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From factors to contexts: analysing changes and interactions in behaviour, venom profiles and body state of Australian funnel-web spiders

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Copyright and collaboration

This thesis does not contain material previously published by any other person. Permission from external copyright holders and collaborators has been obtained when necessary.

Ethics and approvals

This research did not require ethics and approvals from an animal or human ethics committee because funnel-web spiders are not a protected species in Australia. The Department of Environment and Science of Queensland Government advised that a scientific permit was not required. However, our research was conducted within the framework of the Australian Code for the Care and Use of Animals for Scientific Purposes (NHMRC, 2013).

Publications arising from this thesis

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Abstract

Multiple selection pressures shape the variability and evolution of phenotypic traits through the interaction of internal (e.g., genes, physiology) and external (e.g., experience, environment, population density) factors. Behaviour is one factor that can affect the evolution of different traits in multiple taxa. In particular, for venomous animals, behaviour plays an important role in mediating the use and function of venom. However, the role of behaviour, together with other internal and external factors, has been neglected in toxinology studies in general. In this thesis, I used a multidisciplinary approach to understand how behaviour, ecology and morphophysiological traits affect the variability in venom composition in Australian funnel-web spiders, considered to be the most venomous spiders in the world. Funnel-webs show some of the most complex venoms in the animal kingdom, but the evolution and diversification of funnel-web venoms have been poorly understood given that approaches to understanding factors and mechanisms driving venom complexity have only focused on molecular and biochemical tools, overlooking ecological, physiological and behavioural interactions.

This thesis aims to shed light on those fundamental interactions between ecology, physiology and behaviour that must be considered to comprehend the variation and ecological function of venoms. To address these gaps, I used four species of funnel-web spiders: *Hadronyche valida*, *H. infensa*, *H. cerberea*, and *Atrax robustus*. Each species varies in behaviour, habitat and venom composition, thus representing an ideal model for understanding how different factors interact to affect venom diversity within and between species. First, I highlighted the importance of understanding the interactions between environment, experience, ontogeny, physiology (silk and venom) and their effects on spider behaviour across multiple ecological contexts and over time in a comprehensive review.

Then, I started exploring different factors individually to first establish the methodology and provide a framework from which I could build on further. I first explored inter- and intra-individual variation in venom composition over time in *H. valida*, a source of variation not fully considered previously in the study of venomous animals. I highlighted the importance of these findings, not only as a significant source of phenotypic variation in venom composition, which should be included in toxinology studies, but also as a useful tool for species identification. Then, I explored behaviour in the iconic Sydney funnel-web spider *Atrax robustus*, a species for which behavioural studies are considerably lacking. I found that adult females and juveniles varied their behaviour according to the type of stimulus and across different ecological contexts, and that spiders can modify their behavioural responses depending on the life stage, context and threat to which they are exposed. These findings provide important information that should be incorporated in the management and protection of these vulnerable short-range endemic mygalomorphs that are subject to considerable human-wildlife conflict.

To understand species variation in behaviour, and to explore the relative plasticity of behaviour over time and context, I assessed multiple behavioural traits over time and ecological context in all four species of funnel-web spiders. I found behavioural consistency in three behaviours of *H. valida* (risk-taking behaviour, defensiveness against conspecifics, and activity), but only one in *A. robustus* (activity), and none in the other two species (*H. cerberea* and *H. infensa*). When I compared behaviours between species, *A. robustus* showed higher risk-taking behaviour in response to an antipredator stimulus compared to the other species. Defensiveness varied over time and between species. These findings provide insights into how particular traits are species-specific, and how spiders can adjust their behavioural responses depending on the situation, time and ecological context.

Finally, to understand the ecological role of venoms and their interactions with other morphophysiological and behavioural traits. I used multivariate analyses and novel statistical

techniques incorporating behaviour, morphophysiological traits (heart rate as a proxy of metabolic rate, and body condition) and venom components across different ecological contexts and species. I found that behavioural responses, particularly antipredator defence, affected the expression of particular venom molecules, which were also associated with heart rate. Interestingly, species-specific differences in venom composition again provided a chemotaxonomic marker, whereas behaviour and morphophysiological traits, and their interactions with venom components, did not. These results highlight the importance of including multiple approaches to understanding the variability and evolution of venoms, which can have impacts on the biodiscovery of lead drug molecules and conservation of funnel-web spiders in general.

By taking a novel multidisciplinary approach, this thesis contributes to a broader understanding of the evolutionary and ecological processes that shape the evolution of venom composition in funnel-web spiders, and demonstrates the importance of incorporating behaviour, ecology and toxicology into studies of venomous animals. This thesis provides a foundational rigorous methodological framework for exploring complex, synergistic interactions that will allow for direct comparisons between different species of venomous animals in the future.

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Chapter 1: General Introduction

1.1. Introduction

One of the main gaps in studies of evolutionary biology is a lack of consideration that intrinsic (e.g., genetics, physiology) and extrinsic (e.g., environment) factors interact to directly or indirectly affect the evolution, origin and maintenance of phenotypic variation. One of the mechanisms that allows us to understand the synergistic associations between multiple factors and traits is behaviour. Behaviour is considered the evolutionary 'pacemaker' because it is the first response that animals have to different selection pressures (Duckworth, 2009). Changes in behaviour also promote the evolution of other traits, including morphology, life history and physiology (Duckworth, 2009). At the individual level, behavioural types and suites of correlated behaviours or behavioural syndromes (MacKay & Haskell, 2015; Réale et al., 2007; Sih et al., 2004) can influence the origin and maintenance of phenotypic variation in populations, affecting population dynamics (e.g., leading to changes in competition (Wolf & Weissing, 2012), abundance, distribution, and colonization of new habitats (Fogarty et al., 2011).

While behaviour plays an important role in the survival and fitness of all animals, for venomous animals, behaviour is critical because the amount of venom, composition and overall abundance is mediated by a behavioural response (Cooper et al., 2015; Schendel et al., 2019). Depending on the context, venom can be used for different purposes, such as predation, foraging, defence, communication, mating, and intraspecific competition (Williams et al., 2013; Cooper et al., 2017; Schendel et al., 2019). Through the study of behavioural phenotypes, it is possible to determine the underlying factors (i.e., ecology, environment, physiology, experience) affecting venom production, the costs and benefits of venom use (Cooper et al., 2015), and the evolution and diversification of venoms (Cooper et al., 2015).

Behaviour is also linked with intrinsic factors, such as metabolic rate or heart rate frequency (proxy of metabolic rate; Carrel & Heathcote, 1976; Shearer & Pruitt, 2014), which modulate the expression of behavioural phenotypes (Biro & Stamps, 2010; Mathot et al., 2019), and also venom use, production and regeneration (Cooper et al., 2015; Nelsen et al., 2014). For example, individuals of the orb-weaving spider, *Larinioides*, that higher high heart rate frequency (metabolic rates) have been associated with more aggressive behaviours in contexts where they compete for food resources (Biro & Stamps, 2010; Shearer & Pruitt, 2014). These associations between behaviour, metabolic rate and venom, may also depend on the ecological context, the type of activity in which the energy and resources are used (i.e., foraging, predator deterrence), and an individual's body condition or physiological state (Jakob et al., 1996; Mathot et al., 2019; Sih et al., 2015). In venomous animals, the use of their biochemical weapons (i.e., venom) is energetically expensive in terms of peptide production (weeks; Boevé et al., 1995) and temporary impartment to deter predators and catch prey; which suggests that they moderate the use and distribution of this resource (Cooper et al., 2015; Nelsen et al., 2014). However, there are no studies in venomous animals showing the association between metabolic costs of venom use and behavioural traits that can promote or limit the expression of venom. Understanding these associations will provide insights into the ecological use of venom and the conditions in which venomous animals use or limit their biological weapons.

1.2. Motivation for the thesis

Venoms are unique adaptive evolutionary traits that have contributed to the evolution and diversification of many vertebrate and invertebrate taxa (Arbuckle & Harris, 2021; Schendel et al., 2019). Venoms represents an ecological "Swiss army knife" because they can be used in different ecological roles and contexts (Schendel et al., 2019). Venoms have evolved more than 100 times in different taxa, varying from a single toxin to complex mixtures of different toxins (Cooper et al., 2015; Schendel et al., 2019). For decades, venoms have been of medical interest for their impact on human envenomation (Arbuckle, 2017), but also for their bioactive compounds that have made them attractive for drug discovery (Escoubas & Bosmans, 2007; Saez et al., 2010) and bioinsecticide use (Tedford et al., 2004; Windley et al., 2012). The study of behaviour as an evolutionary mechanism is critical for understanding the complexity and variation of venom components because behavioural traits act synergistically with other intrinsic and extrinsic factors to affect the variation, abundance, and function of toxin molecules (Cooper et al., 2015; Schendel et al., 2019; Wullschleger & Nentwig, 2002).

The main objective of my thesis was to understand how morphophysiological and behavioural traits interact to affect venom components in different species of the most venomous spiders in the world, the Australian funnel-webs (Mygalomorphae: Atracidae). To understand the variation in venom composition, I assessed changes at both the inter- and intra-individual levels over time (AIM 1). Then, to understand the role of behaviour in venomous animals (AIM 2), I assessed different behavioural traits (antipredator behaviour, defensiveness and activity) across different ecological contexts (predation, conspecific tolerance, and exploration of a new territory) and stimuli. Thereafter, given that behavioural consistency can cause changes at the population level (Wolf & Weissing, 2012), I assessed whether or not behaviours were consistent (personality) within each of four species of funnel-web spiders, over different ecological contexts (AIM 3). I also compared behavioural differences and contexts between different funnel-web species. Finally, to understand how behavioural traits (defensiveness, risk-taking behaviour and activity) and morphophysiological variables (body condition, heart rate) interact to affect venom components within and between species of funnel-webs (AIM 4), I assessed the relationship between these factors across different ecological contexts.

1.3. Study species

Australian funnel-web spiders are native mygalomorphs from the southern and eastern regions of the continent (Gray, 2010). They are long-lived species, and 34 species have been described (Hedin et al., 2018). Males reach maturity between two and four years, while females can live for more than ten years (Gray, 1992; Levitt, 1961). Due to their largely sedentary nature, these spiders are restricted to specific habitats, with variations in diet and burrow requirements (Gray, 1987, 2010). Funnel-webs build funnel-shaped retreats under rocks, barked trees and crevices on the ground, covering the nearby surroundings with irregular silk trip-lines (Gray, 1987). Females spend most of their lives in this retreat, which is essential for female's survival because it is used for reproduction, feeding and refuge against predators (Mason et al., 2013; Mason et al., 2018). In contrast, adult males roam during the warmer months, seeking females for mating (Bradley, 1993).

Funnel-webs have some of the most complex venoms in the world (Pineda et al., 2020). They are an excellent model species for investigating interactions between behaviour and physiology because of their venom complexity and aggressive behaviour, in addition to their medical importance (Nicholson et al., 1996; Isbister et al., 2005, 2015) and potential for drug biodiscovery (Tedford et al., 2004; Herzig & King, 2015). Furthermore, funnel-webs are short-range endemic species (Harvey, 2002; Mason et al., 2018), meaning they are vulnerable to extinction due to their inability to disperse long distances (range of distribution less than 10.000 km^2), and they have specific habitat requirements that constrain where they inhabit (Mason et al., 2018). Consequently, loss of habitat, fragmentation and urbanisation are important factors that threaten funnel-web spiders, and increase conflict with humans. These characteristics make funnel-web spiders perfect candidates for understanding how multiple factors interact to contribute to the variability and function of funnel-web venoms, and how these spiders can respond to different conditions, all which provide valuable information that will contribute to the management and conservation of these iconic species.

To evaluate differences in venom components within and between species, quantify behavioural traits across different ecological contexts, and assess relationships between behavioural traits and morphophysiological variables, I used four different funnel-web species (Figure 1). These species represent two main genera in the family Atracidae, namely the iconic Sydney funnel-web spider *Atrax robustus*, and three species from the genus *Hadronyche:* two ground-dwelling species, *H. valida* and *H. infensa* (both belonging to the *infensa* species group), and the tree-dwelling species *H. cerberea* (belonging to the *cerberea* species group). *A. robustus* and *H. cerberea* show overlapping distributions, but different habitats (Gray, 2010). *H. valida* and *H. infensa* are closely related species with overlapping distributions and similar habitat characteristics.

A. robustus ***H. valida***

H. cerberea ***H. infensa***

Figure 1.1. The four species of Australian funnel-web spiders used in the thesis.

To date, studies on funnel-web spiders have mainly focused on molecular structure and genetic characterisation of venom molecules involved in the human envenomation syndrome

(Graudins et al., 2002; Nicholson & Graudins, 2002; Pineda et al., 2012). For example, Alewood et al., (2003) and Nicholson et al., (2004) synthesised and characterized δ-Atracotoxin-Ar1a, the lethal neurotoxin found in the Sydney funnel-web spider, while Herzig et al., (2020) studied the transcriptome of 22 funnel-web species, exploring the possible ecological function of the human-lethal δ-hexatoxins. Some studies have focused on the mode of action of these neurotoxins in both vertebrates (Escoubas et al., 2000;Fletcher et al., 1997; Mylecharane et al., 1989; Nicholson et al., 1996) and invertebrates (Wang et al., 2001). Still others have explored the potential use of funnel-web venoms as natural bioinsecticides (King et al., 2002; Tedford et al., 2004) and therapeutic leads (Klint et al., 2012).

In contrasts, studies exploring other aspects of funnel-web biology are rare. Four studies have stressed the use of funnel-web venom profiles as useful chemotaxonomic markers (Gray, 2010; Palagi et al., 2013; Wilson & Alewood, 2004, 2006). Morphological characteristics have only been described in detail by Gray (2010), and current phylogenomics studies have only recently been explored (Hedin et al., 2018). Furthermore, studies related to life-history are primarily limited to *A. robustus* (Bradley, 1993; Levitt, 1961), including only very general descriptions of the habitats of other funnel-web species (Gray, 1981; Gray, 1992; Gray, 2010).

The intention of the study was to obtain equal sample sizes of each species. However, the samples sizes of some of the species used were affected by the COVID-19 pandemic and my inability to conduct additional field work following an initial sampling period. I have used relevant and appropriate statistical methods to analyse the unbalanced samples sizes. Descriptions of the sample sizes and husbandry details are provided in each thesis chapter.

1.4. Thesis outline

The thesis consists of a general introductory chapter (Chapter 1), a literature review (Chapter 2), four experimental (Chapter 3-6) chapters as the main body of the thesis, and a general discussion (Chapter 7). The main experimental chapters are written as separate manuscripts for publication. Chapter 2 (Beyond spider personality: The relationships between behavioural, physiological, and environmental factors) has been published in the journal Ecology and Evolution (vol. 11, pp. 2974-2989). Here, I explored how intrinsic (i.e., genes and physiological traits such as venom, silk and hormones) and extrinsic (i.e., ecology, sociability, environment, experience and density) factors interact to affect spider behavioural and personality types across different ecological contexts. This chapter highlights how the use of spider biological weapons (venoms, silk) and neurohormones might constrain behavioural types (personality traits).

Chapter 3, (Variation in venom composition in the Australian funnel-web spider *Hadronyche valida*) has been published in the journal Toxicon: X (vol. 8, p. 100063). For this first aim of the thesis, I explored variation in venom composition at different levels (intra-individual, and intra- and inter-specific) in the funnel-web spider *H. valida*. This chapter considers some of the mechanisms that may contribute to venom diversification within arachnids, and demonstrates how venom profiles can be used as chemotaxonomic markers to distinguish between closely related species (*H. valida* and *H. infensa*). This chapter provided the methodology for subsequent venom analyses (Chapter 6).

Chapter 4 (Behaviour of the Sydney funnel-web spider *Atrax robustus* over different contexts, time, and stimuli) has been published in the journal Toxicon: X (vol. 13, p.100093). This chapter addresses the second aim of my thesis by exploring behaviour, one of the mechanisms that drive the diversification and evolution of venoms. I used the iconic funnel-web spider *A. robustus*, as previous anecdotal reports have indicated that this spider is one of the most aggressive funnel-webs. I assessed changes in behavioural traits (antipredator behaviour, defensiveness and activity) in juveniles and adult females across different contexts (predation, conspecific tolerance and exploration of a new territory) and stimuli (puff of air versus prod) over time. This chapter provided the methodology for subsequent behavioural analyses (Chapters 5 and 6).

Chapter 5 (Exploring behavioural traits over different contexts in four species of Australian funnel-web spiders) has been accepted for publication in the journal Current Zoology. This chapter focuses on the third aim of my thesis, where I investigated variation in behavioural traits within and between the four species of funnel-webs. I tested repeatability (personality) in risk-taking behaviour, defensiveness and activity across the contexts of predation, conspecific tolerance and exploration of a new territory, the most well-known behavioural axes in spiders (Keiser et al., 2018).

Finally, Chapter 6 addresses the fourth aim of the thesis, exploring the interaction between behavioural traits (defence and risk-taking behaviours towards predators and conspecifics, and activity exploring new territories) and morphophysiological variables (heart rate, body condition) to assess changes in venom components in all four species of funnel-webs. This chapter identifies the ecological function of some venom components and provides insights into understanding the dynamics of multiple traits under different ecological contexts, which contributes to a broader understanding the evolution and diversity of funnel-web venoms.

As the manuscripts have been published separately, these chapters they may contain repetition in background information and methodology. Supplementary material is provided in the tables and figures for each chapter. Tables and figures are numbered separately for each chapter. References are provided separately for each chapter.

1.5. References

Alewood, D., Birinyi-Strachan, L. C., Pallaghy, P. K., Norton, R. S., Nicholson, G. M., & Alewood, P. F. (2003). Synthesis and characterization of δ-Atracotoxin-Ar1a, the lethal neurotoxin from venom of the Sydney funnel-web spider (*Atrax robustus*). *Biochemistry, 42*(44), 12933–12940. https://doi.org/10.1021/bi030091n

Arbuckle, K. (2017). Evolutionary context of venom in animals. In P. Gopalakrishnakone & A. Malhotra (Eds.), *Evolution of Venomous Animals and Their Toxins* (pp. 3–31). Springer Netherlands. https://doi.org/10.1007/978-94-007-6458-3_16

Arbuckle, K., & Harris, R. J. (2021). Radiating pain: venom has contributed to the diversification of the largest radiations of vertebrate and invertebrate animals. *BMC Ecology and Evolution*, *21*(150), 1–12. https://doi.org/10.1186/s12862-021-01880-z

Biro, P. A., & Stamps, J. A. (2010). Do consistent individual differences in metabolic rate promote consistent individual differences in behavior? *Trends in Ecology & Evolution*, *25*(11), 653–659. https://doi.org/10.1016/J.TREE.2010.08.003

Bradley, R. A. (1993). Seasonal activity patterns in Sydney funnel-web spiders, *Atrax* spp. (Araneae: Hexathelidae). *Bulletin of British Arachnological Society*, *9*(6), 189–192.

Carrel, J. E., & Heathcote, R. D. (1976). Heart rate in spiders: Influence of body size and foraging energetics. *Science*, *October*, 148–150. https://doi.org/10.1126/science.935864

Cooper, A. M., Nelsen, D. R., & Hayes, W. K. (2015). The strategic use of venom by spiders. In A. Gopalakrishnakone, P.; Malhotra (Ed.), *Evolution of Venomous Animals and Their Toxins* (pp. 145–163). Springer Netherlands. https://doi.org/DOI 10.1007/978-94-007-6458-3_13

Duckworth, R. A. (2009). The role of behavior in evolution: a search for mechanism. *Evolutionary Ecology*, *23*(4), 513–531. https://doi.org/10.1007/s10682-008-9252-6

Escoubas, P., & Bosmans, F. (2007). Spider peptide toxins as leads for drug development. *Expert Opinion on Drug Discovery*, *2*(6), 823–835. https://doi.org/10.1517/17460441.2.6.823

Fletcher, J. I., Chapman, B. E., Mackay, J. P., Howden, M. E. H., & King, G. F. (1997). The structure of versutoxin (delta-atracotoxin-Hv1) provides insights into the binding of site 3 neurotoxins to the voltage-gated sodium channel. *Structure (London, England : 1993)*, *5*(11), 1525–1535. https://doi.org/10.1016/S0969-2126(97)00301-8

Fogarty, S., Cote, J., & Sih, A. (2011). Social personality polymorphism and the spread of invasive species: A model. *American Naturalist*, *177*(3), 273–287. https://doi.org/10.1086/658174

Graudins, A., Wilson, D., Alewood, P. F., Broady, K. W., & Nicholson, G. M. (2002). Cross-reactivity of Sydney funnel-web spider antivenom: neutralization of the in vitro toxicity of other Australian funnel-web (*Atrax* and *Hadronyche*) spider venoms. *Toxicon : Official Journal of the International Society on Toxinology*, *40*(3), 259–266. https://doi.org/10.1016/S0041-0101(01)00210-0

Gray, M. (1987). Distribution of the funnel-web spiders. In J. Covacevich, P. Davie, & J. Pearn (Eds.), *Toxic plants and animals : a guide for Australia* (pp. 313–321). Queensland Museum.

Gray, M. (2010). A revision of the Australian funnel-web spiders (Hexathelidae: Atracinae). *Records of the Australian Museum*, *62*(2–3), 285–392. https://doi.org/10.3853/j.0067-1975.62.2010.1556

Gray, M. R. (1981). Getting to know funnel webs. *Australian Natural History*, *20*, 265–270.

Gray, M. R. (1988). Aspects of the systematics of the Australian funnel web spiders (Araneae: Hexathelidae: Atracinae) based upon morphological and electrophoretic data. *Australian Entomological Society Miscellaneous Publication*, *5*(1986), 113–125.

Gray, M. R. (1992). Funnel-webs: separating fact from fiction. *Australia’s Leading Nature Magazine*, *24*(3), 32–39.

Harvey, M. S. (2002). Short-range endemism among the Australian fauna: Some examples from non-marine environments. *Invertebrate Systematics*, *16*(4), 555–570. https://doi.org/10.1071/IS02009

Hedin, M., Derkarabetian, S., Ramírez, M. J., Vink, C., & Bond, J. E. (2018). Phylogenomic reclassification of the world’s most venomous spiders (Mygalomorphae, Atracinae), with implications for venom evolution. *Scientific Reports*, *8*(1636), 1–7. https://doi.org/10.1038/s41598-018-19946-2

Herzig, V., & King, G. F. (2015). The cystine knot is responsible for the exceptional stability of the insecticidal spider toxin ω-Hexatoxin-Hv1a. *Toxins*, *7*(10), 4366–4380. https://doi.org/10.3390/toxins7104366

Isbister, G. K., Gray, M. R., Balit, C. R., Raven, R. J., Stokes, B. J., Porges, K., Tankel, A. S., Turner, E., White, J., & McD Fisher, M. (2005). Funnel-web spider bite: a systematic review of recorded clinical cases. In *Medical Journal of Australia* (Vol. 182, Issue 8, pp. 407–411). Med J Aust. https://doi.org/10.5694/j.1326-5377.2005.tb06760.x

Isbister, G. K., Sellors, K. V., Beckmann, U., Chiew, A. L., Downes, M. A., & Berling, I. (2015). Catecholamine-induced cardiomyopathy resulting from life-threatening funnel-web spider envenoming. In *Medical Journal of Australia* (Vol. 203, Issue 7, pp. 302–304). https://doi.org/10.5694/mja15.00279

Jakob, E. M., Marshall, S. D., & Uetz, G. W. (1996). Estimating fitness: a comparison of body condition indices. *Oikos*, *77*(1), 61. https://doi.org/10.2307/3545585

Keiser, C. N., Lichtenstein, J. L. L., Wright, C. M., Chism, G. T., & Pruitt, J. N. (2018). Personality and behavioural syndromes in insects and spiders. In G.-S. I. Cordoba-Aguilar A, Gonzalez-Tokman D (Ed.), *Insect behavior: From mechanisms to ecological and evolutionary consequences* (pp. 236–256). Oxford University Press. https://doi.org/10.1093/oso/9780198797500.003.0016

King, G. F., Tedford, H. W., & Maggio, F. (2002). Structure and function of insecticidal neurotoxins from Australian funnel-web spiders. *Journal of Toxicology: Toxin Reviews*, *21*(4), 361–389. https://doi.org/10.1081/TXR-120014409

Klint, J. K., Senff, S., Rupasinghe, D. B., Er, S. Y., Herzig, V., Nicholson, G. M., & King, G. F. (2012). Spider-venom peptides that target voltage-gated sodium channels: pharmacological tools and potential therapeutic leads. *Toxicon*, *60*(4), 478–491. https://doi.org/10.1016/j.toxicon.2012.04.337

Levitt, V. (1961). The funnel-web spider in captivity. *Proceedings of the Royal Zoological Society of New South Wales, 1958–59*, 80–84. http://www.biodiversitylibrary.org/item/119542

MacKay, J. R. D., & Haskell, M. J. (2015). Consistent individual behavioural variation: The difference between temperament, personality and behavioural syndromes. *Animals, 5*(3), 455–478. https://doi.org/10.3390/ani5030366

Mason, L. D., Bateman, P. W., & Wardell-Johnson, W. (2018). The pitfalls of short-range endemism: high vulnerability to ecological and landscape traps. *PeerJ, 2018*(5), e4715. https://doi.org/10.7717/PEERJ.4715/SUPP-1

Mason, L. D., Tomlinson, S., Withers, P. C., & Main, B. Y. (2013). Thermal and hygric physiology of Australian burrowing mygalomorph spiders (Aganippe spp.). *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology, 183*(1), 71–82. https://doi.org/10.1007/s00360-012-0681-8

Mason, L., Wardell-Johnson, G., Main, B. Y., Mason, L. D., Wardell-Johnson, G., & Main, B. Y. (2018). The longest-lived spider: mygalomorphs dig deep, and persevere. *Pacific Conservation Biology, 24*(2), 203–206. https://doi.org/10.1071/PC18015

Mathot, K. J., Dingemanse, N. J., & Nakagawa, S. (2019). The covariance between metabolic rate and behaviour varies across behaviours and thermal types: meta-analytic insights. *Biological Reviews of the Cambridge Philosophical Society*, *94*(3), 1056–1074. https://doi.org/10.1111/BRV.12491

Mylecharane, E. J., Spence, I., Sheumack, D. D., Claassens, R., & Howden, M. E. H. (1989). Actions of robustoxin, a neurotoxic polypeptide from the venom of the male funnel-web spider (*Atrax robustus*), in anaesthetized monkeys. *Toxicon, 27*(4), 481–492. https://doi.org/10.1016/0041-0101(89)90211-0

Nelsen, D. R., Kelln, W., & 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. https://doi.org/10.1016/j.anbehav.2013.12.019

Nicholson, G. M., & Graudins. (2002). Invited Paper: animal toxins of Asia and Australia spiders of medical importance in the Asia - Pacific: atracotoxin, latrotoxin, and related spider neurotoxins. *Clinical and Experimental Pharmacology and Physiology, 29*(9), 785–794.

Nicholson, G. M., Little, M. J., & Birinyi-Strachan, L. C. (2004). Structure and function of δ-atracotoxins: lethal neurotoxins targeting the voltage-gated sodium channel. *Toxicon, 43*(5), 587–599. https://doi.org/10.1016/j.toxicon.2004.02.006

Nicholson, G. M., Little, M. J., Tyler, M., & Narahashi, T. (1996). Selective alteration of sodium channel gating by Australian funnel-web spider toxins. *Toxicon, 34*(11), 1443–1453. https://doi.org/10.1016/S0041-0101(96)00089-X

Palagi, A., Koh, J. M. S., Leblanc, M., Wilson, D., Dutertre, S., King, G. F., Nicholson, G. M., & Escoubas, P. (2013). Unravelling the complex venom landscapes of lethal Australian funnel-web spiders (Hexathelidae: Atracinae) using LC-MALDI-TOF mass spectrometry. *Journal of Proteomics*, *80*, 292–310. https://doi.org/10.1016/j.jprot.2013.01.002

Pineda, S. S., Chin, Y. K.-Y., Undheim, E. A. B., Senff, S., Mobli, M., Dauly, C., Escoubas, P., Nicholson, G. M., Kaas, Q., Guo, S., Herzig, V., Mattick, J. S., & King, G. F. (2020). Structural venomics reveals evolution of a complex venom by duplication and diversification of an ancient peptide-encoding gene. *Proceedings of the National Academy of Sciences*, *117*(21), 11399 LP – 11408. https://doi.org/10.1073/pnas.1914536117

Pineda, S., Wilson, D., Mattick, J. S., & King, G. F. (2012). The lethal toxin from Australian funnel-web spiders is encoded by an intronless gene. *PLoS ONE*, *7*(8), e43699. https://doi.org/10.1371/journal.pone.0043699

Réale, D., Reader, S. M., Sol, D., McDougall, P. T., & Dingemanse, N. J. (2007). Integrating animal temperament within ecology and evolution. *Biological Reviews*, *82*(2), 291–318. https://doi.org/10.1111/j.1469-185X.2007.00010.x

Saez, N. J., Senff, S., Jensen, J. E., Er, S. Y., Herzig, V., Rash, L. D., & King, G. F. (2010). Spider-venom peptides as therapeutics. *Toxins*, *2*(12), 2851–2871. https://doi.org/10.3390/toxins2122851

Schendel, V., Rash, L. D., Jenner, R. A., & Undheim, E. A. B. (2019). The diversity of venom: the importance of behavior and venom system morphology in understanding its ecology and evolution. *Toxins*, *11*(11), 666. https://doi.org/10.3390/toxins11110666

Shearer, T. A., & Pruitt, J. N. (2014). Individual differences in boldness positively correlate with heart rate in orb-weaving spiders of genus *Larinioides*. *Current Zoology*, *60*(3), 387–391. https://doi.org/10.1093/czoolo/60.3.387

Sih, A., Bell, A. M., Johnson, J. C., & Ziemba, R. E. (2004). Behavioral syndromes: An integrative overview. *Quarterly Review of Biology*, *79*(3), 241–277. https://doi.org/10.1086/422893

Sih, A., Mathot, K. J., Moirón, M., Montiglio, P. O., Wolf, M., & Dingemanse, N. J. (2015). Animal personality and state-behaviour feedbacks: A review and guide for empiricists. *Trends in Ecology and Evolution*, *30*(1), 50–60. https://doi.org/10.1016/j.tree.2014.11.004

Tedford, H. W., Sollod, B. L., Maggio, F., & King, G. F. (2004). Australian funnel-web spiders: Master insecticide chemists. *Toxicon*, *43*(5), 601–618. https://doi.org/10.1016/j.toxicon.2004.02.010

Wang, X.-H., Smith, R., Fletcher, J. I., Wilson, H., Wood, C. J., Howden, M. E. H., & King, G. F. (2001). Structure-function studies of ω-atracotoxin, a potent antagonist of insect voltage-gated calcium channels. *European Journal of Biochemistry*, *264*(2), 488–494.

Williams, G. A., Serena, M., & Grant, T. R. (2013). Age-related change in spurs and spur sheaths of the platypus (*Ornithorhynchus anatinus*). *Australian Mammalogy*, *35*(1), 107–114. https://doi.org/10.1071/AM12011

Wilson, D., & Alewood, P. (2004). Australian funnel-web spider venom analyzed with on-line RP-HPLC techniques. In Aguilar MI. (Ed.), *HPLC of Peptides and Proteins. Methods in Molecular Biology* (Vol. 251, pp. 307–322). Springer, Totowa, NJ. https://doi.org/https://doi.org/10.1385/1-59259-742-4:307

Wilson, D., & Alewood, P. F. (2006). Taxonomy of Australian funnel-web spiders using RP-HPLC/ESI-MS profiling techniques. *Toxicon*, *47*(6), 614–627. https://doi.org/10.1016/j.toxicon.2006.01.014

Windley, M. J., Herzig, V., Dziemborowicz, S. A., Hardy, M. C., King, G. F., & Nicholson, G. M. (2012). Spider-venom peptides as bioinsecticides. *Toxins*, *4*(3), 191–227. https://doi.org/10.3390/toxins4030191

Wolf, M., & Weissing, F. J. (2012). Animal personalities: Consequences for ecology and evolution. *Trends in Ecology and Evolution*, *27*(8), 452–461. https://doi.org/10.1016/j.tree.2012.05.001

Wullschleger, B., & Nentwig, W. (2002). Influence of venom availability on a spider’s prey-choice behaviour. *Functional Ecology*, *16*(6), 802–807. https://doi.org/10.1046/j.1365-2435.2002.00686.x

Chapter 2: Beyond spider personality: The relationships between behavioural, physiological, and environmental factors

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REVIEW

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Beyond spider personality: The relationships between behavioral, physiological, and environmental factors

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2.1. Abstract

Spiders are useful models for testing different hypotheses and methodologies relating to animal personality and behavioural syndromes because they show a range of behavioural types and unique physiological traits (e.g. silk and venom) that are not observed in many other animals. These characteristics allow for a unique understanding of how physiology, behavioural plasticity and personality interact across different contexts to affect spider's individual fitness and survival. However, the relative effect of extrinsic factors on physiological traits (silk, venom and neurohormones) that play an important role in spider survival, and which may impact personality, have received less attention. The goal of this review is to explore how the environment, experience, ontogeny and physiology interact to affect spider personality types across different contexts. I highlight physiological traits, such as neurohormones, and unique spider biochemical weapons, namely silks and venoms, to explore how the use of these traits might, or might not, be constrained or limited by particular behavioural types. I argue that, to develop a comprehensive understanding of the flexibility

and persistence of specific behavioural types in spiders, it is necessary to incorporate these underlying mechanisms into a synthesized whole, alongside other extrinsic and intrinsic factors.

2.2. Introduction

Studies on animal personality and behavioural syndromes (also called coping styles, Table 2.1) have provided significant insights into how sexual selection (e.g. mate choice, sexual cannibalism; Rabaneda-Bueno et al., 2014, and sexual conflict) and natural selection (e.g. environmental conditions, and frequency dependence; Dall et al., 2004) affect the evolution of particular behaviours (Gosling, 2001; Sih et al., 2004a; Sih & Bell, 2008). The expression of repeatable behavioural traits in individuals (i.e. personality axes; Table 2.1), both single behaviours and suites of correlated behaviours (i.e. behavioural syndromes; Table 2.1), can affect the dynamics of populations and communities under different situations and contexts (Koolhaas et al., 2007; Réale et al., 2007; Wolf & Weissing, 2012). Importantly, it was often assumed that the presence of personality is indicative of a lack of behavioural plasticity (Table 2.1). However, it is becoming increasingly apparent that individual behavioural plasticity and/or flexibility can occur, even though behavioural consistency (i.e. personality) might constrain the limits of this plasticity (Briffa et al., 2008), and that the level of plasticity is affected by the selection pressures of particular environments, as well as how much time individuals in a population spend in particular environments (behavioural reaction norm approach; Dingemanse et al., 2010).

Table 2. 1. Glossary of terms.

Term	Definition
Aggressiveness	Degree of aggressiveness towards mates, predators, prey and conspecifics and their response to different stimuli (i.e. aversive or novel) (Pruitt & Riechert, 2012).
Animal personality	Repeatable/consistent individual differences in behaviour that are maintained over time and context (Réale et al., 2007; Sih et al., 2004a; Sih & Bell, 2008). Individual style of behavioural response to stimulus or situations (i.e. the sum of all traits; MacKay & Haskell, 2015). The individual variation in behaviour is related to the variation of between-individual in the intercept of behavioural reaction norm (Dingemanse et al., 2010).
Behavioural axis	Structure for quantifying the behavioural variation within individuals or between populations in one context (i.e. each axis represents a different type of temperament or individual reaction, such as aggressive, bold, docile, active). Personality dimensions refer to populations or species (Réale et al., 2007; MackKay et al., 2015).
Behavioural syndromes	Also called coping styles (see Proactive and Reactive). Correlations involving multiple behavioural and/or physiological traits shown by a set of individuals across time, contexts and situations (Sih et al., 2004a; MackKay et al., 2015). For example, corticosterone concentration and boldness can be correlated within a population (Koolhaas et al., 1999).
Behavioural types	Particular combination on the behavioural axes that an individual can show, and forms part of a behavioural syndrome: boldness (time to react to an aversive stimulus), aggressiveness (towards conspecifics or heterospecifics), or activity level (duration of time spent active) (Sih et al., 2004a; Sih et al., 2004b).
Behavioural plasticity/flexibility	The ability of an animal (at the individual or population level) to change its behaviour depending on prevailing environmental conditions (Briffa & Sneddon, 2016). Animals can adjust their behaviour (e.g. aggressiveness) and be more plastic in different

	contexts and levels of behaviour (Dingemanse & Wolf, 2010) Developmental plasticity is non-reversible, whereas flexibility implies reversible phenotypic change (Piersma & van Gils, 2011; Piersma & Lindstrom, 1997).
Boldness	Measure of the tendency of individuals to take risky behaviours (Sloan Wilson et al., 1994). Boldness is measured as the time that the individual takes to react an aversive stimulus. This behavioural type is measured blowing air on the spider prosoma using a rubber-bulb, which simulates a flying predator (Riechert & Hedrick, 1990).
Context	Domain or behavioural category where an individual performs an activity: foraging, mating, parental care, exploration of new territory, locomotion (Sih et al., 2004a; Sih et al., 2004b).
Environment	Biotic and abiotic conditions in which different selection pressures act on an individual’s phenotype and genotype (Sih et al., 2004a; Sih et al., 2004b).
Experience	Knowledge or skills learned from previous events or situations that can be affected directly or indirectly by the environment (i.e. exposure to predators, food, soil, and space restrictions) (Johnson et al., 2015).
Ontogeny	Development of an organism over its lifetime from conception to maturation (Bosco et al., 2017).
Proactive	One type of coping style (see Behavioural syndromes) in which an animal actively responds to a stimulus (e.g. flight or fight). Often characteristic of aggressive, territorial animals (Koolhaas et al., 1999).
Reactive	One type of coping style (see Behavioural syndromes) in which an animal responds passively to a stimulus (e.g. freezing). Often characteristic of docile, non-territorial animals (Koolhaas et al., 1999).
Situation	Condition in which a context occurs. A situation can occur at one or different points in time (e.g. breeding vs. non-breeding season, high vs low predation risk) (Sih et al., 2004a; Sih et al., 2004b).

