60% solvent B in 120 min, 60–90% solvent B in 5 min, 90% solvent B for 10 min, and 90–0% solvent B in 5 min, at a flow rate of 3 mL/min. The venom component elution was monitored at 214 nm and 280 nm, and 0.5 min fractions were collected.

4.3.3. Liquid Chromatography/Mass Spectrometry (LC/MS)

Five individual scorpion venom samples and serial dilutions of adenosine and adenosine monophosphate (AMP) standards were analysed by liquid chromatography/mass spectrometry (LC/MS) using a Shimadzu LCMS-2020 mass spectrometer coupled to a Shimadzu Prominence HPLC system (Shimadzu, Japan) to allow quantitation of these molecules within the venom. A small amount of pooled venom was also analysed by LC/MS to observe the composition and determine the molecular weights of the molecules present. Then, 8 µL of the individually stored venom samples were injected in triplicate, 5 µL of the adenosine and AMP serial dilutions were injected in triplicate, and 10 µL of pooled venom (3 µL in 7 µL MilliQ water) was injected. The samples were injected via an autosampler (Shimadzu SIL-20AC_{HT}) onto a reversed-phase high-performance liquid chromatography (HPLC) column (Phenomonex Aeris 3.6 µm PEPTIDE XB-C18 100 Å; Phenomenex, Torrence, CA, USA) at 30 °C. Solvent delivery (solvent A: 0.1% formic acid (FA; Sigma-Aldrich, St. Louis, MO, USA)/water; solvent B: 90% acetonitrile (ACN; OPTIMA LCMS grade, Thermo Fisher Scientific, Scoresby, VIC, Australia)/0.09% formic acid/water) was via Shimadzu LC-20AD pumps at a flow rate of 0.250 mL/min. Samples were eluted with a 1% gradient (0-60% solvent B, 60 min; 6090–% solvent B, 5 min; 90% solvent B, 5 min; 90-0% solvent B, 5 min; 0% solvent B, 10 min), and the UV absorbance was observed at 214 nm and 280 nm on a Shimadzu SPD-20A detector. Mass spectra were collected in positive ion mode over a scan range of *m/z* 130-2000 and negative mode with a

scan range of *m/z* 200-2000, with a detector voltage of 1.15 kV, nebulizing gas flow of 1.5 L/min, and drying gas flow of 3.0 L/min. Data were collected and analysed using the Shimadzu LabSolutions v5.96 software (Shimadzu, Japan). The 280 nm peak areas for the peaks containing adenosine and AMP, both in the individual venom samples and serial dilutions, were exported and analysed further in R version 3.6.1 (R Core Team, 2021). The peak areas for standard samples and venom samples run in triplicate were averaged prior to further analysis. A linear model was fit to the serial dilution data to produce a standard curve, which was used to predict the quantity of adenosine and AMP in the 8 µL injection of each venom sample. These quantities were then divided by eight and multiplied by thirty to provide an estimate of the quantity contained within the whole venom samples. As the crude venoms were suspended in 30 µL of water, the total volume would be slightly greater than 30 µL; therefore, we calculated a slight underestimate of the quantity of adenosine and AMP contained within the venom.

4.3.4. Mass Spectrometry and NMR Analysis

NMR spectra were recorded at 290 K on a Bruker Avance III 600 MHz spectrometer (Bruker, Billerica, MA, USA) equipped with a cryoprobe. Samples were dissolved in 90% H_2O/10% D_2O (*v/v*) (100 µM). D_2O (99.9%) was obtained from Cambridge Isotope Laboratories, Woburn, MA, USA, for ^1H NMR measurements. Spectra were referenced to the water signal. Two-dimensional spectra included TOCSY, NOESY, DQF-COSY, HSQC, HMBC, and HSQC-TOCSY. TOCSY and NOESY mixing times of 80 ms and 500 ms, respectively, were used. Spectra were analysed using Topspin v3.6.1(Bruker, Billerica, MA, USA).

High-resolution mass spectrometry (MS) was performed using a SCIEX TOF/TOF™ 5800 MALDI (SCIEX, Framingham, MA, USA) and a SCIEX TripleTOF 6600 (SCIEX, Framingham, MA, USA) mass spectrometers. Matrix-assisted laser desorption ionisation mass spectrometry (MALDI-MS) samples were spotted on 384-well stainless steel target plates using 0.5 µL of sample and 0.5 µL of either α–cyano-4-hydroxycinnamic acid (CHCA; Sigma-Aldrich, St. Louis, MO, USA) matrix (7.5 mg/mL in 50% ACN/0.1% TFA) or a 2,5-dihydroxybenzoic acid (DHB; Sigma-Aldrich, St. Louis, MO, USA) matrix (10 mg/mL in 50% ethanol/0.1% TFA). Calibration was performed before spectra collection for each sample using Calibration Mix Solution 2 (SCIEX, Framingham, MA, USA). Spectra were acquired in reflector positive ion mode from *m/z* 200 to 400, and averaged over 2000 laser shots. Tandem-MS (MS/MS) was performed in 1 kV positive ion mode with collision-induced dissociation (CID) and averaged over 2000 shots. Samples were manually infused to the SCIEX TripleTOF 6600 mass spectrometer equipped with a DuoSpray Ion Source using the syringe pump and a 1 mL 4.61 mm i.d. Hamilton syringe (Reno, NV, USA) at a flow rate of 5 µL/min. Data acquisition was performed

with Analyst® TF 1.7.1 software (SCIEX, Framingham, MA, USA). Product ion mass spectra were collected in positive ion mode with an accumulation time of 500 ms and a mass range of *m/z* 100 to 400. The source temperature was 470 °C, the curtain gas was set to 45 psi, the ion source gas 1 and 2 were set to 40 and 50 psi respectively, and the ion-spray voltage floating set to 4.9 kV. MS/MS data were collected on manually selected product ions under the same conditions with an accumulation time of 250 ms and a mass range of *m/z* 20 to 350.

4.4. Results

4.4.1. Venom Collection and Fractionation

Crude venom pooled from ~15 venom extractions of *H. waigiensis* scorpions was fractionated using RP-HPLC with a C_{18} semi-preparative RP-HPLC column, and 30 sec fractions were collected (see Figure 4.1). A small sample of the pooled venom (3 µL) was also subjected to liquid chromatography/mass spectrometry (LC/MS) analysis, which provided a venom mass profile and identified the presence of a number of small molecules in early eluting peaks.

4.4.2. NMR Analysis of RP-HPLC Fractions

Fractions collected from 5.5 to 23 min were analysed by one-dimensional ^{1}H NMR spectroscopy to observe the presence of small molecules, typified by sharp peaks and an absence of or minimal peaks in the amide region (see Figures 4.2-4.5). Fractions corresponding to the RP-HPLC peaks highlighted in dark blue and red in Figure 4.1 appeared to be relatively clean and produced good signal-to-noise ratios in the ^{1}H NMR spectra. Therefore, these fractions were selected for further analysis by two-dimensional NMR spectroscopy and the structures were elucidated based on correlations observed in the HMBC, HSQC, and COSY spectra. The dark blue peak highlighted in Figure 4.1 represented 1.28% of the total peak area within the 214 nm RP-HPLC chromatogram and was shown to contain glutamic acid and aspartic acid (see Figure 4.2). The red peak (Figure 4.1) represented 7.68% of the total peak area within the 214 nm RP-HPLC chromatogram and contained adenosine (see Figure 4.3). AMP (see Figure 4.4) was present within the light blue peak, which represented 2.42% of the total peak area of the RP-HPLC chromatogram (Figure 4.1). Fractions collected in the pink coloured region (Figure 4.1) contained citric acid, although as it does not have an absorbance profile at 214 nm nor 280 nm, it was not observed in the RP-HPLC chromatogram and was characterised based on NMR data (Figure 4.5). The green peak representing 0.33% of the total peak area of the 214 nm RP-HPLC chromatogram (Figure 4.1) contained a molecule with a mass 1 Da greater than adenosine, suggesting it may be inosine; however, we were unable to confirm its

identity due to its low abundance and coelution with adenosine. A molecule was also observed with the mass of inosine monophosphate (IMP); however, low abundance and coelution with AMP on the RP-HPLC system prevented us from confirming its identity.

4.4.3. High-Resolution Mass Spectrometry

High-resolution mass spectrometry data collected using a SCIEX TripleTOF 6600 (SCIEX, Framingham, MA, USA) and SCIEX TOF/TOF™ 5800 MALDI (SCIEX, Framingham, MA, USA) were consistent with and confirmed the elucidated structures. For the dark blue peak fraction, $[M + H]^+$ ions were observed at *m/z* 148.0350 and *m/z* 134.0187, confirming the presence of glutamic acid (147.053158 Da) and aspartic acid (133.037508 Da). For the red peak fraction, a $[M + H]^+$ ion was observed at *m/z* 268.0783, confirming the presence of adenosine (267.096741 Da). Using a precursor ion of *m/z* 268.1, the red peak fraction showed a strong ion at *m/z* 136.0827 corresponding to adenine following fragmentation and cleavage of the ribose. For the light blue peak fraction, an $[M + H]^+$ ion was observed at *m/z* 348.0649, confirming the presence of AMP (347.063084 Da). Using a precursor ion of *m/z* 348.06 on this fraction showed an ion at *m/z* 136.1006, which also corresponded to adenine following fragmentation and cleavage of the ribose.

4.4.4. Adenosine Quantitation by LC/MS

Five adult scorpions were milked individually to estimate the quantities of adenosine and AMP contained within the total venom by comparing the LC/MS 280 nm absorbance peak areas against those of a serial dilution of standards run under identical chemical conditions. All five scorpion venoms contained adenosine. Quantities of approximately 4.462 µg and 2.448 µg were present within the two male venoms; and 4.555 µg, 2.016 µg, and 5.808 µg were present within the female venoms. The abundance of AMP displayed greater variation between individuals and was absent from two of the female scorpions at detectable levels. The estimated quantity of AMP within the whole venoms of the two males were 0.682 µg and 2.778 µg, while the single female venom contained 0.318 µg. It is important to note that these values are slight underestimates of the true quantity contained within the venom gland. By comparing the volumes of venom collected in the pipette tip against set volumes of water, we estimated that each scorpion produced between 1.5 µL and 3.5 µL during milking, and therefore an average milking contained roughly 2.5 µL of venom. This indicates that the values calculated from 30 µL of the sample are slight underestimates of the true total, but this variation is small in comparison to the large differences observed between individuals.

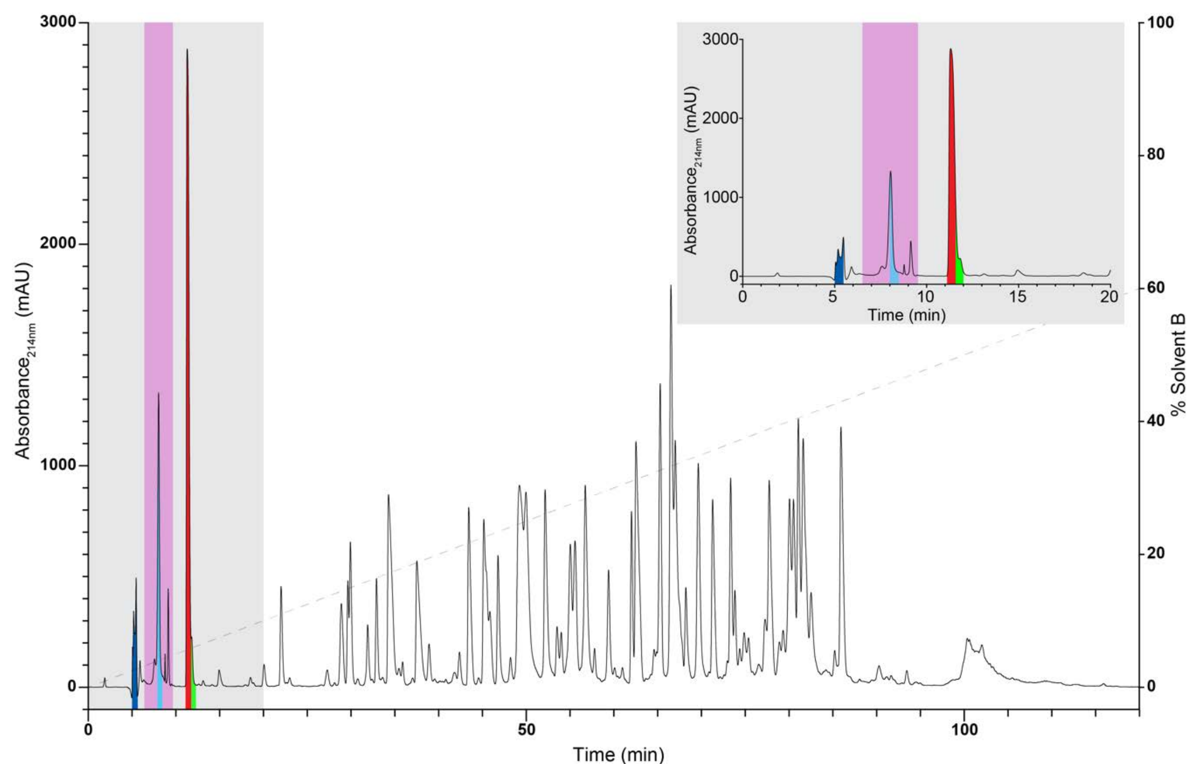


Figure 4. 1 Reversed-phase high performance liquid chromatography (RP-HPLC) chromatogram of pooled crude *Hormurus waigiensis* venom (Phenomenex Jupiter® C_{18} column; 250 × 10 mm, 10 µm, 100 Å; 3 mL/min flow rate; solvent A H_2O/0.05% TFA, solvent B 90% ACN/H_2O/0.045% TFA; 0–60% solvent B in 120 min, 60–90% solvent B in 5 min, 90% solvent B for 10 min, and 90–0% solvent B in 5 min; absorbance at 214 nm). The inset shows an expanded view of the highlighted area in the full chromatogram. The fractions of interest and corresponding peaks in the chromatogram are highlighted in dark blue (glutamic acid and aspartic acid), red (adenosine), and light blue (adenosine monophosphate), respectively. The pink section shows the fractions containing citric acid. The fraction highlighted in green is likely to be inosine. The dashed line shows the solvent B gradient.

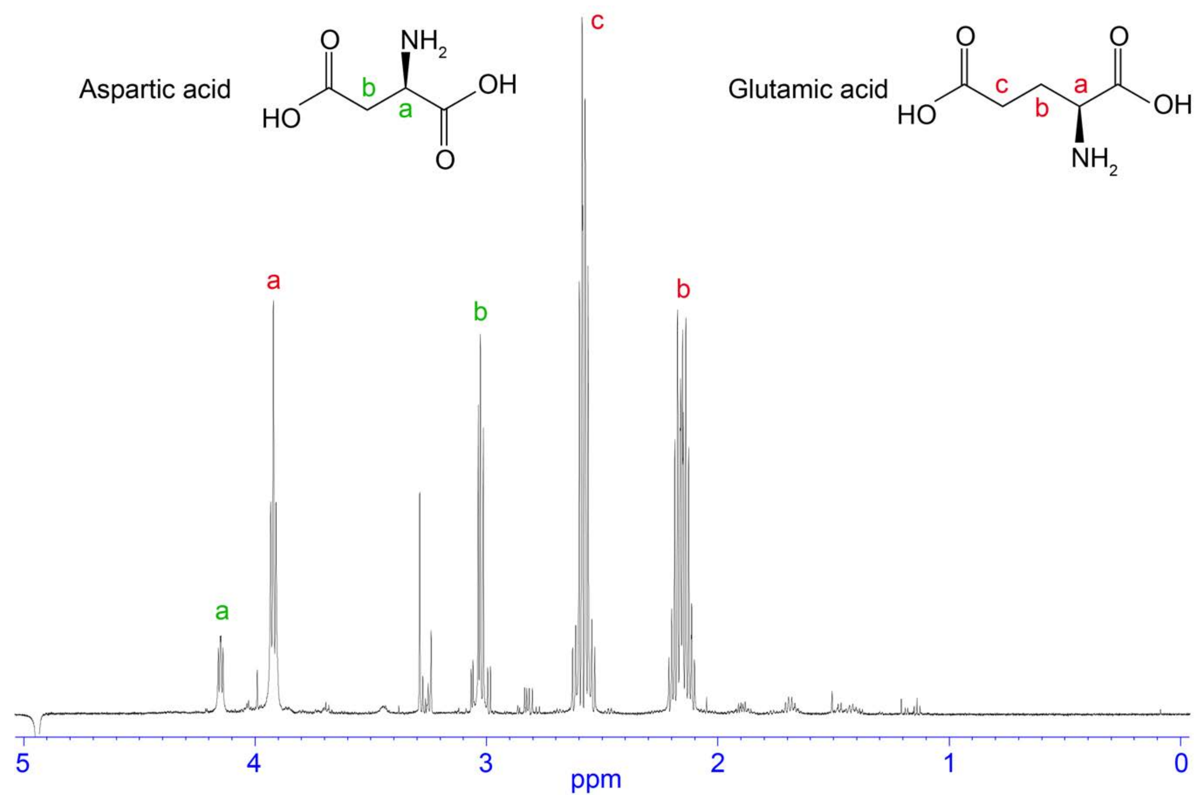


Figure 4. 2 Chemical structure and 1D NMR spectrum of a fraction from *Hormurus waigiensis* venom containing aspartic acid and glutamic acid. The assignments were derived based on two-dimensional NMR spectra and one-dimensional NMR spectra of glutamic acid and aspartic acid standards.

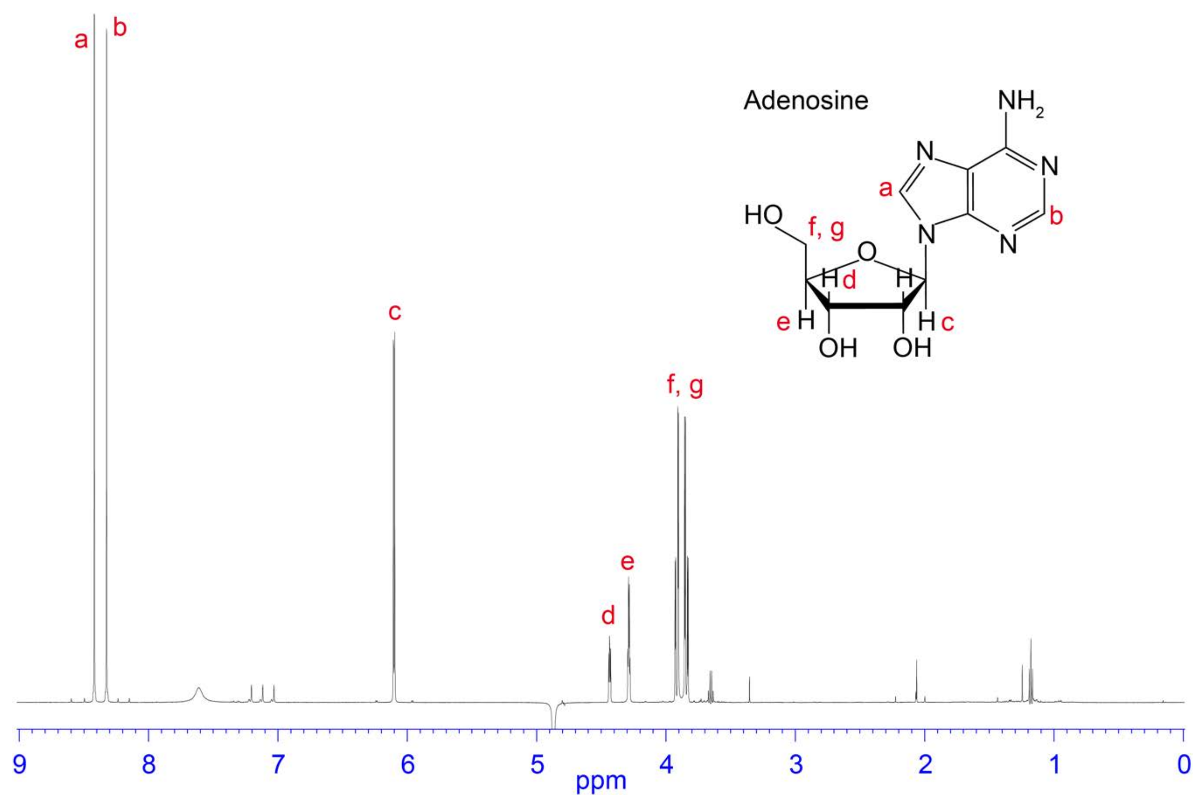


Figure 4. 3 Chemical structure and 1D NMR spectrum of a fraction from *Hormurus waigiensis* venom containing adenosine. The assignments were derived based on one-dimensional NMR spectra of an adenosine standard.

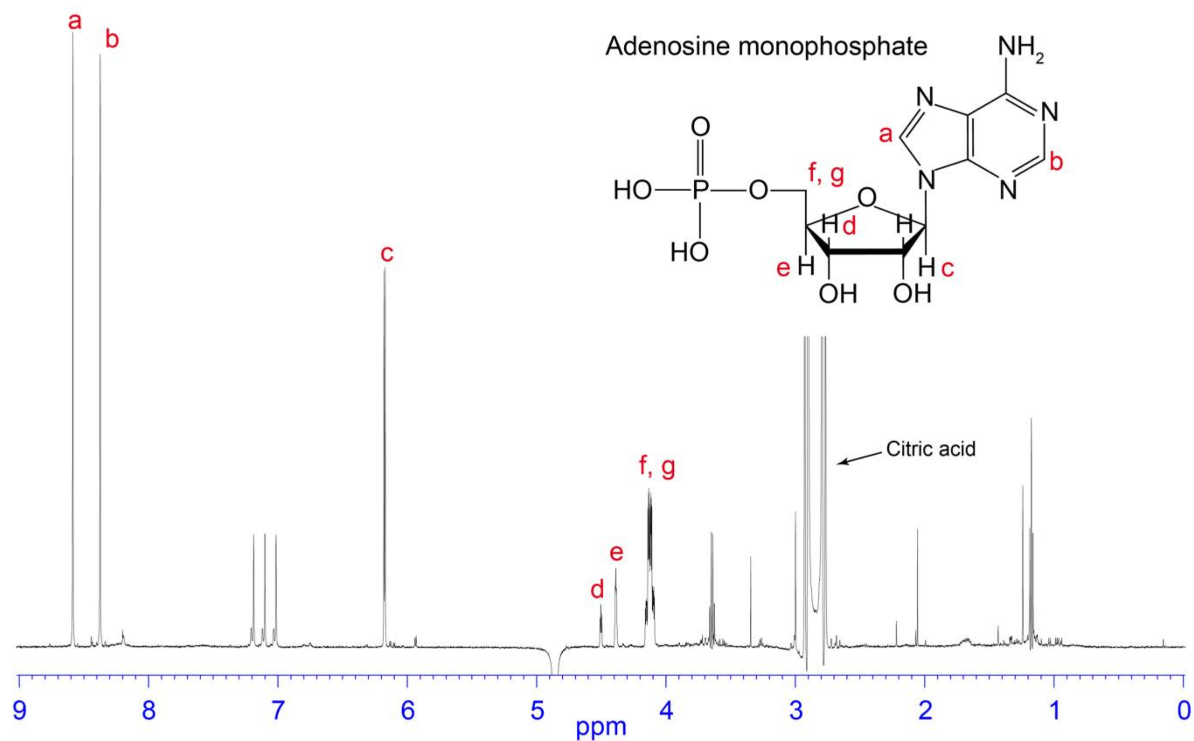


Figure 4. 4 Chemical structure and 1D NMR spectrum of a fraction from *Hormurus waigiensis* venom containing adenosine monophosphate. The assignments were derived based on one-dimensional NMR spectra of an adenosine monophosphate standard. The presence of citric acid is also highlighted.

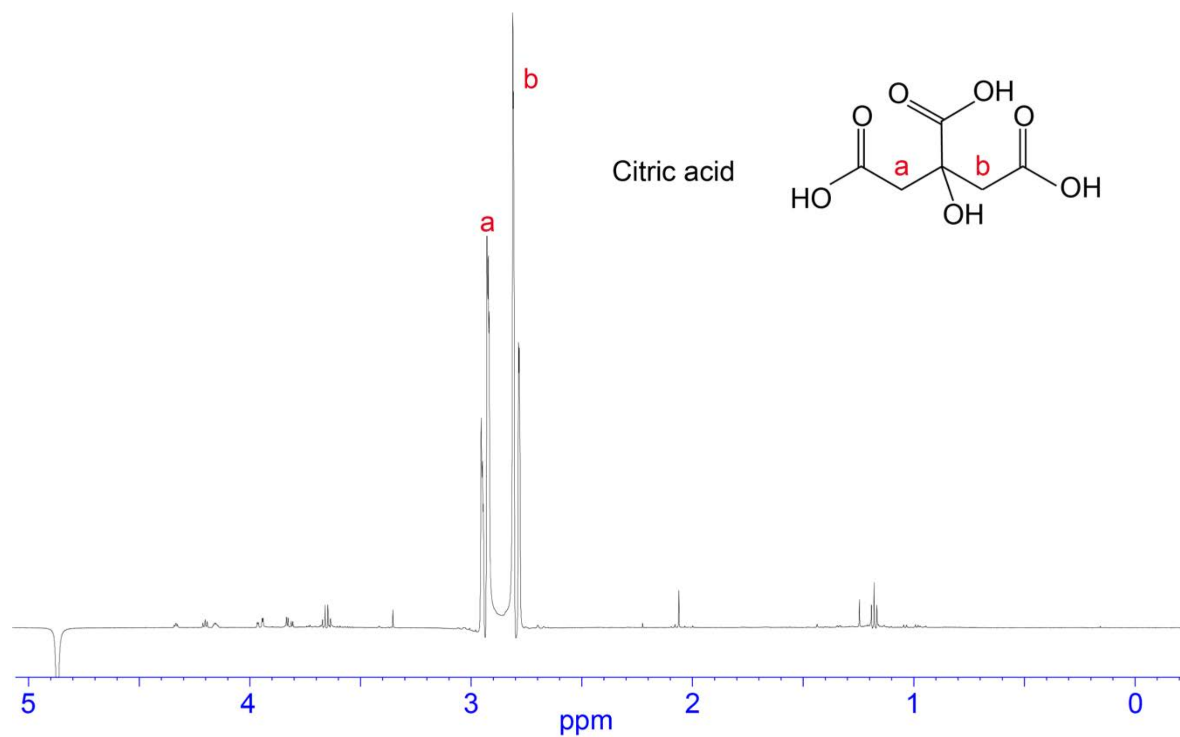


Figure 4. 5 Chemical structure and 1D NMR spectrum of a fraction from *Hormurus waigiensis* venom containing citric acid. The assignments were derived based on one-dimensional NMR spectra of a citric acid standard.

4.5. Discussion

Adenosine, AMP, citric acid, glutamic acid, and aspartic acid were all found to be present within the venom of *H. waigiensis.* Molecules were also observed with masses corresponding to inosine and IMP; however, we were unable to confirm their identity due to low abundances and coelution with adenosine and AMP, respectively.

4.5.1. Adenosine

Adenosine has been previously identified within the venom of the scorpion *Heterometrus laoticus* (Thien et al., 2017;Tran et al., 2017), and is a known constituent of other arthropod venoms, including those belonging to the spiders *Haplopelma lividum* and *Latrodectus menavodi* (Horni et al., 2001;Moore et al., 2009), the wasp *Cyphononyx dorsalis* (Konno et al., 2001), and the ant *Pseudomyrmex triplarinus* (Hink et al., 1989). Inosine was also found to be present within the venom of *L. menavodi* (Horni et al., 2001) and other spider species, including *Parawixia bistriata* (Rodrigues et al., 2004), *Cyriopagopus hainanus* (*Selenocosmia huwena*) (Yimin et al., 1995), *Loxosceles reclusa* (Geren et al., 1975), and six more species (venoMS). Purine nucleosides such as adenosine and inosine are common constituents of elapid and viperine snake venoms and are thought to play important accessory roles in helping snakes envenomate and incapacitate their prey (Aird, 2002;2005;Laustsen, 2016;Villar-Briones and Aird, 2018). Adenosine has a wide range of physiological effects in mammalian targets, including inducing vasodilation, causing increased vascular permeability, increasing blood coagulation time, and inhibiting neurotransmitter release (Aird, 2002;Thien et al., 2017). The effects of inosine are not so well understood, but it is also known to increase vascular permeability and induce hypotension through selective binding with mast cell A_3 receptors (Tilley et al., 2000), as well as having vasodilatory properties (Juhasz-Nagy and Aviado, 1977); reviewed in (Aird, 2002)). Aird (2002) provides an overview of the large body of literature studying the physiological effects of adenosine and inosine in mammalian targets, and summarises how these properties may translate to a functional role within a venom. For example, by inducing vasodilation and increasing vascular permeability venomous organisms may improve their systemic envenomation of the target (Aird, 2002;2005;Laustsen, 2016;Villar-Briones and Aird, 2018).

Whilst the effects of adenosine and inosine in mammalian targets are relatively well understood, scorpions do not solely utilise their venom to defend against mammals (Evans et al., 2019). Unlike elapid and viperid snakes, which frequently target vertebrates, scorpions predominantly hunt invertebrates, although in certain environments they are known to take small vertebrates such as blind snakes (McCormick and Polis, 1982). Whilst Aird (2002) discusses the role of adenosine and inosine in snake venoms that target mammalian prey, it is unclear how closely this

translates to scorpions aiming to incapacitate invertebrate prey. Purinoreceptors evolved at an early date, and therefore structural similarities exist between the receptors that vertebrates and invertebrates possess (Burnstock, 1996). It is, therefore, possible that adenosine will interact with invertebrate and vertebrate purinoreceptors in similar ways (Burnstock, 1996;Aird, 2002), but morphological differences between them may lead to different overall effects. For example, insects and mammals have distinctly different circulatory systems, and therefore the hypotensive effects induced by adenosine in mammals may not directly translate to an insect model. It is, however, likely that other effects of adenosine, such as reducing the release of neurotransmitters, may be more closely paralleled between insects and mammals (Aird, 2005). IMP induces delayed paralysis when injected into termites, but it remains untested whether inosine would have the same effect (Aird, 2005). Because *H. waigiensis* scorpions generally attack invertebrate prey but have to defend themselves against vertebrates, their venom contains components effective specifically against vertebrates and invertebrates, as well as components against both (Gangur et al., 2017). It is currently unclear whether adenosine facilitates the envenomation and incapacitation of invertebrate prey when injected alongside other venom components, or whether it is more heavily involved in defence against vertebrates. It could also function within the venom to aid capture of small vertebrate prey in environments with low invertebrate prey abundance (McCormick and Polis, 1982).

4.5.2. AMP

In addition to adenosine, we identified AMP within the venom of *H. waigiensis.* Similar to adenosine, AMP has hypotensive properties, and therefore could act in a similar way to facilitate envenomation in mammalian aggressors (McCormick and Polis, 1982). Aird (2005) found that only a small number of snake venoms contained AMP compared with adenosine, but stated that the presence of purine monophosphates within venoms is unsurprising due to the hypotensive effects they share with free purines. We are unaware of previous reports of AMP, adenosine diphosphate (ADP), or adenosine triphosphate (ATP) from scorpion venoms; however, they are present within different tarantula venoms, including those belonging to *H. lividum, Lasiodora sp*., *Aphonopelma sp.*, and *Aphonopelma hentzi* (*Dugesiella hentzi* and *Eurypelma californicum*) (Chan et al., 1975;Savel-Niemann, 1989;Moore et al., 2009;Nentwig, 2012;Horta et al., 2013). Unlike adenosine, which binds to P1 receptors, ADP and ATP bind to P2 receptors (Burnstock, 2007), with evidence suggesting that they also have auxiliary roles alongside toxins within venom (Chan et al., 1975;Horta et al., 2013). As with adenosine, ADP and AMP possess hypotensive properties and may fulfil a similar auxiliary function within the venom (Aird, 2002;Horta et al., 2013). On the other hand, ATP is less vasodilatory and can lead to hypertension (Gillespie, 1934;Aird, 2002), and therefore may function differently

within the venom. However, Chan et al. (1975) showed that ATP works synergistically with the specific toxins to increase toxicity towards mice. It is possible that adenosine and AMP may act in a similar way to increase the overall toxicity of scorpion venom. To test this, future work could perform a bioassay injecting adenosine and AMP alongside other venom toxins, looking for synergistic or auxiliary effects. Of the five scorpions we tested, the whole venoms contained between 2.016 µg and 5.808 µg of adenosine. Intravenous injection of much lower quantities (0.1 µg) in mice induced hypotension (Gillespie, 1934), while topically applied 10^{-5} M adenosine solution increased vascular permeability in hamster cheek pouches (Gawlowski and Duran, 1986). This suggests that it is likely that the quantity of adenosine contained within the venom is large enough to elicit such effects at the site of injection.

4.5.3. Citric Acid/Citrate

Citric acid or citrate is a common constituent of venoms, present within spider, snake, bee, wasp, ant, and scorpion venoms (Fenton et al., 1995;Odell et al., 1999;Kuhn-Nentwig et al., 2011). Its presence within spider venoms is particularly well documented, having been recorded from at least 48 species belonging to 16 families (Kuhn-Nentwig et al., 2011). Despite the common occurrence of citrate within venoms, its role is not fully understood, and it may serve multiple functions. One proposed function of venom citrate is the inhibition of toxins within the venom gland, thereby preventing self-harm (Francis et al., 1992). As citrate can act as a chelator and form strong complexes with divalent metal ions such as Ca^{2+}, Mg^{2+}, and Zn^{2+}, it can inhibit venom proteins that are dependent on these metal ions (Francis et al., 1992). Citrate, therefore, inhibits venom proteins such as calcium-ion-dependant phospholipase A_2 (PLA_2) neurotoxins and myotoxins, as well as zinc-ion-dependant venom metalloprotease haemorrhagic toxins (Odell et al., 1999;Kuhn-Nentwig et al., 2011). For example, it has been demonstrated experimentally that honey bee (*Apis mellifera*) venom PLA_2 is at least partially inhibited by citrate (Fenton et al., 1995), and that the citrate concentration present within the fer-de-lance snake (*Bothrops asper)* venom is high enough to likely completely inhibit PLA_2 and 5′-nucleotidase activity, whilst partially inhibiting (75%) phosphodiesterase activity (Francis et al., 1992). Scorpion venoms can contain both PLA_2 proteins (Krayem and Gargouri, 2020) and metalloproteases (Ortiz et al., 2014) within their venom; therefore, citrate may act as an inhibitor of these toxins within the venom gland. One study found that dried Arizona bark scorpion (*Centruroides sculpturatus*) venom contained 7.77 ± 1.2% citrate, but the extent that citrate is present in the venoms of different scorpion species remains unknown (Fenton et al., 1995). It is, therefore, important to test more scorpion venoms for the presence of citrate, particularly those well known to contain PLA_2 or metalloproteases, to identify if this is its primary function within scorpion venom. Other proposed functions of citric acid within the venom gland include having

direct antimicrobial effects or enhancing the effects of antimicrobial molecules (Lee et al., 2001;Kuhn-Nentwig et al., 2011). Furthermore, it has been suggested that in spiders citric acid may be present to counter cationic peptides and acylpolyamines within the venom gland (Kuhn-Nentwig et al., 2011). At the time of writing no acylpolyamines have been characterised from scorpion venoms, but cationic peptides are present (Moerman et al., 2002;Willems et al., 2002;Gao et al., 2010). It is likely that citrate has multiple functions within the venom gland of scorpions, but additionally it may have a role once injected into the target organism. An example of this has been demonstrated with the cardiotoxin A3 (CTX A3) from the Taiwan cobra (*Naja atra*), where heparin-sulphate-mediated cell retention of CTX A3 is citrate dependant (Lee et al., 2005). However, it is currently unknown if citrate acts in conjunction with any scorpion toxins within an envenomated organism.

4.5.4. Free Amino Acids

Free amino acids have been widely reported from snake venoms (Bieber, 1979;Villar-Briones and Aird, 2018) and spider venoms (Schanbacher et al., 1973;Savel-Niemann, 1989;Kuhn-Nentwig et al., 2004), but to our knowledge their possible functions within the venoms remain unknown. We found aspartic acid and glutamic acid to be present within *H. waigiensis* venom, but these molecules were only present in low abundance (Figure 4.1). The fraction containing glutamic acid and aspartic acid contained other small molecules at lower abundance, indicated by the presence of extra peaks within the NMR spectra (Figure 4.2), but we were unable to characterise these molecules. A cocktail of amino acids is naturally found in body fluids and can be released from degrading cells, and these molecules may not have a function contributing to the toxicity of the venom. However, if this was the case we might expect to see a more diverse range of amino acids present within the venom. It is possible that other amino acids are present within the venom of *H. waigiensis* and we were only able to identify aspartic acid and glutamic acid as we were able to attain the cleanest NMR spectra for these molecules, but the presence of these same amino acids in spider venoms suggests they may have some specific function. In tarantulas, glutamic acid has been found in the venom of *H. lividum*, and in two separate studies both aspartic acid and glutamic acid were identified from *A. hentzi* (Schanbacher et al., 1973;Savel-Niemann, 1989). Furthermore, NMR screening has shown that aspartic acid is found within the venom of the spider *Pisaura mirabilis*, while glutamic acid is a common component of many different araneomorph spider venoms (Schroeder et al., 2008). Glutamic acid, or the conjugate base glutamate, is an important neurotransmitter in the central nervous system (Olive et al., 2019), and injection in certain parts of the body will likely disrupt the nervous system. More work is required to explain the presence of these molecules in venom.