While numerous studies on personality and behavioural syndromes have focused on fish (e.g. Dingemanse *et al.*, 2010), birds (e.g. Kluen & Brommer, 2013) and mammals (e.g. Réale et al., 2009), studies on arthropods have only become prevalent within the last 20 years (Modlmeier et al., 2015; Wright et al., 2019). Particularly, spiders have become an interesting group of arthropods to study personality and behavioural syndromes (e.g. Sih & Bell, 2008; Kralj-Fišer & Schneider, 2012; Pruitt & Riechert, 2012; Keiser et al., 2018) because they show a wide range of behavioural types (Table 11.). Individuals fall along continua or axes (Table 1.1), such as boldness and aggressiveness (Kralj-Fišer & Schneider, 2012; Pruitt & Riechert, 2012; Keiser et al., 2018), activity and sociability (Beleyur et al., 2015; Lubin & Bilde, 2007), which can be assessed relatively easily across different contexts and situations (Pruitt & Riechert, 2012). Ecological and behavioural hypotheses related to spider personality (Sih & Bell, 2008; Kralj-Fišer & Schneider, 2012) can be tested across different social groups (social and solitary), clades (Mygalomorph and Araneomorph), life histories (Bonte et al., 2006), and habitats (Foelix, 2011). Spiders also show a variety of strategies for dispersal (Coyle, 1983; Blandenier, 2009), foraging (Jackson, 1992; Michálek et al., 2019) and mating (Jackson, 1992). Importantly, personality in spiders can also be studied in the context of behaviours that are common to these species, but are absent in vertebrates, such as pre-copulatory cannibalism (Arnqvist & Hendrickson, 1997; Rabaneda-Bueno et al., 2008; Kralj-Fišer et al., 2013), and male emasculation during mating (Kralj-Fišer et al., 2011). However, what makes spiders an excellent model system for testing ecological and evolutionary hypotheses related to personality is that spiders have the unique physiological traits of both venom (Cooper et al., 2015; Zobel-Thropp et al., 2018) and silk (Blackledge et al., 2011) production, which are important drivers of behaviours and are critical for spider survival. Importantly, we are not aware of any studies that have specifically explored the relationship between both of these physiological traits and personality in spiders.

To understand how and why spider personality is maintained across different contexts, we need a comprehensive understanding of what intrinsic (i.e. physiological mechanisms, such as hormones, silks and venoms; van Oers & Mueller, 2010) and extrinsic (e.g. environment) factors, and their interactions (Figure 2.1), directly and/or indirectly affect behaviours over the lifetime of individuals (ontogeny; Jandt et al., 2014; Kralj-Fišer & Schuett, 2014). Although, some studies investigating spider personalities have considered some underlying mechanisms independently (Liedtke et al., 2015; Bosco et al., 2017; Langenhof & Komdeur, 2018), there is still a general lack of understanding of how the various intrinsic and extrinsic factors affect the evolution of personality, as well as how they might affect behavioural plasticity (Dingemanse & Wolf, 2010; Dingemanse et al., 2010). Knowing what factors affect morphophysiological traits will provide insights into fitness and general success of spider populations (Figure 2.1), including how populations respond to threats, overcome challenges, and manage the costs and benefits associated with personalities (in particular, behavioural types) occurring under different conditions (Kralj-Fišer & Schuett, 2014; Keiser et al., 2018).

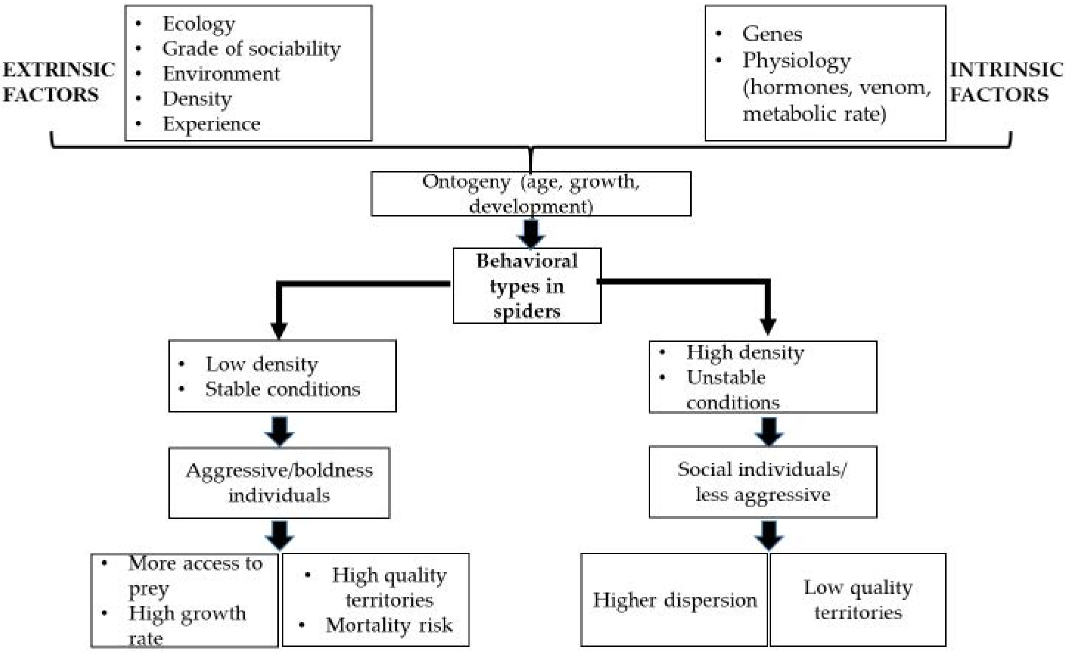


Figure 2.1. Extrinsic (e.g. environment, experience) and intrinsic (e.g. genes and physiology) factors, and their effects on ontogeny and behavioural types in spiders.

Spiders use the information obtained from the environment and conspecifics over the course of their lifetime to maintain and/or modify personality (consistent individual behaviours; Fernández, 2005; Liedtke et al., 2015), and moderate the use of their biochemical weapons, namely silk and venom. Silk and venom are particularly important because they involve multiple physiological processes, regulated by numerous genes and hormones, that affect fitness (both survival and reproductive success), but also involve multiple costs (Nisani et al., 2007; Nisani et al., 2012; Evans *et al.,* 2019) and trade-offs (Nisani et al., 2012; Zobel-Thropp et al., 2018). In addition, these traits are used across different ecological contexts, such as mating, foraging, territory defence, and predation (Blackledge et al., 2011; Santana et al., 2017; Schendel et al., 2019). It has been suggested that personality (Table 1.1) could reflect animal life-history trade-offs (Wolf et al., 2007). Given that venom and silk production are both costly and directly related to prey capture (and thus the rate of food intake), it seems likely that variation between individuals in these specific physiological traits could covary with behavioural differences (Michálek, Řezá, et al., 2019). Thus, investigating links between venom and silk production and behaviour seems like an obvious approach towards increasing our understanding of personality in spiders. Moreover, understanding these potential links in spiders could provide general insights into how life-history trade-offs could underpin animal personality.

Therefore, in this review, I first offer some examples of how environmental conditions, previous experience, and ontogeny induce changes in behavioural types in spiders. Then, I discuss some of the physiological traits and mechanisms, specifically hormones/neurotransmitters, and silk and venom production in relation to personality, areas that are understudied, but which I posit offer fruitful ideas for future study. Connecting these extrinsic and intrinsic factors that may drive the presence or absence of personalities in spiders will contribute to a greater understanding of the evolution and maintenance of behavioural types and behavioural syndromes in spiders, and more broadly.

2.3. The role of environment, experience and ontogeny in shaping spider personalities

The majority of work conducted on personality in spiders has focused on boldness and aggressiveness (e.g. Kralj-Fišer & Schneider, 2012; Pruitt & Riechert, 2012; Keiser et al., 2018), and activity and sociability (e.g. Lubin & Bilde, 2007; Beleyur et al., 2015). Importantly, behavioural types and personalities can be influenced both directly and indirectly by a wide range of extrinsic factors and proximate mechanisms (Table 1.2).

Table 2. 2. Factors inducing changes in behavioural types in spiders, including the physiological traits, body state variables and behaviours that were tested in various studies.

		Factors				
Species	**Behaviour/s**	**Environmental conditions**	**Experience**	**Ontogeny**	**Physiological and body state variables**	**Reference**
Agelenidae						
Agelenopsis aperta	Antipredator behaviour; Agonistic behaviour	X	-	-	-	Riechert & Hedrick, 1993
Agelenopsis lisa	Aggressiveness; Foraging; Exploration	-	-	X	Life history stage; Sex	Bosco et al., 2017
Araneidae						
Larinioides sclopetarius	Aggressiveness; Boldness (plasticity versus personality)	X	-	X	Body condition; Sex	Kralj-Fišer & Schneider, 2012
Parawixia bistriata	Foraging	X	-	-	Fecundity	Fernández, 2005

Eresidae						
Stegodyphus sarasinorum	Sociability (personality task differentiation)	-	-	X	Body condition; Nutritional state	Parthasarathy et al., 2019
Lycosidae						
Lycosa hispanica	Aggressiveness; Voracity	-	-	X	Body mass; Sex	Rabaneda-Bueno et al., 2014
Pardosa agrestis	Activity; Voracity	-	-	X	Life history stage	Rádai et al., 2017
Philodromidae						
Philodromus albidus	Aggressiveness; Boldness	-	-	-	Possibly state dependent?	Michalko et al., 2017
Pisauridae						
Dolomedes triton	Foraging; Antipredator; Voracity	-	-	X	Body condition; Fecundity	Johnson & Sih, 2005, 2007
Salticidae						
Cosmophasis umbratica	Foraging	-	X	-	-	Chang et al., 2017b

Eris militaris	Activity; Aggression; Boldness; Voracity	X	-	-	Body condition; Body size; Sex	Royauté et al., 2014
Marpissa muscosa	Exploration; Social; Learning	X	X	-	Life history stage; Sex	Liedtke & Schneider, 2017; Liedtke et al., 2015
Portia labiata	Foraging; Aggressiveness	-	X	-	-	Chang et al., 2017a; Chang et al., 2017b
Theridae						
Anelosimus studiosus	Boldness (flexibility and individual consistency); Aggressiveness	X	-	X	Nutritional state; Reproductive state; Hormones	Watts et al., 2015; Price, 2016
Latrodectus hesperus	Dispersal; Aggressiveness	X	-	X	Nutritional state	Halpin & Johnson, 2014; Johnson et al., 2015; DiRienzo & Montiglio, 2016

2.3.1. Environment and experience

Environmental and ecological factors (Figure 2.1), such as social context (Webster & Ward, 2011), abiotic conditions (Liedtke et al., 2015; Watts et al., 2015) and food availability (Riechert & Hedrick, 1993), are known to affect the expression of behavioural types across a variety of vertebrate and invertebrate taxa. These environmental and ecological factors have important consequences on individual life history traits including fertility, fecundity (Réale et al., 2007), metabolic rate (Réale et al., 2010), and body size (Vollrath & Rohde-Arndt, 1983; Johnson & Sih 2005; Settepani et al., 2013). Some of these factors have also been shown to affect the expression of behavioural types in spiders. For example, solitary jumping spiders, *Marpissa muscosa*, reared in poor conditions are more reactive to threat stimuli, are less willing to attack prey, and explore new environments, compared to spiders reared under semi-natural conditions. Possibly this occurs because of an absence of natural selection pressures (e.g. predation, conspecific interactions, complex environments) that allow individuals to maintain a behavioural type, or interactions with conspecifics (i.e. family effects) that allow individuals to develop a particular behavioural type, which influences developmental plasticity (Liedtke et al., 2015). Similarly, environmental insecticide treatment leads to a break down in behavioural syndromes (consisting of activity, aggression, boldness, and voracity) of the jumping spider *Eris militaris* via disruptions specific to the activity of the spiders (Royauté et al., 2014). Finally, non-brooding female *Anelosimus studiosus* were found to shift from shy to bold at night, whereas brooding females remained bold regardless of time of day, possibly because brooding females are preparing to increase foraging behaviour, avoid predators and protect spiderlings after birth (Watts et al., 2015).

Broadly, it has been suggested that specific environmental conditions can promote the evolution of intraspecific variation in behavioural types because these local conditions expose individuals within a population to selection pressures that differ to other populations (Sih &

Bell, 2008). As a result, the composition of the group in relation to a behavioural type such as aggressiveness can affect the survival rate, as is seen in *Zygiella x-notata* in urban environments (Kralj-Fišer et al., 2017). This is because intraspecific variation in behavioural types provides the “raw material” on which natural selection can act, and equates to the presence of a diversity of behavioural strategies that can be used to exploit new environments in different ways (Sih et al., 2004a; Sih & Bell, 2008; Kralj-Fišer & Schneider, 2012). Populations of individuals that show differences in behavioural types also have a better chance of coping with environments that have experienced rapid transformations, such as when a habitat changes rapidly because of anthropogenic activities (Sih, 2011).

In general, the behavioural types expressed under specific environmental conditions can also lead to changes in distribution (Sih et al., 2012), dispersion (Cote et al., 2010) and the ability to colonize new habitats (Duckworth & Badyaev, 2007; Hudina et al., 2014; Rehage et al., 2016). Particular life history traits, such as fast growth and short reproduction, as well as personality traits, such as aggressiveness or boldness, could explain high rates of colonization of new environments Fogarty et al., 2011; Kralj-Fišer & Schneider, 2012), and displacement of native species (Fogarty et al., 2011; Wolf & Weissing, 2012). Spiders are well known for these dispersal capabilities (Parthasarathy & Somanathan, 2020) and the ability to colonize new environments through the expression of different behavioural types. For example, a mix of bold and aggressive individuals in a population of *Larinioides sclopetarius* promotes the spread of the population in urban environments (Kralj-Fišer & Schneider, 2012).

Ecotypic variation in individual behaviour can also emerge in response to environmental adaptation (Riechert et al., 2001). For example, some behavioural types in *L. sclopetarius* that are expressed in a particular habitat can be inherited (Kralj-Fišer & Schneider, 2012). The expectation would be that offspring from populations that have experienced different selection pressures would exhibit differences in prey capture, territory defence and anti-predator responses. This has been demonstrated in whip spiders *Phrynus longipes* (an arachnid related to spiders), where individuals from cave environments were more vigilant, less active and less likely to escalate to aggression than individuals from environments on the surface, most likely because of variation in predation pressure, which drives selection for different behavioural types in different environments (Chapin, 2015). A similar pattern has been observed in the colonial spider *Parawixia bistriata*, where spiders from low resource environments show higher levels of group foraging and feeding, and greater plasticity in behaviour than individuals from high resource environments (Fernández, 2005), suggesting prey availability is exerting strong selection pressure on this species' behaviour.

Personalities and behavioural syndromes may manifest under particular environmental conditions (Pinter-Wollman et al., 2012) because conditions experienced by individuals during their ontogeny likely trigger specific physiological cascades (e.g. differential hormone expression; epigenetic regulation) that regulate the expression of particular behaviours that aid survival under those conditions (Stamps & Groothuis, 2010; Sih, 2011). We might expect behavioural syndromes to emerge under stable and predictable environmental conditions because selection pressures acting on individuals from these populations lead to local adaptation, which will persist over generations (Rymer, Pillay, & Schradin, 2013). Particular personalities and/or behavioural syndromes such as boldness-aggressiveness, emerge under particular social conditions. For example, in black widow spiders *Latrodectus hesperus*, a higher number of social interactions during early life were associated with a fast dispersal style, mostly likely because social interactions indicate potential future competition, cannibalism or inbreeding, necessitating a greater need to disperse (Johnson et al., 2015).

In contrast, behavioural flexibility might be more advantageous to allow organisms to respond to rapidly changing environmental conditions. In *L. sclopetarius*, behaviours related to foraging and aggression in novel environments could have lower heritability, at least in the first generation, whereas intrasexual bold-aggressive behaviours have higher heritability, suggesting that plasticity could play a role in the success of these species in urban environments through negative frequency-dependent selection, which acts to generate genetic polymorphisms for aggressiveness and boldness at the population level (Kralj-Fišer & Schneider, 2012). Both behavioural plasticity and consistency facilitate the colonization of new environments; some evidence for this is seen in orb-weaving spiders, where behavioural flexibility and behavioural consistency (i.e. aggressiveness) of spiders in urban environments increase the survival in high density conditions (Kralj-Fišer et al., 2017).

Previous experience in a specific environment has also been broadly suggested to promote changes in individual behaviours to be flexible and adaptive (Figure 2.1) and depending on the circumstances, these changes in individual variation in behaviour, potentially mediated by learning, can either persist over time or shift dynamically with environmental conditions (Dingemanse & Dochtermann, 2013; Sih, 2011). Specifically, for the social spiders *Stegodyphus dumicola* and *A. studiosus,* several reasons have been suggested for an individual's previous experience to affect the plasticity of colony behaviours. Firstly, individuals differ in their ability to respond to new conditions, stimuli or threats, which in turn influences how they respond to these stimuli (Wright et al., 2016). These consistent individual-level responses then influence group-level behavioural responses (Wright et al., 2016). Secondly, individuals may perform specific tasks, and continual experience with the task influences the tasks performed by others. Thirdly, individuals differ in their behavioural types in the colony; these individual differences maintain the behavioural stability of the entire colony (Parthasarathy et al., 2019; Jeanson & Weidenmüller, 2014). However, depending on the behaviours (e.g. collective foraging behaviour in *S. dumicola*), changes in local conditions and density, colonies in different populations need time to adapt to other changes, and to adjust their behaviours to new conditions (Keiser et al., 2014).

2.3.2. Ontogeny

Throughout their lifetime, animals undergo a sequence of physiological changes in response to environmental changes and experience, which affect the development and expression of behavioural and morphological traits (Bosco et al., 2017). Developmental changes in behaviour and physiology that spiders experience from juvenile stages to adulthood can provide us with a better understanding of sexual selection, sexual dimorphism, and sexual conflict, and how apparently non-adaptive behaviours can be maintained in spider populations (Elgar & Schneider, 2004; Johnson & Sih, 2005; Santana et al., 2017). The variation in behavioural types observed over an individual's ontogeny, and the reasons why these may only be present at particular points in time, may allow us to determine what conditions (internal, external and experience) promote and maintain personalities and behavioural syndromes in spider populations. However, adjustments in behaviour come with associated costs. For example, while aggressive spiders under low population density tend to have higher quality territories, they also suffer higher mortality (Riechert & Hedrick 1993; Réale et al., 2007; Fogarty et al., 2011; Sih et al., 2015; Kralj-Fišer et al., 2017; Keiser et al., 2018). As a consequence of how ontogenetic effects impact the expression of particular behaviours (Langenhof & Komdeur, 2018), it is also necessary to understand how ontogenetic factors affect individual personalities and, ultimately, population-level behavioural syndromes (Sih & Bell, 2008; Branch et al., 2015; Bosco et al., 2017). Furthermore, it is important to consider how different behavioural types, exhibited over the course of an individual's development, are affected in response to environmental changes (Langenhof & Komdeur, 2018).

Consistent individual variation in behaviour between adults (personality) and its correlation across different contexts (behavioural syndrome), is not necessarily present during all life stages in some animals (e.g. zebra finches, *Taeniopygia guttata*; Wuerz & Krüger, 2015), including spiders (Bosco et al., 2017; Parthasarathy et al., 2019). Early life stages are often more sensitive to environmental conditions, such as temperature, population density and food availability, which can affect the presence or absence of behavioural syndromes. For example, boldness in the desert funnel-web spider, *Agelenopsis lisa*, tested across different contexts (foraging, placement in a new environment and response to predation) was not consistent across different ontogenetic stages, apart from the penultimate stage (Bosco et al., 2017). The aggressiveness-boldness syndrome observed in these spiders during the penultimate stage is commonly seen in males close to maturity because they need to increase their mass, which is associated with increased mating success as adults (Bosco et al., 2017). Similarly, the repeatability of boldness and aggressiveness declines over time in subadult *S. sarasinorum*, but this is not related to body condition or nutritional state, suggesting underlying ontogenetic effect(s) on the development of personality (Parthasarathy et al., 2019).

Changes in behaviour require time and energy at both neurological and physiological levels (e.g. rewiring neural paths or changing metabolism), so individuals should maintain an intermediate strategy to balance energy and time costs (Bell, 2007a). Consequently, individuals may not be able to exhibit optimal behaviours in every context, which could lead to suboptimal behaviours in different environments, leading to the establishment of conflicts and trade-offs (Sih et al., 2004a; Sih et al., 2004b; Bell, 2007b). However, non-adaptive behaviours, such as sexual cannibalism (e.g. garden spider *Araneus diadematus*, Elgar & Nash, 1988; orb-weaver spider *Argiope aemula*, Sasaki & Iwahashi, 1995), and their incorporation into behavioural syndromes (e.g. voracity and conspecific aggressiveness in foraging and mating) can be explained when ontogeny is taken into consideration (i.e. the aggressive spillover hypothesis; Arnqvist & Henriksson, 1997). Precopulatory sexual cannibalism in spiders occurs when adult females cannibalize males before mating. However, this behaviour is correlated with aggression based on the general feeding voracity developed by juveniles (Elgar & Schneider, 2004;

Johnson & Sih, 2005). Aggression towards conspecifics is present over all spider developmental stages, and is positively correlated with precopulatory sexual cannibalism in adults. Johnson and Sih (2005) also found that foraging voracity is positively correlated with boldness towards predators in fishing spiders, *Dolomedes triton*, with individuals emerging from water faster when they experience an aversive stimulus. In addition, although precopulatory sexual cannibalism in *D. fimbriatus* can lower reproductive success, females have a competitive advantage by increasing their growth rate and fecundity (Arnqvist & Hendrickson, 1997), showing how non-adaptive behaviours in one context may persist over time (Riechert & Hedrick, 1993).

2.4. Proximate mechanisms

Currently, most studies in spiders have focused on determining what extrinsic factors (e.g. environmental conditions) affect behavioural types and personalities throughout the life history of different species (Liedtke et al., 2015; Langenhof & Komdeur, 2018; Parthasarathy et al., 2019). These studies have not included proximate mechanisms (i.e. physiological traits, such as hormone concentrations, silk production, venom composition, metabolic rates, energy reserves and immune responses) that can affect personality (Sih et al., 2004a; Sih et al., 2004b) and play a key role in a spider's development and survival. For example, in some species of myrmecophagous spiders, use of silk and venom as hunting strategies will depend on prey specialization and adaptation to exploiting alternative prey (Michálek, Řezá, et al., 2019). The role that these proximate mechanisms play in the maintenance of individual behavioural differences is important because these mechanisms can induce a myriad of changes during different ontogenetic stages, and over different contexts and situations (Sih, Chang, & Wey, 2014; Briffa & Sneddon, 2016). These physiological variables comprise both morphological and physiological traits, and can affect interspecific and ecological relationships (e.g. sex ratio, density of individuals, predators, competitors, and parasites) that maintain behavioural differences between individuals (Sih et al., 2015).

2.4.1. Hormones and personality in spiders

The endocrine system, and its effects on individual differences in behaviour, has been poorly studied in spiders. However, neuroendocrine traits allow us to understand how an animal behaves in a specific situation or in response to a threat, and how physiological and behavioural traits might be correlated (coping styles; Briffa & Sneddon, 2016). Biogenic amines, namely neurotransmitters, hormones and neuromodulators, act on the central and peripheral nervous systems, allowing arthropods to respond to different stimuli (Roeder, 2005). For example, when an individual is exposed to a threat stimulus, this triggers a response that regulates the release of these biogenic amines that then increase the individual's aggressive or defensive behaviours (Roeder, 2005; Bengston & Jandt, 2014; Jeanson & Weidenmüller, 2014). Additionally, these biogenic amines can be affected by genetic and environmental conditions, mediating changes in different behaviours and personality (Edenbrow & Croft, 2013).

In arthropods in general, the concentration of biogenic amines, such as octopamine and serotonin (Roeder et al., 2003; Roeder, 2005), can influence a wide range of behaviours, including aggression, territory defence and escape behaviours (Jones et al., 2011). For example, in orb-web spiders, *L. cornutus,* increased octopamine concentrations reduce the time to respond when a spider is exposed to a predator or other aversive stimulus (Jones et al., 2011). Although hormones may act differently between species, they can also mediate differences in behavioural tendencies, like aggressiveness, by inducing changes in activity level, or by either reducing or increasing aggression (e.g. *A. studiosus*, *L. cornutus*, Jones et al., 2011; Price, 2016).

Hormones also have direct and indirect effects on the immune system. For example, in cellar spiders, *Physocyclus dugesi*, juvenile hormone (acyclic sesquiterpenoids) down-regulates the immune response during mating (Calbacho-Rosa et al., 2012). Lower concentrations of juvenile hormone are associated with lower aggression in honey bees, *Apis mellifera* (Pearce et al., 2001), and aggressive behaviour is also associated with down-regulation of the immune response, potentially mediated via juvenile hormone, in other arthropods (e.g. beautiful demoiselle, *Calopteryx virgo*, rubyspot damselfly *Hetaerina americana*, Contreras-Garduño et al., 2006, 2009). Neurohormones also regulate different processes (e.g. ontogenesis, sexual maturation, and ecdysis) that may impact the expression of behaviour in spiders in general (Sawadro, Bednarek, & Babczyńska, 2017), and these hormones may also impact other physiological properties, such as venoms and silks, which then further impact behaviour.

2.4.2. Venom properties and personality in spiders

In venomous animals, venom production involves high metabolic costs (Nisani et al., 2007; Nisani et al., 2012; Evans et al., 2019), but it also plays an important role in survival (Cooper et al., 2015). Venom production and composition, and their associated costs, are known to be affected by different extrinsic factors, including diet, habitat, climate (Boevé et al., 1995; Cooper et al., 2015), season (Atkinson & Walker, 1985), niche specialization (Bergmüller & Taborsky, 2010; Michalko & Pekár, 2014; Michalko et al., 2017) and predation risk (Gangur et al., 2017). For example, in the funnel-web spider, *Atrax sutherlandi*, the venom yield from spiders collected in winter is higher than that collected in autumn, suggesting temporal variation in venom production within the species (Wong et al., 2016).

In addition, differences in the quantity and quality of venom are affected by different intrinsic factors (e.g. hunger level, Hayes, 1993; life history stage; Herzig, 2010), metabolic rate (Kowalski & Rychlik, 2018), hormone concentration (Gomes & Palma, 2016; Lira et al., 2017; Zhang et al., 2017), body size (Rocha-e-Silva et al., 2009; Fox, 2018), genes (Casewell et al., 2013; Hargreaves et al., 2014), sex (Zobel-Thropp et al., 2018) and/or ontogeny (Boevé et al., 1995) . For example, sexual dimorphism in venom profiles is seen in the orb-weaver, *Tetragnatha versicolor*, which is thought to play a role in sexual communication (Zobel-Thropp et al., 2018), while venom composition in the tarantula *Phlogius crassipes* changes during development from the juvenile stage through to adulthood, and continues to change throughout adulthood (Santana et al., 2017). However, how these extrinsic and intrinsic factors interact to affect morphology, physiology and behaviour are complex, and potentially species-specific. For example, sex and development affect venom yield in the rainforest tarantula, *Coremiocnemis tropix*, whereas not the availability of food (Herzig, 2010). Orb-web spiders, *Tetragnatha versicolor*, show sex differences in venom properties, ecological functions, and behaviours when they are threatened (Zobel-Thropp et al., 2018). In the funnel-web spider, *A. robustus*, the venom in males has higher mammalian neurotoxin activity than in females, but the toxins that cause the envenomation syndrome are only present during the male adult stage (Gray & Sutherland, 1978; Wilson, 2016; Herzig et al., 2020). While ecological and biological factors can influence these developmental changes in species in general (Sih et al., 2015), we do not yet know what specific intrinsic states trigger these changes in *A. robustus* males.

Venom is used across multiple ecological, contexts including mating, territory defence, feeding/foraging and predator deterrence (Cooper et al., 2015; Schendel et al., 2019). The costs associated with the production and use of venom should be compensated by using the venom in an optimal way through modulation of the quantity, and/or potentially the composition, of venom toxins (venom optimization hypothesis or venom metering, Boevé et al., 1995; Wigger et al., 2002; Morgenstern & King, 2013; Nelsen et al., 2014; Cooper et al., 2015; Schendel et al., 2019). The costs associated with venom use can be direct, such as energy used in the production and storage of toxins, and/or indirect, such as in a reduced capacity to capture prey or deter predators (Evans et al., 2019). For example, in *Cupiennius salei*, the volume and toxicity of venom is not regenerated at equal rates; 50% of the volume of venom can be regenerated in one day, but the toxicity of the venom can take days or weeks to completely regenerate (Boevé et al., 1995). The spiders can compensate for some of these costs by optimizing the use of venom in relation to the amount of venom available in their glands (Wullschleger & Nentwig, 2002). In addition, they show differences in prey capture behaviour, using multiple strategies when different prey are encountered. *C. salei* use only a small amount of venom on small prey, such as crickets, but expend more venom on larger prey (Boevé et al., 1995; Wigger et al., 2002). Similarly, the wandering spider, *Phoneutria nigriventer* uses its chelicerae to cause mechanical damage to small prey and only use venom when the prey is large (Schenberg & Pereira Lima, 1978). Finally, the orb-weaver, *Argiope argentata*, uses short bites when prey is small and long bites when prey is larger (Robinson, 1969).

Unfortunately, the quantity and composition of venom, as well as the physiological costs and the time taken to regenerate the venom (recovery period), have not been studied in relation to personality and behavioural syndromes in spiders. In social spiders, aggressive individuals interact more intensely with both predators and prey than do docile individuals (Riechert, 1993), but, we do not know what and/or how other traits might change during these interactions. We speculate that aggressive spiders would experience a higher metabolic cost, as occurs in scorpions *Parabuthus transvaalicus* (Nisani et al., 2012), because this is associated with a higher concentration and quantity of venom required when subduing prey. It would be interesting, to test if aggressive spiders use different toxins during intraspecific competition, as occurs in the polyps of the aggregating sea anemone *Anthopleura elegantissima* (Macrander et al., 2015) and ants of the genus Monomorium (Westermann et al., 2015). Aggressive anemone polyps show a higher quantity of a particular type of gated potassium ion channel (toxins/Kunitz-type protease inhibitor and type II acrorhagins; Macrander et al., 2015), whereas ants using venom to withstand attack from the invasive Argentine ant *Linepithema humile*, show higher concentrations of toxins compared to populations of ants that do not live in close proximity to these invasive ants (Westermann et al., 2015). In the funnel-web spider *A. robustus*, males are more aggressive and more prone to attack when they are provoked than females, which could be correlated with higher venom toxicity (Mullen & Vetter, 2019). This behavioural type and venom toxicity likely provide a survival advantage for males, as males are more exploratory because they have to search for sedentary females, deter predators, subdue prey and reduce conspecific competition (Stoehr & Kokko, 2006). However, the associated trade-offs of higher aggression and venom toxicity might also include greater exposure to predators, higher metabolic costs and lower immune efficiency (Nisani et al., 2012; Zobel-Thropp et al., 2018). Thus, it is necessary to study the different functions and properties of venom between males and females, and their link with intrinsic and extrinsic factors that shape behavioural types.

Multiple questions can be asked about particular patterns of relationships between behaviour, venoms and their ecological functions. Intra-specific variation in venom composition and regeneration has been reported in funnel-web spiders, *Hadrochyne infensa,* from Toowoomba and Fraser Island in Australia (Palagi et al., 2013). However, we should consider if the regeneration of venom is faster in aggressive individuals. That is, does venom volume and/or composition differ consistently across individuals? Similarly, are the metabolic costs higher for one particular behavioural type, or do different behavioural types adjust their behaviours to compensate for a reduction in venom volume, and is this compensated for in some way? For example, aggressive individuals could waste resources, expelling more venom when a predator is present, but these individuals might have a better ability to colonize new environments (Johnson et al., 2015; Kralj-Fišer & Schneider, 2012). Similarly, spiders might balance the costs of performing a particular behaviour across different contexts, such as mating and foraging. In hairy desert scorpions, *Hadrurus arizonensis*, males use a soft movement of the telson to sting females during courtship, and this movement is also used when scorpions immobilize their prey (Tallarovic et al., 2000; Coelho et al., 2017). Understanding individual variation in venom composition and the costs associated with its use could explain how some personalities are maintained and evolve in different spider species. Likewise, linking behaviours and physiological traits will allow us to explore the ability of individuals to be flexible in response to changing environmental conditions.

2.4.3. Silk properties and spider personality

Silk is a key feature of a spider's biology. The evolution of silk properties and its uses in spiders is related to selective pressures that affect spinning behaviours, ecology and the physiological production of silk (Vollrath, 1999). Different taxonomic groups of spiders have shown modifications in the use of silk, and the variation is linked to the type of habitat, prey capture strategy, predator deterrence and mating (Blackledge et al., 2011; Starrett et al., 2012; Garb, 2013). Although, arguably, the most important factor affecting web properties and architecture is the type of prey that the spider catches, which can change as the spider ages (Sensenig et al., 2011). Mygalomorphs and Mesothele have a morphologically simple and uniform set of silk glands that are related to a sit-and-wait strategy for subduing prey (Starrett et al., 2012; Sensenig et al., 2014). On the other hand, Araneomorph orb-weavers represent the widest diversity of silk types that are functionally distinct (Garb, 2013). Seven to eight glands (Blackledge & Hayashi, 2006; Garb, 2013) produce functionally different types of silks that are used to (a) build the frame, radii and draglines of the web (major ampullate), (b) construct the temporary capture spiral of the web (minor ampulla), (c) make the core fibre of the capture spiral (flagelliform), (d) produce sticky droplets that coat the capture spiral (aggregate glands), (e) make the outer egg case (tuniliform; specific to females), (f) wrap prey and produce the soft inner egg case (actiniform), and (g) secure fibres to substrates (pyriforms). These glands vary in morphology and number between Araneomorph species, which is consistent with the evolution of function, material, mechanical properties of the silk, and the wide diversity of habitats and behaviours that Araneomorphs exhibit (Vollrath, 1999; Blackledge et al., 2011; Garb, 2013).

Spiders use silk during different phases of their life cycle, across a variety of ecological contexts, and for multiple functions (Garb, 2013). Variation in silk production can also differ during development (Moon & Kim, 2005; Garb, 2013). For example, in male orb-weaving spiders, their flagelliform and aggregate glands are lost when they molt to adults (Moon & Kim, 2005; Garb, 2013). Similarly, in the orb-weaver spider, *Neoscona arabesca*, silk properties in webs change with development, where the strength, toughness and web performance change as the spider grows (Sensenig et al., 2011). Silk production and use also varies within species (Sensenig et al., 2011) and between the sexes. In Araneomorph spiders, males produce fewer types of silk than females (Garb, 2013) due to the loss of silk glands (Moon & Kim, 2005). Males also have epiandrous glands that, along with actiniform glands, are used to build the sperm web where sperm is deposited prior to being transferred to the pedipalps for mating (Moon & Kim, 2005).

However, there are some constraints and costs associated with silk production and use (Blackledge et al., 2011; Craig et al., 1999). Synthesizing amino acids is one constraint for silk production, and the amount of energy spent in this process will depend on the metabolic pathway that the spider uses (Blackledge et al., 2011). Additionally, behavioural costs of spinning involve energy consumption, but this differs between orb-weavers, which use viscid glue in their silk to capture prey; in contrast, cribellate spiders produce silk fibres without viscid glue (Blackledge et al., 2011). Cribellate silk is more expensive to produce and demands more time in construction in contrast to webs built by orb-weavers (Craig, 2003; Blackledge et al., 2011). Orb-weaver spiders recycle amino acids from old webs and use them to generate new silk. Recycling silk reduces the costs of spinning by 32% (Craig, 2003). Similarly, the costs of web relocation include exposure of spiders to increased predation risk (Nakata & Ushimaru, 2013), and relocation is time-consuming because it requires that the spider samples prey in a different location for many days until it finds a good location to build the web (Blackledge et al., 2011).

Spiders can adjust their spinning behaviour, biochemical composition and web architecture depending on different factors, such as prey abundance, predation risk, and environmental conditions (Vollrath, 1999; Craig et al., 2000; Vollrath & Selden, 2007; Blackledge et al., 2011). For example, black widow spiders spin two different types of webs depending on prey abundance: starved spiders produce a classic cob-web, while satiated spiders change their behaviour to produce an elaborate network made of supporting threads (Blackledge & Zevenbergen, 2007). Silk also protects spiders against predators. For example, the orb-weaver, *N. arabesca*, adds items such as leaves, silk stabilmenta (web decorations) or barriers to reduce predation risk (Sensenig et al., 2011). However, the behaviours performed to avoid predation can have long lasting impacts on the fitness of both an individual and a population. For example, the social spider, *S. dumicola*, produces special cribellate silk to make a tangled silk barrier during attacks from ants (Henschel, 1998). However, this type of silk is commonly used to repair and construct the web for prey capture, and is costly to produce over extended periods of time, and can contribute to the spread of a fungal disease (Henschel, 1998).

Web-hunting strategies play a role in the type of silk used, as well as the silk's properties (Sensenig et al., 2011). However, other groups of spiders use different strategies for hunting. Instead of investing energy building a capture web, pirate spiders (Araneae, Mimetidae), jumping spiders (*Portia* spp.) and *Poecilochroa senilis* (Araneae, Gnaphosidae) invade the webs of other spiders, using aggressive mimicry or stealthy approach to capture the resident spider (Jackson & Whitehouse, 1986; Li & Jackson, 1996; Michálek et al., 2019). Other spiders, including mysmenids and theridiids (e.g. *Argyrodes*), steal the silk (kleptoparasitism) and prey from other resident spiders without being detected (Tso & Severinghaus, 1998). These araneophagus spiders engage in risky behaviours to use another spider's web and capture the host, which comes with a high cost of being predated. These spiders can assess how dangerous the targeted spider prey is, and make decisions on whether to attack the host spider depending on its size (Chang, Ng, & Li, 2017a).

There has been some consideration of the relationship between personality and silk production in spiders. How species might choose prey, or which type of prey to target, could be associated with specific behavioural types as well as cognitive types/styles (behavioural types related to decision making). It has been proposed for animals in general that bold, aggressive, exploratory and active individuals tend to be faster making decisions related to hunting (Sih & Del Giudice, 2012). This is supported by studies of jumping spiders, *Portia labiata*, where individuals show differences in aggressiveness and speed of prey-choice decision, with aggressive individuals making decisions faster than docile ones (Chang et al., 2017b). Furthermore, the use of silk depends on personality composition, social organization and collective behaviour in colonies of the social spider *S. dumicola*. When attacked by ants, bolder spiders participate in cribellate silk making, while shy individuals carry out body attack and leg immobilization (Wright et al., 2016). Consequently, colonies with a mix of bold-shy personalities show better defence of their webs than monotypic colonies (Wright et al., 2016).