4.6. Conclusions and Future Directions

Whilst scorpion venoms have been subject to intensive study, research has been heavily skewed to focus on peptide venom constituents. Members of the family Buthidae have also been subject to more intensive study than other scorpion families, as Buthidae contains all medically significant species (Santos et al., 2016). *H. waigiensis* belongs to the family Hormuridae (Monod and Prendini, 2015), which has received comparatively little attention from researchers. It may be that members of this family and other neglected families also utilise small molecules within their venom arsenals. To our knowledge, adenosine has previously been reported from just one other scorpion species, *H. laoticus*, which belongs to the family Scorpionidae (Tran et al., 2017). Scorpions show great toxin diversity between species, and the presence of adenosine in two scorpion species belonging to different families suggests that it may be more commonly utilised by other species. Furthermore, to our knowledge this study provides the first direct evidence of nucleotides (AMP) and specific amino acids within a scorpion venom. Our investigation highlights that the small molecules contained within *H. waigiensis* venom are similar to those found in other closely and distantly related venomous organisms, suggesting that such molecules may have important functions in the venoms of a wide range of organisms. Future work should aim to investigate the extent that small molecules are utilised by different scorpion species, paying particular attention to understudied families. Characterising the suite of small molecules within scorpion venoms will help us gain a greater understanding of the biochemical mechanisms involved in scorpion envenomation. This understanding may allow for the development of improved and optimised treatment of scorpion envenomation, or allow facilitation or modulation of the action of developed scorpion-venom-derived therapeutics and bioinsecticides. Furthermore, the abundance and diversity of small-molecule venom constituents in different species of scorpions remains largely unstudied, and uncharacterised bioactive molecules could have potential applications in pharmacology.

4.7. Bibliography

Adam, K.R., and Weiss, C. (1959). Distribution of 5-hydroxytryptamine in scorpion venoms. *Nature* 183**,** 1398.

Ahmadi, S., Knerr, J.M., Argemi, L., Bordon, K.C.F., Pucca, M.B., Cerni, F.A., Arantes, E.C., Çalışkan, F., and Laustsen, A.H. (2020). Scorpion venom: detriments and benefits. *Biomedicines* 8**,** 118.

Aird, S.D. (2002). Ophidian envenomation strategies and the role of purines. *Toxicon* 40**,** 335-393.

Aird, S.D. (2005). Taxonomic distribution and quantitative analysis of free purine and pyrimidine nucleosides in snake venoms. *Comparative Biochemistry and Physiology B: Biochemistry and Molecular Biology* 140**,** 109-126.

Arjunwadkar, A.V., and Reddy, S.R.R. (1983). Spermidine in the venom of the scorpion, *Palamneus phipsoni*. *Toxicon* 21**,** 321-325.

Banerjee, S., Gnanamani, E., Lynch, S.R., ZuñIga, F.Z., JiméNez-Vargas, J.M., Possani, L.D., and Zare, R.N. (2018). An alkaloid from scorpion venom: chemical structure and synthesis. *Journal of Natural Products* 81**,** 1899-1904.

Bieber, A. (1979). "Metal and nonprotein constituents in snake venoms," in *Snake Venoms*. Springer), 295-306.

Burnstock, G. (1996). Purinoceptors: ontogeny and phylogeny. *Drug Development Research* 39**,** 204-242.

Burnstock, G. (2007). Purine and pyrimidine receptors. *Cellular and Molecular Life Sciences* 64**,** 1471.

Carcamo-Noriega, E.N., Sathyamoorthi, S., Banerjee, S., Gnanamani, E., Mendoza-Trujillo, M., Mata-Espinosa, D., Hernández-Pando, R., Veytia-Bucheli, J.I., Possani, L.D., and Zare, R.N. (2019). 1, 4-Benzoquinone antimicrobial agents against *Staphylococcus aureus* and *Mycobacterium tuberculosis* derived from scorpion venom. *Proceedings of the National Academy of Sciences of the United States of America* 116**,** 12642-12647.

Cesar, L.M., Mendes, M.A., Tormena, C.F., Marques, M.R., De Souza, B.M., Saidemberg, D.M., Bittencourt, J.C., and Palma, M.S. (2005). Isolation and chemical characterization of PwTx-II: A novel alkaloid toxin from the venom of the spider *Parawixia bistriata* (Araneidae, Araneae). *Toxicon* 46**,** 786-796.

Chan, T.K., Geren, C., Howell, D., and Odell, G. (1975). Adenosine triphosphate in tarantula spider venoms and its synergistic effect with the venom toxin. *Toxicon* 13**,** 61-66.

Chippaux, J.-P., and Goyffon, M. (2008). Epidemiology of scorpionism: a global appraisal. *Acta Tropica* 107**,** 71-79.

Dai, L., Corzo, G., Naoki, H., Andriantsiferana, M., and Nakajima, T. (2002). Purification, structure–function analysis, and molecular characterization of novel linear peptides from scorpion *Opisthacanthus madagascariensis*. *Biochemical and Biophysical Research Communications* 293**,** 1514-1522.

Daly, N.L., and Wilson, D. (2018). Structural diversity of arthropod venom toxins. *Toxicon* 152**,** 46-56.

Escoubas, P., Diochot, S., and Corzo, G. (2000). Structure and pharmacology of spider venom neurotoxins. *Biochimie* 82**,** 893-907.

Evans, E.R.J., Northfield, T.D., Daly, N.L., and Wilson, D.T. (2019). Venom costs and optimization in scorpions. *Frontiers in Ecology and Evolution* 7**,** 196.

Fenton, A.W., West, P.R., Odell, G.V., Hudiburg, S.M., Ownby, C.L., Mills, J.N., Scroggins, B.T., and Shannon, S.B. (1995). Arthropod venom citrate inhibits phospholipase A2. *Toxicon* 33**,** 763-770.

Francis, B., Seebart, C., and Kaiser, I.I. (1992). Citrate is an endogenous inhibitor of snake venom enzymes by metal-ion chelation. *Toxicon* 30**,** 1239-1246.

Francke, O.F. (2019). Conspectus genericus scorpionorum 1758–1985 (Arachnida: Scorpiones) updated through 2018. *Zootaxa* 4657**,** 1-56.

Gangur, A.N., Smout, M., Liddell, M.J., Seymour, J.E., Wilson, D., and Northfield, T.D. (2017). Changes in predator exposure, but not in diet, induce phenotypic plasticity in scorpion venom. *Proceedings of the Royal Society of London. Series B: Biological Sciences* 284**,** 20171364.

Gao, B., Xu, J., Del Carmen Rodriguez, M., Lanz-Mendoza, H., Hernández-Rivas, R., Du, W., and Zhu, S. (2010). Characterization of two linear cationic antimalarial peptides in the scorpion *Mesobuthus eupeus*. *Biochimie* 92**,** 350-359.

Gawlowski, D.M., and Duran, W.N. (1986). Dose-related effects of adenosine and bradykinin on microvascular permselectivity to macromolecules in the hamster cheek pouch. *Circulation Research* 58**,** 348-355.

Geren, C.R., Chan, T.K., Howell, D.E., and Odell, G.V. (1975). Partial characterization of the low molecular weight fractions of the extract of the venom apparatus of the brown recluse spider and of its hemolymph. *Toxicon* 13**,** 233-238.

Gillespie, J.H. (1934). The biological significance of the linkages in adenosine triphosphoric acid. *Journal of Physiology* 80**,** 345.

Gomes, P.C., De Souza, B.M., Dias, N.B., Cesar-Tognoli, L.M.M., Silva-Filho, L.C., Tormena, C.F., Rittner, R., Richardson, M., Cordeiro, M.N., and Palma, M.S. (2011). Nigriventrine: a low molecular mass neuroactive compound from the venom of the spider *Phoneutria nigriventer*. *Toxicon* 57**,** 266-274.

Hink, W., Romstedt, K., Burke, J., Doskotch, R., and Feller, D. (1989). Inhibition of human platelet aggregation and secretion by ant venom and a compound isolated from venom. *Inflammation* 13**,** 175-184.

Hisada, M., Fujita, T., Naoki, H., Itagaki, Y., Irie, H., Miyashita, M., and Nakajima, T. (1998). Structures of spider toxins: Hydroxyindole-3-acetylpolyamines and a new generalized structure of type-E compounds obtained from the venom of the Joro spider, *Nephila clavata*. *Toxicon* 36**,** 1115-1125.

Horni, A., Weickmann, D., and Hesse, M. (2001). The main products of the low molecular mass fraction in the venom of the spider *Latrodectus menavodi*. *Toxicon* 39**,** 425-428.

Horta, C.C., Rezende, B.A., Oliveira-Mendes, B.B.R., Carmo, A.O., Capettini, L.S.A., Silva, J.F., Gomes, M.T., Chávez-Olórtegui, C., Bravo, C.E.S., and Lemos, V.S. (2013). ADP is a vasodilator component from *Lasiodora* sp. mygalomorph spider venom. *Toxicon* 72**,** 102-112.

Inceoglu, B., Lango, J., Jing, J., Chen, L., Doymaz, F., Pessah, I.N., and Hammock, B.D. (2003). One scorpion, two venoms: prevenom of *Parabuthus transvaalicus* acts as an alternative type of venom with distinct mechanism of action. *Proceedings of the National Academy of Sciences of the United States of America* 100**,** 922-927.

Isbister, G.K., Volschenk, E.S., and Seymour, J.E. (2004). Scorpion stings in Australia: five definite stings and a review. *Internal Medicine Journal* 34**,** 427-430.

Itagaki, Y., Fujita, T., Naoki, H., Yasuhara, T., Andriantsiferana, M., and Nakajima, T. (1997). Detection of new spider toxins from a *Nephilengys borbonica* venom gland using on-line µ-column HPLC continuous flow (FRIT) FAB LC/MS and MS/MS. *Natural Toxins* 5**,** 1-13.

Juhasz-Nagy, A., and Aviado, D.M. (1977). Inosine as a cardiotonic agent that reverses adrenergic beta blockade. *Journal of Pharmacology and Experimental Therapeutics* 202**,** 683-695.

King, G.F. (2011). Venoms as a platform for human drugs: translating toxins into therapeutics. *Expert Opinion on Biological Therapy* 11**,** 1469-1484.

Koch, L. (1977). The taxonomy, geographic distribution and evolutionary radiation of Australo-Papuan scorpions. *Records of the Western Australian Museum* 5.

Konno, K., Hisada, M., Naoki, H., Itagaki, Y., Yasuhara, T., Juliano, M.A., Juliano, L., Palma, M.S., Yamane, T., and Nakajima, T. (2001). Isolation and sequence determination of peptides in the venom of the spider wasp (*Cyphononyx dorsalis*) guided by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry. *Toxicon* 39**,** 1257-1260.

Krayem, N., and Gargouri, Y. (2020). Scorpion venom phospholipases A2: A minireview. *Toxicon*.

Kuhn-Nentwig, L., Schaller, J., and Nentwig, W. (2004). Biochemistry, toxicology and ecology of the venom of the spider *Cupiennius salei* (Ctenidae). *Toxicon* 43**,** 543-553.

Kuhn-Nentwig, L., Stöcklin, R., and Nentwig, W. (2011). "Venom composition and strategies in spiders: is everything possible?," in *Advances in Insect Physiology*. Elsevier), 1-86.

Laustsen, A.H. (2016). Toxin synergism in snake venoms. *Toxin Reviews* 35**,** 165-170.

Lee, S.-C., Guan, H.-H., Wang, C.-H., Huang, W.-N., Tjong, S.-C., Chen, C.-J., and Wu, W.-G. (2005). Structural basis of citrate-dependent and heparan sulfate-mediated cell surface retention of cobra cardiotoxin A3. *Journal of Biological Chemistry* 280**,** 9567-9577.

Lee, Y.L., Thrupp, L., Owens, J., Cesario, T., and Shanbrom, E. (2001). Bactericidal activity of citrate against Gram-positive cocci. *Letters in Applied Microbiology* 33**,** 349-351.

Luna-Ramírez, K., Quintero-Hernandez, V., Juárez-González, V.R., and Possani, L.D. (2015). Whole transcriptome of the venom gland from *Urodacus yaschenkoi* scorpion. *PLoS One* 10**,** e0127883.

Marques, M.R., Mendes, M.A., Tormena, C.F., Souza, B.M., Marcondes Cesar, L.M., Rittner, R., and Palma, M.S. (2005). Structure determination of a tetrahydro-β-carboline of arthropod origin: a novel alkaloid-toxin subclass from the web of spider *Nephila clavipes*. *Chemistry & Biodiversity* 2**,** 525-534.

Mccormick, K.D., and Meinwald, J. (1993). Neurotoxic acylpolyamines from spider venoms. *Journal of Chemical Ecology* 19**,** 2411-2451.

Mccormick, S., and Polis, G.A. (1982). Arthropods that prey on vertebrates. *Biological Reviews of the Cambridge Philosophical Society* 57**,** 29-58.

Moerman, L., Bosteels, S., Noppe, W., Willems, J., Clynen, E., Schoofs, L., Thevissen, K., Tytgat, J., Van Eldere, J., and Van Der Walt, J. (2002). Antibacterial and antifungal properties of α-helical, cationic peptides in the venom of scorpions from southern Africa. *European Journal of Biochemistry* 269**,** 4799-4810.

Monod, L., and Prendini, L. (2015). Evidence for Eurogondwana: the roles of dispersal, extinction and vicariance in the evolution and biogeography of Indo-Pacific Hormuridae (Scorpiones: Scorpionoidea). *Cladistics* 31**,** 71-111.

Monod, L., and Volschenk, E. (2004). *Liocheles litodactylus* (Scorpiones: Liochelidae): an unusual new *Liocheles* species from the Australian wet tropics (Queensland). *Memoirs of the Queensland Museum* 49**,** 675-690.

Moore, S., Smyth, W.F., Gault, V.A., O'kane, E., and Mcclean, S. (2009). Mass spectrometric characterisation and quantitation of selected low molecular mass compounds from the venom of *Haplopelma lividum* (Theraphosidae). *Rapid Communications in Mass Spectrometry* 23**,** 1747-1755.

Nentwig, W. (2012). The species referred to as *Eurypelma californicum* (Theraphosidae) in more than 100 publications is likely to be *Aphonopelma hentzi*. *Journal of Arachnology* 40**,** 128-131.

Odell, G., Fenton, A., Ownby, C., Doss, M., and Schmidt, J. (1999). The role of venom citrate. *Toxicon* 37**,** 407-409.

Olive, M.F., Powell, G., Mcclure, E., and Gipson, C.D. (2019). "Neurotransmitter Systems: Glutamate," in *The Therapeutic Use of N-Acetylcysteine (NAC) in Medicine*. Springer), 19-28.

Ortiz, E., Gurrola, G.B., Schwartz, E.F., and Possani, L.D. (2015). Scorpion venom components as potential candidates for drug development. *Toxicon* 93**,** 125-135.

Ortiz, E., Rendón-Anaya, M., Rego, S.C., Schwartz, E.F., and Possani, L.D. (2014). Antarease-like Zn-metalloproteases are ubiquitous in the venom of different scorpion genera. *Biochimica et Biophysica Acta* 1840**,** 1738-1746.

Palma, M.S., and Nakajima, T. (2005). A natural combinatorial chemistry strategy in acylpolyamine toxins from nephilinae orb-web spiders. *Toxin Reviews* 24**,** 209-234.

Possani, L.D., Becerril, B., Delepierre, M., and Tytgat, J. (1999). Scorpion toxins specific for Na+-channels. *European Journal of Biochemistry* 264**,** 287-300.

R Core Team (2021). "R: A language and environment for statistical computing". (Vienna, Austria: R Foundation for Statistical Computing).

Rodrigues, M.C.A., Guizzo, R., Gobbo-Neto, L., Ward, R.J., Lopes, N.P., and Dos Santos, W.F. (2004). The biological activity in mammals and insects of the nucleosidic fraction from the spider *Parawixia bistriata*. *Toxicon* 43**,** 375-383.

Saidemberg, D.M., Ferreira, M.a.B., Takahashi, T.N., Gomes, P.C., Cesar-Tognoli, L.M.M., Da Silva-Filho, L.C., Tormena, C.F., Da Silva, G.V.J., and Palma, M.S. (2009). Monoamine oxidase inhibitory activities of indolylalkaloid toxins from the venom of the colonial spider *Parawixia bistriata*: functional characterization of PwTX-I. *Toxicon* 54**,** 717-724.

Santos, M.S.V., Silva, C.G.L., Neto, B.S., Júnior, C.R.P.G., Lopes, V.H.G., Júnior, A.G.T., Bezerra, D.A., Luna, J.V.C.P., Cordeiro, J.B., and Júnior, J.G. (2016). Clinical and epidemiological aspects of scorpionism in the world: a systematic review. *Wilderness & Environmental Medicine* 27**,** 504-518.

Savel-Niemann, A. (1989). Tarantula (*Eurypelma californicum*) venom, a multicomponent system. *Biological Chemistry Hoppe-Seyler* 370**,** 485-498.

Schanbacher, F.L., Lee, C.K., Hall, J.E., Wilson, I.B., Howell, D.E., and Odell, G.V. (1973). Composition and properties of tarantula *Dugesiella hentzi* (Girard) venom. *Toxicon* 11**,** 21-29.

Schroeder, F.C., Taggi, A.E., Gronquist, M., Malik, R.U., Grant, J.B., Eisner, T., and Meinwald, J. (2008). NMR-spectroscopic screening of spider venom reveals sulfated nucleosides as major components for the brown recluse and related species. *Proceedings of the National Academy of Sciences of the United States of America* 105**,** 14283-14287.

Skinner, W.S., Dennis, P.A., Lui, A., Carney, R.L., and Quistad, G.B. (1990). Chemical characterization of acylpolyamine toxins from venom of a trap-door spider and two tarantulas. *Toxicon* 28**,** 541-546.

Smith, J.J., Herzig, V., King, G.F., and Alewood, P.F. (2013a). The insecticidal potential of venom peptides. *Cellular and Molecular Life Sciences* 70**,** 3665-3693.

Smith, J.J., Hill, J.M., Little, M.J., Nicholson, G.M., King, G.F., and Alewood, P.F. (2011). Unique scorpion toxin with a putative ancestral fold provides insight into evolution of the inhibitor cystine knot motif. *Proceedings of the National Academy of Sciences* 108**,** 10478-10483.

Smith, J.J., Vetter, I., Lewis, R.J., Peigneur, S., Tytgat, J., Lam, A., Gallant, E.M., Beard, N.A., Alewood, P.F., and Dulhunty, A.F. (2013b). Multiple actions of ϕ-LITX-Lw1a on ryanodine receptors reveal a functional link between scorpion DDH and ICK toxins. *Proceedings of the National Academy of Sciences* 110**,** 8906-8911.