Vollrath and Selden (2007) made the broad observation that individual spiders vary not only in specific morphological and anatomical traits, but also in the way they use different silks (i.e. inter-individual variation in behaviour). Given that spinning behaviour changes according to the conditions in which spiders are exposed (Vollrath & Selden, 2007), we suggest that changes in spinning behaviour could also be interlinked with behavioural repeatability or consistency (i.e. personality), which likely impacts spider survival. This is supported by findings in jumping spider, *M. muscosa*, reared under poor conditions and changed prey availability, where individual variation in behaviour is not consistent (Liedtke et al., 2015). Consistency and/or behavioural plasticity likely differs between species. For example, compared to *M. muscosa, S. sarasinorum* individuals show behavioural consistency and plasticity in prey capture when availability of prey is low (Beleyur et al., 2015). If individual variation in behaviour is adaptive, and there is predictability in environmental conditions, then there is likely a balance between consistency and flexibility, allowing individuals to change the use of resources, such as silk, to respond to changes in conditions (Watts et al., 2015).

2.4.4. How are hormones, venoms and silks related?

Establishing a connection between hormones (e.g. octopamine, serotonin and juvenile hormone) that affect the expression of behavioural types (e.g. aggressiveness), and the use and modulation of venom in a particular context, would aid our understanding of the evolution of personalities in spiders. Recently, Undheim et al., (2015) showed that the evolution of one class of venom peptides in Araneomorph spiders and centipedes was derived from an ancient family of neuropeptide hormones that subsequently became a toxin through structural adaptation. This suggests that hormones and venoms may be closely interlinked and, perhaps, work synergistically in affecting behavioural expression. However, this would require considerable testing to elucidate if these relationships do occur.

Venom and silk are used to capture prey and deter predators (Sensenig et al., 2011). Often these biomolecules are used independently. However, silks and venoms are tightly linked. For example, webs and venom can be used simultaneously to allow spiders to increase the efficiency of prey capture. These physiological adaptations used in conjunction allow spiders to catch prey that can be larger than seven times their own body size (Sanggaard et al., 2014). Similarly, neurotoxins and proteolytic enzymes (similar to those found in the venom from scorpions, wasps and wandering spiders) present in the web silk of golden silk orb-weaver spiders, *Trichonephila clavipes*, likely function to initially induce paralysis of prey, allowing the spider to reduce venom use for prey capture and manipulation (Esteves et al., 2020). Furthermore, both *Nephila antipodiana* (Zhang et al., 2012) and *Trichonephila clavipes* (Knowlton & Kamath, 2018) use chemical weaponry on their webs to deter predators (myrmicine ants). Another unusual strategy is the ability of spitting spiders (family Scytodidae) to eject fibrous venom on prey (Suter & Stratton, 2009).

However, the production and use of silks and venoms involves a high metabolic cost, and the ways in which silk and venom are used have different outcomes for a spider's fitness (Craig et al., 2000; Cooper et al., 2015). These energetic costs must be balanced according to the prey that is targeted or the risk of predation (Evans et al., 2019). For example, silk production involves protein synthesis, energy consumption and behavioural costs of web construction (Blackledge et al., 2011). For venom, depletion of venom and changes in venom composition during regeneration could expose spiders to increased risk of predation in a manner similar to that suggested for thick-tailed scorpions *Parabuthus tranvaalicus* (Nisani et al., 2007). Similarly, newly regenerated venom in the wandering spider, *C. salei*, is characterized by a lower quantity of proteins, and a higher quantity of amino acids, which results in a less acute response in their prey, and could be problematic if the prey is large and difficult to handle (Boevé et al., 1995). These costs can be modulated by adjusting behaviours according to prey availability and type.

The interplay between silk, venom and individual behaviours related with prey capture, predator deterrence and ecological factors should be considered to understand the evolution and adaptation of spider weapons, the compensation of the costs associated with these traits, and the optimization of these mechanisms that have allowed spiders to colonize new habitats and adapt to changing conditions. Currently, these relationships are unstudied.

Individual level behavioural plasticity and the persistence of behavioural types depends on how adaptive or plastic traits are in response to specific conditions (Bengston & Jandt, 2014). However, behavioural plasticity at the population level may be limited when considering what behavioural types are present in that population (Sih et al., 2004a; Sih et al., 2004b; Briffa & Sneddon, 2016). Studying both personality and physiological variables in different species and at different life stages will provide greater insights into how the physiological costs associated with silk production (Sensenig et al., 2011; Craig et al., 1999) and venom production (Evans et al., 2019) might be mitigated through behavioural adjustment (behavioural plasticity).

2.5. Conclusions

Individual behavioural types are seen in spiders, and affect how individuals interact with their environment, and ultimately shape behavioural variation at the population level. While it is broadly understood that both extrinsic and intrinsic factors influence the expression of personalities and the levels of plasticity of behaviours, these factors do not act in isolation, and a broader understanding of the interaction between these factors is currently lacking. In spiders, the physiological factors of silk and venom production, both being unique to this group, could offer unique insights into the evolution and ecology of spider personalities because both venom and silk are quantifiable in terms of metabolic costs, can be managed and manipulated by the individual (i.e. a spider can use different types of silks and alter the volume and composition of venom deployed in different situations), affect growth, fecundity and survival of the individual, and may be impacted by hormone expression. To develop a comprehensive understanding of the flexibility of behaviours, and the persistence or absence of behavioural types in spiders, I argue that it is necessary to incorporate these underlying mechanisms into a synthesized whole alongside other extrinsic and intrinsic factors.

2.6. References

Arnqvist, G. R. & Henriksson, S. (1997). Sexual cannibalism in the fishing spider and a model for the evolution of sexual cannibalism based on genetic constraints. *Evolutionary Ecology*, *11*, 255–273. https://doi.org/10.1023/A:1018412302621

Atkinson, R. K., & Walker, P. (1985). The effects of season of collection, feeding, maturation and gender on the potency of funnel-web spider (*Atrax infensus*) venom. *Australian Journal of Experimental Biology and Medical Science*, *63*, 555–561. https://doi.org/10.1038/icb.1985.59

Beleyur, T., Bellur, D. U., & Somanathan, H. (2015). Long-term behavioural consistency in prey capture but not in web maintenance in a social spider. *Behavioural Ecology and Sociobiology*, *69*, 1019–1028. https://doi.org/10.1007/s00265-015-1915-z

Bell, A. M. (2007a). Future directions in behavioural syndromes research. *Proceedings of the Royal Society of London B*, *274*, 755–761. https://doi.org/10.1098/rspb.2006.0199

Bell, A. M. (2007b). Animal personalities. *Nature*, *447*, 539–540. https://doi.org/doi:10.1038/447539a

Bengston, S. E., & Jandt, J. M. (2014). The development of collective personality: the ontogenetic drivers of behavioural variation across groups. *Frontiers in Ecology and Evolution*, *2*, 81. https://doi.org/10.3389/fevo.2014.00081

Bergmüller, R., & Taborsky, M. (2010). Animal personality due to social niche specialisation. *Trends in Ecology and Evolution*, *25*, 504–511. https://doi.org/10.1016/j.tree.2010.06.012

Blackledge, T. A., & Hayashi, C. Y. (2006). Silken toolkits: biomechanics of silk fibers spun by the orb web spider *Argiope argentata* (Fabricius 1775). *Journal of Experimental Biology*, *209*, 2452–2461. https://doi.org/10.1242/jeb.02275

Blackledge, T. A., & Zevenbergen, J. M. (2007). Condition-dependent spider web architecture in the western black widow, *Latrodectus hesperus*. *Animal Behaviour*, *73*, 855–864. https://doi.org/10.1016/j.anbehav.2006.10.014

Blackledge, T. A., Kuntner, M., & Agnarsson, I. (2011). The form and function of spider orb webs: evolution from silk to ecosystems. *Advances in Insect Physiology*, 41, 175–262). https://doi.org/10.1016/B978-0-12-415919-8.00004-5

Blandenier, G. (2009). Ballooning of spiders (Araneae) in Switzerland: general results from an eleven-year survey. *Arachnology*, *14*, 308–316. https://doi.org/10.13156/arac.2009.14.7.308

Boevé, J. L., Kuhn-Nentwig, L., Keller, S., & Nentwig, W. (1995). Quantity and quality of venom released by a spider (*Cupiennius salei*, Ctenidae). *Toxicon*, *33*, 1347–1357. https://doi.org/10.1016/0041-0101(95)00066-U

Bonte, D., Lens, L., & Maelfait, J. P. (2006). Sand dynamics in coastal dune landscapes constrain diversity and life-history characteristics of spiders. *Journal of Applied Ecology*, *43,* 735–747. https://doi.org/10.1111/j.1365-2664.2006.01175.x

Bosco, J. M., Riechert, S. E., & O’Meara, B. C. (2017). The ontogeny of personality traits in the desert funnel-web spider, *Agelenopsis lisa* (Araneae: Agelenidae). *Ethology*, *123*, 648–658. https://doi.org/10.1111/eth.12639

Branch, C. L., Kozlovsky, D. Y., & Pravosudov, V. V. (2015). Elevation related variation in aggressive response to mirror image in mountain chickadees. *Behaviour*, *152*, 667–676. https://doi.org/10.1163/1568539X-00003248

Briffa, M., Rundle, S. D., & Fryer, A. (2008). Comparing the strength of behavioural plasticity and consistency across situations: animal personalities in the hermit crab *Pagurus bernhardus*. *Proceedings of the Royal Society of London B: Biological Sciences*, *275*, 1305–1311. https://doi.org/10.1098/rspb.2008.0025

Briffa, M., & Sneddon, L. U. (2016). Proximate mechanisms of animal personality among-individual behavioural variation in animals. *Behaviour*, *153*, 1509–1515. https://doi.org/10.1163/1568539x-00003402

Calbacho-Rosa, L., Moreno-García, M. A., Lanz-Mendoza, H., Peretti, A. V, & Córdoba-Aguilar, A. (2012). Reproductive activities impair immunocompetence in *Physocyclus dugesi* (Araneae: Pholcidae). *The Journal of Arachnology*, *40*, 18–22. https://doi.org/10.1636/Hi11-13.1

Casewell, N. R., Wüster, W., Vonk, F. J., Harrison, R. A., & Fry, B. G. (2013). Complex cocktails: the evolutionary novelty of venoms. *Trends in Ecology and Evolution*, *28*, 219–229. https://doi.org/10.1016/j.tree.2012.10.020

Chang, C. C., Ng, P. J., & Li, D. (2017a). Aggressive jumping spiders make quicker decisions for preferred prey but not at the cost of accuracy. *Behavioural Ecology*, *28*, 479–484. https://doi.org/10.1093/beheco/arw174

Chang, C. C., Teo, H. Y., Norma-Rashid, Y., & Li, D. (2017b). Predator personality and prey behavioural predictability jointly determine foraging performance. *Scientific Reports, 7*, 1–8. https://doi.org/10.1038/srep40734

Chapin, K. J. (2015). Cave-epigean behavioural variation of the whip spider *Phrynus longipes* (Arachnida: Amblypygi) evidenced by activity, vigilance, and aggression. *Journal of Arachnology*, 43, 214–219. https://doi.org/10.1636/arac-43-02-214-219.1

Coelho, P., Kaliontzopoulou, A., Rasko, M., & van der Meijden, A. (2017). A ‘striking’ relationship: scorpion defensive behaviour and its relation to morphology and performance. *Functional Ecology, 31*, 1390–1404. https://doi.org/10.1111/1365-2435.12855

Contreras-Garduño, J., Canales-Lazcano, J., & Córdoba-Aguilar, A. (2006). Wing pigmentation, immune ability, fat reserves and territorial status in males of the rubyspot damselfly, *Hetaerina americana*. *Journal of Ethology*, *24*, 165–173. https://doi.org/10.1007/s10164-005-0177-z

Contreras-Garduño, J., Córdoba-Aguilar, A., Lanz-Mendoza, H., & Cordero Rivera, A. (2009). Territorial behaviour and immunity are mediated by juvenile hormone: the physiological basis of honest signalling? *Functional Ecology, 23*, 157–163. https://doi.org/10.1111/j.1365-2435.2008.01485.x

Cooper, A. M., Nelsen, D. R., & Hayes, W. K. (2015). The strategic use of venom by spiders In P. Gopalakrishnakone & A. Malhotra (Eds.). *Evolution of venomous animals and their toxins* (pp. 145–166). Dordrecht, Netherlands: Springer. https://d. oi.org/10.1007/978-94-007-6727-0_13-1

Cote, J., Clobert, J., Brodin, T., Fogarty, S., & Sih, A. (2010). Personality-dependent dispersal: characterization, ontogeny and consequences for spatially structured populations.

Philosophical Transactions of the Royal Society of London B, 365, 4065-4076. https://doi.org/10.1098/rstb.2010.0176

Coyle, F. A. (1983). Aerial dispersal by mygalomorph spiderlings (Araneae, Mygalomorphae). *Journal of Arachnology, 11*, 283-286.

Craig, C. L. (2003). *Spiderwebs and silk: tracing evolution from molecules to genes to phenotypes*. Oxford, UK: Oxford University Press.

Craig, C. L., Hsu, M., Kaplan, D., & Pierce, N. E. (1999). A comparison of the composition of silk proteins produced by spiders and insects. *International Journal of Biological Macromolecules, 24*, 109–118. https://doi.org/10.1016/S0141-8130(99)00006-9

Craig, C. L., Riekel, C., Herberstein, M. E., Weber, R. S., Kaplan, D., & Pierce, N. E. (2000). Evidence for diet effects on the composition of silk proteins produced by spiders. *Molecular Biology and Evolution*, *17*, 1904–1913. https://doi.org/10.1093/oxfordjournals.molbev.a026292

Dall, S. R. X., Houston, A. I., & McNamara, J. M. (2004). The behavioural ecology of personality: consistent individual differences from an adaptive perspective. *Ecology Letters, 7*, 734–739. https://doi.org/10.1111/j.1461-0248.2004.00618.x

Dingemanse, N. J., & Wolf, M. (2010). Recent models for adaptive personality differences: a review. *Philosophical Transactions of the Royal Society of London B, 365*, 3947–3958. https://doi.org/10.1098/rstb.2010.0221

Dingemanse, N. J., Kazem, A. J., Réale, D., & Wright, J. (2010). Behavioural reaction norms: animal personality meets individual plasticity. *Trends in Ecology & Evolution*, 25, 81–89. https://doi.org/10.1016/j.tree.2009.07.013

Dingemanse, N. J., Van der Plas, F., Wright, J., Réale, D., Schrama, M., Roff, D. A., Van der Zee, E., & Barber, I. (2009). Individual experience and evolutionary history of predation affect expression of heritable variation in fish personality and morphology. *Proceedings of the Royal Society of London B, 276*, 1285–1293. https://doi.org/10.1098/rspb.2008.1555

Dingemanse, N. J., & Dochtermann, N. A. (2013). Quantifying individual variation in behaviour: Mixed-effect modelling approaches. *Journal of Animal Ecology*, *82*, 39–54. https://doi.org/10.1111/1365-2656.12013

DiRienzo, N., & Montiglio, P. O. (2016). The contribution of developmental experience vs. condition to life history, trait variation and individual differences. *Journal of Animal Ecology*, *85*, 915–926. https://doi.org/10.1111/1365-2656.12512

Duckworth, R. A., & Badyaev, A. V. (2007). Coupling of dispersal and aggression facilitates the rapid range expansion of a passerine bird. *Proceedings of the National Academy of Sciences*, *104*, 15017-15022. https://doi.org/10.1073/pnas.0706174104

Edenbrow, M., & Croft, D. P. (2013). Environmental and genetic effects shape the development of personality traits in the mangrove killifish *Kryptolebias marmoratus*. *Oikos, 122,* 667–681. https://doi.org/10.1111/j.1600-0706.2012.20556.x

Elgar, M. A., & Nash, D. R. (1988). Sexual cannibalism in the garden spider *Araneus diadematus*. *Animal Behaviour, 36*, 1511-1517. https://doi.org/10.1016/S0003-3472(88)80221-5

Elgar, M. A., & Schneider, J. M. (2004). Evolutionary significance of sexual cannibalism. *Advances in the Study of Behaviour*, *34*, 135–163. https://doi.org/10.1016/S0065-3454(04)34004-0

Esteves, F. G., dos Santos-Pinto, J. R. A., Ferro, M., Sialana, F. J., Smidak, R., Rares, L. C., Nussbaumer, T., Rattei, T., Bilban, M., Bacci Júnior, M., Lubec, G., & Palma, M. S. (2020). Revealing the venomous secrets of the spider's Web. *Journal of Proteome Research, 19*, 3044–3059. https://doi.org/10.1021/acs.jproteome.0c00086

Evans, E. R. J., Northfield, T. D., Daly, N. L., & Wilson, D. T. (2019). Venom costs and optimization in scorpions. *Frontiers in Ecology and Evolution, 7*, 196. https://doi.org/10.3389/fevo.2019.00196

Fernández, M. F. (2005). *Variation in life history and behavioural traits in the colonial spider* Parawixia bistriata *(Araneidae): some adaptive responses to different environments* (Doctoral dissertation, University of Tennessee, Knoxville, USA). Retrieved from https://trace.tennessee.edu/utk_graddiss/1946

Foelix, R. F. (2011). *Biology of spiders* (3rd ed.). Oxford, UK: Oxford University Press.

Fogarty, S., Cote, J., & Sih, A. (2011). Social personality polymorphism and the spread of invasive species: A model. *American Naturalist, 177*, 273–287. https://doi.org/10.1086/658174

Fox, G. A. (2018). *The design of complex weapons systems in scorpions: sexual, ontogenetic, and interspecific variation* (Doctoral disseration, Loma Linda University, California, USA). Retrieved from http://scholarsrepository.llu.edu/etdhttp://scholarsrepository.llu.edu/etd/516

Garb, J. (2013). Spider silk: an ancient biomaterial for the 21st century. In Penney D (Ed.), *Spider research in the 21st century: trends and perspectives* (pp. 252–281). Manchester, UK: Siri Scientific Press.

Gangur, A. N., Smout, M., Liddell, M. J., Seymour, J. E., Wilson, D., & 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 B, 284*, 20171364. https://doi.org/10.1098/rspb.2017.1364

Gomes, P. C., & Palma, M. S. (2016). The nonpeptide low molecular mass toxins from spider venoms. In P. Gopalakrishnakone, G. A., Corzo, M. E., de Lima, & E. Diego-García (Eds.), *Spider venoms* (pp. 3–19). Dordrecht, Netherlands: Springer. https://doi.org/10.1007/978-94-007-6389-0_14

Gosling, S. D. (2001). From mice to men: what can we learn about personality from animal research? *Psychological Bulletin, 127*, 45–86. https://doi.org/10.1037/0033-2909.127.1.45

Gray, M. R., & Sutherland, S. K. (1978). Venoms of Dipluridae. In S. Bettinni (Ed.), *Arthropod venoms* (pp. 121–148). Berlin, Germany: Springer-Verlag. https://doi.org/10.1007/978-3-642-45501-8_7

Halpin, R. N., & Johnson, J. C. (2014). A continuum of behavioural plasticity in urban and desert black widows. *Ethology, 120*, 1237–1247. https://doi.org/10.1111/eth.12297

Hargreaves, A. D., Swain, M. T., Hegarty, M. J., Logan, D. W., & Mulley, J. F. (2014). Restriction and recruitment-gene duplication and the origin and evolution of snake venom toxins. *Genome Biology and Evolution, 6*, 2088–2095. https://doi.org/10.1093/gbe/evu166

Hayes, W. K. (1993). Effects of hunger on striking, prey-handling, and venom expenditure of prairie rattlesnakes (*Crotalus v. viridis*). *Herpetologica, 49*, 305–310.

Henschel, J. R. (1998). Predation on social and solitary individuals of the spider *Stegodyphus dumicola* (Araneae, Eresidae). *Journal of Arachnology, 26*, 61–69. https://doi.org/10.2307/3705451

Herzig, V. (2010). Ontogenesis, gender, and molting influence the venom yield in the spider *Coremiocnemis tropix* (Araneae, Theraphosidae). *Journal of Venom Research*, *1*, 76–83.

Herzig, V., Sunagar, K., Wilson, D. T. R., Pineda, S. S., Israel, M. R., Dutertre, S., McFarland, B. S., Undheim, E. A. B., Hodgson, W.C., Alewood, P.F., Lewis, R.J., Bosmans, F., Vetter, I., King, G.F., Fry, B. G. (2020). Australian funnel-web spiders evolved human-lethal δ-hexatoxins for defense against vertebrate predators. Proceedings of the National Academy of Sciences of the United States of America, 117(40), 24920–24928. https://doi.org/10.1073/pnas.2004516117

Hudina, S., Hock, K., & Žganec, K. (2014). The role of aggression in range expansion and biological invasions. *Current Zoology, 60*, 401–409. https://doi.org/10.1093/czoolo/60.3.401

Jackson, R. R. (1992). Conditional strategies and interpopulation variation in the behaviour of jumping spiders. *New Zealand Journal of Zoology*, *19*, 99–111. https://doi.org/10.1080/03014223.1992.10422314

Jackson, R. R., & Whitehouse, M. E. A. (1986). The biology of New Zealand and Queensland pirate spiders (Araneae, Mimetidae): aggressive mimicry, araneophagy and prey specialization. *Journal of Zoology*, *210*, 279–303. https://doi.org/10.1111/j.1469-7998.1986.tb03635.x

Jandt, J. M., Bengston, S., Pinter-Wollman, N., Pruitt, J. N., Raine, N. E., Dornhaus, A., & Sih, A. (2014). Behavioural syndromes and social insects: personality at multiple levels. *Biological Reviews, 89*, 48–67. https://doi.org/10.1111/brv.12042

Jeanson, R., & Weidenmüller, A. (2014). Interindividual variability in social insects - proximate causes and ultimate consequences. *Biological Reviews, 89,* 671–687. https://doi.org/10.1111/brv.12074

Johnson, J. C, & Sih, A. (2005). Precopulatory sexual cannibalism in fishing spiders (*Dolomedes triton*): A role for behavioural syndromes. *Behavioural Ecology and Sociobiology*, *58*, 390–396. https://doi.org/10.1007/s00265-005-0943-5

Johnson, J. Chadwick, Halpin, R., Stevens, D., Vannan, A., Lam, J., & Bratsch, K. (2015). Individual variation in ballooning dispersal by black widow spiderlings: the effects of family and social rearing. *Current Zoology*, *61*, 520–528. https://doi.org/10.1093/czoolo/61.3.520

Jones, T .C., Akoury, T. S., Hauser, C. K., Neblett II, M. F., Linville, B. J., Edge, A. A., & Weber, N. O. (2011). Octopamine and serotonin have opposite effects on antipredator behaviour in the orb-weaving spider, *Larinioides cornutus*. *Journal of Comparative Physiology*, *197*, 819–825. https://doi.org/10.1007/s00359-011-0644-7

Keiser, C. N., Lichtenstein, J. L. L., Wright, C. M., Chism, G. T., & Pruitt, J. N. (2018). Personality and behavioural syndromes in insects and spiders. In A. Córdoba-Aquilar, D. González-Tokman, & I. González-Santoyo (Eds.), *Insect Behaviour: From Mechanisms to Ecological and Evolutionary Consequences* (pp. 236–256). Oxford, UK: Oxford University Press. https://doi.org/10.1093/oso/9780198797500.003.0016

Keiser, C. N., Modlmeier, A. P., Singh, N., Jones, D. K., & Pruitt, J. N. (2014). Exploring how a shift in the physical environment shapes individual and group behaviour across two social contexts. *Ethology*, *120*, 825–833. https://doi.org/10.1111/eth.12256

Kluen, E., & Brommer, J. E. (2013). Context-specific repeatability of personality traits in a wild bird: a reaction-norm perspective. *Behavioural Ecology*, *24,* 650–658. https://doi.org/10.1093/beheco/ars221

Knowlton, E. D., & Kamath, A. (2018). Ants do not traverse the silk of adult female *Nephila clavipes* (Linnaeus) webs. *Neotropical entomology, 47*, 780-785. https://doi.org/10.1007/s13744-018-0631-6

Koolhaas, J. M., Korte, S. M., De Boer, S. F., Van Der Vegt, B. J., Van Reenen, C. G., Hopster, H., De Jong, I. C., Ruis, M. A. W., & Blokhuis, H. J. (1999). Coping styles in animals: current status in behaviour and stress- physiology. *Neuroscience and Biobehavioural Reviews, 23,* 925–935. https://doi.org/10.1016/S0149-7634(99)00026-3

Koolhaas, J. M., De Boer, S. F., Buwalda, B., & Van Reenen, K. (2007). Individual variation in coping with stress: A multidimensional approach of ultimate and proximate mechanisms. *Brain, Behaviour and Evolution*, *70*, 218–226. https://doi.org/10.1159/000105485

Kowalski, K., & Rychlik, L. (2018). The role of venom in the hunting and hoarding of prey differing in body size by the Eurasian water shrew, *Neomys fodiens*. *Journal of Mammalogy*, *99*, 351–362. https://doi.org/10.1093/jmammal/gyy013

Kralj-Fišer, S., Gregorič, M., Zhang, S., Li, D., & Kuntner, M. (2011). Eunuchs are better fighters. *Animal Behaviour*, *81*, 933–939. https://doi.org/10.1016/j.anbehav.2011.02.010

Kralj-Fišer, S., & Schneider, J. M. (2012). Individual behavioural consistency and plasticity in an urban spider. *Animal Behaviour, 84,* 197–204. https://doi.org/10.1016/j.anbehav.2012.04.032

Kralj-Fišer, S., Schneider, J. M., & Kuntner, M. (2013). Challenging the aggressive spillover hypothesis: is pre-copulatory sexual cannibalism a part of a behavioural syndrome? *Ethology*, *119*, 615–623. https://doi.org/10.1111/eth.12111

Kralj-Fišer, S., & Schuett, W. (2014). Studying personality variation in invertebrates: why bother? *Animal Behaviour, 91*, 41–52. https://doi.org/10.1016/j.anbehav.2014.02.016

Kralj-Fišer, S., Hebets, E. A., & Kuntner, M. (2017). Different patterns of behavioural variation across and within species of spiders with differing degrees of urbanization. *Behavioural Ecology and Sociobiology*, *71*, 1–15. https://doi.org/10.1007/s00265-017-2353-x

Langenhof, M. R., & Komdeur, J. (2018). Why and how the early-life environment affects development of coping behaviours. *Behavioural Ecology and Sociobiology, 72,* 2–23. https://doi.org/10.1007/s00265-018-2452-3

Li, D., & Jackson, R. R. (1996). Prey preferences of *Portia fimbriata*, an araneophagic, web-building jumping spider (Araneae: Salticidae) from Queensland. *Journal of Insect Behaviour*, *9*, 613—642. https://doi.org/10.1007/bf02213884

Liedtke, J., & Schneider, J. M. (2017). Social makes smart: rearing conditions affect learning and social behaviour in jumping spiders. *Animal Cognition*, *20*, 1093–1106. https://doi.org/10.1007/s10071-017-1125-3

Liedtke, J., Redekop, D., Schneider, J. M., & Schuett, W. (2015). Early environmental conditions shape personality types in a jumping spider. *Frontiers in Ecology and Evolution*, *3*, 134. https://doi.org/10.3389/fevo.2015.00134

Lira, A. F. A., Santos, A. B., Silva, N. A., & Martins, R. D. (2017). Threat level influences the use of venom in a scorpion species, *Tityus stigmurus* (Scorpiones, Buthidae). *Acta Ethologica*, *20*, 291–295. https://doi.org/10.1007/s10211-017-0274-3

Lubin, Y., & Bilde, T. (2007). The evolution of sociality in spiders. *Advances in the Study of Behaviour, 37*, 83–145. https://doi.org/10.1016/S0065-3454(07)37003-4

MacKay, J. R. D., & Haskell, M. J. (2015). Consistent individual behavioural variation: the difference between temperament, personality and behavioural syndromes. *Animals*, *5*, 455–478. https://doi.org/10.3390/ani5030366

Macrander, J., Brugler, M. R., & Daly, M. (2015). A RNA-seq approach to identify putative toxins from acrorhagi in aggressive and non-aggressive *Anthopleura elegantissima* polyps. *BMC Genomics*, *16*, 221. https://doi.org/10.1186/s12864-015-1417-4

Michálek, O., Lubin, Y., & Pekár, S. (2019). Nest usurpation: a specialised hunting strategy used to overcome dangerous spider prey. *Scientific Reports, 9,* 5386. https://doi.org/10.1038/s41598-019-41664-6

Michalko, R., & Pekar, S. (2014). Is different degree of individual specialization in three spider species caused by distinct selection pressures? *Basic and Applied Ecology*, *15*, 496–506. https://doi.org/10.1016/j.baae.2014.08.003

Michalko, R., Košulič, O., & Řežucha, R. (2017). Link between aggressiveness and shyness in the spider *Philodromus albidus* (Araneae, Philodromidae): state dependency over stability. *Journal of Insect Behaviour, 30*, 48–59. https://doi.org/10.1007/s10905-017-9596-2

Modlmeier, A. P., Keiser, C. N., Wright, C. M., Lichtenstein, J. L., & Pruitt, J. N. (2015). Integrating animal personality into insect population and community ecology. *Current Opinion in Insect Science*, *9*, 77-85. https://doi.org/10.1016/j.cois.2015.03.008

Moon, M. J., & Kim, T. H. (2005). Microstructural analysis of the capture thread spinning apparatus in orb web spiders. *Entomological Research*, *35*, 133-140. https://doi.org/10.1111/j.1748-5967.2005.tb00149.x

Morgenstern, D., & King, G. F. (2013). The venom optimization hypothesis revisited. *Toxicon, 63*, 120–128. https://doi.org/10.1016/j.toxicon.2012.11.022

Mullen, G. R., & Vetter, R. S. (2019). Spiders (Araneae). In G. R. Mullen & L. A. Durden (Eds.), *Medical and veterinary entomology* (3rd ed.) (pp. 507–531). Cambridge, Massachusetts, USA: Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-814043-7.00025-X

Nakata, K., & Ushimaru, A. (2013). The effect of predation risk on spider's decisions on web-site relocation. *Behaviour*, *150*, 103–114. https://doi.org/10.1163/1568539X-00003039

Nelsen, D. R., Kelln, W., & 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. https://doi.org/10.1016/j.anbehav.2013.12.019

Nisani, Z., Dunbar, S. G., & Hayes, W. K. (2007). Cost of venom regeneration in *Parabuthus transvaalicus* (Arachnida: Buthidae). *Comparative Biochemistry and Physiology A*, *147*, 509–513. https://doi.org/10.1016/j.cbpa.2007.01.027

Nisani, Z., Boskovic, D. S., Dunbar, S. G., Kelln, W., & 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. https://doi.org/10.1016/j.toxicon.2012.04.343

Palagi, A., Koh, J. M. S., Leblanc, M., Wilson, D., Dutertre, S., King, G. F., Nicholson, G. M., & Escoubas, P. (2013). Unravelling the complex venom landscapes of lethal Australian funnel-web spiders (Hexathelidae: Atracinae) using LC-MALDI-TOF mass spectrometry. *Journal of Proteomics*, *80*, 292–310. https://doi.org/10.1016/j.jprot.2013.01.002

Parthasarathy, B., & Somanathan, H. (2020). When and why do sit-and-wait social spiders disperse? *Israel Journal of Ecology and Evolution*, *66*, 15-25. https://doi.org/10.1163/22244662-20191068

Parthasarathy, B., Joshi, C. H., Kalyadan, S. S., & Somanathan, H. (2019). Early ontogenic emergence of personality and its long-term persistence in a social spider. *Behavioural Ecology and Sociobiology*, *73*, 35. https://doi.org/10.1007/s00265-019-2645-4

Pearce, A. N., Huang, Z. Y., & Breed, M. D. (2001). Juvenile hormone and aggression in honey bees. *Journal of Insect Physiology*, *47*, 1243-1247. https://doi.org/10.1016/S0022-1910(01)00109-3

Piersma, T., & Lindström, Å. (1997). Rapid reversible changes in organ size as a component of adaptive behaviour. *Trends in Ecology and Evolution*, *12*, 134–138. https://doi.org/10.1016/S0169-5347(97)01003-3

Piersma, T., & van Gils, J.A. (2011). *The flexible phenotype: a body-centred integration of ecology, physiology, and behaviour*. Oxford, UK: Oxford University Press.

Pinter-Wollman, N., Gordon, D. M., & Holmes, S. (2012). Nest site and weather affect the personality of harvester ant colonies. *Behavioural Ecology*, *23*, 1022-1029. https://doi.org/10.1093/beheco/ars066

Price, J. B. (2016). *Exploring the relationship between behaviour and neurochemistry in the polyphenic spider,* Anelosimus studiosus *(Araneae: Theridiidae)* (Doctoral dissertation, East Tennessee State University, Tennessee, USA). Retrieved from https://dc.etsu.edu/etd/3114

Pruitt, J. N., & Riechert, S. E. (2012). The ecological consequences of temperament in spiders. *Current Zoology*, *58*, 589–596. https://doi.org/10.1093/czoolo/58.4.589

Rabaneda-Bueno, R., Rodríguez-Gironés, M. Á., Aguado-de-la-Paz, S., Fernández-Montraveta, C., De Mas, E., Wise, D. H., & Moya-Laraño, J. (2008). Sexual cannibalism: high incidence in a natural population with benefits to females. *PLoS One*, *3*, e3484. https://doi.org/10.1371/journal.pone.0003484

Rabaneda-Bueno, R., Aguado, S., Fernández-Montraveta, C., & Moya-Laraño, J. (2014). Does female personality determine mate choice through sexual cannibalism? *Ethology*, *120*, 238–248. https://doi.org/10.1111/eth.12197

Rádai, Z., Kiss, B., & Barta, Z. (2017). Pace of life and behaviour: rapid development is linked with increased activity and voracity in the wolf spider *Pardosa agrestis*. *Animal Behaviour*, *126*, 145–151. https://doi.org/10.1016/j.anbehav.2017.02.004

Réale, D., Reader, S. M., Sol, D., McDougall, P. T., & Dingemanse, N. J. (2007). Integrating animal temperament within ecology and evolution. *Biological Reviews*, *82*, 291–318. https://doi.org/10.1111/j.1469-185X.2007.00010.x

Réale, D., Martin, J., Coltman, D. W., Poissant, J., & Festa-Bianchet, M. (2009). Male personality, life-history strategies and reproductive success in a promiscuous mammal. *Journal of Evolutionary Biology*, *22*, 1599–1607. https://doi.org/10.1111/j.1420-9101.2009.01781.x

Réale, D., Garant, D., Humphries, M. M., Bergeron, P., Careau, V., & Montiglio, P. O. (2010). Personality and the emergence of the pace-of-life syndrome concept at the population level. *Philosophical Transactions of the Royal Society of London B, 365*, 4051–4063. https://doi.org/10.1098/rstb.2010.0208

Rehage, J. S., Cote, J., & Sih, A. (2016). The role of dispersal behaviour and personality in post-establishment spread. In J. S. Weis & D. Sol (Eds.). *Biological invasions and animal behaviour* (pp. 96 –115). Cambridge, UK: Cambridge University Press.

Riechert, S. E. (1993). The evolution of behavioural phenotypes: lessons learned from divergent spider populations. *Advances in the Study of Behaviour*, *22*, 103–134. https://doi.org/10.1016/S0065-3454(08)60406-4

Riechert, S., & Hedrick, A. (1993). A test for correlations among fitness-linked behavioural traits in the spider *Agelenopsis aperta* (Araneae, Agelenidae). *Animal Behaviour*, *46*, 669–675. https://doi.org/10.1006/anbe.1993.1243

Riechert, S. E., Singer, F. D., & Jones, T. C. (2001). High gene flow levels lead to gamete wastage in a desert spider system. *Genetica*, *112–113*, 297–319. https://doi.org/10.1023/A:1013356325881

Robinson, M. H. (1969). Predatory behaviour of *Argiope argentata* (Fabricius). *Integrative and Comparative Biology*, *9*, 161–173. https://doi.org/10.1093/icb/9.1.161

Rocha-e-Silva, T. A. A., Collares-Buzato, C. B., da Cruz-Höfling, M. A., & Hyslop, S. (2009). Venom apparatus of the brazilian tarantula *Vitalius dubius* Mello-Leitão 1923 (Theraphosidae). *Cell and Tissue Research*, *335*, 617–629. https://doi.org/10.1007/s00441-008-0738-x

Roeder, T. (2005). Tyramine and octopamine: ruling behaviour and metabolism. *Annual Review of Entomology*, *50*, 447–477. https://doi.org/10.1146/annurev.ento.50.071803.130404

Roeder, T., Seifert, M., Kähler, C., & Gewecke, M. (2003). Tyramine and octopamine: antagonistic modulators of behaviour and metabolism. *Archives of Insect Biochemistry and Physiology*, *54*, 1–13. https://doi.org/10.1002/arch.10102

Royauté, R., Buddle, C. M., & Vincent, C. (2014). Interpopulation variations in behavioural syndromes of a jumping spider from insecticide-treated and insecticide-free orchards. *Ethology*, *120*, 127–139. https://doi.org/10.1111/eth.12185

Rymer, T. L., Pillay, N., & Schradin, C. (2013). Extinction or survival? Behavioural flexibility in response to environmental change in the African striped mouse *Rhabdomys*. *Sustainability, 5*, 163–186. https://doi.org/10.3390/su5010163

Sanggaard, K. W., Bechsgaard, J. S., Fang, X., Duan, J., Dyrlund, T. F., Gupta, V., Jiang, X., Cheng, L., Fan, D., Feng, Y., Han, L., Huang, Z., Wu, Z., Liao, L., Settepani, V., Thøgersen, I. B., Vanthournout, B., Wang, T., Zhu, Y., Funch, P., Enghild, J. J., Schauser, L., Andersen, S. U., Villesen, P., Schierup, M. H., Bilde, T., & Wang, J. (2014). Spider genomes provide insight into composition and evolution of venom and silk. *Nature Communications, 5*, 3765. https://doi.org/10.1038/ncomms4765

Santana, R. C., Perez, D., Dobson, J., Panagides, N., Raven, R. J., Nouwens, A., Jones, A., King, G. F., & 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. https://doi.org/10.3390/toxins9040116

Sasaki, T., & Iwahashi, O. (1995). Sexual cannibalism in an orb-weaving spider *Argiope aemula*. *Animal Behaviour, 49*, 11119–1121. https://doi.org/10.1006/anbe.1995.0140

Sawadro, M., Bednarek, A., & Babczyńska, A. (2017). The current state of knowledge on the neuroactive compounds that affect the development, mating and reproduction of spiders (Araneae) compared to insects. *Invertebrate Neuroscience, 17*, 1–22. https://doi.org/10.1007/s10158-017-0197-8

Schenberg, S., & Pereira Lima, F. A. (1978). Venoms of ctenidae. In S. Bettini (Ed.). *Arthropod venoms* (pp. 217–245). Berlin, Germany: Springer. https://doi.org/10.1007/978-3-642-45501-8_10

Schendel, V., Rash, L. D., Jenner, R. A., & Undheim, E. A. B. (2019). The diversity of venom: the importance of behaviour and venom system morphology in understanding its ecology and evolution. *Toxins*, *11*, 666. https://doi.org/10.3390/toxins11110666

Sensenig, A. T., Agnarsson, I., & Blackledge, T. A. (2011). Adult spiders use tougher silk: ontogenetic changes in web architecture and silk biomechanics in the orb-weaver spider. *Journal of Zoology*, *285*, 28–38. https://doi.org/10.1111/j.1469-7998.2011.00809.x

Settepani, V., Grinsted, L., Granfeldt, J., Jensen, J. L., & Bilde, T. (2013). Task specialization in two social spiders, *Stegodyphus sarasinorum* (Eresidae) and *Anelosimus eximius* (Theridiidae). *Journal of Evolutionary Biology, 26,* 51–62. https://doi.org/10.1111/jeb.12024

Sih, A. (2011). Effects of early stress on behavioural syndromes: an integrated adaptive perspective. *Neuroscience & Biobehavioural Reviews, 35,* 1452–1465. https://doi.org/10.1016/J.NEUBIOREV.2011.03.015

Sih, A., & Bell, A. M. (2008). Insights for behavioural ecology from behavioural syndroms. *Advances in the Study of Behaviour, 38,* 227–281. https://doi.org/10.1016/S0065-3454(08)00005-3

Sih, A., Bell, A., & Johnson, J. (2004a). Behavioural syndromes: an ecological and evolutionary overview. *Trends in Ecology and Evolution, 19,* 372–378. https://doi.org/10.1016/j.tree.2004.04.009

Sih, A., Bell, A. M., Johnson, J. C., & Ziemba, R. E. (2004b). Behavioural syndromes: an integrative overview. *Quarterly Review of Biology, 79,* 241–277. https://doi.org/10.1086/422893

Sih, A., & Del Giudice, M. (2012). Linking behavioural syndromes and cognition: a behavioural ecology perspective. *Philosophical Transactions of the Royal Society of London B, 367*, 2762–2772. https://doi.org/10.1098/rstb.2012.0216

Sih, A., Cote, J., Evans, M., Fogarty, S., & Pruitt, J. (2012). Ecological implications of behavioural syndromes. *Ecology letters, 15*, 278-289. https://doi.org/10.1111/j.1461-0248.2011.01731.x

Sih, A., Chang, A. T., & Wey, T. W. (2014). Effects of behavioural type, social skill and the social environment on male mating success in water striders. *Animal Behaviour*, *94*, 9–17. https://doi.org/10.1016/j.anbehav.2014.05.010

Sih, A., Mathot, K. J., Moirón, M., Montiglio, P. O., Wolf, M., & Dingemanse, N. J. (2015). Animal personality and state-behaviour feedbacks: a review and guide for empiricists. *Trends in Ecology and Evolution, 30,* 50–60. https://doi.org/10.1016/j.tree.2014.11.004

Sloan Wilson, D., Clark, A. B., Coleman, K., & Dearstyne, T. (1994). Shyness and boldness in humans and other animals. Trends in Ecology and Evolution, 9(11), 442–446. https://doi.org/10.1016/0169-5347(94)90134-1

Stamps, J. A., & Groothuis, T. G. (2010). Developmental perspectives on personality: implications for ecological and evolutionary studies of individual differences. *Philosophical Transactions of the Royal Society of London B, 365,* 4029–4041. https://doi.org/10.1098/rstb.2010.0218

Starrett, J., Garb, J. E., Kuelbs, A., Azubuike, U. O., & Hayashi, C. Y. (2012). Early events in the evolution of spider silk genes. *PLoS One, 7,* 38084. https://doi.org/10.1371/journal.pone.0038084

Stoehr, A. M., & Kokko, H. (2006). Sexual dimorphism in immunocompetence: what does life-history theory predict? *Behavioural Ecology, 17,* 751–756. https://doi.org/10.1093/beheco/ark018

Suter, R. B., & Stratton, G. E. (2009). Spitting performance parameters and their biomechanical implications in the spitting spider, *Scytodes thoracica*. *Journal of Insect Science, 9*, 62. https://doi.org/10.1673/031.009.6201

Tallarovic, S. K., Melville, J. M., & Brownell, P. H. (2000). Courtship and mating in the giant hairy desert scorpion, *Hadrurus arizonensis* (Scorpionida, Iuridae). *Journal of Insect Behaviour, 13,* 827–838. https://doi.org/10.1023/A:1007858400402

Tso, I. M., & Severinghaus, L. L. (1998). Silk stealing by *Argyrodes lanyuensis* (Araneae: Theridiidae): a unique form of kleptoparasitism. *Animal Behaviour, 56*, 219–225. https://doi.org/10.1006/anbe.1998.0770

Undheim, E. A. B., Grimm, L. L., Low, C. F., Morgenstern, D., Herzig, V., Zobel-Thropp, P., Pineda, S. S., Habib, R., Dziemborowicz, S., Fry, B. G., Nicholson, G. M., Binford, G. J., Mobli, M., & King, G. F. (2015). Weaponization of a hormone: convergent recruitment of hyperglycemic hormone into the venom of arthropod predators. *Structure, 23*, 1283–1292. https://doi.org/10.1016/j.str.2015.05.003

van Oers, K., & Mueller, J. C. (2010). Evolutionary genomics of animal personality. *Philosophical Transactions of the Royal Society of London B*, *365,* 3991–4000. https://doi.org/10.1098/rstb.2010.0178

Vollrath, F., & Rohde-Arndt, D. (1983). Prey capture and feeding in the social spider *Anelosimus eximius*. *Zeitschrift Für Tierpsychologie*, *61*(4), 334–340. https://doi.org/10.1111/j.1439-0310.1983.tb01348.x

Vollrath, F. (1999). Biology of spider silk. *International Journal of Biological Macromolecules, 24*, 81–88. https://doi.org/10.1016/S0141-8130(98)00076-2

Vollrath, F., & Selden, P. (2007). The role of behaviour in the evolution of spiders, silks, and webs. *Annual Review of Ecology, Evolution, and Systematics*, *38*, 819–846. https://doi.org/10.1146/annurev.ecolsys.37.091305.110221

Watts, J. C., Ross, C. R., & Jones, T. C. (2015). Diel and life-history characteristics of personality: consistency versus flexibility in relation to ecological change. *Animal Behaviour, 101*, 43–49. https://doi.org/10.1016/j.anbehav.2014.12.020

Webster, M. M., & Ward, A. J. W. (2011). Personality and social context. *Biological Reviews, 86,* 759–773. https://doi.org/10.1111/j.1469-185X.2010.00169.x

Westermann, F. L., Mcpherson, I. S., Jones, T. H., Milicich, L., & Lester, P. J. (2015). Toxicity and utilization of chemical weapons: does toxicity and venom utilization contribute to the formation of species communities? *Ecology and Evolution*, *5*, 3103–3113. https://doi.org/10.1002/ece3.1595

Wigger, E., Kuhn-Nentwig, L., & Nentwig, W. (2002). The venom optimisation hypothesis: a spider injects large venom quantities only into difficult prey types. *Toxicon*, *40,* 749–752. https://doi.org/10.1016/S0041-0101(01)00277-X

Wilson, D. T. R. (2016). The venom of Australian spiders. In P. Gopalakrishnakone, G. Corzo, M. E., de Lima, & E. Diego-García (Eds.), *Spider venoms* (pp. 21–46). Dordrecht, Netherlands: SpringerReference. https://doi.org/10.1007/978-94-007-6389-0_21

Wolf, M., & Weissing, F. J. (2012). Animal personalities: consequences for ecology and evolution. *Trends in Ecology and Evolution, 27,* 452–461. https://doi.org/10.1016/j.tree.2012.05.001

Wolf, M., van Doorn, G. S., Leimar, O., & Weissing, F. J. (2007). Life-history trade-offs favour the evolution of animal personalities. Nature, 447(7144), 581–584. https://doi.org/10.1038/nature05835

Wong, M. K. L., Woodman, J. D., & Rowell, D. M. (2016). Temporal variation in venom yield of the Australian funnel-web spider *Atrax sutherlandi* (Hexathelidae: Atracinae). *Arachnology*, *17*, 7–9. https://doi.org/10.13156/arac.2006.17.1.7

Wright, C. M., Keiser, C. N., & Pruitt, J. N. (2016). Colony personality composition alters colony-level plasticity and magnitude of defensive behaviour in a social spider. *Animal Behaviour*, *115*, 175–183. https://doi.org/10.1016/j.anbehav.2016.03.002

Wright, C. M., Lichtenstein, J. L., Doering, G. N., Pretorius, J., Meunier, J., & Pruitt, J. N. (2019). Collective personalities: present knowledge and new frontiers. *Behavioural Ecology and Sociobiology*, *73*, 31. https://doi.org/10.1007/s00265-019-2639-2

Wuerz, Y., & Krüger, O. (2015). Personality over ontogeny in zebra finches: long-term repeatable traits but unstable behavioural syndromes. *Frontiers in Zoology*, *12*, S9. https://doi.org/10.1186/1742-9994-12-S1-S9

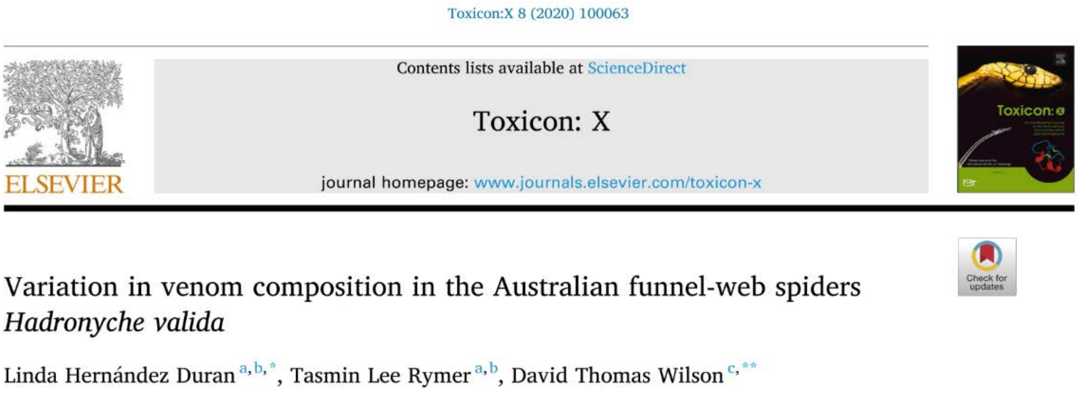
Wullschleger, B., & Nentwig, W. (2002). Influence of venom availability on a spider's prey-choice behaviour. *Functional Ecology*, *16*, 802–807. https://doi.org/10.1046/j.1365-2435.2002.00686.x

Zhang, S., Gao, B., & Zhu, S. (2017). Independent origins of scorpion toxins affecting potassium and sodium channels. In P. Gopalakrishnakone P., & A. Malhotra (Eds.), *In evolution of venomous animals and their toxins*. (pp. 167–186). Dordrecht, Netherlands: Springer. https://doi.org/10.1007/978-94-007-6458-3_12

Zhang, S., Koh, T. H., Seah, W. K., Lai, Y. H., Elgar, M. A., & Li, D. (2012). A novel property of spider silk: chemical defence against ants. *Proceedings of the Royal Society of London B*, *279,* 1824-1830. https://doi.org/10.1098/rspb.2011.2193

Zobel-Thropp, P. A., Bulger, E. A., Cordes, M. H. J., Binford, G. J., Gillespie, R. G., & Brewer, M. S. (2018). Sexually dimorphic venom proteins in long-jawed orb-weaving spiders (*Tetragnatha*) comprise novel gene families. *PeerJ*, *6*, e4691. https://doi.org/10.7717/peerj.4691

Chapter 3: Variation in venom composition in the Australian funnel-web spiders *Hadronyche valida*



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Variation in venom composition in the Australian funnel-web spiders *Hadronyche valida*

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3.1. Abstract

Mygalomorph venom properties and active components, which have importance in medicine, agronomy, venomics, ecology and evolution, have been widely studied, but only a small fraction have been characterised. Several studies have shown inter-individual variation in the composition of venom peptides based on ontogeny, sexual dimorphism, season and diet. However, intra-individual variation in venom composition, which could play a key role in the evolution, diversification and function of toxins, is poorly understood. In this study, I demonstrate significant intra- and inter-individual variation in venom composition in the Australian funnel-web spider *Hadronyche valida*, highlighting that individuals show different venom profiles over time. Fourteen (four juvenile and ten adult females) funnel-web spiders, maintained under the same environmental conditions and diet, were milked a total of four times, one month apart. I then used reversed-phase high performance liquid chromatography/electrospray ionisation mass spectrometry to generate venom fingerprints containing the retention time and molecular weights of the different toxin components in the venom. Across all individuals, I documented a combined total of 83 individual venom components. Only 20% of these components were shared between individuals. Individuals

showed variation in the composition of venom peptides, with some components consistently present over time, while others were only present at specific times. When individuals were grouped using the Jaccard clustering index and Kernel Principal Component Analysis, spiders formed two distinct clusters, most likely due to their origin or time of collection. This study contributes to the understanding of variation in venom composition at different levels (intra-individual, and intra- and inter-specific) and considers some of the mechanisms of selection that may contribute to venom diversification within arachnids. In addition, inter-specific variation in venom composition can be highly useful as a chemotaxonomic marker to identify funnel-web species.

3.2. Introduction

Spider venoms are a complex blend of peptides, proteins and small molecules (e.g. polyamines) that induce a variety of biological activities across a wide range of biological targets (Nentwig & Kuhn-Nentwig, 2013) Spider venom components commonly modulate ion channels, such as voltage-gated sodium (NaV) and calcium channels (CaV) (Gomes & Palma, 2016; Klint et al., 2012; Rash & Hodgson, 2002), affecting excitatory and inhibitory neurotransmission, neuronal and neuromuscular transduction in both vertebrates and invertebrates (Alewood et al., 2003; Langenegger et al., 2019; Nunes et al., 2008; Ushkaryov et al., 2004). For example, nucleosides block kainate receptors and L-type Ca2+ channels (Langenegger et al., 2019), while peptide toxins, such as hexatoxins from Australian funnel-web spiders, target NaV channels (Nicholson et al., 1996). Some protein toxins, acting as neurotoxins, affect Ca2+ channels and neurotransmitter release (Ushkaryov et al., 2004; Shatursky et al., 1995) or the extracellular matrix, causing necrotoxic effects in humans (Binford et al., 2009; Lopes et al., 2019). This molecular complexity and variety of potent activities across numerous targets has generated significant interest in the potential of spider venoms as an extensive source of natural, active molecules for use as therapeutic and bioinsecticide leads (Herzig et al., 2020a; Robinson et al., 2017; Saez et al., 2010; Wilson et al., 2017).

The variation in molecular complexity of spider venoms has also been studied. Venom composition of spiders varies between species, and provides sufficient consistency and resolution to be used as a chemotaxonomic marker down to the species variant level (Binford, 2001; Wilson and Alewood, 2006; Palagi et al., 2013). In addition, venom composition varies between individuals of the same species (Wilson & Alewood, 2006). However, while variation within the same individual over time has been reported to a limited degree in snakes (Ryabinin et al., 2019; Casewell et al., 2020; Tasoulis et al., 2020), scorpions (Pimenta et al., 2003), and cone snails (Jakubowski et al., 2005; Biass et al., 2009; Dutertre et al., 2010); this variation has not been reported in spiders. Identification and awareness of the level of variation in venom composition, both within and between individuals and species, is important for reproducibility in lead molecule discovery, and also medically. For example, variation in venom composition in the production of antivenoms, frequently still performed by inoculation of hosts with crude venom (e.g. funnel-web spider antivenom, https://www.seqirus.com.au/products), may impact the efficacy of these antivenoms (Isbister et al., 2014; Casewell et al., 2020).

Variation in the venom amount delivered and the composition observed within and between species (Atkinson & Wright, 1992; Arbuckle, 2017; Dutertre et al., 2014) can be affected by multiple factors. Some sources of this variation which can work synergistically and/or independently are: seasonality (variations in temperature and microhabitat conditions; Wong et al., 2016); sex (male and female lifestyles; Binford, 2001ab; Herzig, 2010; Wilson, 2016; Santana et al., 2017; Zobel-Thropp et al., 2018; Herzig et al., 2020b); type and size of prey (Kuhn-Nentwig et al., 2004; Barlow et al., 2009; Morgenstern & King, 2013; Nelsen et al., 2014); and age (selection pressures affect the availability of prey over development; Herzig, 2010; Cooper et al., 2015; Arbuckle, 2017). Depending on the stimulus spiders are experiencing, behaviour can trigger changes in spider responses, and the way they use venom (e.g. defense or predation; Schendel et al., 2019). Other sources of variation in venom components are related to geographic origin (Gomes & Palma, 2016), which can promote the divergence of species and communities, leading to changes at the genetic level (Escoubas et al., 2002). For example, different families of toxins in some scorpions differ based on their geographical locality; toxins belonging to the αNaScTx family that act on the NaV receptor site 3, are a characteristic of Asian and Mediterranean scorpions belonging to the Buthidae family (Morgenstern, 2013).

The diversity and complexity of venoms can also vary depending on function, either for defense or predation (Casewell et al., 2013; Schendel et al., 2019). Predatory venoms are generally more complex and variable in composition, showing toxicity across a broad range of biological targets (Casewell et al., 2013; Arbuckle, 2017; Dutertre et al., 2014; Schendel et al., 2019). In contrast, defensive venoms are generally relatively simple in composition in some animals, such as bees and fish (Casewell et al., 2013), although in cone snails, defensive venoms can be more complex than predatory venoms (Dutertre et al., 2014). Divergence in predatory venom is linked to the ecological role of the venom, which may be driven by dietary differences and/or prey specialisation (Boevé et al., 1995; Wigger et al., 2002; Schendel et al., 2019). In general, variation in the complexity of defensive and predatory venoms, the modes of action, and biological targets, is present across all taxonomic levels, and can also occur in closely related species (Abdel-Rahman et al., 2009; Touchard et al., 2015; Zancolli et al., 2019).

Historically, Australian funnel-web spiders have been of research interest primarily because their venom components are responsible for an envenomation syndrome in humans that can lead to death, but also due to the extraordinary breadth and the potential of the toxin libraries they possess for commercial bioactive lead discovery (Gray & Sutherland, 1978; Nicholson & Graudins, 2002; Nicholson et al., 2004; Tedford et al., 2004; Chassagnon et al., 2017; Ikonomopoulou et al., 2018; Pineda et al., 2020). Funnel-web spiders show high complexity in venom composition (Palagi et al., 2013; Pineda et al., 2020), which is likely related to prey availability in different microhabitats, trophic adaptations, predator deterrance (Beavis et al., 2011; Pekár et al., 2018; Herzig et al., 2020b), genetics, molecular diversity (Pineda et al., 2020a), ecological factors, and behaviour (Cooper et al., 2015).

While intra- and inter-specific variation in spider venoms has been previously reported (Escoubas et al., 1997; Palagi et al., 2013), the level of individual variation over time, and the conditions and factors that affect individual variation in venom properties, has not been established. Therefore, in this study, I analysed venom fingerprints of female funnel-web spiders *H. valida* to assess the inter- and intra-individual differences in venom components of individual spiders over time. I then compared the venom components between *H. valida* and the closely related species *H. infensa*, which contributes to a broader understanding of the evolution of venom components, and the extent of the potential for the identification and characterisation of possible bioactive leads. Both species belong to a species complex group, the infensa group (Gray, 2010), where some of the species share similar morphological traits (Gray, 2010) and overlapping distributions, but differ in microhabitat, behaviour (Hernandez, unpub. obs.), and toxin composition (Palagi et al., 2013).

3.3. Materials and Methods

3.3.1. Spider collection and husbandry

Fourteen *H. valida* (four juveniles and ten adult females; collected by manual excavation of burrows in the Currumbin Valley and Mount Tamborine) were purchased from Thargomindah Man Productions in 2019 (Varsity Lakes, QLD, Australia). Eighteen *H. infensa* (nine adult females and nine juveniles), were also collected manually in Toowoomba at Blue Meadow court and Ravensbourne (-27.5028782°S, 151.953638°E; -27.3665311°S, 151.1792198°E) in 2019. Sex differences in venom composition are known for these species (Wilson & Alewood, 2004), and I attempted to remove this variation by focusing on adult females. The spiders were transported alive in small plastic containers with damp cotton wool to the laboratory of the Australian Institute of Tropical Health and Medicine (AITHM), James Cook University Cairns campus, Queensland, Australia. Each spider was housed individually in a 3 L plastic container containing coconut coir peat as a substrate. The spiders were housed in a climate-controlled room (temperature: 20 ± 2 °C; relative humidity: 60%) on a reverse light:dark cycle (12L:12D; lights on at 6 pm). Each spider received one house cricket (*Acheta domestica*) once a week.

Adult females were identified by epigyne sclerotisation and the opening in the epigastric furrow (gonoslit, Zhan et al., 2019), which is very apparent in adult females, but is absent in juveniles (F. Perez-Miles, pers. comm.). Cephalothorax width was measured to assess spider size (Supplementary material Table S3.1) after each repetition (see below). To obtain size, I photographed the dorsal aspect of each spider under Leica stereomicroscope, and processed the images using Image J 1.8.0 Software.

The research was conducted within the framework of the Australian Code for the Care and Use of Animals for Scientific Purposes (NHMRC, 2013). Funnel web spiders are not a protected species in Australia. Consequently, the Department of Environment and Science of the Queensland Government advised that a scientific permit for collection and holding was not required.

3.3.2. Venom collection and analysis

The spiders were milked one week after they arrived at the laboratory to obtain a baseline venom profile. Venom expelled on the tips of the fangs of aggravated individuals was collected using a 200 µL Gilson P200 pipette with polypropylene micropitpette tips. To aggravate the spiders, I touched the first pair of legs using tweezers until the venom was expressed on the fang tips (Wilson & Alewood, 2006). The process was repeated at short intervals for 10 minutes. The venom was then placed in a 1.5 mL microcentrifuge tube with 40 µL of Milli-Q water and stored at -20 °C. Thereafter, I milked the spiders three more times, one month apart, for a total of four venom samples per individual. Venom samples from each milking were kept separate. In each case, the spiders were not fed two weeks prior to venom extraction to minimise venom depletion and to reduce the potential effects of feeding on venom composition (Wigger et al., 2002).

Liquid chromatography/electrospray ionisation mass spectrometry (LC/ESI-MS) analysis of the venom peptides of each individual from each species was performed to generate venom fingerprint profiles and observe differences in venom composition at the intra- and inter-individual levels, as well as the inter-specific level. To detect variation in venom composition, and to obtain venom profiles, the samples were injected via an autosampler (Shimadzu SIL-20AC HT) onto a reversed-phase high-performance liquid chromatography (RP-HPLC) column (Phenomenex Aeris 150 x 2.1mm 3.6µm PEPTIDE XB-C18 100Å) at 30 °C. Solvent (buffer A: 0.1% formic acid/water; buffer B: 90% acetonitrile/0.09% formic acid/water) was delivered via Shimadzu LC-20AD pumps at a flow rate of 0.250 mL/min. 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 250-2000 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 analyzed using the Shimadzu LabSolutions v 5.96 software.

3.3.3. Statistical analyses

Statistical analyses were conducted using RStudio (version 1.0.153; https://www.rproject.org; R version 3.5.0, https://cran.rstudio.com). To compare the number of venom peptides within and between individuals of *H. valida* (Supplementary material Table S3.2), I used the package UpSet plot (Lex et al., 2014). The package allowed me to quantify the number of sets (i.e. individuals) and intersections that are shared between elements (i.e. venom component masses shared between individuals).

To determine intra-individual variation in venom composition in *H. valida*, I plotted the venom fingerprint profile of each spider and its replicates (Supplementary material Table S3.3, Figure S3.1). Juveniles (A2, A4, A6, A10) and adults were separated (M, J, S, A1t, A5t, A7t, A8t, A9t, A11t, A12t) to quantify the number of venom components shared between each group. To obtain an UpSet plot with all the individuals, I binned the venom components found for each individual over all replicates. Some of the venom peptides were identified based on retention times from previous studies and mass and sequence information available in the Arachnoserver database (http://www.arachnoserver.org/). The other components currently remain unidentified.

A Jaccard matrix was constructed from the venom components obtained from each peak in the chromatograms of each individual. To measure the similarity of venom components within, and between, individuals of *H. valida*, I used the Jaccard similarity coefficient and the average linkage method to measure the distance between clusters (venom component masses of each individual). Dunn’s index was used to determine the suitable average method to calculate the clusters. The correlation coefficient cophenetic distances were used to assess the best possible dendogram generated. Using the Jaccard matrix I carried out a Kernel Principal Component Analysis (KPCA) using the package mixKernel (Mariette & Villa-Vialaneix, 2017) to visualise in a better manner the relationships between how individuals were grouping, and the distances between them. The ten most important venom components that explained the majority of the variance of the first principal component of KPCA are shown in Supplementary Figures S3.2a and S3.2b. I used the package factoextra to build a hierarchical cluster for all indiviudals (Altman & Krzywinski, 2017).

3.4. Results

3.4.1. LC/ESI-MS venom analysis

Venom fingerprints for each individual were generated from the LC/ESI-MS chromatograms of H. valida venom, which provided retention time and mass data of the venom components present (Figure 3.1). The venom components found ranged in mass from 295.030 Da to 8420.294 Da, with a predominant bimodal mass distribution in the ranges 3863.399 - 4854.311 Da and 6733.890 - 8420.294 Da.

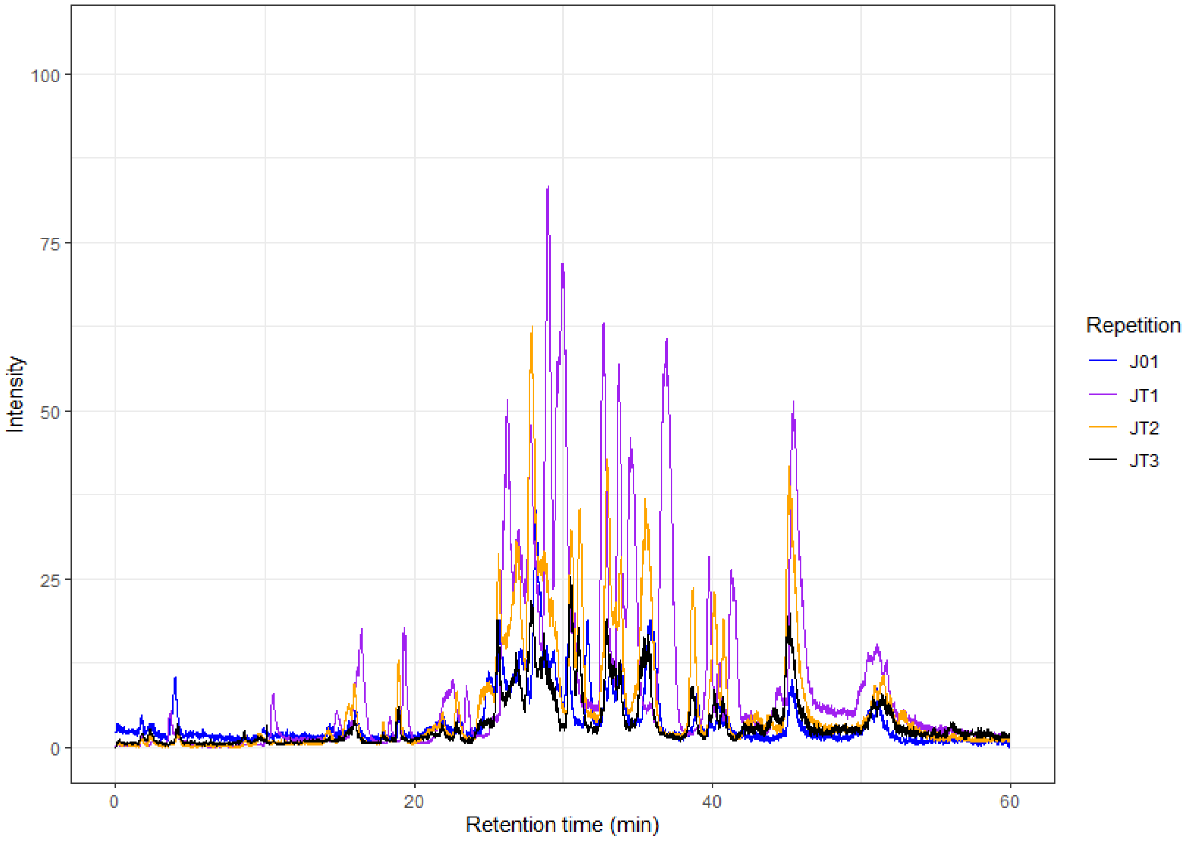


Figure 3.1. Total ion chromatograms (TICs) of LC/ESI-MS analysis of venom sample repetitions over time from a female *H. valida* specimen (individual J). The chro- matograms were visualised using the ‘ggplot2’ package (Wickham, 2009) in R version 4.0.1, using the normalised intensities and retention time from venom components obtained from the individual.

3.4.2. Intra-individual variation

All individuals showed variation in venom composition over time (i.e. individual variation between replicates; Supplementary material Figure S3.1). In addition, the total number of venom components shared between each replicate varied depending on the individual (Figure 3.2). Some components only appeared in a specific replicate and were not shared between replicates (Figure 3.2; Supplementary materials Figure S3.1). For example, in individual A1, the number of components shared between the baseline (A1) sample and replicates was 18 out of 42 (Figure 3.2). Moreover, in the same individual, the baseline and replicates one (A1T1) and two (A1T2) shared only eight venom components overall, and seven venom components were exclusively present in the baseline sample.

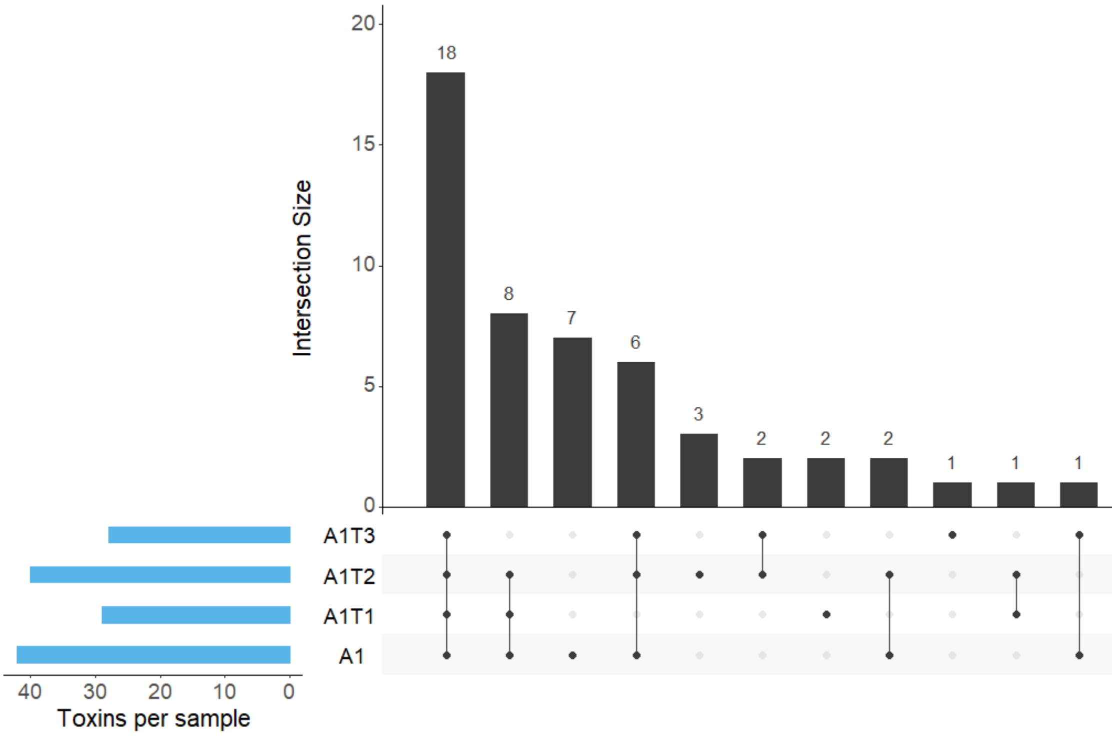


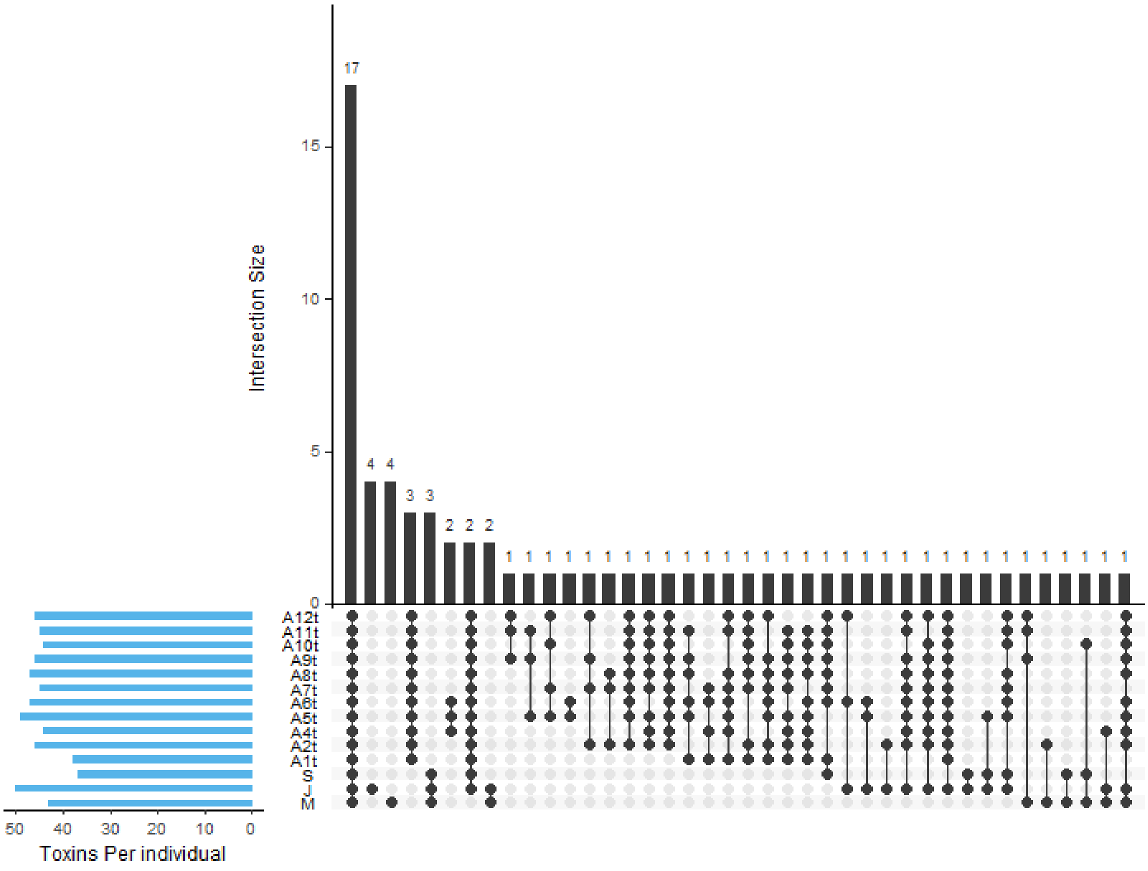
Figure 3.2. Intra-individual variation in venom composition of a juvenile female *H. valida* (spider A1) over time. The Upsetplot shows the total number of uniquely individual venom components present in all replicates (bottom left: set size). A1 represents the baseline venom sample taken one week after the spider was collected. The order of the replicates is shown by the letter T (A1T1, A1T2, A1T3). The black dots show the venom components (intersections) shared between replicates (e.g. 18 peptides are present in all replicates).

3.4.3. Inter-individual variation

The total venom composition found in *H. valida* (when all four venom samples from each individual were considered) showed that, across all the spiders tested, a total of 83 discrete venom components were present, with up to 50 venom component masses evident in some individuals (see M, Figure 3.3a) and only 37 in other individuals (Figure 3.3a). All spiders shared 17 venom components; however, some of the individuals also showed specific components. For example, the individuals M and J each showed four components that were unique to each of these individuals (Figure 3.3a; Supplementary material Figure S3.1).

Comparing the venom fingerprints between just the juvenile specimens revealed 31 shared venom components (Figure 3.3b), while a comparison between just the adult specimens showed only 18 shared venom components (Figure 3.3c). A total of eighteen venom components were unique to the adult specimens. The Jaccard analysis of similarity (Supplementary materials Table S3.4) showed that three individuals (Cluster B, including individuals M, J and S) formed a separate cluster to the remaining individuals (Cluster A; Figure 3.4). Interestingly, the component of mass 4079.420 Da was unique to individuals belonging to cluster A and was absent in individuals belonging to cluster B. Juveniles did not form a separate cluster to adults (Figure 3.4a). In addition, the KPCA showed similar results to the ones returned by the hierarchical cluster, where Cluster B was completely separate from Cluster A (Figure 3.4b). In addition, I found a sub-cluster grouping three individuals (A4, A6 and A5) inside cluster A, which are more separated from the rest of the cluster (Figure 3.4b).

a.