Taggi, A.E., Meinwald, J., and Schroeder, F.C. (2004). A new approach to natural products discovery exemplified by the identification of sulfated nucleosides in spider venom. *Journal of the American Chemical Society* 126**,** 10364-10369.

Thien, T.V., Anh, H.N., Trang, N.T.T., Van Trung, P., Khoa, N.C., Osipov, A., Dubovskii, P., Ivanov, I., Arseniev, A., and Tsetlin, V. (2017). Low-molecular-weight compounds with anticoagulant activity from the scorpion *Heterometrus laoticus* venom. *Doklady Biochemistry and Biophysics* 476**,** 316-319.

Tilley, S.L., Wagoner, V.A., Salvatore, C.A., Jacobson, M.A., and Koller, B.H. (2000). Adenosine and inosine increase cutaneous vasopermeability by activating A3 receptors on mast cells. *The Journal of Clinical Investigation* 105**,** 361-367.

Tran, T., Hoang, A., Nguyen, T., Phung, T., Nguyen, K., Osipov, A., Ivanov, I., Tsetlin, V., and Utkin, Y. (2017). Anticoagulant activity of low-molecular weight compounds from *Heterometrus laoticus* scorpion venom. *Toxins* 9**,** 343.

Vassilevski, A.A., Kozlov, S.A., and Grishin, E.V. (2009). Molecular diversity of spider venom. *Biochemistry (Moscow)* 74**,** 1505-1534.

Venoms. Available: https://www.venoms.ch/ [Accessed 30/06/2020].

Villar-Briones, A., and Aird, S. (2018). Organic and peptidyl constituents of snake venoms: the picture is vastly more complex than we imagined. *Toxins* 10**,** 392.

Willems, J., Noppe, W., Moerman, L., Van Der Walt, J., and Verdonck, F. (2002). Cationic peptides from scorpion venom can stimulate and inhibit polymorphonuclear granulocytes. *Toxicon* 40**,** 1679-1683.

Wilson, D., Boyle, G., Mcintyre, L., Nolan, M., Parsons, P., Smith, J., Tribolet, L., Loukas, A., Liddell, M., and Rash, L. (2017). The aromatic head group of spider toxin polyamines influences toxicity to cancer cells. *Toxins* 9**,** 346.

Yimin, S., Bingkun, X., Songping, L., and Shunyi, L. (1995). Separation and identification of inosine in the *Selenocosmia huwena* spider. *Chinese Journal of Analytical Chemistry***,** 25.

Yoshioka, M., Narai, N., Teshima, T., Matsumoto, T., Wakamiya, T., Shiba, T., Tokoro, N., Okauchi, T., and Kono, Y. (1992). Characterization of a new insecticide, clavamine, from the venom of a spider, *Nephila clavata* by use of a synthetic compound. *Chemical and Pharmaceutical Bulletin* 40**,** 3005-3008.

Urodacus yaschenkoi adult – Edward Evans

Chapter 5. You Get What You Deserve: Effect of Threat Level and Repetitive Attacks on the Defensive Stinging Behaviours of the Desert Scorpion (*Urodacus yaschenkoi*)

5.1. Abstract

Scorpions adapt their defensive behaviours in different contexts, which can allow them to use their venom more efficiently and effectively. For example, they can 'dry' sting without injecting venom. This can help avoid wasting venom, which requires both time and energy to replenish. I studied the defensive behaviours of *Urodacus yaschenkoi* in response to a repeated attack sequence under low- and high-threat (attack frequency) treatments. In response to each attack, I recorded if a scorpion attempted to sting, if a dry sting was performed, and whether the venom appeared clear, opalescent, or milky, which I assigned to a level of defensive investment. Under both threat treatments defensive investment increased as the threat persisted. Defensive investment per sting was greater in high- versus low-threat treatments and increased at a faster rate across the sting sequence under high-threat. I additionally analysed all stings using liquid chromatography/mass spectrometry (LC/MS) to test for compositional differences between venom that was visually clear, opalescent, or milky. The analysis showed clear samples contained the fewest molecules, whilst milky samples contained the most. The majority of molecules were shared between clear, opalescent, and milky samples, but some were unique to each visual classification. A large number were shared between clear/opalescent and opalescent/milky, which suggests a compositional change from clear to milky. Finally, my analysis showed that clear samples contained the highest proportions of small peptides 1000-2500 Da, whilst milky samples contained the highest proportions of large peptides (>5000 Da). The lower proportion of large, energetically expensive, toxins in the clear samples provides evidence that the initial clear venom secretions may have evolved as a cost-reducing mechanism.

5.2. Introduction

Many organisms participate in chemical warfare against their prey or predators by producing toxins that act as poisons or venoms. Whilst poisons must typically contact or be ingested by a predator, venoms are injected into either prey or potential predators (Wüster, 2010;Casewell et al., 2013;Nelsen et al., 2014b;Jared et al., 2021). Venom can often serve a dual function for an animal, assisting in both prey-capture and defensive contexts. The use of venom can be considered an extremely successful evolutionary trait, with ~15% of the currently described animal species being venomous (Holford et al., 2018). Over 2500 of these species comprise scorpions (Rein, 2022), which, having evolved over 400 million years ago, are one of the oldest venomous lineages (Dunlop and Selden, 2009). Scorpions possess complex venom mixtures (de la Vega et al., 2010) and a highly mobile and specialised stinging apparatus (van der Meijden et al., 2013;van der Meijden and Kleinteich, 2017), which they frequently use against both prey and potential predators (Gangur et al., 2018;Simone and Meijden, 2021).

There is a large amount of evidence to suggest that scorpions, like other venomous animals, control expenditure of their venom when facing different prey and predators, optimising efficiency of their venom use (Hayes et al., 2002;Cooper et al., 2015;Evans et al., 2019;Nelsen et al., 2020). This often involves assessment of a potential prey or predator in each situation, to calculate the appropriate response. For example, some scorpions have been observed to sting smaller, more easily subdued prey items less frequently (Rein, 1993;Edmunds and Sibly, 2010), and a study on the defensive behaviours of *Parabuthus transvaalicus* found they injected over twice the volume of venom per sting in high versus low threat encounters (Nisani and Hayes, 2011). By controlling the use of venom in different situations, scorpions can avoid wasting their venom unnecessarily. This is advantageous, as the replenishment of venom toxins requires energy, but also time when the scorpion could be left with reduced defences (Nisani et al., 2007;Nisani et al., 2012;Carcamo-Noriega et al., 2019a). Metabolic costs of venom replenishment appear to be high in scorpions, with one study on *P. transvaalicus* recording a 39% increase in metabolic rate during the first 72 h of venom regeneration (Nisani et al., 2007).

Scorpions modulate their defensive behaviours on multiple levels, which allows them to vary their level of defensive investment in different scenarios. Firstly, when encountering a potential predator they can choose to act defensively or flee (Miller et al., 2016). If a defensive stance is taken, they can deliver a ‘dry’ sting, which may scare off a potential threat without using venom (Nisani and Hayes, 2011;Lira et al., 2017;Rasko et al., 2018). When a venomous sting is performed, scorpions can control the volume of venom they inject, which will affect their energetic investment (Nisani and

Hayes, 2011;van der Meijden et al., 2015;Evans et al., 2019). An additional level of complexity arises when considering how the volume injected affects the composition injected, as larger injections may also include different types of molecules. It was first observed in *Leiurus quinquestriatus* that the initial venom expelled is clear, and becomes opalescent as it is continued to be released, finally turning milky and viscous (Yahel-Niv and Zlotkin, 1979). This visual change corresponded to changes in both the composition and toxicity of the venom (Yahel-Niv and Zlotkin, 1979). An in-depth analysis of *P. transvaalicus* venom found the initial clear secretion, 'prevenom', contained a six-fold lower peptide/protein concentration, and a sixteen-fold higher concentration of potassium salt (K^+) than the milky secretion, whilst still effectively paralysing invertebrates and causing pain in vertebrates (Inceoglu et al., 2003). As prevenom is likely sufficient for many interactions scorpions have with their prey and predators, it has been proposed it may have evolved as a metabolically inexpensive way for them to use their venom without committing to the use of their full, metabolically expensive, toxin arsenal (Inceoglu et al., 2003;Nisani and Hayes, 2011;Nisani et al., 2012;Evans et al., 2019). Although sequential changes from clear to milky have been observed in other species of scorpion (Gopalakrishnakone et al., 1995;Abdel-Rahman et al., 2009;Lira et al., 2017;Evans et al., 2019), only a small number of studies have examined chemical differences. One experiment with *Parabuthus leiosoma* similarly found the first sting contained the lowest protein concentration and fewest protein bands, and also documented large compositional variation throughout the series of nine stings tested (Sarhan et al., 2012). Sequential changes in the visual characteristics of scorpion venoms appear to be widespread across taxonomically distant species in different families (Gopalakrishnakone et al., 1995;Abdel-Rahman et al., 2009;Lira et al., 2017;Evans et al., 2019), but it is unknown if heterogenous venom compositions are ubiquitous in scorpions, and how comparisons made in one species translate to others.

Studies on scorpion defensive behaviours support the notion that prevenom has evolved as a cost reducing mechanism, and that scorpions vary their defensive investment based on levels of threat (Nisani and Hayes, 2011;Lira et al., 2017). When faced with repetitive attacks under low- and high-threat treatments, *P. transvaalicus* (Buthidae) produced milky venom earlier in the sting sequence under high-threat (Nisani and Hayes, 2011). Similarly, a later study performed using the same threat treatments found *Tityus stigmurus* (Buthidae) only produced milky venom under high-threat, with only prevenom produced in the low-threat treatment (Lira et al., 2017). Whilst these studies show that two species of buthid scorpions modulate the use of prevenom and milky venom when faced with different defensive scenarios, it remains unknown if other species behave in a similar way, particularly those in other families.

The Australian desert scorpion *Urodacus yaschenkoi* (Scorpiones: Urodacidae) is a large and robust species, with a widespread distribution in arid regions of Australia (Koch, 1977;Shorthouse and Marples, 1982). Although the venoms of many Australian scorpions are poorly described, *U. yaschenkoi* venom has been subject to proteomic and transcriptomic analysis (Luna-Ramírez et al., 2013;Luna-Ramírez et al., 2015); however, no comparison has been made between clear and milky venom secretions. In this study I examined the defensive behaviours of *U. yaschenkoi* in response to repetitive attacks in low- versus high-threat encounters. I tested defensive investment in response to each attack, by documenting if a sting response was elicited, if a dry sting was performed or venom was injected, and whether the venom appeared clear/opalescent/milky. Each sting was collected, and individually analysed by liquid chromatography/mass spectrometry (LC/MS) to test for differences in chemical complexity and composition associated with the three visual classifications of the venom.

5.3. Methods

5.3.1. Scorpion Husbandry

Fourteen desert scorpions (*U. yaschenkoi*) were purchased from a commercial collector (Thargomindah Man Productions (Varsity Lakes, QLD, Australia)). All scorpions were housed in plastic containers (22 cm length x 14.5 cm width x 14.5 cm height) containing ~10 cm depth of substrate (coco-fibre and sand mix) and kept in darkness inside a temperature-controlled room (25 °C). The scorpions were offered one adult cricket every 1-2 weeks, and uneaten prey were removed. They were maintained under these conditions for eight months before trials began.

5.3.2. Defensive Stinging Trial

Scorpions were exposed to a sequence of six simulated attacks under either high- or low-threat treatments, to elicit a defensive response. The ‘attacks’ were identical under high- and low-threat conditions but were performed at a faster rate in the high-threat condition. Each scorpion was randomly assigned into two groups at the beginning of the experiment, and in each trial half the scorpions were exposed to each threat condition. A total of four trials were performed, so that each scorpion experienced the two threat treatments twice.

Trials were performed mid-afternoon to late evening in a temperature-controlled room (25 °C). The room was kept in darkness, but a red light was used for illumination. Scorpions were selected in a random order for each trial, gently removed from their container using a plastic cup, placed into an empty plastic storage container arena (24 cm length x 21.5 cm width x 10.5 cm

height), and left to acclimatise for 15 min. The scorpion was then subject to six ‘attacks’, simulated by briefly touching/pressing a parafilm covered plastic cup held with forceps onto the dorsal mesosoma. This ‘attack’ was kept constant across trials as touching different parts of a scorpion can elicit different responses (Nelsen et al., 2020). Scorpions were exposed to high- and low-threat treatments as described by (Nisani and Hayes, 2011), and thereafter used by (Lira et al., 2017). Half of the scorpions were exposed to a ‘high-threat’ situation (each attack separated by 5 sec), and the other half were exposed to ‘low-threat’ (attacks separated by 5 min). In most cases the attack stimulus elicited a stinging response into the cup, and if venom was produced it was pipetted off the parafilm and the inside of the cup, suspended in 10 μL of Type 1 water (T1W) water, and stored at -20 °C. For each attack in the sequence, it was documented whether the scorpion attempted to defensively sting the cup or not, and whether it was a dry sting or if venom was injected. Additionally, when venom was expelled into the cup it was noted whether it appeared clear, opalescent (slightly opaque white), or milky (very opaque white) in appearance. A small number of stings were categorised as clear/opalescent or opalescent/milky when it was difficult to assign them to a specific category, particularly in instances when a very small volume of venom was produced.

The second trial was performed 14 days after the first, which followed the exact same method, with each scorpion experiencing the same threat treatment as in the first trial. After this, the scorpions were then kept under normal husbandry conditions for 6 weeks, and the above method repeated for each scorpion under the opposite threat conditions. Therefore, every scorpion was exposed to both the low and high threat condition two times each across the four trials. The only exception was three individuals that died between trials.

5.3.3. Liquid Chromatography/Mass Spectrometry (LC/MS)

To test for compositional differences between each sting, the collected samples were analysed individually using liquid chromatography/mass spectrometry (LC/MS) with an Eksigent ekspert nanoLC 415 system coupled to a SCIEX TripleTOF 6600 equipped with a DuoSpray Ion Source (SCIEX, Framingham, MA, USA). Due to the sensitivity of the instrument, and the large variation in the amount of venom produced between each sting, I standardised the amount of venom injected into the LC/MS. 1 μL of the samples were each diluted in 20 μL T1W, and 3 μL of these mixtures were injected into the LC/MS. Chromatography was performed with a C_{18} column (Trajan ProteCol C_{18} 250 mm x 300 μm, 3μm, 200 Å), in an oven set at 40 °C. Solvent A used was: H_2O/0.1% formic acid (FA); and B: acetonitrile (ACN)/0.1% FA. Flow rate was set at 4 μL/min, with the following solvent gradient: 0-1 min/3-3% B; 1-20 min/3-80% B; 20-27min/80-80% B; 27-28min/80-3% B; 28-35 min/3-3% B. TOF-MS was performed, and spectra collected in positive mode with an accumulation

time of 250 ms and a scan range of 250-2000 *m/z*. The source temperature was set to 300 °C, the ion-spray voltage floating field set to 5 kV, and the declustering potential set to 60 V. Curtain gas was set at 30 psi, ion source gas 1 at 25 psi, and ion source gas 2 at 30 psi. Data acquisition was performed with Analyst® TF 1.7.1 software (SCIEX, Framingham, MA, USA).

5.3.4. Data Processing/Analysis

5.3.4.1. Data selection

Prior to analysis, data were removed from sting sequences where the experimental conditions had varied. For example, in three simulated attack sequences, the scorpions were able to grab hold of the cup with their pedipalps and stung the cup repeatedly. Retrieving the cup from the scorpion involved a tussle that would likely have been perceived as an extra threat, therefore the data collected for these sequences were not included. However, the venom samples collected in each sequence prior to the one where the scorpion grabbed the cup were included in the LC/MS analysis. Additionally, in four attack sequences the scorpions missed the cup with one or more of their stings (three scorpions missed one sting each, one missed two stings). The data for these sequences were not included in the behavioural analysis, but the venom samples collected for the other stings were analysed by LC/MS.

During the experiment, three of the fourteen scorpions died between trials, two of which succumbed in the six-week gap between trial two and three. These animals appeared to behave normally in trials prior to their death, therefore the data collected in the first two trials were included. The third scorpion died between trial three and four, but it was noted in trial three that its behaviour was sluggish – atypical of *U. yaschenkoi* which usually acts defensively in an alert and twitchy fashion. This scorpion also had only produced clear stings in the first two trials, and it was therefore deemed that this scorpion may have been affected by illness, and the data collected for this scorpion was therefore not included in any of the behavioural analysis.

5.3.4.2. Behavioural analysis

All statistical analyses were performed in R version 4.0.5 (R Core Team, 2021). The defensive behaviours observed were each assigned a value as a measure of defensive ‘investment’. Defensive investment was assigned based on previous work in other scorpion species that showed milky venom contains a higher concentration of peptides/proteins than clear venom, and therefore likely require greater investment of energy and time to replenish (Inceoglu et al., 2003;Nisani et al., 2012). The following values of defensive investment were assigned to each behaviour: no attempt to sting = 0, dry sting = 1, sting with clear venom = 2, sting with opalescent venom = 3, and sting with milky

venom = 4. Samples that were recorded as clear/opalescent or opalescent/milky were assigned values of 2.5 and 3.5 respectively. As each scorpion was tested twice under each threat condition, the defensive investment in response to each attack in the sequence for each scorpion under the two threat levels was averaged across the trials. For scorpions with viable data collected in only one attack sequence, the single values were used. Defensive investment was then analysed using a general linear mixed model (LMM) with the lme4 R package (Bates et al., 2015), to test the effect of attack sequence as a continuous fixed effect, and threat level as a categorical fixed effect, on defensive investment. Scorpion identity was incorporated into the model as a random factor with a random intercept. I additionally tested for non-linear responses by incorporating polynomial variables into the model, and the model that best fit the data was selected by comparing Akaike information criterion (AIC) values.

5.3.4.3. LC/MS analysis

LC/MS data was processed using the LCMS peptide reconstruct function in the Bio Tool Kit package for PeakView 2.2.0 software (SCIEX, Framingham, MA, USA). This tool was used to automate mass lists for each sample based on the following parameters: Retention time = 5-22 min, approximate peak width = 15 sec, minimum intensity (counts) = 5, mass tolerance = 0.1 Da, and maximum charge = 10. The generated mass lists were then output for analysis in R (R Core Team, 2021).

The auto-generated mass lists frequently contained duplicates of molecules with the same/similar mass. Additionally, many were present with one mass unit difference where the automation had also picked isotope peaks to reconstruct the parent mass. I worked under the assumption that molecules with similar masses and retention times were the same, and therefore processed each sample using an algorithm using a ‘for loop’ in R to remove duplicate molecules. This algorithm identified molecules within ±1.5 Da of each other and eluted within a ±30 sec retention time window. When these conditions were fulfilled, the mean of the two masses was calculated, and both values in the list reassigned as the mean of the two. Additionally, any values previously averaged with the value being averaged were reassigned the new mean value. After this algorithm had averaged all similar masses in the list and assigned them the mean values, exact replicates were removed from the list. This code was executed on each sample independently to remove duplicates of molecules in each mass list produced by the PeakView software.