Intersection Size
0
5
10
15
17
4
4
3
3
2
2
2
1
1
1
1
1
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1
1
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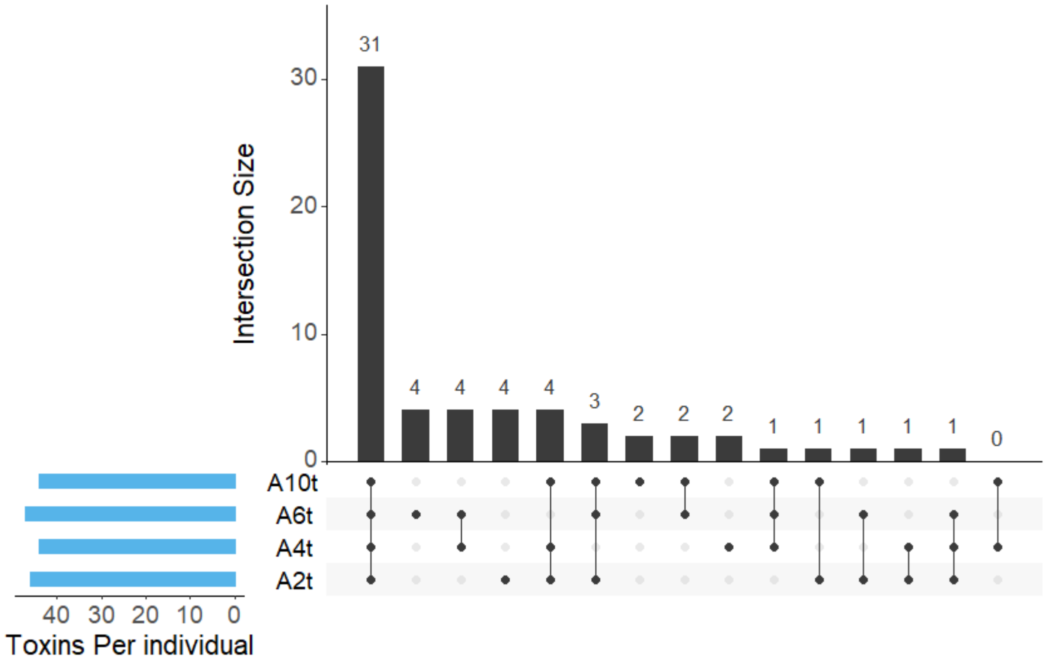
A12t
A11t
A10t
A9t
A8t
A7t
A6t
A5t
A4t
A2t
A1t
S
J
M
50
40
30
20
10
0
Toxins Per individual

b.


Intersection Size
0
10
20
30
31
4
4
4
4
3
2
2
2
1
1
1
1
1
0
A10t
A6t
A4t
A2t
40
30
20
10
0
Toxins Per individual

c.

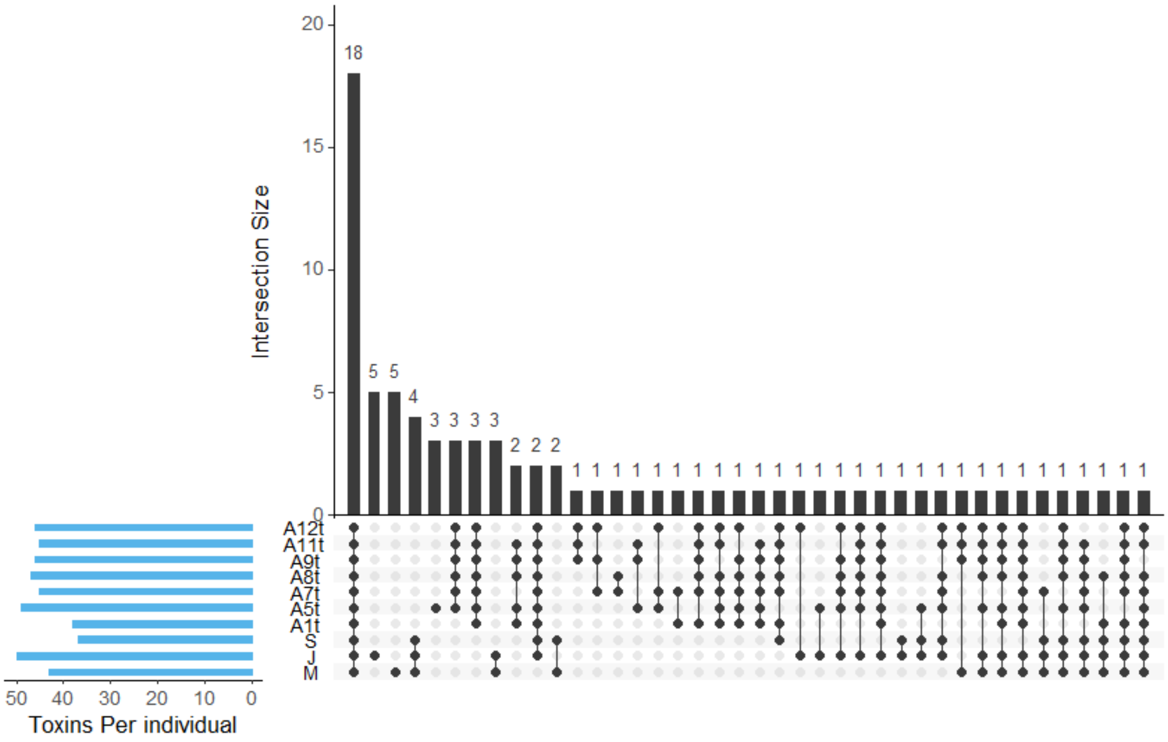


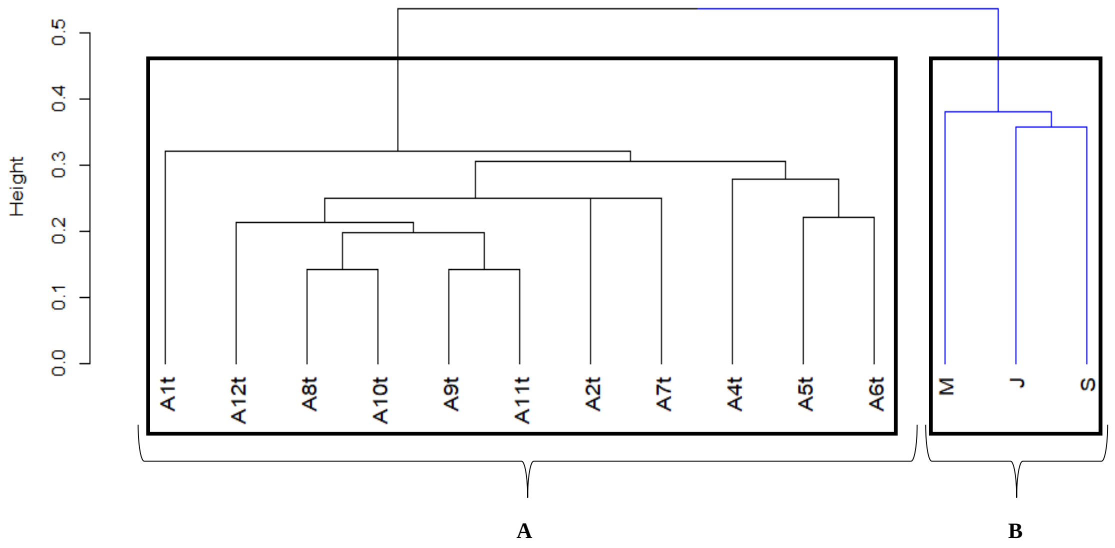
Figure 3. 3. (a) Upsetplot showing the number of toxins shared by all individual *H. valida*. The bars representing the intersection size show the number of venom components shared by the individuals highlighted by black dots in the matrix panel below. The bars in the panel to the left of the dot matrix panel show the total number of venom components per individual. (b) Upsetplots of juvenile and (c) adult specimen venom components.

3.4.4.Variation in venom components between H. valida and H. infensa

Analysis of the venom fingerprints between specimens of the closely related *H. valida* and *H. infensa* species revealed that 26 venom components were shared between the species (Table 3.1). However, *H. valida* showed numerous species-specific compounds (50 venom components) that were not found in *H. infensa* and can be used as markers to characterise the species (e.g. 7069.219 Da at 15 min and 4175.818 Da at 36 min; Table 3.1). Similarly, *H. infensa* showed species-specific compounds (48 venom components) and specific markers characteristic of the species, such as 4795.449 Da (retention time 29.567 to 30.220 min) and

7120.841 Da (retention time 34.740 to 35.207 min). Similar to *H. valida*, the bimodal distribution in *H. infensa* showed that the majority of masses were found in the range of 3354.319 - 5216 Da, and a smaller group of masses in the range of 6764.1457–8420.2943 Da.

a.



b.

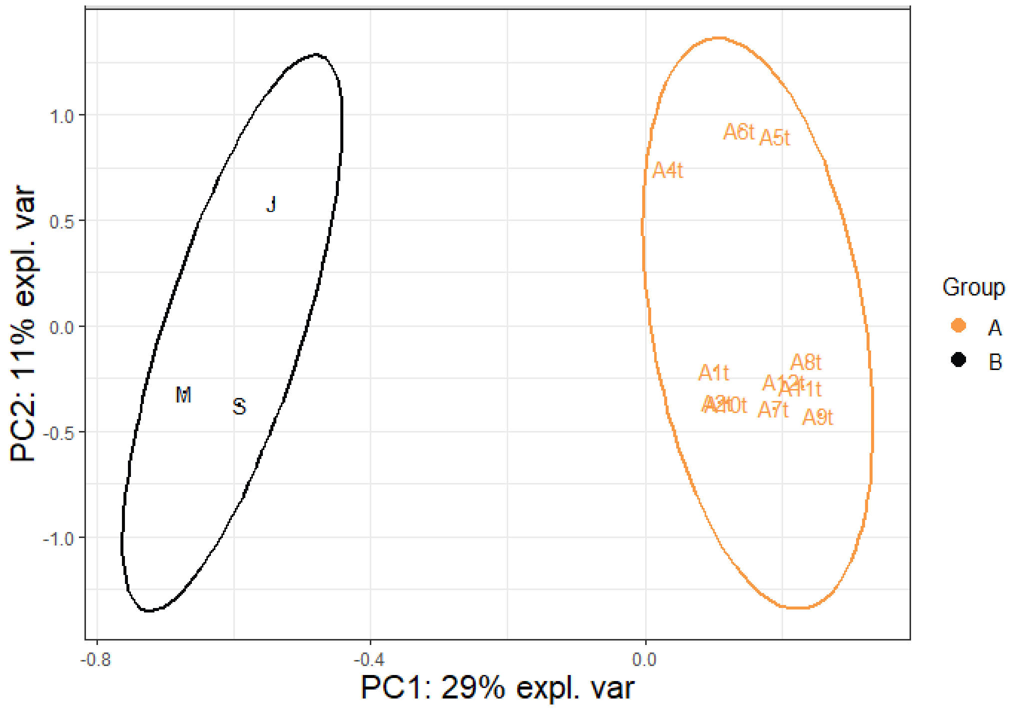


Figure 3. 4. (a) Hierarchical cluster of *H. valida* individuals based on the presence/absence of venom component masses (Da) using the Jaccard matrix and the average linkage method. Cluster A is clearly delineated from the Cluster B. (b) Projection of individuals of *H. valida* on the first two KPCA axes.

Table 3. 1. List of venom components of *H. valida* and the respective retention times, mass, sharing of toxins within individuals and with *H. infensa*, and the name of the component (if known). Masses indicated in red are the main peaks observed in the chromatogram.

Retention time (min)	***H. valida* (Mass Da)**	**Toxins shared between individuals (n=17)**	***H. infensa***	**Toxins**
2.273	294.0422	X	X	
3.713	437.8880	X	X	
3.907	346.9548	X	X	
3.907	307.4829	X	X	
5.267->5.600	362.9748			
8.613	266.9519	X	X	
8.613	277.9618			
13.713->14.633	3918.4483		X	
13.953->14.860	3863.3993			
14.773->15.127	4050.0898		X	
15.213->16.220	3992.5309		X	
15.367->16.068	4079.4200			
15.647->16.000	3921.1337	X	X	ω-hexatoxin-Hi1a (*H. infensa*)
16.027->16.173	4035.5376			
17.840->18.247	3950.1693			
18.967->19.280	4047.9459		X	ω-hexatoxin-Hi1b (*H. infensa*)
18.900->19.220	8095.2821		X	
20.400->22.133	4221.9554			
21.607->22.367	6968.5368			
21.920->22.132	7069.1648	X		
22.473->22.687	4249.3587			
22.753->23.153	6733.8906			
22.753->23.153	6764.1457	X	X	
24.327->25.033	4728.3849		X	
24.480->25.133	7066.5963			
24.840->25.387	4009.7675			

3.5. Discussion

3.5.1. Intra-individual variation

Through analysis of venom fingerprint profiles, I found considerable variation in venom components within *H. valida* individuals over time. Each individual spider showed the presence of unique components over time, some of which were present only once in one sample. Prey type and abiotic factors can affect variation in venom composition (Barlow et al., 2009; Casewell et al., 2013; Schendel et al., 2019). However, the individual *H. valida* specimens in this study still showed variation despite experiencing the same diet (house crickets) and housing under the same environmental conditions. To understand intra-individual variation, it is necessary to consider if multiple components in the venom are playing a particular role or have several functions in the individual (Casewell et al., 2013; Schendel et al., 2019). However, it is also necessary to consider the drivers of venom variation that cannot only be explained based on local diets (Schendel et al., 2019; Zancolli et al., 2019). For example, in the rattlesnake *Crotalus scutulatus*, neither diet nor genetic population structure explained intra-individual variation in venom composition, whereas both temperature and habitat conditions were the main drivers of variation in venom composition in this species (Zancolli et al., 2019).

Although the aggravation process used to milk spiders was identical for all individuals, changes in the behavioural responses associated with the aggravation process could trigger differences in the way that spiders respond to the threat stimulus, which in turn could lead to changes in venom composition (Nelsen et al., 2014). Variation in venom composition could be related to ecological function (Schendel et al., 2019). For example, Morgenstern (2013) reported unique peptide masses in different secretion series in *H. infensa,* suggesting that spiders can qualitatively and quantitatively modulate venom secretions for each stimulus they receive. In our study, spiders were most likely using defensive venom, which can have a higher complexity of components and greater variation in composition than offensive venom (Escoubas et al., 2006; Casewell et al., 2013; Schendel et al., 2019). In addition, the spiders could be showing a plastic response by varying venom properties after being exposed to a threatening stimulus (Nelsen et al., 2014), as occurs in the orb-web spider *Tetragnatha versicolor* (Zobel-Thropp et al., 2018) and Australian rainforest scorpion *Hormurus waigiensis* (previously *Liocheles waigiensis*) (Gangur et al., 2017). More studies testing intra-individual variation in venom composition including different factors (environment, predator/stimuli over time) are necessary to understand the underlying factors that lead to variation in venom composition.

3.5.2. Inter-individual variation

Juvenile and adult *H. valida* individuals had different venom compositions, and only shared a small number of venom components. This variation may be the result of the intra-individual variation observed but could also be complemented by other factors. For example, the variation in venom composition between developmental stages has been widely documented in spiders (Santana et al., 2017), scorpions (Fox, 2018), gastropods (Conoidea; Puillandre et al., 2017), and snakes (Andrade & Abe, 1999). Ontogenetic shifts in spider venoms can occur throughout a spider’s development (e.g. tarantula venoms; Guette et al., 2006; Santana et al., 2017). Juveniles could have different predatory/prey interactions affected by different selection pressures that lead to variations in venom composition (Gibbs et al., 2011; Santana et al., 2017). The number of venom components in common between juveniles of *H. valida* were higher than in adults. However, juveniles did not form a specific cluster in the Jaccard similarity coefficient/matrix and KPCA. This lack of clustering may be a consequence of small sample size. However, it is also possible that venom could be continually changing in young individuals as they mature to adulthood, which would indicate that age is a factor affecting variation in venom composition, such as that observed in tarantulas of *Brachypelma* species (Escoubas et al., 2002) and *Phlogius crassipes* (Elias et al., 2006).

Although the juvenile and adults showed individual variation in venom components, most of the individuals were clustered together in the Jaccard similarity coefficient/matrix and KPCA, possibly due to geographic origin (Chippaux et al., 1991; Núñez et al., 2009; Touchard et al., 2015) or time of collection. Geographic origin could be a source of increased venom composition variation due to specific microhabitat differences and genetic diversity leading to intra-specific venom plasticity, as has been seen in the scorpion *Scorpio maurus palmatus* (Escoubas et al., 1997; Touchard et al., 2015). Unfortunately, as the *H. valida* spiders used here were collected by a commercial collector, we do not have specific locality information. The season of collection could also promote differences in venom components between individuals belonging to cluster A compared to the individuals belonging to cluster B, particularly as individuals from cluster A were collected in April while individuals from cluster B were collected in June. Differences in venom properties have been found in funnel-web spiders collected during different seasons. For example, in the funnel-web spider *Atrax sutherlandi*, specimens collected during winter showed a higher venom yield than those collected in autumn, although venom composition was not investigated (Keegan et al., 1960; Wong et al., 2016). However, venom yield and venom composition are not mutually exclusive and changes in both are possible (Morgenstern & King, 2013; Schendel et al., 2019).

3.5.3. Inter-specific variation in venom peptides

The complexity of venom in closely related species of funnel-webs such as *H. valida* and *H. infensa* has revealed a high degree of heterogeneity in venom components between species. Although similarities in the presence of different venom peptides exist between the two species

(Table 1), both *H. valida* and *H. infensa* venom profiles can be easily distinguished by the variation of specific components.

Venoms from species belonging to the *infensa* species group, such as *H. valida* and *H. infensa*, may have venom components in common because of genetic and/or ecological factors (Palagi et al., 2013). However, each species also has unique venom components that vary in both composition and abundance, which could be related to venom adaptations specific to habitat and/or ecological function (Palagi et al., 2013; Cooper et al., 2015; Schendel et al., 2019). For example, Wilson and Alewood (2006) previously reported differences in venom components between similar species of the *infensa* species group collected from different geographical locations in South East Queensland. However, each species may have unique venom components simply due to genetic divergence over time. Without knowledge of the properties and function of each venom component, it is difficult to ascertain whether there is active selection driving differences between the species, or whether the differences are simply due to genetic drift. Nonetheless, my findings highlight the importance of venom fingerprint profiling for identification, which can be a useful tool for identifying and classifying closely related species. Similar findings have been observed in different species of *Brachypelma*, where common venom components are shared between closely related species, but each species retains venom components specific to that species (Escoubas et al., 1997).

While I observed substantial intra-individual, inter-individual and inter-specific variation in *H. valida* funnel-web venom composition, there is still sufficient consistency in the venom components present to identify specific character markers to use venom fingerprint profiles as chemotaxonomic tools. This level of variation, from the intra-individual to inter-specific levels, may also have medical implications in the production of antivenoms and efficacy in the treatment of envenomations. For example, the controversy surrounding the efficacy and use of antivenom to treat latrodectism caused by envenomation by widow spiders *Latrodectus* sp.

(Isbister et al., 2014) may be a result of intra-individual or inter-specific variation in venom composition in the specimens sourced for antivenom production. More studies testing intra-individual variation in venom composition including different factors (environment, predator/stimuli over time) are necessary for understanding the underlying factors that could lead to variation in venom composition.

3.6. Conclusions

Different factors can trigger both intra- and inter-individual variation in venom composition in spiders, such as geographic origin, genetics, predator-prey interactions, behaviour and age. My findings suggest that intra-individual variation in venom composition is likely a result of the way individuals respond to a particular stimulus over time, but more experiments including different predators and stimuli are necessary. Understanding inter- and intra-individual variation in venom composition in one species contributes to a broader understanding of the evolution and adaptation of venom in general. Venom fingerprint profiles can be used as chemotaxonomic markers to identify species, and possibly particular geographical populations, allowing the discrimination of species complexes such as in the genus *Hadronyche*.

3.7. References

Abdel-Rahman, M. A., Omran, M. A. A., Abdel-Nabi, I. M., Ueda, H., & McVean, A. (2009). Intraspecific variation in the Egyptian scorpion *Scorpio maurus palmatus* venom collected from different biotopes. *Toxicon*, *53*(3), 349–359. https://doi.org/10.1016/j.toxicon.2008.12.007

Alewood, D., Birinyi-Strachan, L. C., Pallaghy, P. K., Norton, R. S., Nicholson, G. M., & Alewood, P. F. (2003). Synthesis and characterization of δ-Atracotoxin-Ar1a, the lethal neurotoxin from venom of the Sydney funnel-web spider (*Atrax robustus*). *Biochemistry, 42*(44), 12933–12940. https://doi.org/10.1021/bi030091n

Altman, N., & Krzywinski, M. (2017). Points of Significance: Clustering. *Nature Methods, 14*(6), 545–546. https://doi.org/10.1038/nmeth.4299

Andrade, D. V., & Abe, A. S. (1999). Relationship of venom ontogeny and diet in *Bothrops*. *Herpetologica, 55*(2), 200–204. https://www.jstor.org/stable/3893080?seq=1#metadata_info_tab_contents

Australian code for the care and use of animals for scientific purposes 8th edition, 2013. https://www.nhmrc.gov.au/about-us/publications/australian-code-care-and- use-animals-scientific-purposes/. (Accessed 21 August 2020).

Arbuckle, K., 2017. Evolutionary context of venom in animals. In: Gopalakrishnakone, P., Malhotra, A. (Eds.), Evolution of Venomous Animals and Their Toxins. Springer Netherlands, Dordrecht, pp. 3–31. https://doi.org/10.1007/ 978-94-007-6458-3_16.

Atkinson, R. K., & Wright, L. G. (1992). The modes of action of spider toxins on insects and mammals. *Comparative Biochemistry and Physiology. Part C, Comparative, 102*(3), 339–342. https://doi.org/10.1016/0742-8413(92)90124-P

Barlow, A., Pook, C. E., Harrison, R. A., & 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*(1666), 2443–2449. https://doi.org/10.1098/rspb.2009.0048

Beavis, A. S., Sunnucks, P., & Rowell, D. M. (2011). Microhabitat preferences drive phylogeographic disparities in two Australian funnel web spiders. *Biological Journal of the Linnean Society*, *104*(4), 805–819. https://doi.org/10.1111/j.1095-8312.2011.01753.x

Binford, G. J. (2001)a. An analysis of geographic and intersexual chemical variation in venoms of the spider *Tegenaria agrestis* (Agelenidae). Toxicon, 39(7), 955–968. https://doi.org/10.1016/S0041-0101(00)00234-8

Binford, G. J. (2001)b. Differences in venom composition between orb-weaving and wandering Hawaiian *Tetragnatha* (Araneae). *Biological Journal of the Linnean Society*, *74*(4), 581–595. https://doi.org/10.1006/bijl.2001.0592

Boevé, J. L., Kuhn-Nentwig, L., Keller, S., & Nentwig, W. (1995). Quantity and quality of venom released by a spider (*Cupiennius salei*, Ctenidae). Toxicon, 33(10), 1347–1357. https://doi.org/10.1016/0041-0101(95)00066-U

Binford, G. J., Bodner, M. R., Cordes, M. H. J., Baldwin, K. L., Rynerson, M. R., Burns, S. N., & Zobel-Thropp, P. A. (2009). Molecular evolution, functional variation, and proposed nomenclature of the gene family that includes sphingomyelinase D in sicariid spider venoms. *Molecular Biology and Evolution*, *26*(3), 547–566. https://doi.org/10.1093/molbev/msn274

Casewell, N. R., Jackson, T. N. W., Laustsen, A. H., & Sunagar, K. (2020). Causes and Consequences of Snake Venom Variation. *Trends in Pharmacological Sciences*, *41*(8), 570–581. https://doi.org/10.1016/j.tips.2020.05.006

Casewell, N. R., Wüster, W., Vonk, F. J., Harrison, R. A., & Fry, B. G. (2013). Complex cocktails: The evolutionary novelty of venoms. *Trends in Ecology and Evolution*, *28*(4), 219–229. https://doi.org/10.1016/j.tree.2012.10.020

Chassagnon, I. R., McCarthy, C. A., Chin, Y. K. Y., Pineda, S. S., Keramidas, A., Mobli, M., Pham, V., De Silva, T. M., Lynch, J. W., Widdop, R. E., Rash, L. D., & King, G. F. (2017). Potent neuroprotection after stroke afforded by a double-knot spider-venom peptide that inhibits acid-sensing ion channel 1a. *Proceedings of the National Academy of Sciences of* *the United States of America*, 114(14), 3750–3755. https://doi.org/10.1073/pnas.1614728114

Casewell, N. R., Jackson, T. N. W., Laustsen, A. H., & Sunagar, K. (2020). Causes and Consequences of snake venom variation. *Trends in Pharmacological Sciences*, 41(8), 570–581. https://doi.org/10.1016/j.tips.2020.05.006

Chippaux, J. P., Williams, V., & White, J. (1991). Snake venom variability: methods of study, results and interpretation. *Toxicon*, *29*(11), 1279–1303. https://doi.org/10.1016/0041-0101(91)90116-9

Cooper, A. M., Nelsen, D. R., & Hayes, W. K. (2015). The strategic use of venom by spiders. In A. Gopalakrishnakone, P.; Malhotra (Ed.), *Evolution of Venomous Animals and Their Toxins* (pp. 145–163). Springer Netherlands. https://doi.org/DOI 10.1007/978-94-007-6458-3_13

Dutertre, S., Biass, D., Stöcklin, R., & Favreau, P. (2010). Dramatic intraspecimen variations within the injected venom of *Conus consors*: An unsuspected contribution to venom diversity. *Toxicon*, *55*(8), 1453–1462. https://doi.org/10.1016/j.toxicon.2010.02.025

Dutertre, S., Jin, A. H., Vetter, I., Hamilton, B., Sunagar, K., Lavergne, V., Dutertre, V., Fry, B. G., Antunes, A., Venter, D. J., Alewood, P. F., & Lewis, R. J. (2014). Evolution of separate predation-and defence-evoked venoms in carnivorous cone snails. *Nature Communications*, *5*(1), 1–9. https://doi.org/10.1038/ncomms4521

Elias, D. O., Hebets, E. A., & Hoy, R. R. (2006). Female preference for complex/novel signals in a spider. *Behavioural Ecology, 17*(5), 765–771. https://doi.org/10.1093/beheco/arl005

Escoubas, P., Célérier, M. L., & Nakajima, T. (1997). High-performance liquid chromatography matrix-assisted laser desorption/Ionization time-of-flight mass spectrometry peptide fingerprinting of tarantula venoms in the genus *Brachypelma*: Chemotaxonomic and biochemical applications. *Rapid Communications in Mass Spectrometry*, *11*(17), 1891–1899. https://doi.org/10.1002/(SICI)1097-0231(199711)11:17<1891::AID-RCM94>3.0.CO;2-X

Escoubas, P., Corzo, G., Whiteley, B. J., Célérier, M. L., & 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*(5), 403–413. https://doi.org/10.1002/rcm.595

Escoubas, P., Sollod, B., & King, G. F. (2006). Venom landscapes: mining the complexity of spider venoms via a combined cDNA and mass spectrometric approach. Toxicon, 47(6), 650–663. https://doi.org/10.1016/j.toxicon.2006.01.018

Fox, G. A. (2018). *The Design of Complex Weapons Systems in Scorpions: Sexual, Ontogenetic, and Interspecific Variation* [Loma Linda University Electronic Theses, Dissertations & Projects.]. http://scholarsrepository.llu.edu/etdhttp://scholarsrepository.llu.edu/etd/516

Gangur, A. N., Smout, M., Liddell, M. J., Seymour, J. E., Wilson, D., & Northfield, T. D. (2017). Changes in predator exposure, but not in diet, induce phenotypic plasticity in scorpion venom. *Proceedings of the Royal Society B: Biological Sciences*, *284*(1863), 20171364. https://doi.org/10.1098/rspb.2017.1364

Gibbs, H. L., Sanz, L., Chiucchi, J. E., Farrell, T. M., & 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*(10), 2169–2179. https://doi.org/10.1016/j.jprot.2011.06.013

Gomes, P. C., & Palma, M. S. (2016). The Nonpeptide Low Molecular Mass Toxins from

Spider Venoms. In P. Gopalakrishnakone, G. A. Corzo, M. E. de Lima, & E. Diego-García (Eds.), *Spider Venoms* (pp. 3–19). Springer Netherlands. https://doi.org/10.1007/978-94-007-6389-0_14

Gray, M. (2010). A revision of the Australian funnel-web spiders (Hexathelidae: Atracinae). *Records of the Australian Museum*, *62*(2–3), 285–392. https://doi.org/10.3853/j.0067-1975.62.2010.1556

Gray, M. R., & Sutherland, S. K. (1978). Venoms of Dipluridae. In S. Bettinni (Ed.), *Arthropod Venoms* (pp. 121–148). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-45501-8_7

Guette, C., Legros, C., Tournois, G., Goyffon, M., & Célérier, M. L. (2006). Peptide profiling by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry of the *Lasiodora parahybana* tarantula venom gland. *Toxicon*, *47*(6), 640–649. https://doi.org/10.1016/j.toxicon.2006.01.017

Herzig, V. (2010). Ontogenesis, gender, and molting influence the venom yield in the spider *Coremiocnemis tropix* (Araneae, Theraphosidae). *Journal of Venom Research*, *1*, 76–83. http://www.ncbi.nlm.nih.gov/pubmed/21544186

Herzig, V., Cristofori-Armstrong, B., Israel, M. R., Nixon, S. A., Vetter, I., & King, G. F. (2020)a. Animal toxins — Nature’s evolutionary-refined toolkit for basic research and drug discovery. *Biochemical Pharmacology*, *181*(114096), 1–14. https://doi.org/10.1016/j.bcp.2020.114096

Herzig, V., Sunagar, K., Wilson, D. T. R., Pineda, S. S., Israel, M. R., Dutertre, S., McFarland, B. S., Undheim, E. A. B., Hodgson, W. C., Alewood, P. F., Lewis, R. J., Bosmans, F., Vetter, I., King, G. F., & Fry, B. G. (2020)b. Australian funnel-web spiders evolved human-lethal δ-hexatoxins for defense against vertebrate predators. *Proceedings of the*

National Academy of Sciences of the United States of America, *117*(40), 24920–24928. https://doi.org/10.1073/pnas.2004516117

Ikonomopoulou, M. P., Fernandez-Rojo, M. A., Pineda, S. S., Cabezas-Sainz, P., Winnen, B., Morales, R. A. V., Brust, A., Sánchez, L., Alewood, P. F., Ramm, G. A., Miles, J. J., & King, G. F. (2018). Gomesin inhibits melanoma growth by manipulating key signaling cascades that control cell death and proliferation. *Scientific Reports*, *8*(11519), 1–14. https://doi.org/10.1038/s41598-018-29826-4

Isbister, G. K., Page, C. B., Buckley, N. A., Fatovich, D. M., Pascu, O., MacDonald, S. P. J., Calver, L. A., & Brown, S. G. A. (2014). Randomized controlled trial of intravenous antivenom versus placebo for latrodectism: The second redback antivenom evaluation (RAVE-II) study. *Annals of Emergency Medicine*, *64*(6), 620–628. https://doi.org/10.1016/j.annemergmed.2014.06.006

Jakubowski, J. A., Kelley, W. P., Sweedler, J. V., Gilly, W. F., & Schulz, J. R. (2005). Intraspecific variation of venom injected by fish-hunting *Conus* snails. *Journal of Experimental Biology*, *208*(15), 2873–2883. https://doi.org/10.1242/jeb.01713

Keegan, H. L., Hedeen, R. A., & Whittemore, F. W. (1960). Seasonal variation in venom of black widow spiders. *The American Journal of Tropical Medicine and Hygiene, 9*(5), 477–479. https://doi.org/10.4269/ajtmh.1960.9.477

Klint, J. K., Senff, S., Rupasinghe, D. B., Er, S. Y., Herzig, V., Nicholson, G. M., & King, G. F. (2012). Spider-venom peptides that target voltage-gated sodium channels: pharmacological tools and potential therapeutic leads. *Toxicon*, *60*(4), 478–491. https://doi.org/10.1016/j.toxicon.2012.04.337

Kuhn-Nentwig, L., Schaller, J., & Nentwig, W. (2004). Biochemistry, toxicology and ecology of the venom of the spider *Cupiennius salei* (Ctenidae). *Toxicon*, *43*(5), 543–553.

https://doi.org/10.1016/j.toxicon.2004.02.009

Langenegger, N., Nentwig, W., & Kuhn-Nentwig, L. (2019). Spider venom: Components, modes of action, and novel strategies in transcriptomic and proteomic analyses. *Toxins, 11*(10), 611. https://doi.org/10.3390/toxins11100611

Lex, A., Gehlenborg, N., Strobelt, H., Vuillemot, R., & Pfister, H. (2014). UpSet: Visualization of intersecting sets. *IEEE Transactions on Visualization and Computer Graphics, 20*(12), 1983–1992. https://doi.org/10.1109/TVCG.2014.2346248

Lopes, P. H., Murakami, M. T., Portaro, F. C. V., Mesquita Pasqualoto, K. F., van den Berg, C., & Tambourgi, D. V. (2019). Targeting *Loxosceles* spider Sphingomyelinase D with small-molecule inhibitors as a potential therapeutic approach for loxoscelism. *Journal of Enzyme Inhibition and Medicinal Chemistry, 34*(1), 310–321. https://doi.org/10.1080/14756366.2018.1546698

Mariette, J., & Villa-Vialaneix, N. (2017). Unsupervised multiple kernel learning for heterogeneous data integration. *BioRxiv*, 139287. https://doi.org/10.1101/139287

Morgenstern, D. (2013). *The bio-logic of venom complexity: A chemical and evolutionary investigation into the role of venom complexity in two orders of venomous animals* [PhD Thesis, Institute for Molecular Bioscience, The University of Queensland.]. https://doi.org/10.1017/CBO9781107415324.004

Morgenstern, D., & King, G. F. (2013). The venom optimization hypothesis revisited. In *Toxicon* (Vol. 63, Issue 1, pp. 120–128). https://doi.org/10.1016/j.toxicon.2012.11.022

Nelsen, D. R., Kelln, W., & 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. https://doi.org/10.1016/j.anbehav.2013.12.019

Nentwig, W., & Kuhn-Nentwig, L. (2013). Main components of spider venoms. In W Nentwig (Ed.), *Spider Ecophysiology* (pp. 191–202). Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-33989-9_14

Nicholson, G. M., Little, M. J., Tyler, M., & Narahashi, T. (1996). Selective alteration of sodium channel gating by Australian funnel-web spider toxins. *Toxicon, 34*(11), 1443–1453. https://doi.org/10.1016/S0041-0101(96)00089-X

Nicholson, G. M., & Graudins. (2002). Invited Paper: animal toxins of Asia and Australia spiders of medical importance in the Asia - Pacific: atracotoxin, latrotoxin, and related spider neurotoxins. *Clinical and Experimental Pharmacology and Physiology*, *29*(9), 785–794.

Nicholson, G. M., Little, M. J., & Birinyi-Strachan, L. C. (2004). Structure and function of δ-atracotoxins: lethal neurotoxins targeting the voltage-gated sodium channel. *Toxicon*, *43*(5), 587–599. https://doi.org/10.1016/j.toxicon.2004.02.006

Nunes, K. P., Costa-Gonçalves, A., Lanza, L. F., Cortes, S. F., Cordeiro, M. N., Richardson, M., Pimenta, A. M. C., Webb, R. C., Leite, R., & De Lima, M. E. (2008). Tx2-6 toxin of the *Phoneutria nigriventer* spider potentiates rat erectile function. *Toxicon*, *51*(7), 1197–1206. https://doi.org/10.1016/j.toxicon.2008.02.010

Núñez, V., Cid, P., Sanz, L., De La Torre, P., Angulo, Y., Lomonte, B., Gutiérrez, J. M., & Calvete, J. J. (2009). Snake venomics and antivenomics of *Bothrops atrox* venoms from Colombia and the Amazon regions of Brazil, Perú and Ecuador suggest the occurrence of geographic variation of venom phenotype by a trend towards paedomorphism. *Journal of Proteomics*, *73*(1), 57–78. https://doi.org/10.1016/j.jprot.2009.07.013

Palagi, A., Koh, J. M. S., Leblanc, M., Wilson, D., Dutertre, S., King, G. F., Nicholson, G. M., & Escoubas, P. (2013). Unravelling the complex venom landscapes of lethal Australian funnel-web spiders (Hexathelidae: Atracinae) using LC-MALDI-TOF mass spectrometry. *Journal of Proteomics, 80*, 292–310. https://doi.org/10.1016/j.jprot.2013.01.002

Pekár, S., Bočánek, O., Michálek, O., Petráková, L., Haddad, C. R., Šedo, O., & Zdráhal, Z. (2018). Venom gland size and venom complexity-essential trophic adaptations of venomous predators: A case study using spiders. *Molecular Ecology, 27*(21), 4257–4269. https://doi.org/10.1111/mec.14859

Pimenta, A. M. C., Almeida, F. D. M., de Lima, M. E., Martin-Eauclaire, M. F., & 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*(5), 413–418. https://doi.org/10.1002/rcm.934

Pineda, S. S., Chin, Y. K.-Y., Undheim, E. A. B., Senff, S., Mobli, M., Dauly, C., Escoubas, P., Nicholson, G. M., Kaas, Q., Guo, S., Herzig, V., Mattick, J. S., & King, G. F. (2020). Structural venomics reveals evolution of a complex venom by duplication and diversification of an ancient peptide-encoding gene. *Proceedings of the National Academy of Sciences, 117*(21), 11399–11408. https://doi.org/10.1073/pnas.1914536117

Puillandre, N., Fedosov, A. E., & Kantor, Y. I. (2017). *Evolution of venomous animals and their toxins* (A. M. P. Gopalakrishnakone (ed.)). Springer Netherlands, Dordrecht. https://doi.org/10.1007/978-94-007-6458-3_19

Rash, L. D., & Hodgson, W. C. (2002). Pharmacology and biochemistry of spider venoms. *Toxicon, 40*(3), 225–254. https://doi.org/10.1016/S0041-0101(01)00199-4

Robinson, S. D., Undheim, E. A. B., Ueberheide, B., & King, G. F. (2017). Venom peptides as therapeutics: advances, challenges and the future of venom-peptide discovery Venom peptides as therapeutics: advances, challenges and the future of venom-peptide discovery. *Expert Review of Proteomics, 14*(10), 931–939.

https://doi.org/10.1080/14789450.2017.1377613

Ryabinin, V. V., Ziganshin, R. H., Starkov, V. G., Tsetlin, V. I., & Utkin, Y. N. (2019). Intraspecific Variability in the Composition of the Venom from Monocled Cobra (*Naja kaouthia*). *Russian Journal of Bioorganic Chemistry*, *45*(2), 107–121. https://doi.org/10.1134/S1068162019020109

Saez, N. J., Senff, S., Jensen, J. E., Er, S. Y., Herzig, V., Rash, L. D., & King, G. F. (2010). Spider-venom peptides as therapeutics. *Toxins*, *2*(12), 2851–2871. https://doi.org/10.3390/toxins2122851

Santana, R. C., Perez, D., Dobson, J., Panagides, N., Raven, R. J., Nouwens, A., Jones, A., King, G. F., & 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*(4), 1–13. https://doi.org/10.3390/toxins9040116

Schendel, V., Rash, L. D., Jenner, R. A., & Undheim, E. A. B. (2019). The diversity of venom: the importance of behaviour and venom system morphology in understanding its ecology and evolution. *Toxins*, *11*(11), 666. https://doi.org/10.3390/toxins11110666

Shatursky, O. Y., Pashkov, V. N., Bulgacov, O. V, & Grishin, E. V. (1995). Interaction of α-latroinsectotoxin from *Latrodectus mactans* venom with bilayer lipid membranes. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, *1233*(1), 14–20. https://doi.org/https://doi.org/10.1016/0005-2736(94)00226-F

Tasoulis, T., Silva, A., Veerati, P. C., Baker, M., Hodgson, W. C., Dunstan, N., & Isbister, G. K. (2020). Intra-Specific Venom Variation in the Australian Coastal Taipan *Oxyuranus scutellatus*. *Toxins*, *12*(8), 485. https://doi.org/10.3390/toxins12080485

Tedford, H. W., Sollod, B. L., Maggio, F., & King, G. F. (2004). Australian funnel-web spiders:

Master insecticide chemists. *Toxicon*, *43*(5), 601–618. https://doi.org/10.1016/j.toxicon.2004.02.010

Touchard, A., Dejean, A., Escoubas, P., & Orivel, J. (2015a). Intraspecific variations in the venom peptidome of the ant *Odontomachus haematodus* (Formicidae: Ponerinae) from French Guiana. *Journal of Hymenoptera Research*, *47*, 87–101. https://doi.org/10.3897/JHR.47.6804

Touchard, A., Dejean, A., Escoubas, P., & Orivel, J. (2015b). Intraspecific variations in the venom peptidome of the ant Odontomachus haematodus (Formicidae: Ponerinae) from French Guiana. *Journal of Hymenoptera Research*, *47*, 87–101. https://doi.org/10.3897/JHR.47.6804

Ushkaryov, Y. A., Volynski, K. E., & Ashton, A. C. (2004). The multiple actions of black widow spider toxins and their selective use in neurosecretion studies. *Toxicon*, *43*(5), 527–542. https://doi.org/10.1016/j.toxicon.2004.02.008

Wickham, H, 2009. ggplot2: elegant graphics for data analysis. Springer, New York. W

Wigger, E., Kuhn-Nentwig, L., & Nentwig, W. (2002). The venom optimisation hypothesis: A spider injects large venom quantities only into difficult prey types. *Toxicon*, *40*(6), 749–752. https://doi.org/10.1016/S0041-0101(01)00277-X

Wilson, D. (2016). The Venom of Australian Spiders. In & E. D.-G. P. Gopalakrishnakone, G. Corzo, M. E. de Lima (Ed.), *Spider Venoms* (pp. 21–46). Springer Netherlands. https://doi.org/10.1007/978-94-007-6389-0_21

Wilson, D., & Alewood, P. (2004). Australian funnel-web spider venom analyzed with on-line RP-HPLC techniques. In Aguilar MI. (Ed.), *HPLC of Peptides and Proteins. Methods in Molecular Biology* (Vol. 251, pp. 307–322). Springer, Totowa, NJ.

https://doi.org/https://doi.org/10.1385/1-59259-742-4:307

Wilson, D., & Alewood, P. F. (2006). Taxonomy of Australian funnel-web spiders using RP-HPLC/ESI-MS profiling techniques. *Toxicon*, *47*(6), 614–627. https://doi.org/10.1016/j.toxicon.2006.01.014

Wilson, D., Boyle, G. M., McIntyre, L., Nolan, M. J., Parsons, P. G., Smith, J. J., Tribolet, L., Loukas, A., Liddell, M. J., Rash, L. D., & Daly, N. L. (2017). The aromatic head group of spider toxin polyamines influences toxicity to cancer cells. *Toxins*, *9*(11), 346. https://doi.org/10.3390/toxins9110346

Wong, M. K. L., Woodman, J. D., & Rowell, D. M. (2016). Temporal variation in venom yield of the Australian funnel-web spider *Atrax sutherlandi* (Hexathelidae: Atracinae). *Arachnology*, *17*(1), 7–9. https://doi.org/10.13156/arac.2006.17.1.7

Zancolli, G., Calvete, J. J., Cardwell, M. D., Greene, H. W., Hayes, W. K., Hegarty, M. J., Herrmann, H. W., Holycross, A. T., Lannutti, D. I., Mulley, J. F., Sanz, L., Travis, Z. D., Whorley, J. R., Wüster, C. E., & Wüster, W. (2019). When one phenotype is not enough: Divergent evolutionary trajectories govern venom variation in a widespread rattlesnake species. *Proceedings of the Royal Society B: Biological Sciences*, *286*(1898), 20182735. https://doi.org/10.1098/rspb.2018.2735

Zhan, Y., Jiang, H., Wu, Q., Zhang, H., Bai, Z., Kuntner, M., & Tu, L. (2019). Comparative morphology refines the conventional model of spider reproduction. *PLoS ONE*, *14*(7). https://doi.org/10.1371/journal.pone.0218486

Zobel-Thropp, P. A., Bulger, E. A., Cordes, M. H. J., Binford, G. J., Gillespie, R. G., & Brewer, M. S. (2018). Sexually dimorphic venom proteins in long-jawed orb-weaving spiders (*Tetragnatha*) comprise novel gene families. *PeerJ*, *2018*(6), e4691. https://doi.org/10.7717/peerj.4691

3.8. Appendix Chapter 3

Table S3.1. Size of cephalothorax of *H. valida* and *H. infensa*.

ID	Size		Stage
	Mean	**Sd**	
Hadronyche valida			
A1	9.470	0.383	Adult
A2	8.760	0.176	Juvenile
A4	7.780	0.054	Juvenile
A5	8.960	0.281	Adult
A6	8.870	0.131	Juvenile
A7	8.170	0.244	Adult
A8	8.500	0.156	Adult
A9	10.300	0.304	Adult
A10	8.040	0.214	Juvenile
A11	8.310	0.199	Adult
A12	9.490	0.311	Adult
J	8.800	0.241	Adult
M	10.400	0.049	Adult
S	8.630	0.441	Adult
Hadronyche infensa			
LOC1	9.119	0.453	Juvenile
LOC2	9.312	0.168	Juvenile
LOC4	9.237	1.674	Juvenile
LOC5	7.334	0.203	Juvenile
LOC6	6.922	0.135	Juvenile
LOC7	9.386	0.137	Adult
LOC8	11.035	0.872	Adult
LOC10	9.662	0.324	Adult
LOC11	10.747	0.927	Adult
LOC13	9.165	0.819	Juvenile
LOC14	8.812	0.138	Adult
LOC15	10.579	0.138	Adult
LOC17	8.362	0.471	Juvenile
LOC18	6.766	0.567	Adult
LOC19	8.136	0.706	Adult
LOC20	9.393	0.386	Adult
LOC21	10.463	0.252	Adult
Hi13	6.161	0.126	Juvenile

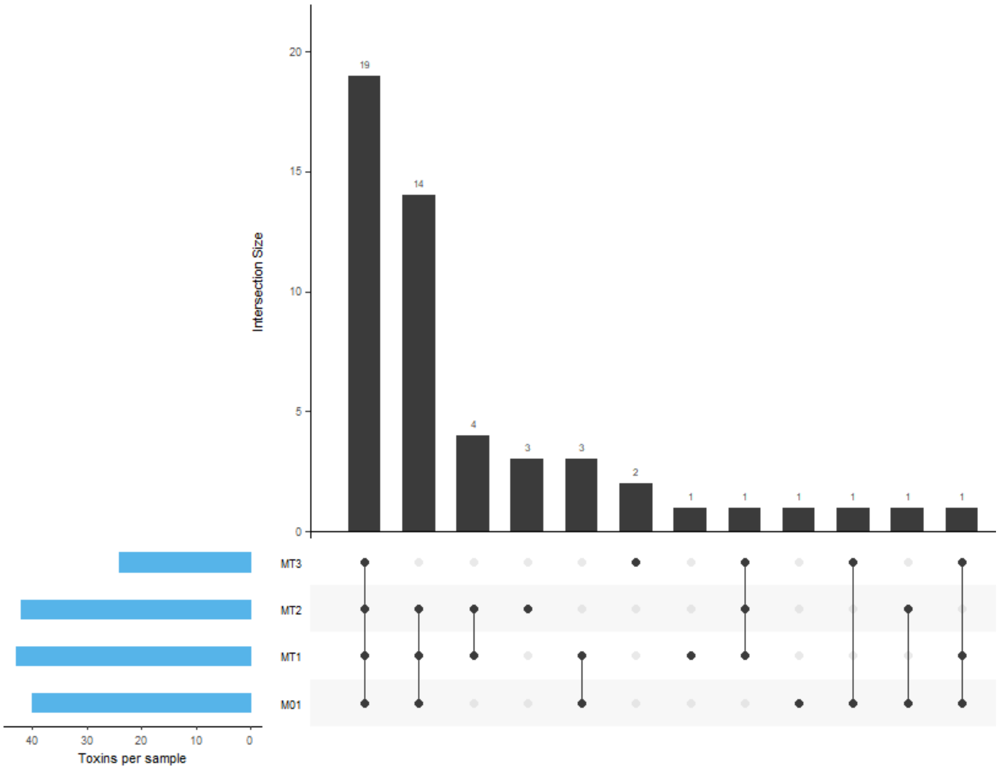
Table S3.2. Matrix presence absence venom components *H. valida.*
https://raw.githubusercontent.com/LICA0731/JSON-TEST1/master/validaJSON.csv

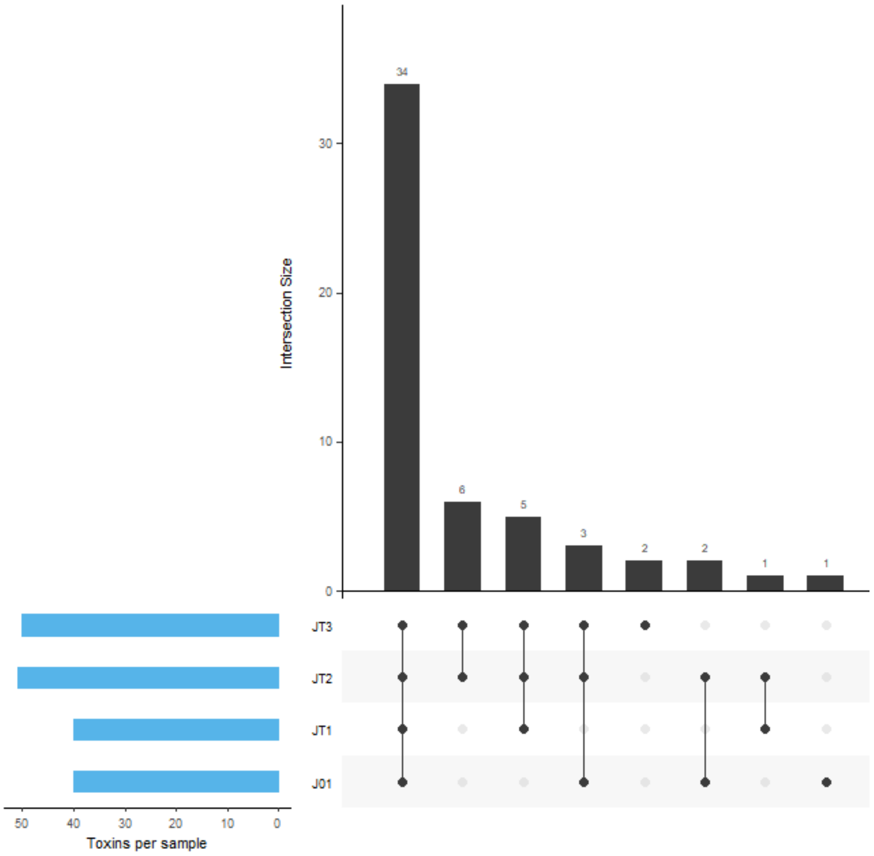
Table S3.3. Percentage of venom components shared in all repetitions within each individual of *H. valida*.

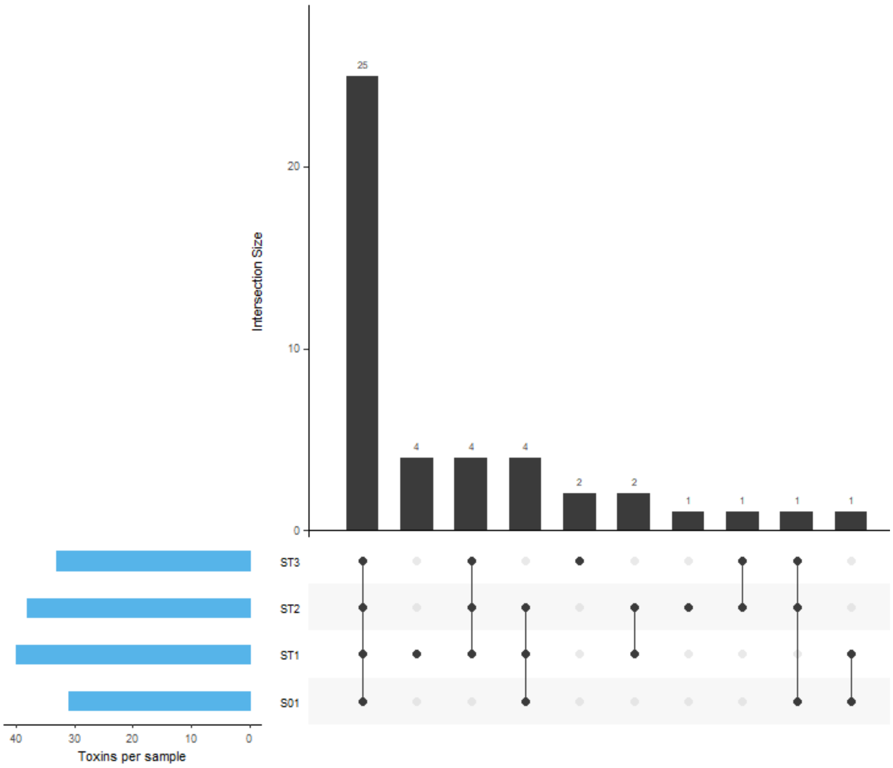
Id	**Total number of toxins**	**Percentage Venom components share between all repetitions (%)**
M	43	44
J	50	68
S	37	67.60
A1	38	47
A2	46	69.5
A4	44	66
A5	42	76
A6	47	70
A7	45	60
A8	47	70
A9	46	67
A10	44	68
A11	45	76
A12	46	76

Table S3.4. Jaccard similarity index between individuals of *H. valida*.

	M	J	S	A1t	A2t	A4t	A5t	A6t	A7t	A8t	A9t	A10t	A11t
J	0.486												
S	0.400	0.364											
A1t	0.576	0.528	0.448										
A2t	0.486	0.368	0.500	0.394									
A4t	0.514	0.389	0.485	0.419	0.294								
A5t	0.610	0.425	0.514	0.457	0.342	0.212							
A6t	0.590	0.395	0.486	0.375	0.395	0.324	0.229						
A7t	0.650	0.500	0.514	0.300	0.286	0.353	0.257	0.361					
A8t	0.553	0.395	0.441	0.267	0.206	0.273	0.229	0.235	0.156				
A9t	0.568	0.447	0.500	0.333	0.156	0.281	0.286	0.343	0.161	0.129			
A10t	0.528	0.405	0.406	0.333	0.265	0.333	0.286	0.343	0.219	0.129	0.194		
A11t	0.583	0.459	0.469	0.345	0.324	0.290	0.294	0.353	0.226	0.133	0.200	0.200	
A12t	0.625	0.436	0.528	0.375	0.306	0.371	0.278	0.235	0.156	0.182	0.188	0.242	0.250


Intersection Size
20
15
10
5
0
19
14
4
3
3
2
1
1
1
1
1
1
MT3
MT2
MT1
M01
40
30
20
10
0
Toxins per sample


Intersection Size
30
20
10
0
34
6
5
3
2
2
1
1
JT3
JT2
JT1
J01
50
40
30
20
10
0
Toxins per sample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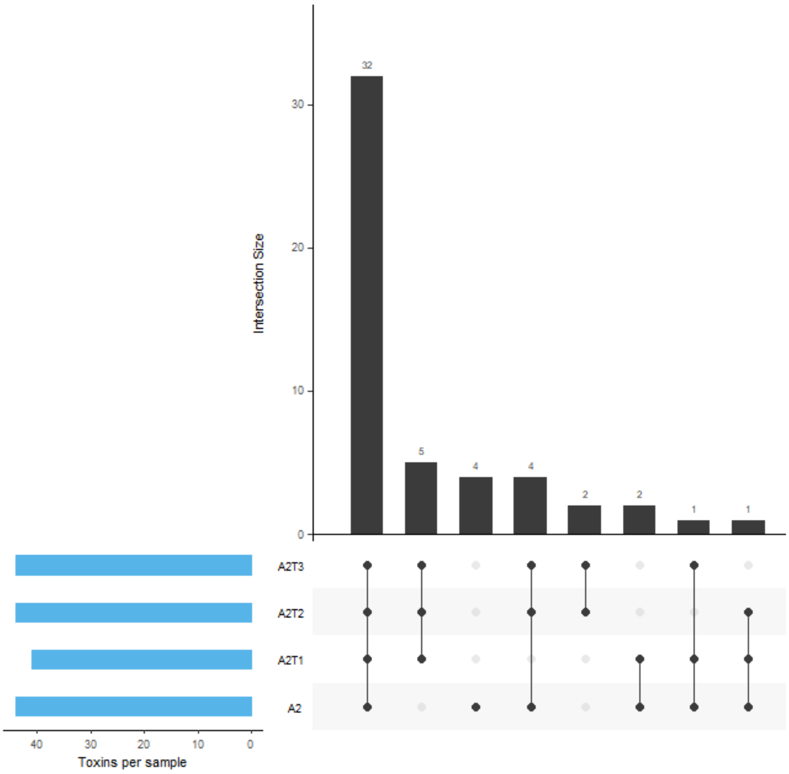
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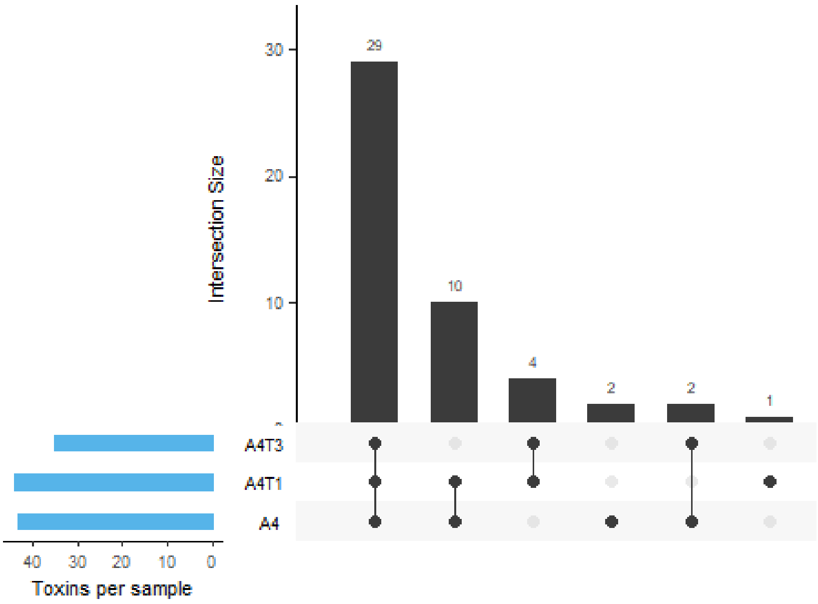
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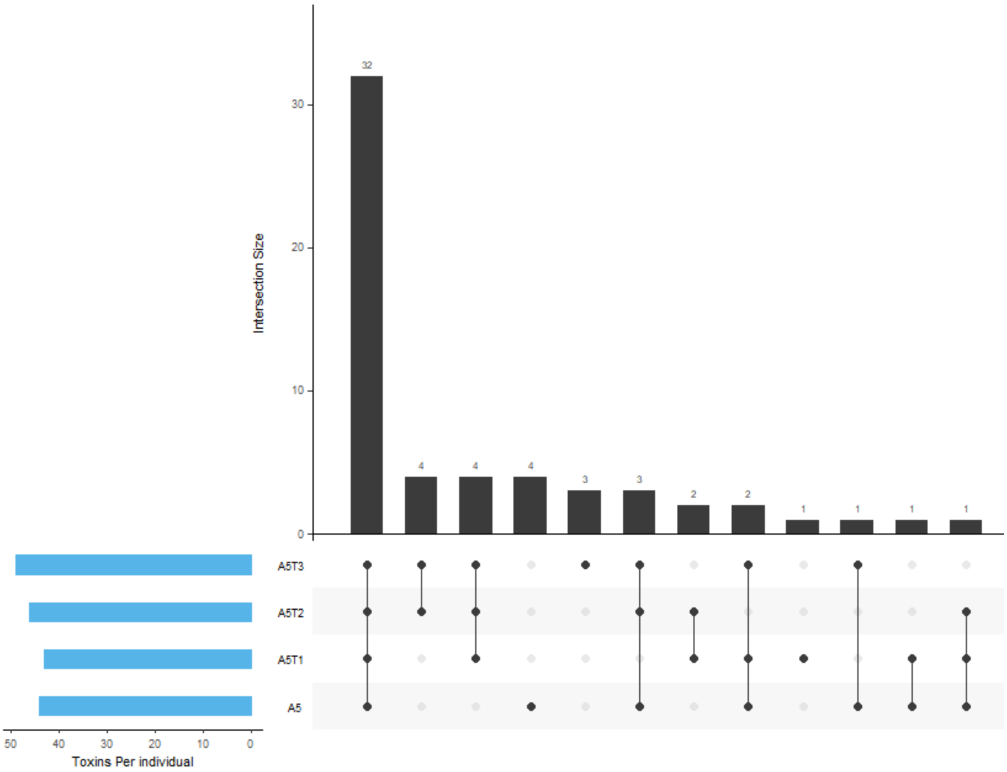
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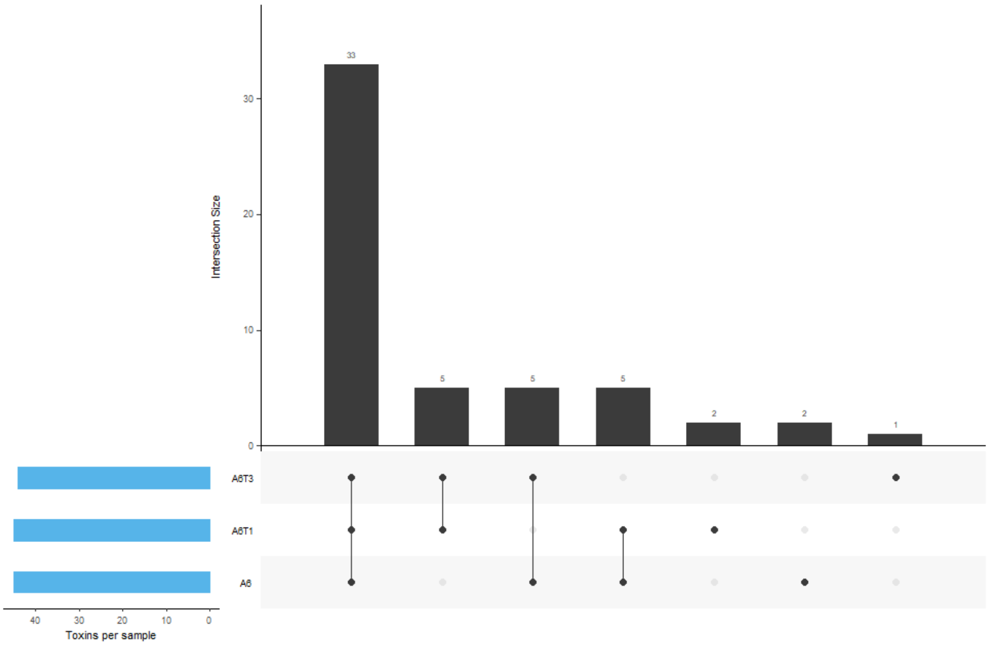
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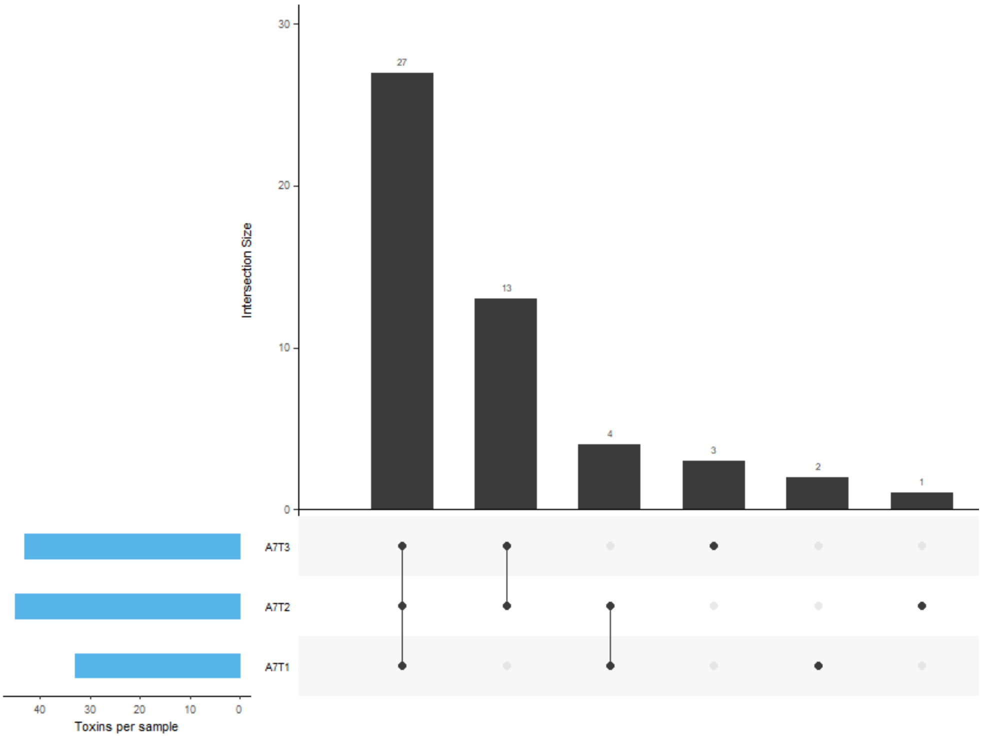
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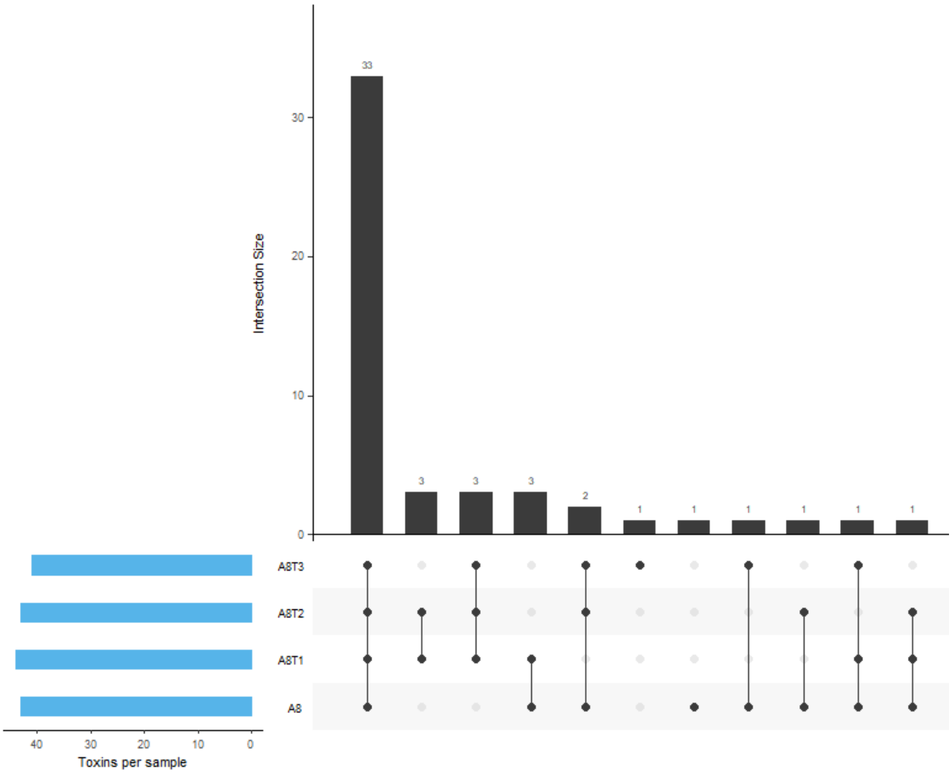
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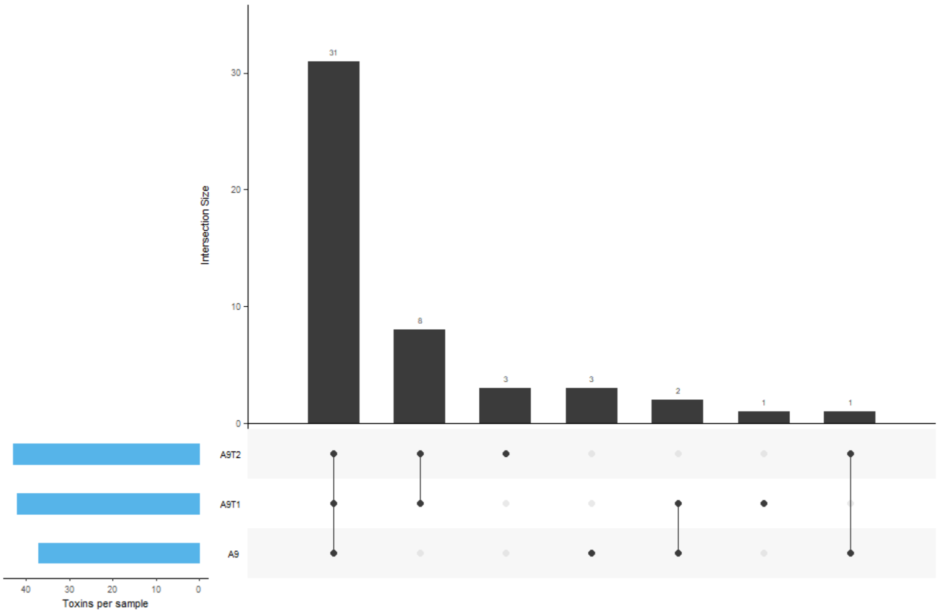
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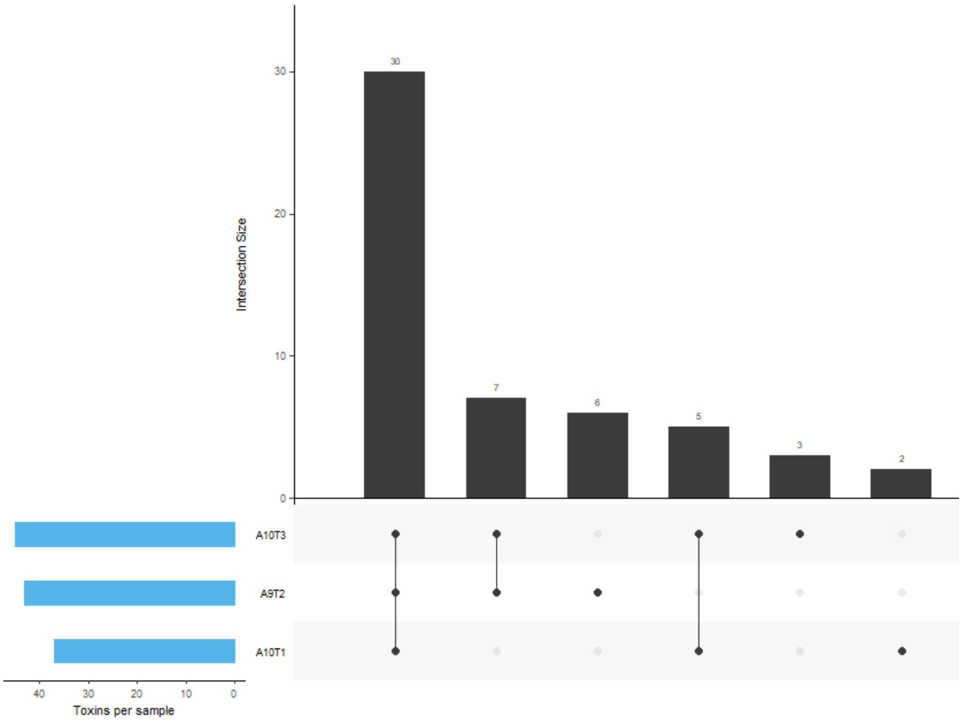
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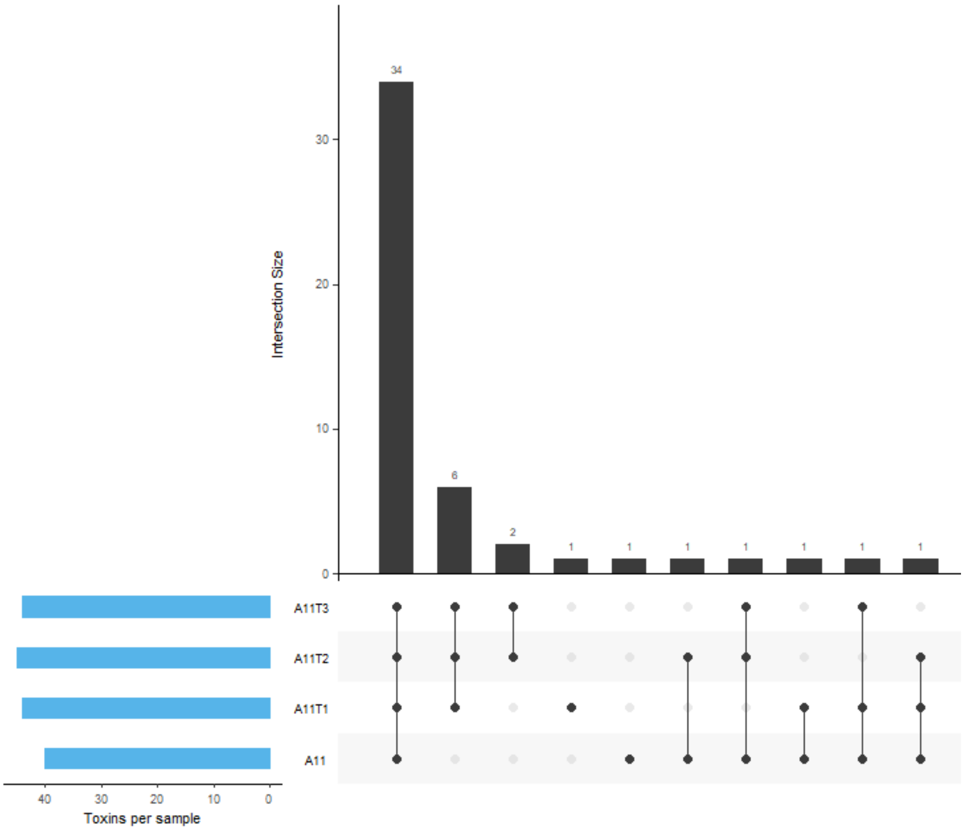
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Toxins per sample

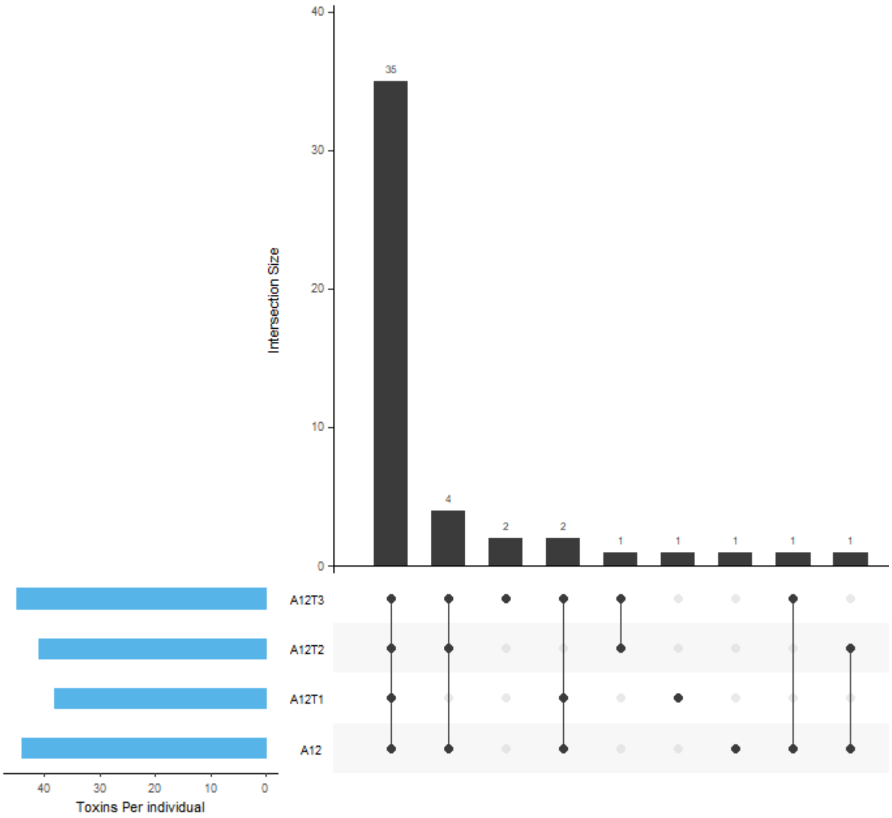
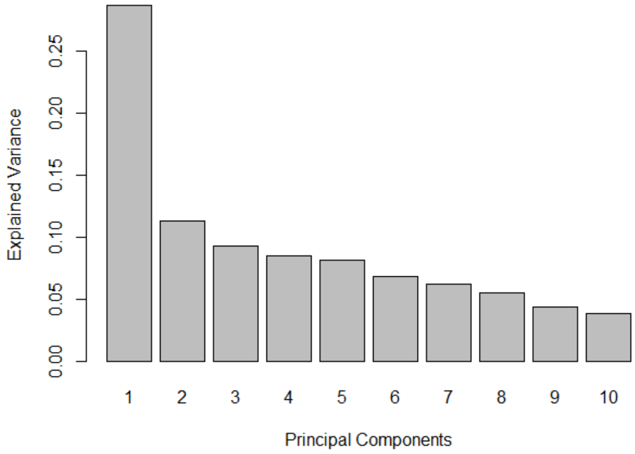


Figure S3.1. Venom components found in each individual of *H. valida* and their replicates. The intersections are sorted by frequency, revealing variation in toxins between replicates within each individual.

a.



b.