The algorithm described above resulted in a mass list for each sample, which were then combined to obtain a master mass list. As masses in the lists for each sample were similar but not exactly the same as in other samples, another algorithm similar to the one described above was

used to remove duplicates from the master list. The masses of molecules within a ±1.5 Da and a ±1 min retention time window were averaged, and the two values replaced with the mean. Any values in the list previously averaged with the same mass were also assigned the new mean value. Exact duplicates were removed from the list. As the master list contained many rows prior to the removal of duplicates (>36000 molecules in all the lists combined) some molecules remained in the output list with very similar masses and retention times. Repeating the same algorithm on the reduced list further removed duplicated molecules and produced a list of 4821 masses.

The mass list of all molecules found across all samples was used to generate a 4821 (molecules) x 210 (samples) matrix describing the presence or absence of each molecule in each sample. This was generated using another algorithm that read down the mass lists for each sample and checked against the master list for molecules within ±1.5 Da and ±1 min retention time windows. If these criteria were met, the molecule was considered present in the sample, otherwise it was considered absent. This presence/absence matrix was finally subset by removing molecules that were present in less than four samples, as these molecules were likely to be rare, in low abundance, or possibly erroneously calculated/generated in the automation process. This final matrix contained 1938 molecules.

The presence/absence matrix of molecules was analysed to test whether chemical complexity was associated with the visual characteristics of the venom sample using general LMMs. A model that included scorpion identity and trial as random effects with random intercepts was used to directly test the effect of visual classification on the chemical complexity of the samples, and pairwise Tukey tests performed to compare between groups using the multcomp R package (Hothorn et al., 2008). Additionally, a LMM incorporating visual classification and sting number in the sequence as fixed effects, and scorpion and trial as random effects was used to compare the effects of visual classification of samples when attack sequence was accounted for in the model. I additionally tested how chemical complexity per sting responded to repeated attacks and threat level irrespective of its visual classification with a LMM that included sting number, threat level, and sting number x threat level as fixed effects, and scorpion identity with a random intercept. Trial number was not included as a random effect in this model, as the structure of the model became too complex.

Compositional differences associated with clear, opalescent and milky samples were visualised using Venn diagrams created with the VennDiagram R package (Chen and Boutros, 2011). Finally, I compared the relative abundance of molecules in different size classes between clear, opalescent, and milky venoms. Samples that were found to contain zero molecules (n = 2) were not

included in the analysis. Molecules were split into molecular weight bins <1000 Da, 1000-2500 Da, 2500-5000 Da, 5000-8500 Da, and >8500 Da. These bins were selected based on molecular weight groups that loosely correspond to known families of scorpion toxins. <1000 Da would mostly contain small molecules and small linear peptides (Shalabi et al., 2004;Banerjee et al., 2018;Carcamo-Noriega et al., 2019b;Evans et al., 2020), 1000-2500 Da would include non-disulfide bonded peptides (Zeng et al., 2005;Almaaytah and Albalas, 2014), 2500-5000 Da would represent short-chain toxins (Possani et al., 2000;de la Vega and Possani, 2004), 5000-9000 Da would largely represent long-chain toxins (de la Vega and Possani, 2005), and >9000 Da would contain all larger peptides/proteins (Luna-Ramírez et al., 2013;Delgado-Prudencio et al., 2022). Visualisation of data was performed using the ggplot2 R package (Wickham, 2016).

5.4. Results

5.4.1. Defensive Investment

The level of defensive investment exhibited by *U. yaschenkoi*, as measured using sting responses, dry sting rates, and the visual representation of the venom samples, increased with repeated attacks (Figure 5.1). Defensive investment was higher in response to each attack under the high-threat treatment, and additionally increased at a faster rate across the attack sequence when compared against the low-threat treatment (Figure 5.1). A LMM testing the effect of attack number, threat level, and attack number x threat level showed that attack number described significant variation in the mean defensive investment ($F_{1, 122.05} = 96.4433$, $p < 0.0001$), increasing as the attack sequence progressed (Figure 5.1). Threat level did not describe significant variation in the model ($F_{1, 122.86} = 0.3125$, $p = 0.5772$), but defensive investment was significantly described by the interaction of attack number x threat level ($F_{1, 122.05} = 7.9005$, $p = 0.0058$), showing that as sting sequence progressed, the defensive investment increased at a faster rate in the high threat treatment (Figure 5.1). Under both high and low threat, the mean investment in response to each attack appeared to respond in a curvilinear fashion when plotted, plateauing in the later stings (Figure 5.1). Addition of a quadratic term ($sting^2$) to a LMM to test if investment responded in a non-linear fashion showed the curvilinear model had a higher AIC value than models with only linear terms, suggesting it is a less-good fit of the data.

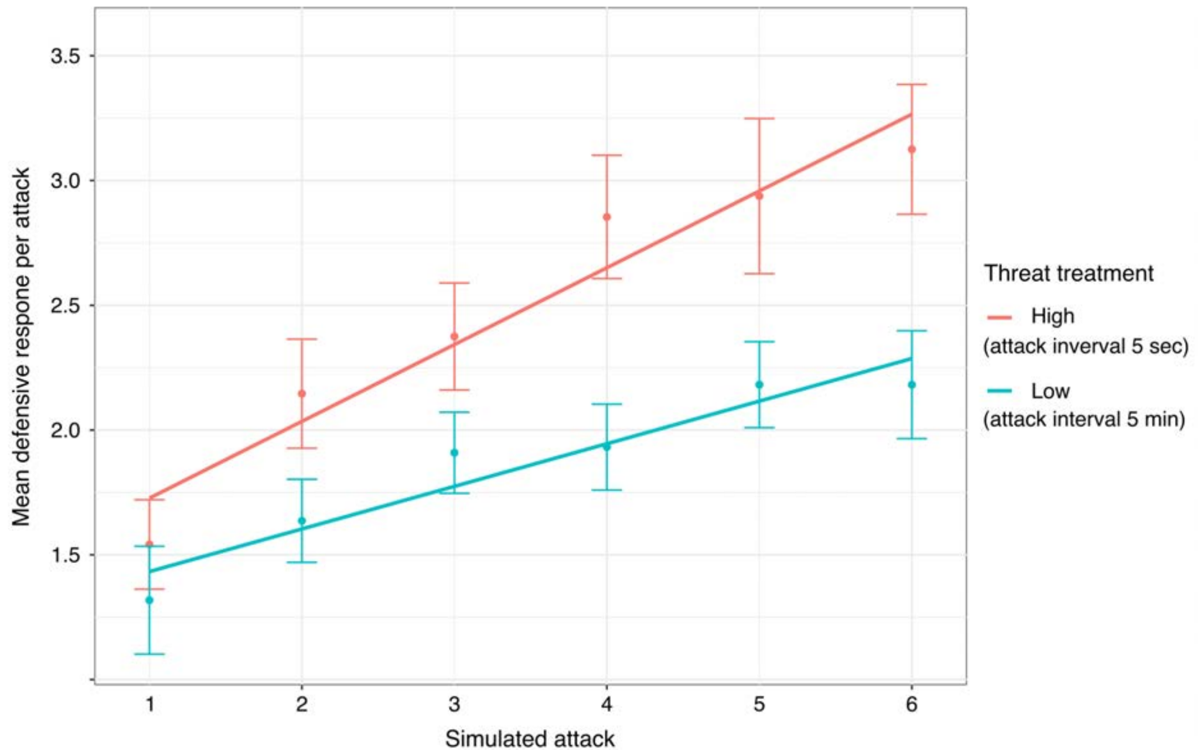


Figure 5. 1 Mean defensive response of *Urodacus yaschenkoi* per simulated 'attack' in the sequence (n = 6) under low- and high-threat (attack frequency) treatments. The level of defensive response assigned to each behaviour was as follows: no sting = 0; dry sting = 1; clear venom = 2; clear/opalescent venom = 2.5; opalescent venom = 3, opalescent/milky venom = 3.5; milky venom = 4. In the high-threat treatment attacks were separated by 5 sec, and under the low-threat treatment the interval was 5 min. Data collected under the high-threat condition is plotted in pink, and low-threat in blue. The plotted lines represent a linear mixed model incorporating threat condition, sting number, and their interaction as fixed effects, and scorpion identity as a random effect. Error bars represent the standard error of the mean.

5.4.2. Chemical Complexity

LC/MS analysis showed the mean number of molecules (chemical complexity) per individual sting sample increased from clear, to opalescent, to milky venom (Figure 5.2). Variation in mean chemical complexity was significantly described by the visual classification (LMM: $F_{4,\ 199.67} = 30.932$, $p < 0.0001$), and pairwise Tukey tests identified significant differences between both clear and opalescent samples ($z = 6.612$, $p < 0.001$), and opalescent and milky samples ($z = 5.392$, $p < 0.001$). As visual classification was correlated with number in the attack sequence, it was not incorporated in the previous model, but a second model was fit that included visual classification to identify any effects on chemical composition that were not correlated with changes in the visual characteristics of the venom. In this case, chemical complexity was significantly associated with visual classification ($F_{4,\ 187.57} = 5.3730$, $p = 0.0004$), whilst sting number did not describe significant variation in the model ($F_{1,\ 187.74} = 0.0188$, $p = 0.8911$), and neither did their interaction ($F_{4,\ 187.70} = 1.2092$, $p = 0.3083$), suggesting that the visual representation of the venom was representative of chemical complexity.

When considering changes in chemical composition over repeated stings, sting order described a significant amount of variation in chemical complexity ($F_{1,\ 190.53} = 26.9611$, $p < 0.0001$), whilst threat level did not ($F_{1\ 187.96} = 0.3534$, $p = 0.5529$), but the interaction of sting number x threat level was significant ($F_{1,\ 187.27} = 4.7823$, $p = 0.0300$). As the attack sequence progressed, the number of molecules per sample increased at a steady rate in the high-threat treatment, whereas in the low-threat treatment the mean chemical complexity per sting stayed relatively constant (and low) until increasing at sting number five (Figure 5.3).

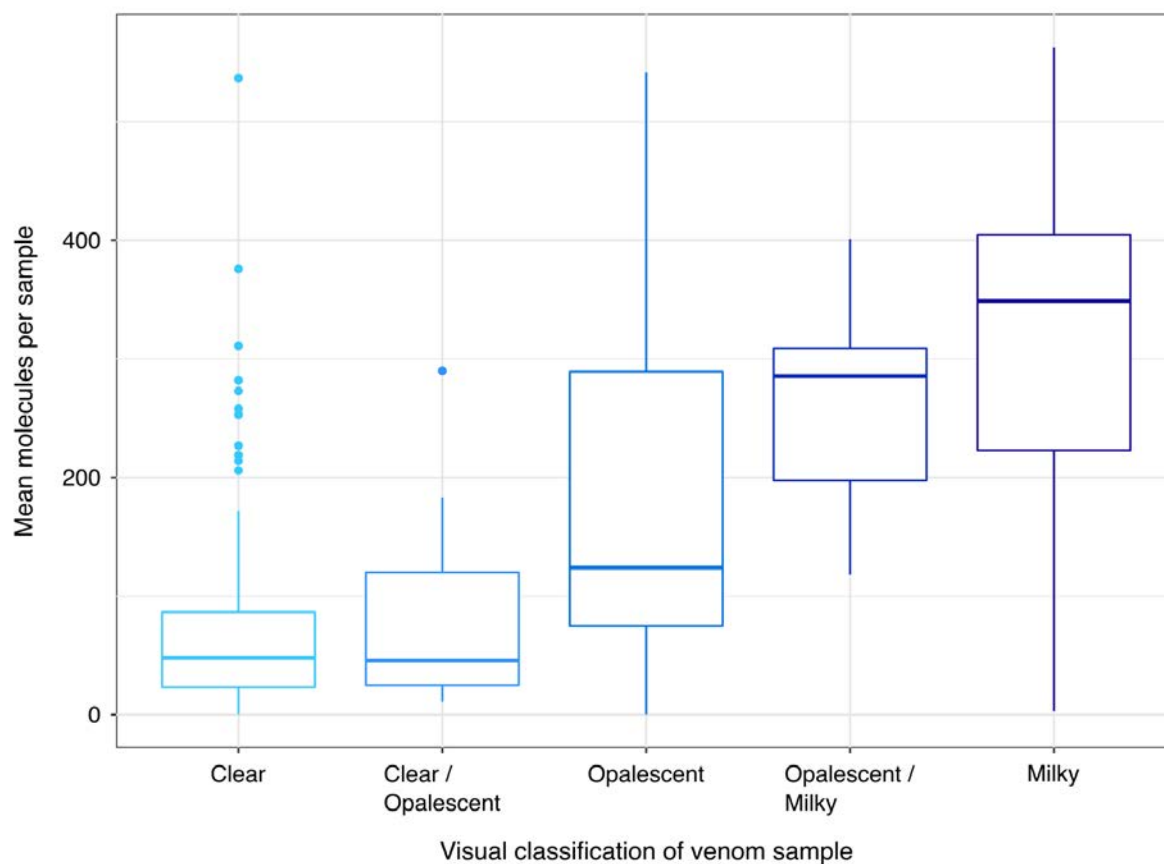


Figure 5. 2 Box and whisker plot showing chemical complexity (total molecules) in each *Urodacus yaschenkoi* venom sample associated for each visual classification of sample. The number of molecules per sample is correlated with the visual classification, with chemical complexity increasing from clear-opalescent-milky. Data points plotted represent outliers.

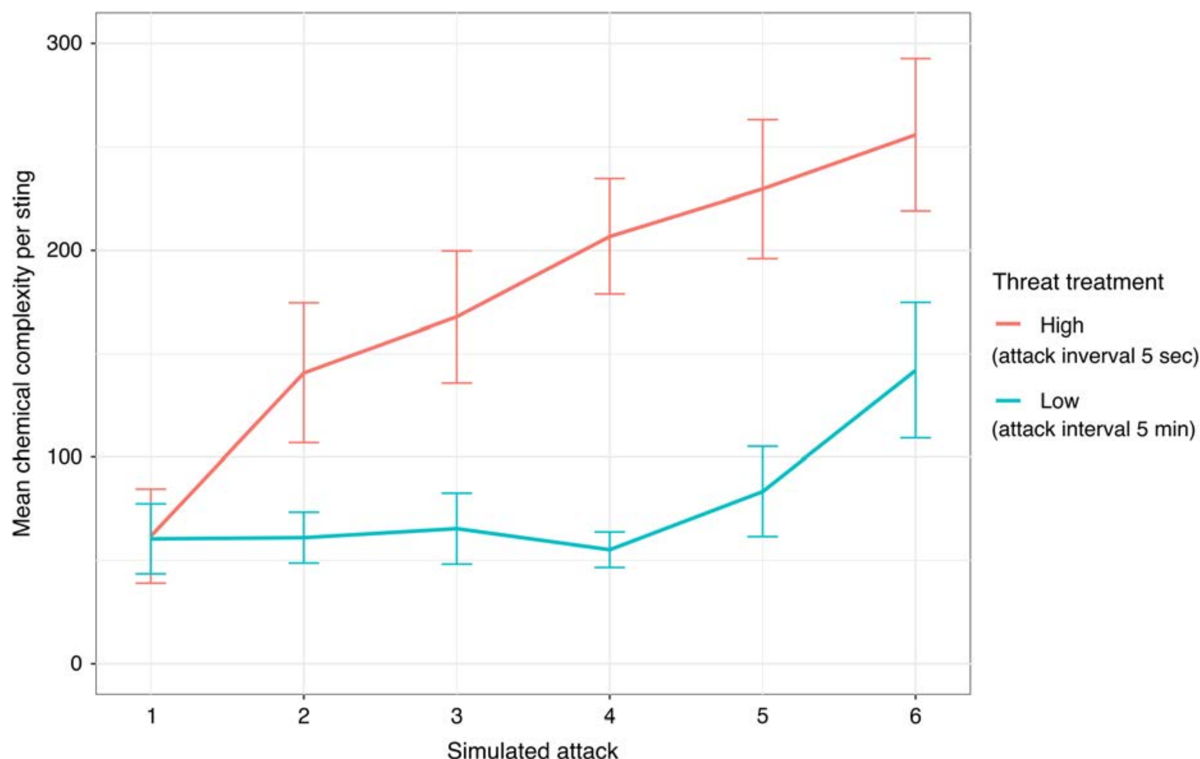


Figure 5. 3 Mean chemical complexity (total molecules) of *Urodacus yaschenkoi* venom samples collected in response to each simulated 'attack' in the sequence (n = 6) under low- and high-threat treatments. In the high-threat treatment attacks were separated by 5 sec, and under the low-threat treatment the interval was 5 min. Data collected under the high-threat condition is plotted in pink, and low-threat in blue. Error bars represent the standard error of the mean.

5.4.3. Compositional Comparisons

The chemical composition between clear, opalescent, and milky venoms was described by assessing the number of molecules shared between each visual classification, based on the generated molecular weight lists. Most molecules (1189/1938) that were present in more than three samples were shared across clear, opalescent, and milky samples (Figure 5.4). Only a small number of molecules were unique to each visual classification; 22 molecules only appeared in clear samples, 28 were only in opalescent samples, and 14 were only documented in milky samples. Whilst the number unique to each visual classification was relatively low, many molecules were shared between only clear and opalescent samples (290 molecules), and only opalescent and milky samples (313). Clear and milky samples shared 82 molecules that were absent in opalescent. Whilst milky venom contained the most molecules per sample, opalescent venom contained the greatest diversity of molecules across the samples. As most molecules in clear and milky samples were additionally also detected in the opalescent samples, it suggests that a transitional change in venom composition occurs from clear to milky. It also shows that although clear samples contained fewer molecules on average, they contain components that were not detected in the complex milky samples. In addition, this evaluation when combined with the result that clear venom samples contain on average fewer molecules suggests that while a large number of different molecules can be present in clear venom, the probability of observing each molecule in clear venom samples is lower than in opalescent or milky samples.

Comparisons made between molecules present in more than five samples, and ten samples, showed a similar pattern as described above, but with fewer molecules representing each group (Supplementary Materials 5.1). This provides further support for the notion that opalescent venom is a transitionary phase between clear and milky venoms and contains a mixture of both.

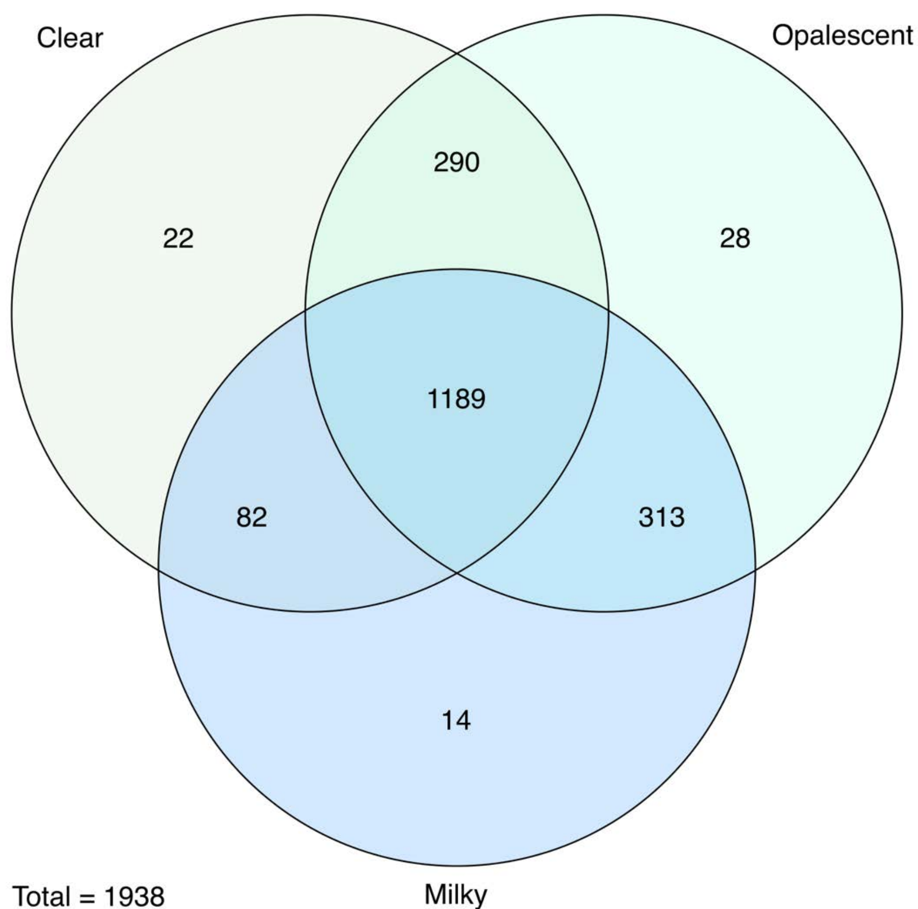


Figure 5. 4 Venn diagram of molecules shared between clear, opalescent, and milky *Urodacus yaschenkoi* venom samples. Only molecules present in more than three samples were included in the analysis, and only samples that were confidently assigned to clear, opalescent or milky (no interim classifications e.g. clear/opalescent). The analysis identified a total of 1938 potential different molecules present in more than three different samples, the majority of which (1189) were shared across the different visual classifications. 22 molecules were only recorded from clear, 28 from opalescent, and 14 from milky samples. 290 molecules were uniquely shared between clear and opalescent, and 313 between opalescent and milky, and 82 between milky and clear samples.

5.4.4. Toxin Families

I further examined differences between clear, opalescent, and milky samples, by visualising the relative number of molecules in each venom sample belonging to different molecular weight classes. Of the 208 venom samples, three were not included as they were found to contain zero molecules in the previous analysis. As venoms changed from clear to milky, the largest relative differences were observed in the proportion of molecules in the mass ranges 1000-2500 Da, 5000-9000 Da, and > 9000 Da (Figure 5.5). Clear venoms contained the highest relative proportions of molecules 1000-2500 Da, whilst milky venoms contained the highest proportions of larger peptides >5000. Thus, milky venoms were typically characterised by having a higher relative preponderance of larger peptides.

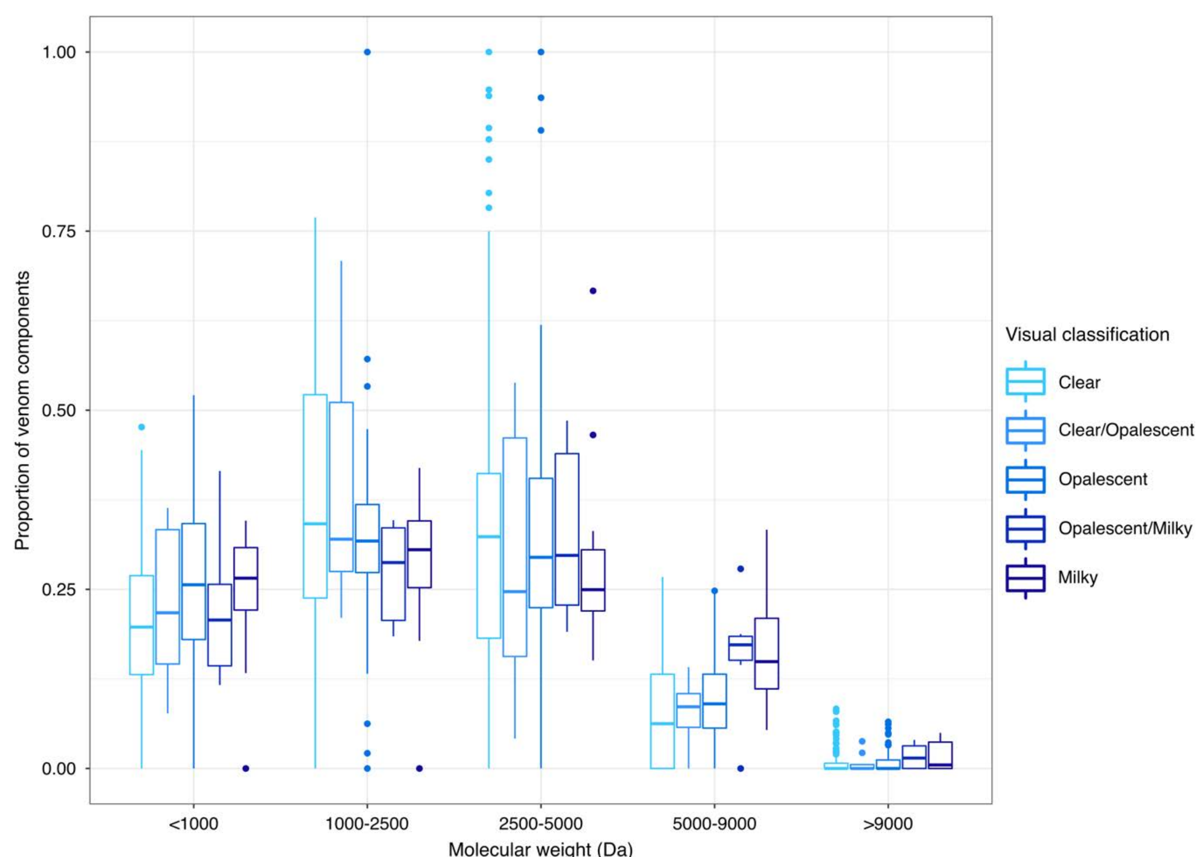


Figure 5. 5 Boxplot showing the proportion of molecules in different molecular weight classes associated with the visual classification of *Urodacus yaschenkoi* venom samples. Molecules were binned into size groups of <1000 Da, 1000-2500 Da, 2500-5000 Da, 5000-9000 Da, and >9000 Da. Milky venoms contained the greatest proportions of molecules in the weight classes 5000-9000 and >9000 Da. Correspondingly, clear venoms contained the highest proportions of smaller peptides 1000-2500 Da.

5.5. Discussion

U. yaschenkoi displayed an increased level of defensive investment (as measured by attempted sting rate, dry sting rate, and whether expelled venom appeared clear, opalescent, or milky) in response to persistent attacks by predators, independent of the rate of repeated attacks (low- versus high-threat treatment). Defensive investment was overall higher in response to the high-threat treatment, which simulated a more aggressive predator by repeating attacks more rapidly. Additionally, under the high-threat treatment defensive investment in response to successive attacks increased at a faster rate between attacks in the sequence than in low-threat treatments (Figure 5.1). The best fit model described a linear increase of defensive investment as the attack sequence progressed. However, if a longer attack sequence was tested, defensive investment may not continue to increase in a linear fashion beyond six attacks as they would presumably inject full venom complements when the stimulus is sufficiently intense and/or prolonged.

A level of defensive 'investment' was first assigned to the defensive responses based on previous studies that have demonstrated in other species that clear venom is lower in protein concentration than milky venom (Inceoglu et al., 2003;Sarhan et al., 2012). Clear venom is secreted first, and is thought to be a metabolically 'cheap' mixture that can be used initially, allowing milky secretions that likely require more energy and time to replenish to be saved for encounters with prey or predators that require more venom (Inceoglu et al., 2003;Nisani and Hayes, 2011;Nisani et al., 2012). This study contains the first comparison of clear versus milky *U. yaschenkoi* venom composition and provides more evidence that prevenom, an injection of fewer molecules, has likely evolved as a cost reducing mechanism in scorpions. Clear *U. yaschenkoi* samples contained the fewest toxins per sample, and a lowest relative proportion of larger peptides (>5000 Da). The larger chemical complexity and proportion of large, complex peptides >5000 Da in milky venoms suggests that their replenishment will presumably require more time and energy (Nisani et al., 2012), and the use of milky venom in defensive context will correlate to greater defensive investment.

The combination of these behavioural and chemical analyses shows *U. yaschenkoi* adapt their defensive behaviours in response to aggressors, modulating their venom use in a way that appears to allow them to avoid wasting their chemically complex milky venom when facing less aggressive predators, and turn to its use when facing more frequent and persistent attackers. The venom optimisation hypothesis proposes that animals will be frugal in their use of venom and avoid using it in situations that do not necessitate its use, as only a limited quantity of venom can be stored, and replenishment requires both energy and time (Hayes, 1995;Wigger et al., 2002;Pimenta

et al., 2003;Hayes, 2008;Morgenstern and King, 2013;Nelsen et al., 2014a;Cooper et al., 2015). In scorpions, the metabolic costs of venom production appear to be relatively high (Nisani et al., 2007), and they can control expenditure of their venom via multiple behaviours when facing different prey and predators (Cushing and Matherne, 1980;Rein, 1993;2003;Nisani and Hayes, 2011;Carlson et al., 2014;Nisani and Hayes, 2015;Miller et al., 2016;Lira et al., 2017;Rasko et al., 2018;Evans et al., 2019;de Albuquerque and de Araujo Lira, 2021). The results of my study provide the first evidence of venom optimisation in *U. yaschenkoi*, as the early stings in the sequence were more chemically simplistic, with complexity increasing as the attacks persisted. In addition, when the observed defensive behaviours were assigned a value of defensive investment ranging from no sting response (0) to milky venom injection (4), the overall defensive investment in response to each attack was higher in response to both persistent and more frequent attacks. The lower defensive investment associated with early defensive responses and low-intensity attacks suggests that *U. yaschenkoi* are being frugal in their venom use when facing less-threatening aggressors.

The few studies that have thoroughly investigated how scorpions modulate their defensive use of venom in response to different threats show contrasting results, suggesting different species may respond to predation risk in different ways (Nisani and Hayes, 2011;Lira et al., 2017;Rasko et al., 2018). Under high-threat experimental conditions, *P. transvaalicus* injected on average over twice as much venom per sting, dry stung less frequently, and injected chemically complex milky venom earlier in repetitive sting sequences when compared to low-threat treatments (Nisani and Hayes, 2011). This is indicative of an increased defensive investment in response to more intense and persistent threats. Correspondingly, Lira et al. (2017) found that in high-threat experimental conditions, *T. serrulatus* injected milky venom more frequently (low-threat conditions only elicited clear venom expulsions), whilst no significant difference in the rate of dry stings was recorded between the threat levels (Lira et al., 2017). My findings suggest *U. yaschenkoi* responds to threats in similar ways to *P. transvaalicus* and *T. serrulatus*, by investing more heavily in its defences under high threat, and in response to threat persistence. Similarly, behavioural studies specifically investigating sting rates have shown *Tityus pusillus* responded to attacks performed at a faster rate by stinging more often (de Albuquerque and de Araujo Lira, 2021), and *Vaejovis carolinianus* more frequently performed stings as attacks were repeated (Nelsen et al., 2020). On the contrary, the opposite was recorded in a study on *Hadrurus arizonensis* defensive behaviour (Rasko et al., 2018). In response to a sequence of ten attacks, simulated by pressing a latex covered tube onto the prosoma for 5 sec, *H. arizonensis* were found to dry sting more frequently, and inject lower volumes of venom per sting as the attack sequence progressed (Rasko et al., 2018). The apparent reduction in defensive investment may be due to fatigue, the simulated attack may not have been sufficiently

vigorous/representative of a natural predation attempt, or the ineffectiveness of early stings may prevent the scorpion from persisting in this means of defence (Rasko et al., 2018). Depletion of the venom supply is unlikely to have been the explanatory factor, as it was found that on average the scorpions only used 7.9% of their total venom supplies in each trial (Rasko et al., 2018). It would be beneficial to know if the venom expelled was clear or milky, as the reduced volume of venom produced in later stings may have been balanced with an increase in peptide/protein concentration. The study by Rasko et al. (2018) highlights that much is still to be learnt about the defensive behaviours of different scorpion species, and observations made in one species may not be representative of others.

The chemical comparisons of clear versus milky *U. yaschenkoi* venoms made in this study are the first of their kind in this species. Although successive visual changes from clear to milky have been documented in the venoms of different scorpion species (Yahel-Niv and Zlotkin, 1979;Gopalakrishnakone et al., 1995;Inceoglu et al., 2003;Abdel-Rahman et al., 2009;Nisani and Hayes, 2011;Lira et al., 2017;Evans et al., 2019), only a small number of studies have investigated how visual changes correspond to chemical composition and toxicity (Yahel-Niv and Zlotkin, 1979;Inceoglu et al., 2003;Nisani et al., 2012;Sarhan et al., 2012). The most rigorous analyses of clear and milky venom secretions have previously been performed in two members of the family Buthidae: *P. transvaalicus* and *P. leiosoma* (Inceoglu et al., 2003;Sarhan et al., 2012). The organism in the current study, *U. yaschenkoi*, is a member of the Urodacidae, distantly related to *Parabuthus* spp., and to my knowledge this study is the first to assess the chemical composition of prevenom in any urodacid scorpion species. The observed chemical simplicity and lower proportion of large (>5000 Da) peptides in *U. yaschenkoi* prevenom appears to mirror the low protein concentration of *P. transvaalicus* and *P. leiosoma* prevenom (Inceoglu et al., 2003;Sarhan et al., 2012), and given the two species belong to different scorpion families, it is likely that chemically simplistic prevenom may be widespread in scorpions. It is important to note, that as the same volume of each *U. yaschenkoi* sample was analysed using LC/MS, and they were processed with the same parameters, the lower chemical complexity observed in clear samples could also have arisen if the clear stings were consistently lower in volume or peptide concentration.

Whilst the majority of molecules appeared in clear, opalescent and milky samples, a small number were only recorded from each visual classification. Of the 1938 masses detected in the analysis that were present in more than three samples, 22 were only present in clear samples, 28 only in opalescent, and 14 only in milky samples. Clear and opalescent samples shared 290 masses that were absent in milky samples, whilst opalescent and milky samples shared 313 that were not detected in the clear samples. As opalescent samples independently shared a large number of

molecules with both clear and milky it suggests that the opalescent venoms contained a mixture of clear and milky venoms. Clear and milky samples contained 82 masses that were absent in opalescent samples, a surprisingly large number if opalescent venom is a transitionary phase from clear to milky. However, this analysis was run with a relaxed threshold that included all molecules present in more than three samples. If a molecule present in low concentration was just detected in four samples, it could be chance that it was only observed in clear and milky samples. Tightening the threshold to compare only molecules present in over five and ten samples drastically reduced the total molecules in each class, but the greatest number was still shared between clear/opalescent and opalescent/milky (apart from those shared between all visual classifications)(Supplementary Materials 5.1).

Overall, molecules in the mass groups 1000-2500 Da and 2500-5000 Da constituted the largest proportion of *U. yaschenkoi* venom (Figure 5.5). This is similar to the observations of Luna-Ramírez et al. (2013), who reported most molecules fell in the weight classes 1000-2000 Da and 4000-6000 Da. Comparison of clear, opalescent, and milky samples also revealed differences in the relative proportions of molecules in different size classes which have not been examined in past studies. Milky *U. yaschenkoi* venoms contained the highest proportion of large peptides (5000-9000 Da), followed by opalescent, with clear samples containing the lowest. Although the overall proportion of molecules >9000 Da was low in all samples, they constituted a greater proportion of milky samples than clear samples.

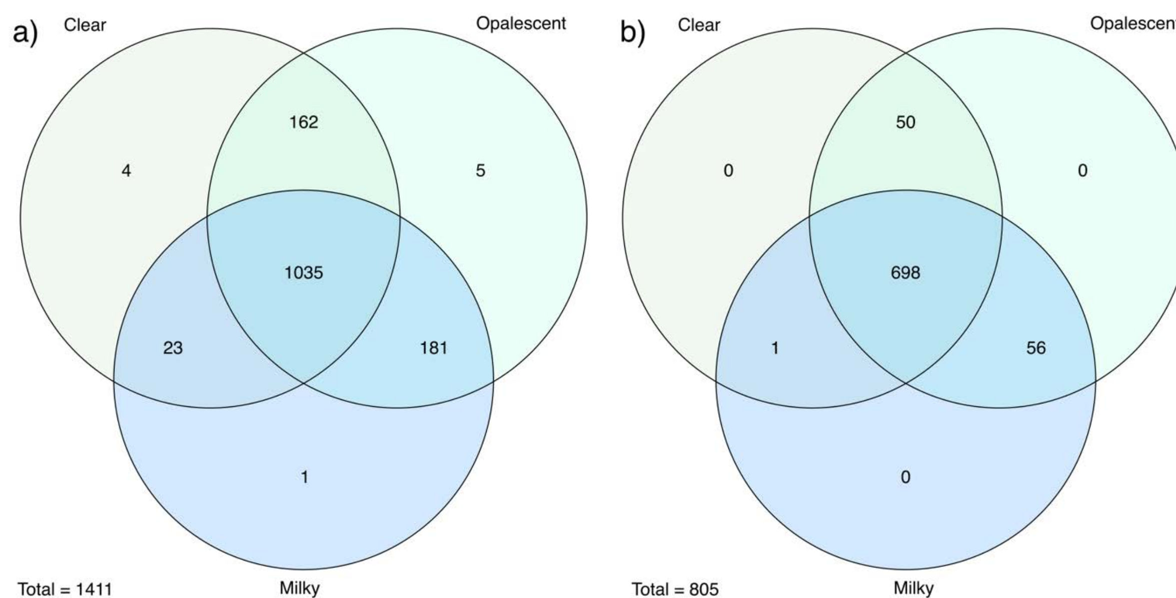
A total of 1938 molecules were identified in the analysis when incorporating all molecules present in more than three samples, which is many more than the 274 identified in a past analysis of *U. yaschenkoi* venom by Luna-Ramírez et al. (2013). The automated analysis of the LC/MS data may have generated an overestimate of the true number of different molecules within the venom, as some peaks automatically picked by the LC/MS software may correspond to adducts. Additionally, some molecules may be duplicated where masses were additionally reconstructed from isotope peaks of ions. Although I reduced the number of duplicated molecules in the mass lists by assuming molecules with similar masses and retention times were the same, some may still be present if molecules fell outside of the threshold for averaging (for example, if a molecule eluted over a long period of time). Whilst the most complex sample contained 709 molecules, variation in the venom composition of individual scorpions also likely contributed to the overall toxin diversity. When the list of molecules was restricted to those present in over five, and over ten samples, the total number fell to 1411 and 805 respectively. If the overall molecular weight lists generated do overestimate the total number of different molecules present in the venom samples, it should not impact the comparisons made between the samples, as all data was processed under the same method.