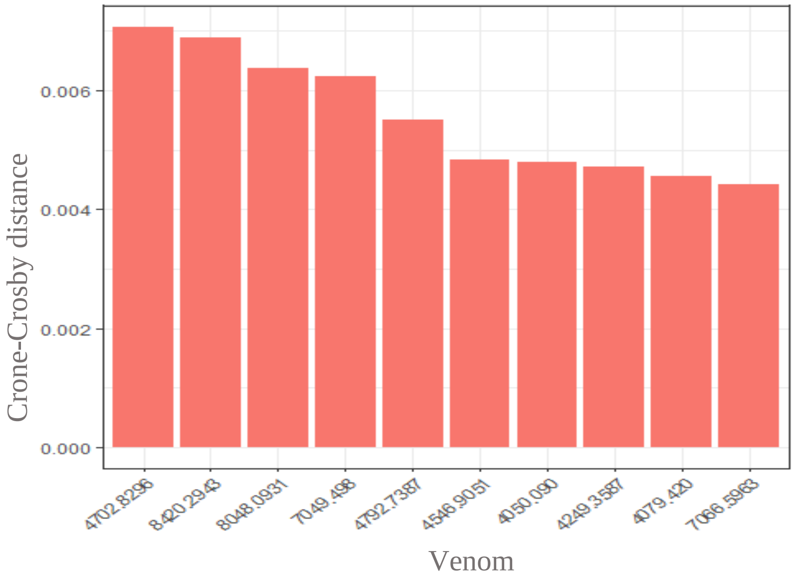


Figure S3.2. (a) Summary of the variance explained (~25%) by the first two axes of the KPCA. (b) The ten most important venom components for the first KPCA axis ranked by decreasing Crone-Crosby distance.

Chapter 4: Behaviour of the Sydney funnel-web spider *Atrax robustus* over different contexts, time, and stimuli

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Behaviour of the Sydney funnel-web spider *Atrax robustus* over different contexts, time, and stimuli

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4.1. Abstract

Atrax robustus is an iconic Australian spider because the venom can be lethal to humans. Moreover, some of the venom biomolecules have promise as therapeutic and bioinsecticidal leads. Nonetheless, aspects related to the life history and behaviour of this species, which might influence changes in venom components, have been overlooked. I assessed different behavioural traits (antipredator behaviour, defensiveness and activity) of juveniles and adult females across different contexts (predation, conspecific tolerance and exploration of a new territory) and stimuli (puff of air versus prod) over time. Adults responded to a puff of air faster than juveniles, but in response to a prod, both juveniles and adults become more defensive over time. No differences were observed between adults and juveniles for conspecific tolerance and exploration. Understanding behaviour of venomous species is important because behaviours may affect physiological traits, such as venom, and the ability of spiders to adapt to different conditions.

4.2. Introduction

Australian funnel-web spiders are of considerable research interest because the venom can cause a fatal envenomation syndrome in humans (Nicholson et al., 2004; Nicholson & Graudins, 2002), but also because some of the bioactive components in their venom have shown potential as therapeutic and agrochemical leads (Chassagnon et al., 2017; Ikonomopoulou et al., 2018; Nicholson & Graudins, 2002; Schendel et al., 2019). However, ecological, biological, and behavioural aspects of funnel-webs have been largely overlooked.

The study of behaviour is critical for understanding the complexity and variation of venom components because behavioural traits can act synergistically with other factors to affect variation, abundance, and function of spider venoms (Cooper et al., 2015; Hernández Duran et al., 2021). The behaviours associated with venom deployment affect fitness because spiders depend on venom for the acquisition of resources (Boevé et al., 1995; Nelsen et al., 2014), territory defence, predator deterrence, and reproduction (Boevé et al., 1995; Nelsen et al., 2014; Cooper et al., 2015; Zobel-Thropp et al., 2018; Schendel et al., 2019; Herzig et al., 2020). For example, spiders may deploy only small amounts of venom when subduing small prey, but may need to deploy larger amounts when subduing larger prey, as seen in the tiger wandering spider, *Cupiennius salei* (Boevé et al., 1995).

The ability of spiders to assess threats and modulate their defensive behaviours could explain how spiders use different physiological resources, such as venom, to respond or adapt to different environmental conditions and threats, considering the costs and benefits that these threats could have to their fitness (e.g. high predation risk or competition from conspecifics) (Nelsen et al., 2014). The selection pressures that affect individual responses depend on the context and situation, which involve the survivorship, life stage and reproduction of individuals (Dillon & Pruitt, 2014). To understand how spiders respond in different situations, it is necessary to study their behaviour in different contexts, such as foraging, interactions with conspecific (social behaviour), antipredator behaviour, and exploration of new environments (Riechert & Hedrick, 1990; Hernández Duran et al., 2021). This information provides insight into individual performance capacities. For example, individuals with faster responses or higher frequency of defensive behaviours are associated with greater survivorship; aggressive behaviours (more bites) also may help individuals to defend their territory and obtain access to prospective mates (Dillon & Pruitt, 2014).

The majority of behavioural work on spiders testing threatening stimuli or studying risk assessment has been conducted on species belonging to the infraorder Araneomorphae (Riechert & Hedrick, 1990; Lohrey et al., 2009; Pruitt & Husak, 2010; Dillon & Pruitt, 2014; Nelsen et al., 2014). Less work has been conducted on spiders in the infraorder Mygalomorphae (Jackson & Pollard, 1990; Bengston et al., 2014; Hernández Duran et al., 2021). In Australian funnel-web spiders (Mygalomorphae), behaviour has received less attention. The Sydney funnel-web spider, *A. robustus*, is one of the most venomous species in the world (Nicholson & Graudins, 2002). However, despite its reputation, aspects related to the ecology, biology and behaviour of this species are sparse (Bradley, 1993). Studies on *A. robustus* have mainly focused on the characterisation, identification and mode of action of δ-hexatoxin-Ar1a (δ-HXTX- Ar1a), the lethal neurotoxin found in the venom of mature males (Nicholson et al., 1996; Alewood et al., 2003; Klint et al., 2012; Wilson, 2016). Mature male *A. robustus* use δ-HXTX-Ar1a as defence against vertebrate predators through inhibition of the inactivation of voltage-gated sodium ion channels involved in nociceptive signalling (Herzig, Sunagar, et al., 2020). The lethal effects on humans are seemingly an unfortunate evolutionary coincidence (Herzig, Sunagar, et al., 2020). No studies have explored the behaviour of females, which have a different lifestyle to males (Bradley, 1993), and also show different venom profiles (Nicholson et al., 1996; Wilson, 2016). Females spend their lives in a burrow (Gray, 1981), which they aggressively defend against conspecifics and predators, rarely moving unless forced to do so.

In this study, I measured different behavioural traits of juveniles and adult females of *A. robustus* across different ecological contexts (response to predation, conspecific tolerance and exploration of a new territory) to assess spider responses over time. I assessed spider responses to different types of predator threat stimuli, a puff of air (simulated approach of an aerial predator such as a wasp or bird) and prodding (mechanical stimulus: simulated encounter with a predator). I also assessed spider responses to conspecific in the same special area, and how spiders responded when placed into a novel environment. This information contributes to understanding the relationships between contexts and the environmental cues that affect the spiders' ability to modulate their behavioural responses under different levels of threat.

4.3. Methods

4.3.1. Study species and husbandry

Eighteen individuals of *A. robustus* (n = 13 adult females, body length: 9.75 ± 1.46; n = 5 juveniles, body length: 7.09 ± 1.31) were collected by burrow excavation from the Gosford/Central Coast region, New South Wales, Australia, and transported them alive in small plastic containers with damp cotton wool to the laboratory of the Australian Institute of Tropical Health and Medicine (AITHM), James Cook University Nguma-bada (Cairns) campus, Queensland, Australia. Spiders were kept individually in 5 L plastic containers (L: 25 cm; W: 17.5 cm; H: 10 cm) in a climate-controlled room (temperature: 20 ± 2 °C; relative humidity: 60%) on a reverse light: dark cycle (12L:12D; lights on at 6 pm). The spiders were acclimated for one month before the start of behavioural assays. Each spider received one house cricket, *Acheta domestica*, and water (spraying the container) once a week.

A. robustus has a long life span; females can live for more than eight years, and both males and females generally only reach maturity in the fifth year (Gray, 1981; Levitt, 1961). Adult females were identified by epigyne sclerotisation and the opening of the epigastric furrow (gonoslit, Zhan et al., 2019), which is very apparent in adult females but is absent in juveniles (F. Perez-Miles, pers. comm.). Cephalothorax width was measured to assess spider size. To obtain the size, we photographed the cephalothorax of each spider under a Leica stereomicroscope and processed the images using Image J 1.8.0 Software. To determine whether the size of individuals was related to their life stage, we ran a repeated measures ANOVA (juvenile vs. adult). I found a significant effect, with juveniles being consistently smaller than adults (Supplementary material Figure S4.1, Table S4.1). Then, we assessed the correlation between stage and size, using point-biserial correlation (package lmt; Rizopoulos, 2006), where we found a positive correlation between both variables (Supplementary material Figure S4.1b, Table S4.1b). Therefore, I chose to use the life stage as a measurement of size in all further statistical analyses.

The research was conducted within the framework of the Australian Code for the Care and Use of Animals for Scientific Purposes (NHMRC, 2013). Funnel-web spiders are not a protected species in Australia. Spider collection was carried out in collaboration with the Australian Reptile Park (Somersby, New South Wales).

4.3.2. Behavioural assays

After the one-month acclimation period, food was withheld from the spiders for two weeks prior to behavioural testing (see below). Thereafter, following modified methods of Riechert & Hedrick, (1990) and Stankowich, (2009), we assessed antipredator behaviour and defensiveness of all individuals in the following contexts: response to predation, conspecific tolerance and exploration of a new territory. To avoid habituation, only one test was carried out each day, allowing the spiders to rest for 24 h between tests. While tests were conducted on consecutive days for each spider, the order of tests was randomised for each spider, except for the prod test, which was always presented first due to venom collection (venom profiles were not included in this study). Behavioural tests were repeated three times for each individual, one month apart, to measure changes over time. All assays were carried out between 6 am and 12 pm during the dark phase, and were video recorded under red light (Supplementary material Table S4.2). Behaviours were analysed using BORIS version 7.8.2 (Friard & Gamba, 2016).

Predation

The variation in behavioural responses to an antipredator stimulus has been linked with fitness components in different species (Dillon & Pruitt, 2014; Nelsen et al., 2014). Spiders use their different senses (visual, chemical, mechanical) to acquire information from the environment, habitat and they also use previous experiences to help them respond to different types of stimuli (Eiben & Persons, 2007). To measure spider responses to different types of antipredator stimuli, we used two types of aversive stimulus: the puff of air stimulus and the prodding stimulus. Both stimuli represent a predatory cue (e.g. avian predator; Riechert & Hedrick, 1990; Dillon & Pruitt, 2014), but the puff of air produces an avoidance response because the spider is not physically touched (Riechert & Hedrick, 1990), whereas the prod provokes a defensive response because the spider is physically touched (Nelsen et al., 2014). The puff of air was conducted in each individual's home container. Lifting the first pair of legs, moving the fangs and expelling venom are common behavioural responses to predatory threats in Australian funnel-web spiders (Wilson & Alewood, 2004, 2006). We use fang movements to measure spider responses to this aversive stimulus as frequency of fang movements was positively correlated with lifting the first pair of legs (Kendall's tau = 0.744, $z = 6.8145$, $P = < 0.001$, and negatively correlated with huddling (Kendall's tau = -0.51, $z = - 4.710$, $P = < 0.001$) . Each spider was gently moved out of its retreat using tweezers. After a 60 s acclimation period (a longer period resulted in spiders returning to the burrow), we applied three rapid puffs of air on the spider's anterior prosoma using a camera air blower and recorded fang movements for 420 s (Jackson et al., 1990; Riechert & Hedrick, 1990).

The prod test was also conducted in the home container and followed the modified methodology of Bengston et al. (2014). The individual was moved out of the retreat, as for the "puff test", and after a 60 s acclimation period, I gently prodded the first pair of legs with blunt tweezers continuously for 240 s. Thereafter, I recorded the number of fang movements of the individual for a further 360 s, totalling 600 s. Using the same behavioural response (frequency of fang movements) for the two different stimuli (puff of air and prod) allowed me to directly compare if the stimulus affected the spiders’ behaviour in a different way.

Conspecific tolerance

A. robustus is usually a solitary species; however, it is possible to find female retreats located spatially close to one another (Gray, 1992), suggesting that competition might occur between females for food. In addition, males wander in search of females, and female aggression towards males may lead to precopulatory sexual cannibalism (Johnson & Sih, 2005). To test defensiveness towards conspecifics I follow the modified protocol of Bengtson *et al* (2014). I placed an individual in a new arena (L: 23 cm; W: 16.5 cm; H: 10.5) that was divided into two equal halves with a mesh barrier (mesh diameter: 2 x 2 mm). This prevented direct contact between individuals, reducing the risk of fighting and death, but the mesh allowed spider to detect olfactory (and possibly mechanical cues) from other individuals. I randomly selected pairs of spiders (we attempted to size match but this was not always possible) and video recorded the whole arena for 1800 s. I measured the number of times the spider climbed the barrier as a measure of a conspecific defence response, which usually occurred when one spider perceived the other and tried to move closer by climbing the barrier. I previously observed that defensive spiders tried to cross the barrier and attack the other spider. Therefore, I chose climbing as a measurement of defensiveness against conspecifics. While this test could potentially measure a spider's propensity to escape using the mesh barrier, initial observations indicated that some spiders never approached or climbed the mesh, regardless of whether a conspecific was present or not, and others only moved towards the mesh when a conspecific was present, often displaying aggressive behaviours (e.g. leg waving and lunging) towards them. Therefore, I suggest that this test measures a response to conspecifics rather than a motivation to escape.

Exploration of a new territory

The willingness of spiders to disperse or move could affect spider survival because females can relocate their burrow in order to acquire resources, colonise new areas and avoid predators (Nakata and Ushimaru, 2013; Bengston et al., 2014). I assessed exploration of a new territory using activity level as the individual's willingness to explore a new environment (fear of novel objects or environment; Bengston et al., 2014). I placed an individual spider in a novel arena (L: 34.5cm; W: 22 cm; H: 10.7cm). I then measured the activity of the spiders as the time the individual spent moving around the container for 900 s.

4.3.3. Statistical methods

All statistical analyses were conducted using the statistical software R version 4.1.0 (R Core Team 2021; https://www.rproject.org).

Changes in behaviour over time

I used rank-based non-parametric analyses for longitudinal data instead of generalized linear mixed models (GLMM) to test for changes in behaviour between individuals over time. This analysis offers a robust framework for non-continuous variables, small sample size and skewed data (Noguchi et al., 2012). I used this analysis to assess the effects of life stage (juvenile and adult) on the behavioural variables (fang movements, number of climbs and duration of activity) measured in each context (antipredator behaviour, conspecific tolerance and exploration of a new territory), and over three time periods (T1, T2, T3). The design used was F1-LD-F1 in the nparLD package (Noguchi et al., 2012). The first F1, refers to the number of factors in each group, in this case juveniles and adults (whole-plot factor group) of *A. robustus*. LD refers to the term longitudinal data, and the last F1, refers to the time level (sub-plot factor). The random effect of individual identity was included as a subject in this model. I performed a Bonferroni correction to adjust *p*-values for repetitions. I also used the F1-LD-F1 design to test for differences between the type of stimulus (puff of air and prod) used, considering fang movements as the response variable in the antipredator context. For each group (stage), the rank mean of overall rank, observations (Nobs), the point estimates of the relative treatment effect (RTE), and confidence intervals for each behavioural variable are shown in the Supplementary material Table S4.3.

To validate my hypotheses related to the effects of repetition and differences between stages over each behavioural variable in each context, I conducted a randomization test (1000 times) for each longitudinal model (nparLD), where the ANOVA-Type statistic was used to assess whether the values obtained in the tests were simply a result of chance or were indicative of true significant differences (Supplementary material Figure S4.2).

4.4. Results

Changes in behaviour over time

For response to predation, the frequency of fang movements of all individuals was compared over time in response to the puff of air (Table 4.1). I found no significant difference over repetitions (ATS = 0.270; df =1.711; P = 0.729). However, there was a significant difference between adults and juveniles (ATS = 9.157; df =1; P = 0.002, Table 1; Figure 4.1a; Supplementary material Figure S4.2a). Adults showed more fang movements than juveniles at T1 and T3. In contrast, adults and juveniles showed a similar frequency of fang movements at T2 (Figure 4.1a).

Table 4. 1. Output of rank-based non-parametric analyses for longitudinal data models of different spider behavioural traits, and the effects of repetitions and life stage. The * refers to results that are significant at the α = 0.05 level.

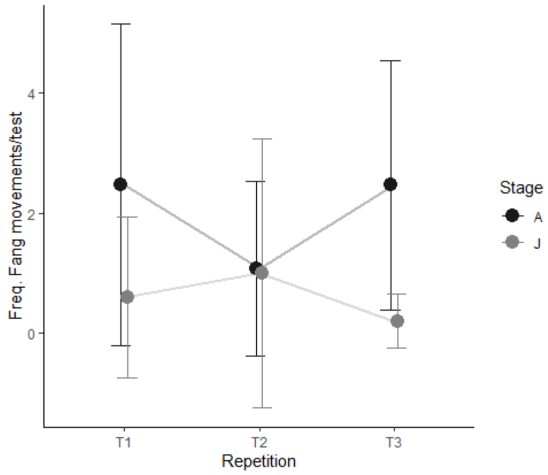
	ANOVA-Type Statistic (ATS)		
Model	Frequency of fang movements (puff of air) ~ Repetition + Stage, subject= factor (ID)		
	Statistic	**df**	**p-value**
Stage	9.157	1	0.002 *
Repetition	0.270	1.711	0.729
Model	Frequency of fang movements (prod) ~ Repetition + Stage, subject= factor (ID)		
Stage	2.247	1	0.195
Repetition	26.308	1.989	<0.001 *
Model	Frequency of climbs ~ Repetition + Stage, subject= factor (ID)		
Stage	0.516	1	0.472
Repetition	0.468	1.828	0.607
Model	Activity ~ Repetition + Stage, subject= factor (ID)		
Stage	0.145	1	0.703
Repetition	1.590	1.723	0.207

In contrast, when I measured the frequency of fang movements of all individuals over time in response to the prod stimulus (Table 4.1), I found a significant increase in the frequency of fang movements from repetitions T1 to T3 (ATS = 26.308; df =1.989; *P* = <0.001; Figure 4.1b). However, I did not find differences in the frequency of fang movements between adults and juveniles (ATS = 2.247; df =1; *P* = 0.195; Table 4.1; Supplementary material S4.2b).

For differences in fang movements between the type of stimulus, puff of air and prod in the context of a predatory cue, I found significant differences in the frequency of fang movements between the different types of stimuli (puff vs. prod; ATS = 220.673; df =1; *P* =

<0.001), and over repetitions (ATS = 10.495; df = 1; *P* = <0.001). Spiders showed a significantly higher frequency of fang movements when prodded than when exposed to the puff of air (Figure 4.2), and there was a significant increase in fang movements over time in response to the prod stimulus from T1to T3 (Figure 4.2).

a.



b.

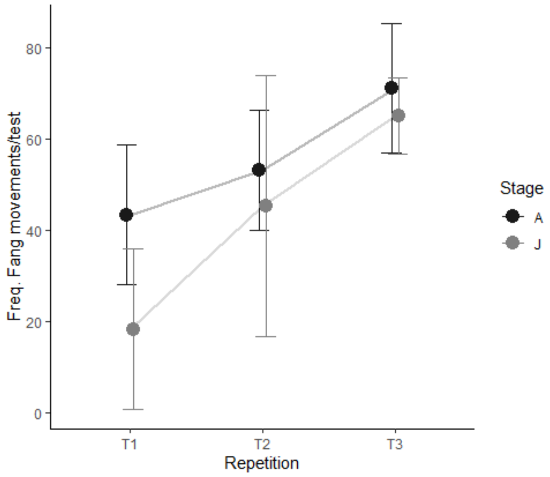


Figure 4. 1. Frequency of fang movements made by adult female and juvenile *Atrax robustus* in response to (a) a puff of air and (b) a prod stimulus.

For conspecific tolerance, there were no significant differences in the climbing frequency over time (ATS = 0.471; df =1.827; *P* = 0.606) and life stage (ATS = 0.544; df =1; *P* = 0.460) (Supplementary material S4.2c). Similarly, in the context of exploration of a new territory, we found no significant differences in activity over time (ATS = 1.589; df =1.722; *P* = 0.206) and life stage (ATS = 0.145; df =1; *P* = 0.702) (Supplementary material S4.2d).

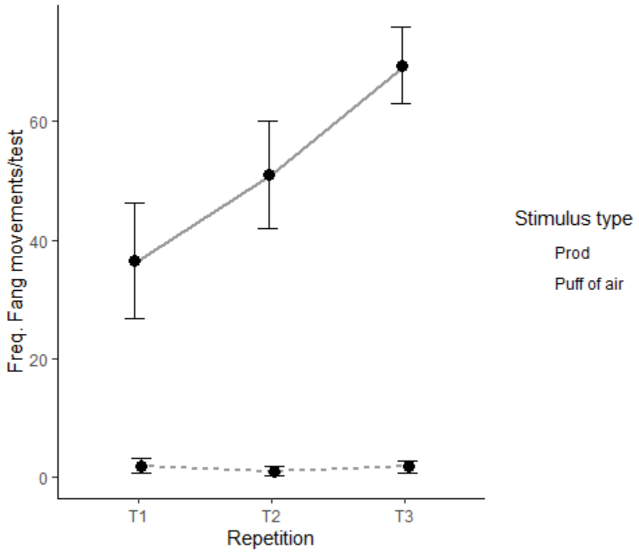


Figure 4. 2. Mean frequency of fang movements made by *Atrax robustus* in response to either a puff of air (dotted line: antipredator behaviour) and or a prod stimulus (solid line: defensiveness) over three-time repetitions (T1-T3).

4.5. Discussion

This is the first study to assess changes in behavioural traits of *A. robustus* over different contexts and time. I found that adult female spiders made more fang movements than juveniles in response to a puff of air over all time periods. Juveniles appeared to show a different behavioural response compared to adults. I observed that juveniles tended to huddle rather than move their fangs, whereas adults tended to lift the first pair of legs and move the fangs in response to the puff of air. Although I must treat these differences with caution because of the small sample size of juveniles, the trend is maintained, even with randomization (Supplementary material Figure S2). The different responses between adults and juveniles could be a consequence of the developmental stage, reproductive status (e.g. females are more aggressive when they reach maturity to deter predators and conspecifics), and/or investment in offspring (e.g. sexually mature females are more aggressive as they invest more energy into egg production) (Bessékon & Horel, 1996; Mooney & Haloin, 2006). Broadly, for spiders, juveniles tend to respond to a threatening stimulus by fleeing or huddling (to blend in to the environment or feign death), reducing the likelihood of being predated or being injured (Stankowich, 2009), which in turn can also reduce the metabolic costs related to the use of venom (Cooper et al., 2015). In contrast, adults are more likely to take risks to defend their burrow or web and guard their offspring (Mooney & Haloin, 2006). Adults are also more likely to show a higher number of defensive behaviours when threatened because they are larger than juveniles, and have different concentrations of toxins (Escoubas et al., 2002) that could help them to deter predators (Herzig, Sunagar, et al., 2020), conspecifics and a diverse range of parasitoids (Stankowich, 2009).

In contrast, when I assessed the spiders' responses to the prod stimulus, I found that both juveniles and adults increased the number of fang movements over time, suggesting that they became more defensive. This type of stimulus likely affects the behavioural response because it is more similar to a direct conspecific or predator encounter (Jackson et al., 1990; Stankowich, 2009) than the puff of air, which elicits another type of antipredator response (predator escape). In support, I found that spiders responded differently to the different types of stimuli, showing an increased frequency of fang movements in response to the prod compared to the puff stimulus. My results are consistent with those found in *Pholcus phalangioides*, where larger juveniles and adult females whirled for longer periods in respond to a mechanical stimulus compared to when they received a puff of air (Jackson et al., 1990).

Spiders can optimise their responses to different threatening stimuli (Cooper et al., 2015) depending on their life stage, size or the approach of the stimulus (e.g. how fast the threatening stimulus is moving, or how close it is to the spider) (Lohrey et al., 2009; Stankowich, 2009). In addition, antipredator behaviour can change over time (Glaudas et al., 2006). Animals may be more flexible if they are continuously exposed to a threat, where they can learn to discriminate between potential harmful and non-harmful threats (Glaudas et al., 2006; Lohrey et al., 2009; Nelsen et al., 2014). For example, in populations of *Agelenopsis aperta* with high predation pressure, the latency to return to foraging was faster compared to the populations with lower predator pressure (Riechert & Hedrick, 1990).

Spiders can also modulate their behaviour according to the level of threat that they are facing (Lohrey et al., 2009; Stankowich, 2009; Nelsen et al., 2014). For example, in the black tunnel-web spider, *Porrhothele antipodiana*, individuals display a gaping behaviour, which consists of raising the body and moving the fangs into a defensive position against conspecifics and predators (Jackson & Pollard, 1990). Funnel-webs also show similar gaping displays, lifting the first pair of legs and deploying venom from their fangs (Wilson & Alewood, 2004, 2006). The ability to use biochemical weapons might also influence the way spiders assess the level of threat (Nelsen et al., 2014; Cooper et al., 2015). Spiders can choose to expel venom as a mechanism of defence when they do not see a way of escaping the threat (Cooper et al., 2015). For example, an aggravation stimulus of prodding in Australian funnel-web spiders (personal observation) and species of mouse spider, *Missulena*, (Herzig et al., 2008) resulted in large amounts of venom being expended in initial bites. However, the spiders tended to reduce the amount of venom expended in subsequent bites in response to the same stimulus. Furthermore, some spiders (e.g. mouse spiders, black widows) can use dry bites instead of deploying venom when they are threatened, reducing the metabolic costs of expending venom (Nelsen et al., 2014; Cooper et al., 2015). In my study, although not quantified here, venom expenditure appeared to be lower in juveniles than adults, as juveniles rarely deployed venom on the fangs, whereas adults readily deployed venom.

For the contexts of conspecific tolerance and exploration of a new territory, I did not find differences between life stages and repetitions in climbing behaviour and activity level, which suggests that both behaviours are likely not affected by life stage, and do not change over time. This could be due to both juveniles and adults sharing similar types of prey and microhabitats, and engaging in the same types of con- and interspecific interactions (Riechert, 1984). Spiders under natural conditions can alter their agonistic behaviour when resources are manipulated (Riechert, 1978). It would be worthwhile in future to test spiders in their home containers, as aggressiveness against conspecifics could be affected by their fidelity to a burrow or territory.

In this study, I followed established methodology that largely required active, mechanical stimuli, and found variation in behavioural responses to these stimuli. However, chemical cues also play an important role in how spiders respond behaviourally to predators and potential prey (Eiben & Persons, 2007; Montiglio & DiRienzo, 2016). Studies that have explored personality in spiders with consideration of chemical cues have all been conducted on Araneomorph spiders that build webs, which serve as an extended phenotype (Montiglio & DiRienzo, 2016). To my knowledge, chemical cues have not yet been explored in the context of personality in Mygalomorphs. Female funnel-webs remain in their burrows for their whole lives (Gray, 2010), so future studies into whether chemical cues elicit behavioural changes, or whether behavioural responses to these chemical cues are repeatable over time, would be a valuable area for future research.

Another avenue that needs more exploration is the relationship between venom and different behaviours. These associations will provide important information for understanding the effects of venom and its function on the natural prey of spiders, as well as the role played in other contexts such as mating and intraspecific competition (Hernández Duran et al., 2021; Schendel et al., 2019). Behavioural and ecological factors can influence the quality of venoms (as has been observed in bees *Apis mellifera*; Scaccabarozzi et al., 2021), having a direct or indirect effect on an individual’s fitness. For example, aggressive individuals could experience higher metabolic costs associated with venom expenditure, but aggression could also be associated with higher venom concentration and quantity when they are exposed to predators and when the level of threat is higher (Westermann et al., 2015; Lira et al., 2017; Hernández Duran et al., 2021), increasing the likelihood that they can mount a stronger aggressive response. Studying these relationships will provide insights into understanding how venoms evolve, and could contribute to the identification of molecular changes in toxins, which are relevant in pharmacological applications and drug discovery (Herzig, Cristofori-Armstrong, et al., 2020; Schendel et al., 2019).

In summary, this study highlights how juveniles and adult females of *A. robustus* show variation in behavioural responses when different types of threatening stimuli are assessed over time and across different contexts. Differences in behaviour were observed between life stages, suggesting that previous experiences, physiological traits and/or environmental conditions may trigger or inhibit responses in juveniles and adults differently. Adults were more likely to show a defensive response when they received a puff of air or prod, and this could be related to hormonal changes and/or energy invested in egg production when spiders reach sexual maturity. More studies are required to assess the relationship between venom components, hormones, metabolic rates, and behaviours in different contexts. We suggest that changes in behavioural responses could potentially affect variation in venom components, and how these biological weapons are used over different developmental stages. This is an exciting avenue for future investigation.

4.6. References

Alewood, D., Birinyi-Strachan, L. C., Pallaghy, P. K., Norton, R. S., Nicholson, G. M., & Alewood, P. F. (2003). Synthesis and characterization of δ-Atracotoxin-Ar1a, the lethal neurotoxin from venom of the Sydney funnel-web spider (*Atrax robustus*). *Biochemistry, 42*(44), 12933–12940. https://doi.org/10.1021/bi030091n

Assi Bessékon, D., & Horel, A. (1996). Social-maternal relations in *Coelotes terrestris* (Araneae, Agelenidae): Influence of the female reproductive state on its tolerance towards conspecific spiderlings. *Behavioural Processes*, *36*(1), 19–25. https://doi.org/10.1016/0376-6357(95)00012-7

Australian code for the care and use of animals for scientific purposes 8th edition, 2013. https://www.nhmrc.gov.au/about-us/publications/australian-code-care-and- use-animals-scientific-purposes/.

Bengston, S. E., Pruitt, J. N., & Riechert, S. E. (2014). Differences in environmental enrichment generate contrasting behavioural syndromes in a basal spider lineage. *Animal Behaviour*, *93*, 105–110. https://doi.org/10.1016/J.ANBEHAV.2014.04.022

Boevé, J. L., Kuhn-Nentwig, L., Keller, S., & Nentwig, W. (1995). Quantity and quality of venom released by a spider (*Cupiennius salei*, Ctenidae). *Toxicon*, *33*(10), 1347–1357. https://doi.org/10.1016/0041-0101(95)00066-U

Bradley, R. A. (1993). Seasonal activity patterns in Sydney funnel-web spiders, *Atrax* spp. (Araneae: Hexathelidae). *Bull. Br. Arachnol. Soc.*, *9*(6), 189–192.

Chassagnon, I. R., McCarthy, C. A., Chin, Y. K. Y., Pineda, S. S., Keramidas, A., Mobli, M., Pham, V., De Silva, T. M., Lynch, J. W., Widdop, R. E., Rash, L. D., & King, G. F. (2017). Potent neuroprotection after stroke afforded by a double-knot spider-venom peptide that inhibits acid-sensing ion channel 1a. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(14), 3750–3755. https://doi.org/10.1073/pnas.1614728114

Cooper, A. M., Nelsen, D. R., & Hayes, W. K. (2015). The strategic use of venom by spiders. In A. Gopalakrishnakone, P.; Malhotra (Ed.), *Evolution of Venomous Animals and Their Toxins* (pp. 145–163). Springer Netherlands. https://doi.org/DOI 10.1007/978-94-007-

6458-3_13

Dillon, J. A., & Pruitt, J. N. (2014). Use of locomotor performance capacities reflects the risk level associated with specific cue types in two cursorial spider species. *Journal of Arachnology, 42*(1), 79–85. https://doi.org/10.1636/J13-68.1

Eiben, B., & Persons, M. (2007). The effect of prior exposure to predator cues on chemically-mediated defensive behaviour and survival in the wolf spider *Rabidosa rabida* (Araneae: Lycosidae). *Behaviour, 144*(8), 889–906. https://doi.org/10.1163/156853907781492681

Escoubas, P., Corzo, G., Whiteley, B. J., Célérier, M. L., & 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*(5), 403–413. https://doi.org/10.1002/rcm.595

Friard, O., & Gamba, M. (2016). BORIS: a free, versatile open-source event-logging software for video/audio coding and live observations. *Methods in Ecology and Evolution*, *7*(11), 1325–1330. https://doi.org/10.1111/2041-210X.12584

Glaudas, X., Winne, C. T., & Fedewa, L. A. (2006). Ontogeny of anti-predator behavioural habituation in cottonmouths (*Agkistrodon piscivorus*). *Ethology*, *112*(6), 608–615. https://doi.org/10.1111/j.1439-0310.2005.01183.x

Gray, M. (2010). A revision of the Australian funnel-web spiders (Hexathelidae: Atracinae). *Records of the Australian Museum*, *62*(2–3), 285–392. https://doi.org/10.3853/j.0067-1975.62.2010.1556

Gray, M. R. (1981). Getting to know funnel webs. *Australian Natural History*, *20*, 265–270.

Gray, M. R. (1992). Funnel-webs: separating fact from fiction. *Australia's Leading Nature*

Magazine, *24*(3), 32–39.

Hernández Duran, L., Wilson, D. T., Briffa, M., & Rymer, T. L. (2021). Beyond spider personality: The relationships between behavioural, physiological, and environmental factors. *Ecology and Evolution*, *11*(7), 2974–2989. https://doi.org/https://doi.org/10.1002/ece3.7243

Herzig, V., Cristofori-Armstrong, B., Israel, M. R., Nixon, S. A., Vetter, I., & King, G. F. (2020a). Animal toxins — Nature's evolutionary-refined toolkit for basic research and drug discovery. *Biochemical Pharmacology*, *181*(114096), 1–14. https://doi.org/10.1016/j.bcp.2020.114096

Herzig, V., Khalife, A. A., Chong, Y., Isbister, G. K., Currie, B. J., Churchill, T. B., Horner, S., Escoubas, P., Nicholson, G. M., & Hodgson, W. C. (2008). Intersexual variations in Northern (*Missulena pruinosa*) and Eastern (*M. bradleyi*) mouse spider venom. *Toxicon*, *51*(7), 1167–1177. https://doi.org/10.1016/j.toxicon.2008.02.001

Herzig, V., Sunagar, K., Wilson, D. T. R., Pineda, S. S., Israel, M. R., Dutertre, S., McFarland, B. S., Undheim, E. A. B., Hodgson, W. C., Alewood, P. F., Lewis, R. J., Bosmans, F., Vetter, I., King, G. F., & Fry, B. G. (2020b). Australian funnel-web spiders evolved human-lethal δ-hexatoxins for defense against vertebrate predators. *Proceedings of the National Academy of Sciences of the United States of America*, *117*(40), 24920–24928. https://doi.org/10.1073/pnas.2004516117

Ikonomopoulou, M. P., Fernandez-Rojo, M. A., Pineda, S. S., Cabezas-Sainz, P., Winnen, B., Morales, R. A. V., Brust, A., Sánchez, L., Alewood, P. F., Ramm, G. A., Miles, J. J., & King, G. F. (2018). Gomesin inhibits melanoma growth by manipulating key signaling cascades that control cell death and proliferation. *Scientific Reports*, *8*(11519), 1–14. https://doi.org/10.1038/s41598-018-29826-4

Jackson, R. R., Brassington, R. J., & Rowe, R. J. (1990). Anti-predator defences of *Pholcus phalangioides* (Araneae, Pholcidae), a web-building and web-invading spider. *Journal of Zoology*, *220*(4), 543–552. https://doi.org/10.1111/j.1469-7998.1990.tb04733.x

Jackson, Robert R, & Pollard, S. D. (1990). Intraspecific interactions and the function of courtship in mygalomorph spiders: a study of *Porrhothele antipodiana* (Araneae: Hexathelidae) and a literature review. *New Zealand Journal of Zoology*, *17*(4), 499–526. https://doi.org/10.1080/03014223.1990.10422949

Johnson, J. C., & Sih, A. (2005). Precopulatory sexual cannibalism in fishing spiders (*Dolomedes triton*): A role for behavioural syndromes. *Behavioural Ecology and Sociobiology*, *58*(4), 390–396. https://doi.org/10.1007/s00265-005-0943-5

Klint, J. K., Senff, S., Rupasinghe, D. B., Er, S. Y., Herzig, V., Nicholson, G. M., & King, G. F. (2012). Spider-venom peptides that target voltage-gated sodium channels: pharmacological tools and potential therapeutic leads. *Toxicon*, *60*(4), 478–491. https://doi.org/10.1016/j.toxicon.2012.04.337

Levitt, V. (1961). The funnel-web spider in captivity. *Proceedings of the Royal Zoological Society of New South Wales*, *1958–59*, 80–84. http://www.biodiversitylibrary.org/item/119542

Lira, A. F. A., Santos, A. B., Silva, N. A., & Martins, R. D. (2017). Threat level influences the use of venom in a scorpion species, *Tityus stigmurus* (Scorpiones, Buthidae). *Acta Ethologica*, *20*(3), 291–295. https://doi.org/10.1007/s10211-017-0274-3

Lohrey, A. K., Clark, D. L., Gordon, S. D., & Uetz, G. W. (2009). Antipredator responses of wolf spiders (Araneae: Lycosidae) to sensory cues representing an avian predator. *Animal Behaviour*, *77*, 813–821. https://doi.org/10.1016/j.anbehav.2008.12.025

Montiglio, P.-O., & DiRienzo, N. (2016). There's no place like home: the contribution of direct and extended phenotypes on the expression of spider aggressiveness. *Behavioural Ecology*, *27*(6), 1880–1888. https://doi.org/10.1093/beheco/arw094

Mooney, K. A., & Haloin, J. R. (2006). Spider size and guarding of offspring affect *Paraphidippus aurantius* (Araneae, Salticidae) response to predation threat. *The Journal of Arachnology*, *34*, 34–98. https://doi.org/10.1636/S02-64.1

Nakata, K., & Ushimaru, A. (2013). The effect of predation risk on spider's decisions on web-site relocation. *Behaviour*, *150*(1), 103–114. https://doi.org/10.1163/1568539X-00003039

Nelsen, D. R., Kelln, W., & 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. https://doi.org/10.1016/j.anbehav.2013.12.019

Nicholson, G. M., & Graudins. (2002). Invited Paper: animal toxins of Asia and Australia spiders of medical importance in the Asia - Pacific: atracotoxin, latrotoxin, and related spider neurotoxins. *Clinical and Experimental Pharmacology and Physiology*, *29*(9), 785–794.