5.6. Conclusions and Future Directions

U. yaschenkoi responded to predation risk in a similar way to past studies on *P. transvaalicus* and *T. serrulatus* (Nisani and Hayes, 2011;Lira et al., 2017). The scorpions responded to repeated attacks, and more frequent attacks, with greater levels of defensive investment; measured through sting response rate, dry sting rate, and whether expelled venom appeared clear, opalescent, or milky. LC/MS analysis of the venom revealed that the milky venom secretions were more complex than the clear 'prevenom'. Additionally, the milky samples contained a higher proportion of peptides >5000 Da, which will likely require more energy and resources to replenish than smaller peptides (Nisani et al., 2012). These results provide strong evidence that *U. yaschenkoi* control use of their venom when facing different predators, saving their more 'expensive' milky venoms for situations that necessitate its use.

The magnitude of the costs associated with venom use are very difficult to calculate experimentally. Measuring changes in metabolic rate during venom regeneration provides insight into the energetic costs of replenishment, though large differences have been observed in different organisms (Nisani et al., 2007;Pintor et al., 2010;Smith et al., 2014). On the other hand, the fitness costs associated with reduced ability to capture prey or defend against potential predators whilst venom supplies are depleted are almost impossible to measure in natural settings. However, in future studies, a potential avenue to examine venom costs could be to follow behavioural studies such as this with starvation treatments applied to measure the additive effects of venom use and starvation on mortality rates. For example, if future experiments are performed with similar methods to these behavioural trials, following up with a starvation event would enable future researchers to test if larger defensive investment had direct impacts on survivorship.

5.7. Supplementary Materials



Supplementary Materials 5. 1 Venn diagrams of the molecules shared between clear, opalescent, and milky *Urodacus yaschenkoi* venom samples. **a)** Analysis included molecules present in more than five samples, totalling 1411 molecules. **b)** Analysis included molecules present in more than ten samples, totalling 805 molecules.

5.8. Bibliography

Abdel-Rahman, M.A., Omran, M.a.A., Abdel-Nabi, I.M., Ueda, H., and Mcvean, A. (2009). Intraspecific variation in the Egyptian scorpion *Scorpio maurus palmatus* venom collected from different biotopes. *Toxicon* 53**,** 349-359.

Almaaytah, A., and Albalas, Q. (2014). Scorpion venom peptides with no disulfide bridges: a review. *Peptides* 51**,** 35-45.

Banerjee, S., Gnanamani, E., Lynch, S.R., ZuñIga, F.Z., JiméNez-Vargas, J.M., Possani, L.D., and Zare, R.N. (2018). An alkaloid from scorpion venom: chemical structure and synthesis. *Journal of Natural Products* 81**,** 1899-1904.

Bates, D., Mächler, M., Bolker, B., and Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software* 67**,** 1-48.

Carcamo-Noriega, E.N., Possani, L.D., and Ortiz, E. (2019a). Venom content and toxicity regeneration after venom gland depletion by electrostimulation in the scorpion *Centruroides limpidus*. *Toxicon* 157**,** 87-92.

Carcamo-Noriega, E.N., Sathyamoorthi, S., Banerjee, S., Gnanamani, E., Mendoza-Trujillo, M., Mata-Espinosa, D., Hernández-Pando, R., Veytia-Bucheli, J.I., Possani, L.D., and Zare, R.N. (2019b). 1, 4-Benzoquinone antimicrobial agents against *Staphylococcus aureus* and *Mycobacterium tuberculosis* derived from scorpion venom. *Proceedings of the National Academy of Sciences of the United States of America* 116**,** 12642-12647.

Carlson, B.E., Mcginley, S., and Rowe, M.P. (2014). Meek males and fighting females: sexually-dimorphic antipredator behavior and locomotor performance is explained by morphology in bark scorpions (*Centruroides vittatus*). *PLoS One* 9**,** e97648.

Casewell, N.R., Wüster, W., Vonk, F.J., Harrison, R.A., and Fry, B.G. (2013). Complex cocktails: the evolutionary novelty of venoms. *Trends in Ecology & Evolution* 28**,** 219-229.

Chen, H., and Boutros, P.C. (2011). VennDiagram: a package for the generation of highly-customizable Venn and Euler diagrams in R. *BMC Bioinformatics* 12**,** 35.

Cooper, A.M., Nelsen, D.R., and Hayes, W.K. (2015). The strategic use of venom by spiders. *Evolution of Venomous Animals and Their Toxins***,** 1-18.

Cushing, B.S., and Matherne, A. (1980). Stinger utilization and predation in the scorpion *Paruroctonus boreus*. *The Great Basin Naturalist***,** 193-195.

De Albuquerque, K.B.C., and De Araujo Lira, A.F. (2021). Sex-based defensive behavior influenced by threat level in the scorpion *Tityus pusillus* (Scorpiones: Buthidae). *The Journal of Arachnology* 49**,** 402-406.

De La Vega, R.C.R., and Possani, L.D. (2004). Current views on scorpion toxins specific for K+-channels. *Toxicon* 43**,** 865-875.

De La Vega, R.C.R., and Possani, L.D. (2005). Overview of scorpion toxins specific for Na+ channels and related peptides: biodiversity, structure–function relationships and evolution. *Toxicon* 46**,** 831-844.

De La Vega, R.C.R., Schwartz, E.F., and Possani, L.D. (2010). Mining on scorpion venom biodiversity. *Toxicon* 56**,** 1155-1161.

Delgado-Prudencio, G., Cid-Uribe, J.I., Morales, J.A., Possani, L.D., Ortiz, E., and Romero-Gutiérrez, T. (2022). The enzymatic core of scorpion venoms. *Toxins* 14**,** 248.

Dunlop, J.A., and Selden, P.A. (2009). Calibrating the chelicerate clock: a paleontological reply to Jeyaprakash and Hoy. *Experimental and Applied Acarology* 48**,** 183.

Edmunds, M.C., and Sibly, R.M. (2010). Optimal sting use in the feeding behavior of the scorpion *Hadrurus spadix*. *Journal of Arachnology* 38**,** 123-125.

Evans, E.R.J., Mcintyre, L., Northfield, T.D., Daly, N.L., and Wilson, D.T. (2020). Small molecules in the venom of the scorpion *Hormurus waigiensis*. *Biomedicines* 8**,** 259.

Evans, E.R.J., Northfield, T.D., Daly, N.L., and Wilson, D.T. (2019). Venom costs and optimization in scorpions. *Frontiers in Ecology and Evolution* 7**,** 196.

Gangur, A.N., Seymour, J.E., Liddell, M.J., Wilson, D., Smout, M.J., and Northfield, T.D. (2018). When is overkill optimal? Tritrophic interactions reveal new insights into venom evolution. *Theoretical Ecology* 11**,** 141-149.

Gopalakrishnakone, P., Cheah, J., and Gwee, M.C.E. (1995). Black scorpion (*Heterometrus longimanus*) as a laboratory animal: maintenance of a colony of scorpion for milking of venom for research, using a restraining device. *Laboratory Animals* 29**,** 456-458.

Hayes, W.K. (1995). Venom metering by juvenile prairie rattlesnakes, *Crotalus v. viridis*: effects of prey size and experience. *Animal Behaviour* 50**,** 33-40.

Hayes, W.K. (2008). "The snake venom-metering controversy: levels of analysis, assumptions, and evidence," in *The Biology of Rattlesnakes*.), 191-220.

Hayes, W.K., Herbert, S.S., Rehling, G.C., and Gennaro, J.F. (2002). "Factors that influence venom expenditure in viperids and other snake species during predatory and defensive contexts," in *Biology of the Vipers*.), 207-233.

Holford, M., Daly, M., King, G.F., and Norton, R.S. (2018). Venoms to the rescue. *Science* 361**,** 842-844.

Hothorn, T., Bretz, F., and Westfall, P. (2008). Simultaneous inference in general parametric models. *Biometrical Journal: Journal of Mathematical Methods in Biosciences* 50**,** 346-363.

Inceoglu, B., Lango, J., Jing, J., Chen, L., Doymaz, F., Pessah, I.N., and Hammock, B.D. (2003). One scorpion, two venoms: prevenom of *Parabuthus transvaalicus* acts as an alternative type of venom with distinct mechanism of action. *Proceedings of the National Academy of Sciences of the United States of America* 100**,** 922-927.

Jared, C., Luiz Mailho-Fontana, P., and Maria Antoniazzi, M. (2021). Differences between poison and venom: An attempt at an integrative biological approach. *Acta Zoologica* 102**,** 337-350.

Koch, L.E. (1977). *The taxonomy, geographic distribution and evolutionary radiation of Australo-Papuan scorpions.* Western Australian Museum.

Lira, A.F.A., Santos, A.B., Silva, N.A., and Martins, R.D. (2017). Threat level influences the use of venom in a scorpion species, *Tityus stigmurus* (Scorpiones, Buthidae). *Acta Ethologica* 20**,** 291-295.

Luna-Ramírez, K., Quintero-Hernandez, V., Juárez-González, V.R., and Possani, L.D. (2015). Whole transcriptome of the venom gland from *Urodacus yaschenkoi* scorpion. *PLoS One* 10**,** e0127883.

Luna-Ramírez, K., Quintero-Hernández, V., Vargas-Jaimes, L., Batista, C.V., Winkel, K.D., and Possani, L.D. (2013). Characterization of the venom from the Australian scorpion *Urodacus yaschenkoi*: Molecular mass analysis of components, cDNA sequences and peptides with antimicrobial activity. *Toxicon* 63**,** 44-54.

Miller, D.W., Jones, A.D., Goldston, J.S., Rowe, M.P., and Rowe, A.H. (2016). Sex differences in defensive behavior and venom of the striped bark scorpion *Centruroides vittatus* (Scorpiones: Buthidae). *Integrative and Comparative Biology* 56**,** 1022-1031.

Morgenstern, D., and King, G.F. (2013). The venom optimization hypothesis revisited. *Toxicon* 63**,** 120-128.

Nelsen, D.R., David, E.M., Harty, C.N., Hector, J.B., and Corbit, A.G. (2020). Risk assessment and the effects of refuge availability on the defensive behaviors of the southern unstriped scorpion (*Vaejovis carolinianus*). *Toxins* 12**,** 534.

Nelsen, D.R., Kelln, W., and Hayes, W.K. (2014a). Poke but don't pinch: risk assessment and venom metering in the western black widow spider, *Latrodectus hesperus*. *Animal Behaviour* 89**,** 107-114.

Nelsen, D.R., Nisani, Z., Cooper, A.M., Fox, G.A., Gren, E.C.K., Corbit, A.G., and Hayes, W.K. (2014b). Poisons, toxungens, and venoms: redefining and classifying toxic biological secretions and the organisms that employ them. *Biological Reviews* 89**,** 450-465.

Nisani, Z., Boskovic, D.S., Dunbar, S.G., Kelln, W., and Hayes, W.K. (2012). Investigating the chemical profile of regenerated scorpion (*Parabuthus transvaalicus*) venom in relation to metabolic cost and toxicity. *Toxicon* 60**,** 315-323.

Nisani, Z., Dunbar, S.G., and Hayes, W.K. (2007). Cost of venom regeneration in *Parabuthus transvaalicus* (Arachnida: Buthidae). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 147**,** 509-513.

Nisani, Z., and Hayes, W.K. (2011). Defensive stinging by *Parabuthus transvaalicus* scorpions: risk assessment and venom metering. *Animal Behaviour* 81**,** 627-633.

Nisani, Z., and Hayes, W.K. (2015). Venom-spraying behavior of the scorpion *Parabuthus transvaalicus* (Arachnida: Buthidae). *Behavioural Processes* 115**,** 46-52.

Pimenta, A.M., Almeida, F.D.M., De Lima, M.E., Martin-Eauclaire, M.F., and Bougis, P.E. (2003). Individual variability in *Tityus serrulatus* (Scorpiones, Buthidae) venom elicited by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Communications in Mass Spectrometry* 17**,** 413-418.

Pintor, A.F., Krockenberger, A.K., and Seymour, J.E. (2010). Costs of venom production in the common death adder (*Acanthophis antarcticus*). *Toxicon* 56**,** 1035-1042.

Possani, L.D., Merino, E., Corona, M., Bolivar, F., and Becerril, B. (2000). Peptides and genes coding for scorpion toxins that affect ion-channels. *Biochimie* 82**,** 861-868.

R Core Team (2021). "R: A language and environment for statistical computing". (Vienna, Austria: R Foundation for Statistical Computing).

Rasko, M., Coelho, P., Simone, Y., and Van Der Meijden, A. (2018). How to attack a scorpion: venom metering during a repeated attack. *Animal Behaviour* 145**,** 125-129.

Rein, J.O. (1993). Sting use in two species of *Parabuthus scorpions* (Buthidae). *Journal of Arachnology***,** 60-63.

Rein, J.O. (2003). Prey capture behavior in the East African scorpions *Parabuthus leiosoma* (Ehrenberg, 1828) and *P. pallidus* Pocock, 1895 (Scorpiones: Buthidae). *Euscorpius* 2003**,** 1-8.

Rein, J.O. (2022). *The Scorpion Files. Trondheim: Norwegian University of Science and Technology* [Online]. Available: https://www.ntnu.no/ub/scorpion-files/ [Accessed 12/04/2022 2022].

Sarhan, M.M., Fouda, M.M., Elbitar, A.M., Aly, H.A., and Sayed, A.B. (2012). Variation of protein profile among consecutive stings of the scorpion *Parabuthus leiosoma* (family: Buthidae)

from Egypt, supports the venom-metering hypothesis in scorpions. *Al-Azhar Bulletin of Science* 23**,** 61-72.

Shalabi, A., Zamudio, F., Wu, X., Scaloni, A., Possani, L.D., and Villereal, M.L. (2004). Tetrapandins, a new class of scorpion toxins that specifically inhibit store-operated calcium entry in human embryonic kidney-293 cells. *Journal of Biological Chemistry* 279**,** 1040-1049.

Shorthouse, D.J., and Marples, T.G. (1982). The life stages and population dynamics of an arid zone scorpion *Urodacus yaschenkoi* (Birula 1903). *Australian Journal of Ecology* 7**,** 109-118.

Simone, Y., and Meijden, A.V.D. (2021). Armed stem to stinger: a review of the ecological roles of scorpion weapons. *Journal of Venomous Animals and Toxins including Tropical Diseases* 27.

Smith, M.T., Ortega, J., and Beaupre, S.J. (2014). Metabolic cost of venom replenishment by prairie rattlesnakes (*Crotalus viridis viridis*). *Toxicon* 86**,** 1-7.

Van Der Meijden, A., Coelho, P., and Rasko, M. (2015). Variability in venom volume, flow rate and duration in defensive stings of five scorpion species. *Toxicon* 100**,** 60-66.

Van Der Meijden, A., Coelho, P.L., Sousa, P., and Herrel, A. (2013). Choose your weapon: defensive behavior is associated with morphology and performance in scorpions. *PLoS one* 8**,** e78955.

Van Der Meijden, A., and Kleinteich, T. (2017). A biomechanical view on stinger diversity in scorpions. *Journal of Anatomy* 230**,** 497-509.

Wickham, H. (2016). *ggplot2: elegant graphics for data analysis.* springer.

Wigger, E., Kuhn-Nentwig, L., and Nentwig, W. (2002). The venom optimisation hypothesis: a spider injects large venom quantities only into difficult prey types. *Toxicon* 40**,** 749-752.

Wüster, W. (2010). What's your poison? *Heredity* 104**,** 519.

Yahel-Niv, A., and Zlotkin, E. (1979). Comparative studies on venom obtained from individual scorpions by natural stings. *Toxicon* 17**,** 435-446.

Zeng, X.C., Corzo, G., and Hahin, R. (2005). Scorpion venom peptides without disulfide bridges. *IUBMB Life* 57**,** 13-21.

Overall Summary and Future Directions

The experiments included in this thesis were performed to gain a greater overall understanding of scorpion venom chemistry in neglected species/families, to further our knowledge of less well-characterised molecular classes within scorpion venoms, and additionally reveal new insights as to how scorpion venoms are shaped by ecological processes. Furthermore, scorpion venoms possess several unique properties which are poorly described/understood, specifically the role of venom in mating and the presence of chemically simplistic 'prevenom', and these topics were both examined in Australian species. By combining ecological and behavioural studies with high-resolution molecular analyses, the experiments undertaken add a new depth to past research. For example, past analytical studies characterising small molecules frequently do not thoroughly discuss how the observed activities could translate to a role within the whole venom in the context of prey capture/predator deterrence, and additionally behavioural/ecological studies often do not attempt to describe the underlying biochemical mechanisms at play. In each chapter of the thesis, I attempted to bridge the gap between the fields of biochemistry and ecology, to gain the most comprehensive understanding of the results of each individual experiment in a wider context.

Drivers of Venom Variation

There is still much to learn about the complex interaction of ecological and evolutionary processes on venom variability, and the results reported in Chapter 2 reinforce this point. Though scorpion venoms are well-known to display both geographic and intersexual variation (Omran and McVean, 2000;Newton et al., 2007;Rodríguez-Ravelo et al., 2015;Miller et al., 2016;Carcamo-Noriega et al., 2018), the effects of other factors that cause variation in other venomous taxa are not as well-described in scorpions (Keegan et al., 1960;Andrade and Abe, 1999;Herzig et al., 2004;Nisani et al., 2012;Santana et al., 2017;Duran et al., 2020;Sachkova et al., 2020). The results of Chapter 2 demonstrate that *Lychas variatus* show ontogenetic shifts in their venom compositions, both relating to the presence of specific molecules at particular life stages, and changes in the relative proportion of molecules in different molecular weight classes. Furthermore, the identification of adult male-specific components exemplifies the complex nature of intraspecific variation, as intersexual variation can be further affected by ontogeny. Much research currently being undertaken on scorpion venoms is performed focussing on biodiscovery of molecules with useful activities (Veiseh et al., 2007;King, 2011;Ortiz et al., 2015), and the results from this thesis demonstrate that considering factors such as the age and sex of animals may significantly increase the number of potential molecular candidates. Additionally, future examination of dangerously

venomous species could identify if the most toxic components vary among the different life stages or the sexes. Understanding the drivers of variation in medically important species may illuminate avenues to improve medical treatment of sting victims, but also be useful to select optimal animals used for antivenom production.

As the results of Chapter 2 show, it can be difficult to isolate and understand the mechanisms promoting intraspecific venom variation, particularly without experimental manipulation of individual factors to evaluate their effect, and especially when the interaction of different factors may have unpredictable effects. Future work should aim to focus not only on 'how' scorpion venoms vary, but additionally 'why' they vary. For example, are differences observed between populations due to local adaptation or genetic drift? Whilst I discuss why *L. variatus* may show ontogenetic change in Chapter 2, for example due to a change in pressures associated with prey or predators, future work is required to fully understand the processes driving ontogenetic changes in scorpion venoms.

Sexual Stinging

Perhaps the least well-understood, and in my opinion most fascinating, aspect of venom usage in scorpions relates to its involvement in mating: sexual stinging. Sexual stings vary between species in their presence, duration, and location of the sting; suggesting that there are likely to be differences in the underlying biochemical mechanisms in different species (Tallarovic et al., 2000;Peretti and Carrera, 2005;Toscano-Gadea, 2010;Olguín-Pérez et al., 2021). Speculative functions of sexual stings include subduing the female to prevent predation of the male, hormonal modulation of the female, chemical signalling, voiding past mating events, or acting as an honest indicator of male fitness (Inceoglu et al., 2003;Jiao and Zhu, 2010;Peretti, 2014;Olguín-Pérez et al., 2021); however, the 'true' function has not been confirmed. A recent study showed for the first time that the sexual stings of *Megacormus gertschi* involve venom transference, but it is still a mystery if a specific set of molecules (e.g., prevenom) are injected, or a complete set of toxins (Olguín-Pérez et al., 2021). Sexual dimorphism in the venom gland of a sexually stinging species, *Euscorpius alpha*, suggests that prevenom may have a role in sexual stings (Sentenská et al., 2017), but future work is necessary to support this idea.

The three novel peptides I labelled Lvar1, Lvar2, and Lvar3 isolated from *L. variatus* were selected as candidates to possibly function in sexual stings as they were only observed in the venom of adult males, were much smaller than most scorpion venom peptides, and were found to be structurally more similar to neuropeptides (cell-signalling molecules) than known scorpion toxins.

The lack of toxicity observed in both crickets and *L. variatus* females injected with the three peptides, and the absence of activity observed on the Na_V and K_V channels tested, suggest that they may not be conventional toxins evolved for prey capture/defence. Whilst these peptides provide an exciting lead into molecules with possible functions in sexual stinging, confirmation of this role would require more information elucidating their molecular targets and activities. The three peptides possess similar properties to neuropeptides, but they do not share similar sequences to those characterised in past studies. If future research is performed aiming to isolate and investigate if particular venom components have roles specifically associated with sexual stinging, neuropeptide-like venom components are an avenue worth exploring.

Non-Proteinaceous Small Molecules

The small molecule components of scorpion venoms largely remain a mystery, but the results presented in Chapter 4 represent a significant step forward in understanding the types of small molecules that can be present in scorpion venoms. Prior to the publication of Chapter 4, very few non-peptide small molecules had been described from the venom of any scorpion species. These studies were performed across numerous species, and typically only identified few molecules per species (Adam and Weiss, 1959;Arjunwadkar and Reddy, 1983;Fenton et al., 1995;Tran et al., 2017;Banerjee et al., 2018;Carcamo-Noriega et al., 2019). Although organic small molecules are frequently reported to be constituents of scorpion venoms (Ortiz et al., 2015;Ahmadi et al., 2020), there is a distinct lack of published work attempting to identify what they are. It is likely that the venoms of most scorpions contain small molecules, and researchers have simply not identified them; but it is also possible that *Hormurus waigiensis* (the study organism in Chapter 4) and other hormurid scorpions could contain more (or less) than members of other families. Since the publication of Chapter 4, other studies have been published reinforcing the idea that small molecules are likely important constituents of scorpion venoms. Analysis performed in *Hottentotta saulcyi* showed that non-proteinaceous small molecules were abundant within the venom, and specifically lipids constituted ~1.2% of the dry weight (Ghezellou et al., 2022). As future work is performed characterising the non-peptide small molecules of scorpions, it will not only help understand how venoms have evolved to act as combinational attacks on biological systems, but also will provide another avenue for biodiscovery of novel molecules with useful activities. It remains to be investigated if the few small molecules that have already been characterised provide a good representation of the composition within other scorpion species, or if there is significant variation occurring within the order Scorpiones. It is also possible that intraspecific variation, such as

ontogenetic shifts discussed in Chapter 2, have further impacts on the presence and/or abundance of small molecules in scorpion venoms. The small molecules identified in Chapter 4 were isolated from a pooled sample containing the venom of multiple adult *H. waigiensis*, and future analysis of individual venoms could identify if ontogenetic or intersexual differences occur in the presence/abundance of these molecules.

Threat-Perception and Modulation of Venom Use

The results presented in Chapter 5 identify that the initial clear venom secretions (prevenom) of *Urodacus yaschenkoi* is more chemically simplistic and contains a lower proportion of large peptides (>5000 Da) than the subsequent opalescent and milky venom secretions. This finding is very similar to comparisons made between clear and milky *Parabuthus transvaalicus* and *Parabuthus leiosoma* venom (Inceoglu et al., 2003;Sarhan et al., 2012). It is surprising that, despite being observed to occur in numerous scorpion species, few compositional comparisons have been made between clear and milky scorpion venom secretions (Gopalakrishnakone et al., 1995;Inceoglu et al., 2003;Abdel-Rahman et al., 2009;Lira et al., 2017;Evans et al., 2019). With modern LC/MS techniques requiring very small quantities of venom, it would be relatively easy to assess prevenom and venom in a wide range of species. A future assessment made across a taxonomically broad range of species would make it evident if the prevenom of different species differs to the venom in a general manor, or if each species has its own special differences. For species that have currently been examined, prevenoms appear to be chemically simplistic yet still effective at targeting both invertebrate and vertebrate systems (Inceoglu et al., 2003;Sarhan et al., 2012). This has led to the idea that they may have evolved as a metabolically cheap mixture to use in low-threat encounters with predators or against easily subdued prey (Inceoglu et al., 2003;Nisani and Hayes, 2011). Past studies assessing the use of prevenom in defensive contexts and the results presented in Chapter 5 provide data to support this idea. The controlled use of prevenom versus venom in response to different threats is perhaps the strongest evidence to point towards prevenom having evolved as a method to avoid using more chemically complex venom unnecessarily; therefore, if future studies do make compositional comparisons between prevenom and venom in new species, supporting these analyses with behavioural studies assessing how these species use prevenom in different contexts will be invaluable in confirming or contradicting prevenom's proposed function.

Another approach for future studies would be to examine how certain factors, such as sex or ontogeny, may affect threat perception and the use of prevenom versus venom. The size of scorpion, either associated with ontogeny or sexual dimorphism, may affect the relative costs of

venom use, and therefore could lead to different defensive strategies being adopted. The experiments in Chapter 5 were performed on unsexed adult *U. yaschenkoi*, but past studies have shown that there can be differences in the defensive behaviours of different sized (adult versus juvenile) and sexed scorpions (Miller et al., 2016;Lira et al., 2020). Future studies could build upon the results of Chapter 5 to now determine if juvenile *U. yaschenkoi* display different, or similar, modulation of their venom in response to predators. Alternatively, as *L. variatus* show ontogenetic variation in their venom composition (Chapter 2), it could be tested if they display ontogenetic differences in their defensive behaviours associated with venom use. *U. yaschenkoi* was selected as a study organism in Chapter 5 as the large volumes of venom it produces allowed for individual stings to be analysed separately. However, as LC/MS instruments become increasingly sensitive, it may be possible to evaluate single stings even in small scorpion species such as *L. variatus*.

Final Words

By applying behavioural, ecological, and evolutionary theories to chemical analyses of venom it is possible to not only describe 'how' venom composition varies, but additionally gain understanding of the processes driving this variation. For example, the reported geographic and intersexual differences observed in numerous studies could arise in response to many different factors as discussed in this thesis. Understanding these processes is not simply interesting from a theoretical standpoint, but it can have direct impacts in clinical, commercial and research settings. Whilst the experiments presented in this thesis fulfil the initial aim of filling knowledge gaps in understudied aspects of scorpion venoms, plenty of new questions have arisen from the project. Some questions are as simple as: how do the results translate to other species of scorpion? Whilst others are more complex, such as: do neuropeptide-like molecules have roles in sexual stings? There is ultimately still much to learn about many aspects of venom use in scorpions, but I hope that the experiments presented in this thesis inspire future research to answer the new and remaining questions.

Bibliography

Abdel-Rahman, M.A., Omran, M.a.A., Abdel-Nabi, I.M., Ueda, H., and Mcvean, A. (2009). Intraspecific variation in the Egyptian scorpion *Scorpio maurus palmatus* venom collected from different biotopes. *Toxicon* 53**,** 349-359.

Adam, K.R., and Weiss, C. (1959). Distribution of 5-hydroxytryptamine in scorpion venoms. *Nature* 183**,** 1398.

Ahmadi, S., Knerr, J.M., Argemi, L., Bordon, K.C.F., Pucca, M.B., Cerni, F.A., Arantes, E.C., Çalışkan, F., and Laustsen, A.H. (2020). Scorpion venom: detriments and benefits. *Biomedicines* 8**,** 118.

Andrade, D.V., and Abe, A.S. (1999). Relationship of venom ontogeny and diet in *Bothrops*. *Herpetologica***,** 200-204.

Arjunwadkar, A.V., and Reddy, S.R.R. (1983). Spermidine in the venom of the scorpion, *Palamneus phipsoni*. *Toxicon* 21**,** 321-325.

Banerjee, S., Gnanamani, E., Lynch, S.R., ZuñIga, F.Z., JiméNez-Vargas, J.M., Possani, L.D., and Zare, R.N. (2018). An alkaloid from scorpion venom: chemical structure and synthesis. *Journal of Natural Products* 81**,** 1899-1904.

Carcamo-Noriega, E.N., Olamendi-Portugal, T., Restano-Cassulini, R., Rowe, A., Uribe-Romero, S.J., Becerril, B., and Possani, L.D. (2018). Intraspecific variation of *Centruroides sculpturatus* scorpion venom from two regions of Arizona. *Archives of Biochemistry and Biophysics* 638**,** 52-57.

Carcamo-Noriega, E.N., Sathyamoorthi, S., Banerjee, S., Gnanamani, E., Mendoza-Trujillo, M., Mata-Espinosa, D., Hernández-Pando, R., Veytia-Bucheli, J.I., Possani, L.D., and Zare, R.N. (2019). 1, 4-Benzoquinone antimicrobial agents against *Staphylococcus aureus* and *Mycobacterium tuberculosis* derived from scorpion venom. *Proceedings of the National Academy of Sciences of the United States of America* 116**,** 12642-12647.

Duran, L.H., Rymer, T.L., and Wilson, D.T. (2020). Variation in venom composition in the Australian funnel-web spiders *Hadronyche valida*. *Toxicon: X* 8**,** 100063.

Evans, E.R.J., Northfield, T.D., Daly, N.L., and Wilson, D.T. (2019). Venom costs and optimization in scorpions. *Frontiers in Ecology and Evolution* 7**,** 196.

Fenton, A.W., West, P.R., Odell, G.V., Hudiburg, S.M., Ownby, C.L., Mills, J.N., Scroggins, B.T., and Shannon, S.B. (1995). Arthropod venom citrate inhibits phospholipase A2. *Toxicon* 33**,** 763-770.

Ghezellou, P., Jakob, K., Atashi, J., Ghassempour, A., and Spengler, B. (2022). Mass-spectrometry-based lipidome and proteome profiling of *Hottentotta saulcyi* (Scorpiones: Buthidae) venom. *Toxins* 14**,** 370.

Gopalakrishnakone, P., Cheah, J., and Gwee, M.C.E. (1995). Black scorpion (*Heterometrus longimanus*) as a laboratory animal: maintenance of a colony of scorpion for milking of venom for research, using a restraining device. *Laboratory Animals* 29**,** 456-458.

Herzig, V., Ward, R.J., and Dos Santos, W.F. (2004). Ontogenetic changes in *Phoneutria nigriventer* (Araneae, Ctenidae) spider venom. *Toxicon* 44**,** 635-640.

Inceoglu, B., Lango, J., Jing, J., Chen, L., Doymaz, F., Pessah, I.N., and Hammock, B.D. (2003). One scorpion, two venoms: prevenom of *Parabuthus transvaalicus* acts as an alternative type of

venom with distinct mechanism of action. *Proceedings of the National Academy of Sciences of the United States of America* 100**,** 922-927.

Jiao, G.B., and Zhu, M.S. (2010). Courtship and mating of *Scorpiops luridus* Zhu Lourenço & Qi, 2005 (Scorpiones: Euscorpiidae) from Xizang province, China. *Journal of Venomous Animals and Toxins including Tropical Diseases* 16**,** 155-165.

Keegan, H.L., Hedeen, R.A., and Whittemore Jr, F.W. (1960). Seasonal variation in venom of black widow spiders. *The American Journal of Tropical Medicine and Hygiene* 9**,** 477-479.

King, G.F. (2011). Venoms as a platform for human drugs: translating toxins into therapeutics. *Expert Opinion on Biological Therapy* 11**,** 1469-1484.

Lira, A.F.A., Almeida, F.M.F., and Albuquerque, C.M.R. (2020). Reaction under the risk of predation: effects of age and sexual plasticity on defensive behavior in scorpion *Tityus pusillus* (Scorpiones: Buthidae). *Journal of Ethology* 38**,** 13-19.

Lira, A.F.A., Santos, A.B., Silva, N.A., and Martins, R.D. (2017). Threat level influences the use of venom in a scorpion species, *Tityus stigmurus* (Scorpiones, Buthidae). *Acta Ethologica* 20**,** 291-295.

Miller, D.W., Jones, A.D., Goldston, J.S., Rowe, M.P., and Rowe, A.H. (2016). Sex differences in defensive behavior and venom of the striped bark scorpion *Centruroides vittatus* (Scorpiones: Buthidae). *Integrative and Comparative Biology* 56**,** 1022-1031.

Newton, K.A., Clench, M.R., Deshmukh, R., Jeyaseelan, K., and Strong, P.N. (2007). Mass fingerprinting of toxic fractions from the venom of the Indian red scorpion, *Mesobuthus tamulus*: biotope-specific variation in the expression of venom peptides. *Rapid Communications in Mass Spectrometry* 21**,** 3467-3476.

Nisani, Z., Boskovic, D.S., Dunbar, S.G., Kelln, W., and Hayes, W.K. (2012). Investigating the chemical profile of regenerated scorpion (*Parabuthus transvaalicus*) venom in relation to metabolic cost and toxicity. *Toxicon* 60**,** 315-323.

Nisani, Z., and Hayes, W.K. (2011). Defensive stinging by *Parabuthus transvaalicus* scorpions: risk assessment and venom metering. *Animal Behaviour* 81**,** 627-633.

Olguín-Pérez, L., Francke, O.F., and Carbajal-Saucedo, A. (2021). Evidence of piercing and sexual differences in venom composition in a sexual stinging scorpion (Scorpiones: Euscorpiidae). *The Journal of Arachnology* 49**,** 98-107.

Omran, M.A., and Mcvean, A. (2000). Intraspecific variation in scorpion *Leiurus quinquestriatus* venom collected from Egypt (Sinai and Aswan deserts). *Journal of Toxicology: Toxin Reviews* 19**,** 247-264.

Ortiz, E., Gurrola, G.B., Schwartz, E.F., and Possani, L.D. (2015). Scorpion venom components as potential candidates for drug development. *Toxicon* 93**,** 125-135.

Peretti, A.V. (2014). "Chapter 2 - Sexual selection in Neotropical species: rules and exceptions," in *Sexual Selection,* eds. R.H. Macedo & G. Machado. (San Diego: Academic Press), 33-52.

Peretti, A.V., and Carrera, P. (2005). Female control of mating sequences in the mountain scorpion *Zabius fuscus*: males do not use coercion as a response to unreceptive females. *Animal Behaviour* 69**,** 453-462.

Rodríguez-Ravelo, R., Batista, C.V.F., Coronas, F.I.V., Zamudio, F.Z., Hernández-Orihuela, L., Espinosa-López, G., Ruiz-Urquiola, A., and Possani, L.D. (2015). Comparative proteomic analysis of male and female venoms from the Cuban scorpion *Rhopalurus junceus*. *Toxicon* 107**,** 327-334.

Sachkova, M.Y., Macrander, J., Surm, J.M., Aharoni, R., Menard-Harvey, S.S., Klock, A., Leach, W.B., Reitzel, A.M., and Moran, Y. (2020). Some like it hot: population-specific adaptations in venom production to abiotic stressors in a widely distributed cnidarian. *BMC Biology* 18**,** 121.

Santana, R., Perez, D., Dobson, J., Panagides, N., Raven, R., Nouwens, A., Jones, A., King, G., and Fry, B. (2017). Venom profiling of a population of the theraphosid spider *Phlogius crassipes* reveals continuous ontogenetic changes from juveniles through adulthood. *Toxins* 9**,** 116.

Sarhan, M.M., Fouda, M.M., Elbitar, A.M., Aly, H.A., and Sayed, A.B. (2012). Variation of protein profile among consecutive stings of the scorpion *Parabuthus leiosoma* (family: Buthidae) from Egypt, supports the venom-metering hypothesis in scorpions. *Al-Azhar Bulletin of Science* 23**,** 61-72.

Sentenská, L., Graber, F., Richard, M., and Kropf, C. (2017). Sexual dimorphism in venom gland morphology in a sexually stinging scorpion. *Biological journal of the Linnean Society* 122**,** 429-443.

Tallarovic, S.K., Melville, J.M., and Brownell, P.H. (2000). Courtship and mating in the giant hairy desert scorpion, *Hadrurus arizonensis* (Scorpionida, Iuridae). *Journal of insect behavior* 13**,** 827-838.

Toscano-Gadea, C.A. (2010). Sexual behavior of *Bothriurus buecherli* (Scorpiones: Bothriuridae) and comparison with the *B. prospicuus* group. *The Journal of Arachnology* 38**,** 360-363.

Tran, T., Hoang, A., Nguyen, T., Phung, T., Nguyen, K., Osipov, A., Ivanov, I., Tsetlin, V., and Utkin, Y. (2017). Anticoagulant activity of low-molecular weight compounds from *Heterometrus laoticus* scorpion venom. *Toxins* 9**,** 343.

Veiseh, M., Gabikian, P., Bahrami, S.-B., Veiseh, O., Zhang, M., Hackman, R.C., Ravanpay, A.C., Stroud, M.R., Kusuma, Y., and Hansen, S.J. (2007). Tumor paint: a chlorotoxin: Cy5. 5 bioconjugate for intraoperative visualization of cancer foci. *Cancer Research* 67**,** 6882-6888.