Nicholson, G. M., Little, M. J., & Birinyi-Strachan, L. C. (2004). Structure and function of δ-atracotoxins: lethal neurotoxins targeting the voltage-gated sodium channel. *Toxicon*, *43*(5), 587–599. https://doi.org/10.1016/j.toxicon.2004.02.006

Nicholson, G. M., Little, M. J., Tyler, M., & Narahashi, T. (1996). Selective alteration of sodium channel gating by Australian funnel-web spider toxins. *Toxicon*, *34*(11), 1443–1453. https://doi.org/10.1016/S0041-0101(96)00089-X

Noguchi, K., Gel, Y. R., Brunner, E., & Konietschke, F. (2012). nparLD : An R Software Package for the Nonparametric Analysis of Longitudinal Data in Factorial Experiments.

Journal of Statistical Software, *50*(12), 1–23. https://doi.org/10.18637/jss.v050.i12

Pruitt, J. N., & Husak, J. F. (2010). Context-dependent running speed in funnel-web spiders from divergent populations. *Functional Ecology, 24*(1), 165–171. https://doi.org/10.1111/j.1365-2435.2009.01623.x

Riechert, S. E. (1978). Games spiders play: behavioural variability in territorial disputes. *Behavioural Ecology and Sociobiology*, *3*(2), 135–162. https://doi.org/10.1007/BF00294986

Riechert, S. E. (1984). Games spiders play. III: Cues underlying context-associated changes in agonistic behaviour. *Animal Behaviour, 32*(1), 1–15. https://doi.org/10.1016/S0003-3472(84)80318-8

Riechert, S. E., & Hedrick, A. (1990). Levels of predation and genetically based anti-predator behaviour in the spider, *Agelenopsis aperta*. *Animal Behaviour*, *40*(4), 679–687. https://doi.org/https://doi.org/10.1016/S0003-3472(05)80697-9

R Core Team, 2021. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Rizopoulos, D. (2006). ltm: an R package for latent variable modeling and item response analysis. *Journal of Statistical Software, 1*(15), 1–25. https://doi.org/10.18637/jss.v017.i05

Scaccabarozzi, D., Dods, K., Le, T. T., Gummer, J. P. A., Lussu, M., Milne, L., Campbell, T., Wafujian, B. P., & Priddis, C. (2021). Factors driving the compositional diversity of *Apis mellifera* bee venom from a *Corymbia calophylla* (marri) ecosystem, Southwestern Australia. *PLOS ONE*, *16*(6), e0253838. https://doi.org/10.1371/JOURNAL.PONE.0253838

Schendel, V., Rash, L. D., Jenner, R. A., & Undheim, E. A. B. (2019). The diversity of venom: the importance of behaviour and venom system morphology in understanding its ecology and evolution. *Toxins*, *11*(11), 666. https://doi.org/10.3390/toxins11110666

Stankowich, T. (2009). When predators become prey: flight decisions in jumping spiders. *Behavioural Ecology*, *20*(2), 318–327. https://doi.org/10.1093/beheco/arp004

Westermann, F. L., Mcpherson, I. S., Jones, T. H., Milicich, L., & Lester, P. J. (2015). Toxicity and utilization of chemical weapons: Does toxicity and venom utilization contribute to the formation of species communities? Ecology and Evolution, 5(15), 3103–3113. https://doi.org/10.1002/ece3.1595

Wilson, D. (2016). The Venom of Australian Spiders. In & E. D.-G. P. Gopalakrishnakone, G. Corzo, M. E. de Lima (Ed.), *Spider Venoms* (pp. 21–46). Springer Netherlands. https://doi.org/10.1007/978-94-007-6389-0_21

Wilson, D., & Alewood, P. (2004). Australian funnel-web spider venom analyzed with on-line RP-HPLC techniques. In Aguilar MI. (Ed.), *HPLC of Peptides and Proteins. Methods in Molecular Biology* (Vol. 251, pp. 307–322). Springer, Totowa, NJ. https://doi.org/https://doi.org/10.1385/1-59259-742-4:307

Wilson, D., & Alewood, P. F. (2006). Taxonomy of Australian funnel-web spiders using RP-HPLC/ESI-MS profiling techniques. *Toxicon*, *47*(6), 614–627. https://doi.org/10.1016/j.toxicon.2006.01.014

Zhan, Y., Jiang, H., Wu, Q., Zhang, H., Bai, Z., Kuntner, M., & Tu, L. (2019). Comparative morphology refines the conventional model of spider reproduction. *PLoS ONE*, *14*(7). https://doi.org/10.1371/journal.pone.0218486

Zobel-Thropp, P. A., Bulger, E. A., Cordes, M. H. J., Binford, G. J., Gillespie, R. G., & Brewer,

M. S. (2018). Sexually dimorphic venom proteins in long-jawed orb-weaving spiders (*Tetragnatha*) comprise novel gene families. *PeerJ*, *2018*(6), e4691. https://doi.org/10.7717/peerj.4691

4.7. Appendix Chapter 4

Table S4. 1. (a) Output repeated measures ANOVA showing the effect of repetition and stage on size. (b) Output Pearson correlation between stage (juveniles and adults)

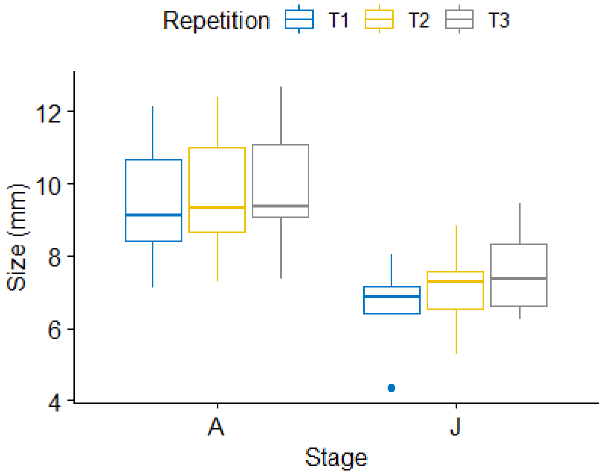
a.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Repetition	2	3.12	1.56	0.736	0.484
Stage	1	76.89	76.89	36.252	0.001 ***
Repetition:Stage	2	0.72	0.36	0.169	0.845
Residuals	48	101.81	2.12		
Signif. codes: 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1**					

b.

t	3.5081
df	16
p-value	0.002
CI	0.278; 0.861
r	0.6593

a.



b.

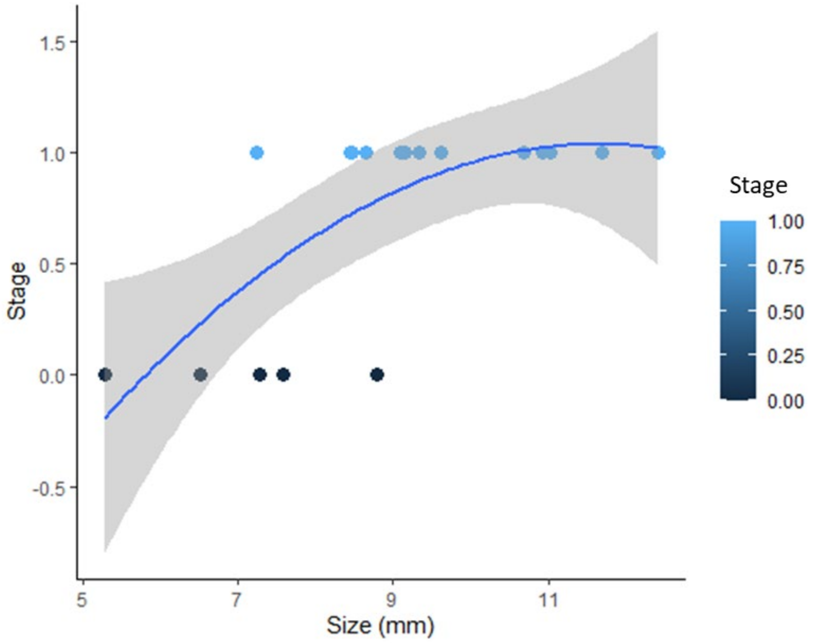


Figure S4.1. (a) Relationship between size and stage (A: adults; J: juveniles) over time. (b) Correlation between Stage and Size. The dark dots represent the juveniles and the clear blue dots represent the adults.

Table S4.2. Behavioural assays were conducted each month for three months. For each assay, individuals were first tested in one context, then rested for a 24 hour rest period before the second context. The presentation of different contexts was randomly selected with the exception of the prod stimulus. This was always the first assay due to venom collection (venom profiles were not included in the study). The colour of conspecific tolerance indicates the pair of spiders that were assessed. Prod stimulus (4), Puff of air (1), Exploration of new territory (3), Conspecific of tolerance (5).

Atrax robustus				
Test I				
Date				
ID	**27/04/2020**	**28/04/2020**	**29/04/2020**	**30/04/2020**
R8	4	1	5	3
R11	4	1	3	5
R7	4	5	3	1
H12B	4	1	5	3
H19B	4	5	1	3

R4	4	5	3	1
H13B	4	5	1	3
R2	4	1	5	3
H17B	4	5	1	3
R5	4	5	1	3
H10B	4	3	1	5
R3	4	3	1	5
H18B	4	1	5	3
H16B	4	3	1	5
R10	4	3	1	5
H15B	4	3	1	5
R1	4	1	3	5
R9	4	3	1	5

Test II

	30/05/2020	**31/05/2020**	**1/06/2020**	**2/06/2020**
R8	4	3	5	1
R11	4	3	5	1
R7	4	1	3	5
H12B	4	1	5	3
H19B	4	1	5	3
R4	4	5	1	1
H13B	4	5	3	1
R2	4	5	1	3
H17B	4	5	1	3
R5	4	3	1	5
H10B	4	1	3	5
R3	4	5	3	1
H18B	4	5	3	1
H16B	4	1	3	5
R10	4	3	1	5
H15B	4	1	5	1
R1	4	3	1	5
R9	4	1	5	3

Test III

ID	**30/06/2020**	**7/01/2020**	**7/02/2020**	**7/03/2020**

R8	4	1	3	5
R11	4	3	5	1
R7	4	3	5	1
H12B	4	3	1	5
H19B	4	1	5	3
R4	4	3	5	1
H13B	4	5	1	3
R2	4	5	3	1
H17B	4	5	3	1
R5	4	5	3	1
H10B	4	3	5	1
R3	4	1	5	3
H18B	4	5	1	3
H16B	4	5	3	1
R10	4	5	3	1
H15B	4	3	5	1
R1	4	3	5	1
R9	4	5	3	1

Table S4.3. For each group (stage), the rank mean of overall rank, observations (Nobs) and the point estimates of the relative treatment effect (RTE) are displayed for each behavioural variable

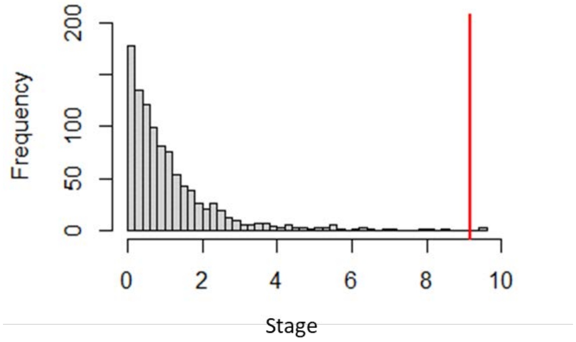
Model (aggression): F1LDF1	**Frequency of fang movements ~ Repetition + Stage, subject= factor (ID)**		
Relative Treatment Effect (RTE): defensive behaviour conspecific and predators			
	RankMeans	**Nobs**	**RTE**
StageA	29.307	39	0.533
StageJ	22.800	15	0.412
RepetitionT1	12.884	18	0.229
RepetitionT2	25.330	18	0.459
RepetitionT3	39.946	18	0.730
Relative Treatment Effect (RTE): antipredator behaviour puff of air			
	RankMeans	Nobs	RTE
StageA	30.794	39	0.561
StageJ	18.933	15	0.34
RepetitionT1	26.150	18	0.475
RepetitionT2	22.776	18	0.412
RepetitionT3	25.665	18	0.466
Relative Treatment Effect (RTE): defensive behaviour against conspecifics (climb)			

	RankMeans	Nobs	RTE
StageA	26.269	39	0.477
StageJ	30.700	15	0.559
RepetitionT1	26.292	18	0.477
RepetitionT2	30.923	18	0.563
RepetitionT3	28.238	18	0.513
Relative Treatment Effect (RTE): activity			
	RankMeans	Nobs	RTE
StageA	27.743	39	0.504
StageJ	26.866	15	0.488
RepetitionT1	23.476	18	0.425
RepetitionT2	31.330	18	0.570
RepetitionT3	27.107	18	0.492

a.

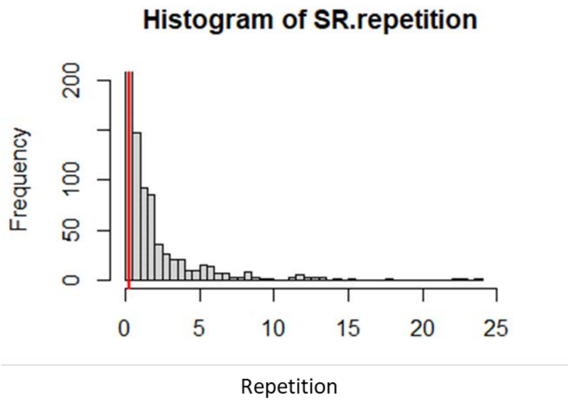
Stage statistic (observed value) = 9.157

PvalueR Stage (simulated value) = 0.002



Repetition statistic (observed value) = 0.2697

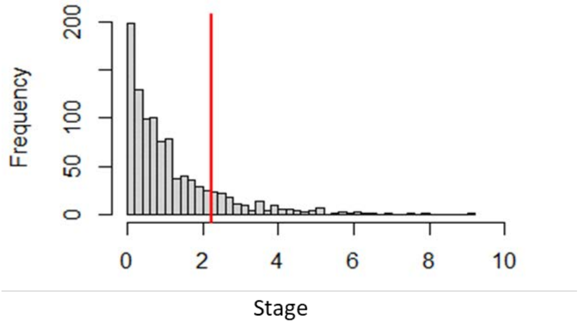
Pvalue Repetition (simulated value) = 0.635



b.

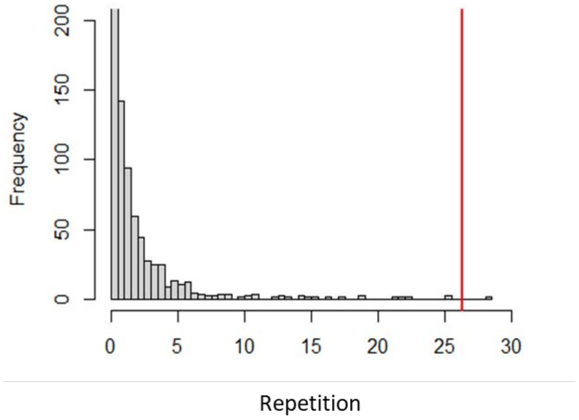
Stage statistic (observed value) = 2.247

P-value for Stage (simulated value) = 0.147



Repetition statistic (observed value) = 0.001

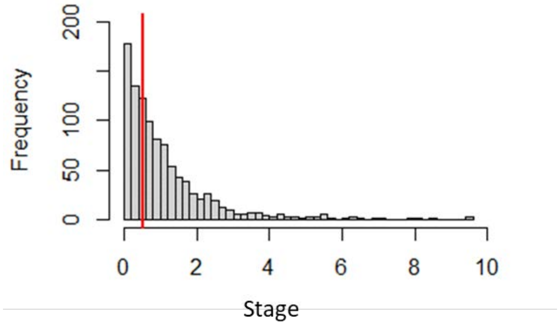
P_value Repetition (simulated value) = 26.308



c.

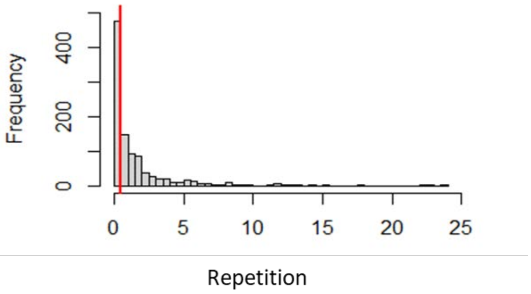
Stage statistic (observed value) = 0.516

Pvalue Stage (simulated value) = 0.613



Repetition statistic (observed value) = 0.469

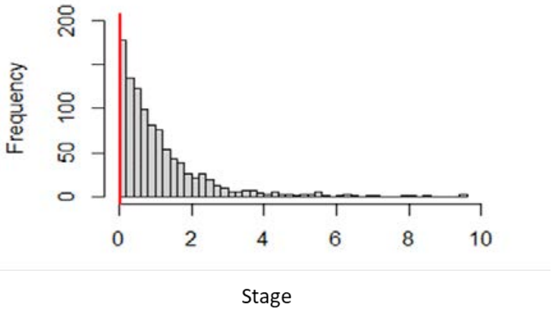
P_value Repetition (simulated value) = 0.536



d.

Stage statistic (observed value) = 0.0251

P_value for Stage (simulated value) = 0.972



Repetition statistic (observed value) = 1.323

P_value Repetition (simulated value) = 0.314

Figure S4.2. Randomization tests using nparLD to validate the hypotheses related to the effects of repetition and differences between stages over each behavioural variable in each context. The randomization test was run 1000 times. When the simulated value does not fall inside the distribution of the observed value (statistic), the probability of finding differences between stages, behaviours and repetitions are not expected by chance. In contrast, if the simulated value falls on, or overlaps with, the observed value, it means that the result obtained is by chance. a) Randomization test for antipredator behaviour (puff of air), testing the effects of the variables stage and repetition. b) Randomization test for defensiveness (prod stimulus), testing the effects of the variables stage and repetition. c) Randomization test for territory defence, testing the effects of the variables stage and repetition. d) Randomization test for exploration of a new territory, testing the effects of the variables stage and repetition.

Chapter 5: Exploring behavioural traits over different contexts in four species of Australian funnel-web spiders

JOURNAL ARTICLE ACCEPTED MANUSCRIPT

Exploring behavioural traits over different contexts in four species of Australian funnel-web spiders

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5.1. Abstract

Australian funnel-web spiders are arguably the most venomous spiders in the world, with much research focusing on this aspect of their biology. However, other aspects related to their life history, ecology and behaviour have been overlooked. For the first time, I assessed repeatability, namely risk-taking behaviour, aggressiveness and activity in the contexts of predation, conspecific tolerance and exploration of a new territory in four species of Australian funnel-web spiders: two are closely related, *Hadronyche valida* and *H. infensa*, and two have overlapping distributions but occupy different habitats, *H. cerberea* and *Atrax robustus*. I also compared behaviours between species. At the species level, I found that *H. valida* showed consistency in risk-taking behaviour when exposed to a predator stimulus, aggressiveness against conspecifics, and exploration of a new territory. In contrast, in the other species, only *A. robustus* showed repeatability in the context of exploration of a new territory. These results suggest that some behavioural traits are likely more flexible than others, and that the repeatability of behaviours may be species-specific in funnel-webs. When I compared species, I found differences in risk-taking behaviour and defensiveness. This study provides novel insights to understanding variation in behavioural traits within and between species of funnel-web spiders, suggesting that some behavioural traits are likely context- and/or species-

dependent, as a result of their evolutionary history. These findings provide key insights for understanding the ecological role of behaviour and venom deployment in venomous animals, and a greater understanding of behaviour in these medically significant and iconic spiders that are of conservation concern.

5.2. Introduction

Australian funnel-web spiders (Araneae: Mygalomorphae: Atracidae) are endemic species from eastern Australia (Gray, 2010; Opatova et al., 2020). Funnel-webs are mostly forest ground-dwelling species, although some species do occur in other habitat types, such as montane herbland, open woodlands and closed forests (Gray, 2010). Their microhabitat can vary from ground burrow retreats to logs, rocks, stumps, trees, and sand (Gray, 2010). Funnel-webs are considered the most venomous spiders in the world because they can cause severe envenomation in humans, which is life-threatening (Isbister et al., 2005, 2015). More recently, funnel-web venom has also been a focus of research because individual components from different species possess therapeutic (Chassagnon et al., 2017; Ikonomopoulou et al., 2018) and natural bioinsecticide properties (Herzig & King, 2015; Windley et al., 2012).

While funnel-webs are iconic species, the majority of studies, including studies relating to their evolutionary history, have focused only on venom properties (Nicholson & Graudins, 2002; Alewood et al., 2003; Herzig et al., 2020). Despite multiple studies on venom properties, the ecological function of venoms remains largely unexplored, and it is surprising that behaviour and ecology have not been included in toxinology studies, particularly as both play a critical role in the evolution and diversification of venoms (Schendel et al., 2019; Hernández Duran et al., 2021). The study of behaviour is critical for understanding the complexity and variation of venom components because behavioural traits act synergistically with other intrinsic (genetics, physiology, neurohormones) and extrinsic factors (environment, experience, level of sociability) to affect the variation, abundance, and function of toxin molecules (Hernández Duran et al., 2021; Nelsen et al., 2014; Schendel et al., 2019; Wullschleger & Nentwig, 2002). For example, in *Apis mellifera*, both ecological factors (temperature, plant flowering stage) and behavioural responses to a venom stimulating device affected the protein composition of bee venom in the species (Scaccabarozzi et al., 2021).

For funnel-web spider behaviour in particular, only anecdotal or field collection observations have been registered previously (Levitt, 1961; Gray, 1987; Gray, 1981, 1992). This is surprising because behaviours associated with venom deployment, such as antipredator behaviour (Dutertre et al., 2014; Nelsen et al., 2014; Arbuckle, 2017; Hernández Duran et al., 2022), defensive and aggressive behaviours (Nekaris et al., 2020), courtship and mating (Arbuckle, 2017), among others, affect fitness, as venomous animals depend on venom for the acquisition of resources and protection (Boevé et al., 1995; Nelsen et al., 2014; Cooper et al., 2015; Zobel-Thropp et al., 2018; Schendel et al., 2019; Herzig et al., 2020). Furthermore, funnel-webs are short-range endemic species, meaning they have a higher risk of extinction due to their restricted ability to disperse and specific habitat conditions, which makes them vulnerable to habitat loss (i.e., fires, floodings, urbanisation; Mason et al., 2018). Therefore, given their potential conservation significance, it is surprising that ecological and behavioural studies have ignored these spiders in general.

Two particularly relevant avenues for behavioural studies in spiders are repeatability, or consistency, of behaviours and behavioural flexibility. Repeatability, or temporally consistent individual differences in behaviour (Wolf & Weissing, 2012), could explain how spiders use different resources, such as venom, to respond or adapt to different environmental conditions or threats (e.g., high predation risk or conspecific competition). Different spider behavioural types (defined as differences in behavioural traits among individuals), such as aggressive-docile (Gosling, 2001), are likely the result of intrinsic (i.e., physiological traits such as venom, neurohormones, DiRienzo, McDermott, & Pruitt, 2015; metabolic rate, Shearer & Pruitt, 2014; silk production; Wullschleger & Nentwig, 2002) and extrinsic factors (i.e., environment and niche specialisation; Kralj-Fišer, Hebets, & Kuntner, 2017; Kralj-Fišer & Schneider, 2012) that work synergistically to affect spider survival (Chang et al., 2017; Hernández Duran et al., 2021). For example, in the grass spider, *Agelenopsis aperta* (Agelenidae), females show different levels of aggression across different contexts (i.e., reproduction, prey capture, and conspecific interaction). More aggressive females are able to secure higher quality territories and capture more prey, but also suffer higher mortality (Riechert & Hedrick, 1993). The balance of different behavioural types in a population could affect the ability of spiders to adapt to, and colonise, new environments.

Behavioural flexibility, which is compatible with repeatability, allows individuals to modulate their behaviour across different contexts and conditions over time. While repeatability might be a consequence of strong selection pressure (i.e., changes in repeatability leading to higher fitness costs, Halpin & Johnson, 2014), behavioural flexibility may occur when species are exposed to variable selection pressures (i.e., individuals can exhibit distinct flexibility levels for different behavioural traits; Briffa et al., 2008 ; Kralj-Fišer & Schneider, 2012; Sih, Bell, & Johnson, 2004). The constraints that limit behavioural flexibility include morphology, physiology, and environmental conditions (Briffa et al., 2008). For example, in the black widow spider, *Latrodectus hesperus* (Theridiidae), plasticity and repeatability are both present when a specific context is assessed, and both are part of an individual's collective behaviours (Halpin & Johnson, 2014). This suggests that the strength of plasticity and consistency may be the result of a trade-off between costs and benefits to cope with different conditions (Briffa et al., 2008). In spiders, both behavioural plasticity and consistency have implications for the use of resources, limiting or increasing the ability to colonise new environments (Kralj-Fišer et al., 2017; Kralj-Fišer & Schneider, 2012), as well as using or constraining the use of their biological weapons (Nelsen et al., 2014).

Given that different species of Australian funnel-web spiders can be found along a continuous gradient of habitats, I explored different behavioural characteristics in females of four different species (*Hadronyche infensa*, *H. valida*, *H. cerberea*, and *Atrax robustus* - all species belong to the Atracidae family that includes the *Atrax* and *Hadronyche* genera; Hedin et al., 2018) across different ecological contexts (response to predation, conspecific tolerance, and exploration of a new territory) to assess the consistency (i.e., repeatability) of spider behaviours over time. In addition, we compared behaviours across species, firstly because of the general public assumption that all funnel-webs behave the same (i.e. aggressively), and secondly because their ecologies suggest differing selection pressures that could affect their behaviours, venom composition and, ultimately, overall fitness. Each species occupies a different microhabitat, although some have overlapping distributions. *H. infensa* and *H. valida* belong to the *infensa* species group (Gray, 2010). Both species have overlapping distributions (highlands ground-dwelling), show similar morphological characteristics, and share similar venom components, although each also has distinctive venom molecules (Hernández Duran et al., 2020). *H. cerberea* belongs to the *cerberea* group, and is a tree-dwelling species. In contrast to the other species of *Hadronyche, H. cerberea* shows distinct morphological characteristics (Gray, 2010) and venom profiles. Finally, *A. robustus*, most famously known for the lethal neurotoxin (δ-hexatoxin-Ar1a) found in the venom of mature males (Alewood et al., 2003; Klint et al., 2012; Nicholson et al., 1996; Nicholson & Graudins, 2002), shares an overlapping distribution with *H. cerberea* in the Sydney and Central Coast regions of New South Wales (Gray, 2010). However, unlike *H. cerberea*, *A. robustus* is a ground-dwelling spider, similar to *H. valida* and *H. infensa*.

I predicted that the closely related species, *H. infensa* and *H. valida*, would show similar and consistent behavioural responses across all different contexts due to both species sharing similar habitats and remaining confined in their ground burrows. For *H. cerberea*, given the characteristics of its arboreal habitat and exposure to predators, such as birds, I expected more flexible risk-taking behaviours and aggressiveness towards predator stimuli and conspecifics. However, I predicted consistency in activity due to confinement in their tree burrows. Finally, because *A. robustus* are regularly found in urban and sub-urban environments (Gray, 1992), I expected more flexible behaviour in response to predator stimuli due to their habitat being more open and exposed to predators and other threats. However, I predicted consistency in aggressiveness against conspecifics and in activity because, as for the other species, *A. robustus* females are confined to a burrow. When comparing between species, I expected that if *A. robustus* is indeed more defensive than other species (based on anecdotal evidence), then *A. robustus* would show higher frequencies of fang movements (defensive behaviour), higher frequencies of climbing, resume their movements faster and be more active compared to *Hadronyche* species.

5.3. Methods

5.3.1. Study species and locations

A total of 75 spiders were used in this study. Females are ground burrow-dwellers (*H. valida*, *H. infensa* and *A. robustus*) or tree-dwellers (*H. cerberea*). Burrow-dwellers can dig burrows of more than 1 m deep (pers. obs.). Therefore, it is difficult to know whether the spider being collected is an adult or juvenile prior to collection. Digging up an individual spider can take hours, so I collected any spiders I could find, regardless of size or stage, which led to unbalanced sample sizes. Twenty-three adult female *H. valida* (collected by manual excavation of burrows in the Currumbin Valley and Mount Tamborine) were purchased from Thargomindah Man Productions in 2019 (Varsity Lakes, QLD, Australia). Sixteen adult female *H. infensa* were collected manually in Toowoomba and Ravensbourne in 2019 (collection permit SA 2016/08/55). Eighteen *H. cerberea* (nine adult females; nine juveniles) were collected across three *Eucalyptus regnans* in Gosford, New South Wales in 2019. Eighteen *A. robustus* (thirteen adult females; five juveniles) were collected from the Gosford/Central Coast region, New South Wales (collection carried out in cooperation with the Australian Reptile Park). The spiders were transported alive in plastic containers with damp cotton wool to the laboratory of the Australian Institute of Tropical Health and Medicine (AITHM), James Cook University Nguma-Bada (Cairns) campus, Queensland, Australia.

Spiders were kept individually in 5 L plastic containers with a 7 cm layer of coconut peat (L: 25 cm; W: 17.5 cm; H: 10 cm) in a climate-controlled room (temperature: 20 ± 2 °C; relative humidity: 60%) on a reverse light: dark cycle (12L:12D; lights on at 6 pm). Funnel-web spiders are solitary in nature, thus solitary housing is reflective of their natural social organisation. The spiders were acclimated for one month before the start of behavioural assays (see below). Each spider received one house cricket, *Acheta domestica*, once a week. Compared to Araneomorph spiders, funnel-web spiders take a long time to mature and have a long life span. For example, males reach maturity between two and four years, while females can live for more than ten years (Levitt, 1961; Gray, 1992). We did not include adult males in the study because once they reach maturity, they leave the burrow and wander in search of females (Bradley, 1993). Furthermore, males have a significantly shorter life span than females, particularly in captivity, making it difficult to obtain repeated behavioural measurements over extended periods. Adult females were identified by epigyne sclerotisation and the opening of the epigastric furrow (gonoslit, Zhan et al., 2019). Cephalothorax width was measured to assess spider size. It is impossible to tell males and females apart prior to the final moult to maturity.

5.3.2. Behavioural tests

Following the one-month acclimation period, food was withheld from the spiders for two weeks before behavioural assays commenced, and the same procedure was repeated before each bout of testing. I withheld the food to control for venom state and to ensure all individuals were at the same motivational state prior to behavioural tests. I assessed aggressiveness, risk-taking behaviour and activity of each individual of each species in the context of predation (two separate tests), conspecific tolerance and exploration of a new territory (see below). Individuals were tested separately and tests were conducted on consecutive days (spiders were rested for 24 h between tests). The order of tests was randomised for each spider, with the exception of the prod test (see below), which always came first due to collection of venom samples (which we did not include here). Behavioural tests were repeated three times per individual, separated by one month between testing sessions, to measure repeatability. All assays were carried out between 6 am and 12 pm during the dark phase, and were video recorded under red light. Behaviours were analysed using the behavioural analysis software BORIS version 7.8.2. (Friard & Gamba, 2016).

Predation context

The willingness of a spider to take risks was assessed using an aversive stimulus (a puff of air), which represents an air current that resembles the wing beat movements of a predator (e.g., a bird) or a parasite (e.g., a wasp; Keiser, Lichtenstein, Wright, Chism, & Pruitt, 2018; Riechert & Hedrick, 1993). Following the methodology described in Hernández Duran et al. (2022), I gently moved the spider out of its retreat, and waited 60 s before commencing the test. Then, each spider received three rapid puffs of air to the anterior prosoma using a camera air blower. Thereafter, I recorded the spider’s huddling behaviour (a standard antipredator posture;

Riechert & Hedrick, 1993; DiRienzo et al., 2015; Parthasarathy et al., 2022) for 420 s, following the modified protocol of Riechert & Hedrick (1993) and Shearer & Pruitt (2014). Huddling was recorded as a binary response because the distribution of the data suggested a binomial distribution. Spiders that did not resume movement within 15 s of receiving the stimulus (i.e., that remained huddled) were assigned 0 (designated low risk-taking individual), while those that moved within 15 s were assigned 1 (designated high risk- taking individual).

Aggression against conspecifics and heterospecifics is common in spiders (Keiser et al., 2018). As for the puff test, I gently moved the spider out of its retreat, and waited 60 s before commencing the test. Following the protocols outlined in Hernández Duran et al. (2020) and Hernández Duran et al. (2022), I gently prodded the first pair of legs with blunt tweezers for 240 s, and then recorded each individual's behaviour for a further 360 s (600 s in total). Australian funnel-webs respond to threating stimuli by raising the forelegs, moving the fangs and expelling venom directly on the fangs (Wilson & Alewood, 2004, 2006). I collected expelled venom from aggravated individuals during the first 240 s using a 200 µL Gilson P200 pipette with polypropylene micropipette tips (Hernández Duran et al., 2020). I did not collect venom after the second 360 s. As there was a strong positive correlation between lifting the first pair of legs and fang movements, and a negative correlation between huddling and fang movements in *A. robustus* (Hernández Duran et al., 2022), I used the number of fang movements as a measure of the aggressive response in all species. Spiders that showed a greater number of fang movements were considered aggressive, while spiders that showed fewer fang movements were considered submissive.

Conspecific tolerance

Aggressiveness and boldness can affect spider survival and fitness during foraging and mating (Riechert & Hedrick, 1990). In funnel-webs, female retreats are often located close to each other (Gray, 1992), indicating that burrow defence, mate choice, and food competition are likely occurring between females. To assess conspecific tolerance, following the methodology of Hernández Duran et al. (2022), I placed two individuals in a new container (L: 23 cm; W: 16.5 cm; H: 10.5), divided into two equal halves with a mesh barrier. This reduced the risk of fighting and death, but permitted detection of olfactory and mechanical cues. I randomly selected pairs of similar-sized spiders and video recorded the whole arena for 1800 s. I measured aggressiveness against conspecifics as the number of times the spider climbed the barrier. Aggressive behaviours (e.g., leg waving and lunging) were observed when spiders climbed the barrier, but not when moving around the arena, indicating that climbing behaviour occurs as a direct response to other individuals rather than an attempt to escape.

Exploration of a new territory

Spiders can disperse or relocate burrows to reduce predation risk and/or increase the chance of prey capture (Bengston et al., 2014; Nakata & Ushimaru, 2013). To assess exploration of a new territory (fear of novel objects or environment, Bengston et al., 2014; Réale, Reader, Sol, McDougall, & Dingemanse, 2007), I assessed the activity level of individuals in a new arena (L: 34.5cm; W: 22 cm; H: 10.7cm) by measuring the total time the spider spent moving around the novel arena for 900 s (Hernández Duran et al. 2022).

Statistical analyses

All statistical analyses were conducted using R (version 4.1.0, Core R Team 2021). We had both adult females and juveniles for only two species, *H. cerberea* and *A. robustus*. Following Hernández Duran et al. (2022), I tested for stage effects in these two species on the magnitude of individual behavioural measurements (risk-taking behaviour and aggressiveness) between adults and juveniles in each context and over time using rank-based non-parametric analyses for longitudinal data (Table S5.1). These analyses offer a robust framework for non-continuous variables, small sample sizes and skewed data (Noguchi et al., 2012). The design used was F1-LD-F1 in the nparLD package (Noguchi et al., 2012), where the first F1 refers to the number of factors in each group (juveniles and adults; whole-plot factor group) of each species. LD specifies the nature of the data (i.e., longitudinal), and the second F1 refers to the time level (sub-plot factor). I included the random effect of individual identity as a subject in this model. I did not observe differences between adults and juveniles for either *H. cerberea* or *A. robustus* for behavioural measurements and contexts (Table S5.1). Therefore, I pooled juvenile and adult data for these two species to calculate repeatability within species and then to compare differences between species.

To assess repeatability in risk-taking behaviour and aggressiveness across different contexts (Dingemanse & Wright, 2020; Nakagawa & Schielzeth, 2010; Schielzeth et al., 2020; Stoffel et al., 2017), I used separate generalised linear-mixed effect models (GLMM) for each species separately using the package glmmADMB (Stoffel et al., 2017). For risk-taking behaviour (puff of air), given the distribution of the data, I used a binomial distribution with a logit-link function (1= moved, 0 = huddled). For aggressiveness in the context of predation (frequency of fang movements) and conspecific tolerance (frequency of climbs), I used a negative binomial distribution with a log-link function. For activity in a novel field, I used a gamma distribution (log-link function) for *H. infensa*, *H. valida* and *A. robustus*. However, I could not calculate activity for *H. cerberea* because only three out of 18 individuals (17%) moved. There is individual variation in the size at which individuals moult to maturity, so I included size as a continuous predictor of stage, in preference to a categorical predictor of stage (Padilla et al., 2018; Santana et al., 2017). Therefore, in all models, the behaviour (huddling, frequency of fang movements, frequency of climbs and activity) was the response variable, size was a continuous fixed effect, and spider identity (ID) was included as a random effect. I report effect sizes for all tests (due to relatively small sample sizes).

For each species, I determined the proportion of variance explained by the fixed effects (marginal R^2), the proportion of variance explained by both the fixed and random effects (conditional R^2), and the intra-class correlation (ICC) without (see Supplementary material Table S5.2a; ICC:Null-model) and with fixed effects (see Supplementary material Table S5.2a; ICC: Full-model) following Nakagawa & Schielzeth (2013) and Stoffel et al. (2017). For models with negative binomial and gamma distributions, I used the trigamma function to calculate the marginal and conditional R^2, as well as the ICC (Nakagawa et al., 2017). I tested the significant among-individual variation using likelihood ratio tests (LRT), comparing the model with the individual random effect to a model without it (Pinheiro & Bates, 2000; Stoffel, Nakagawa, & Schielzeth, 2019). To obtain the observation level variance in the negative binomial and gamma models, I used the trigamma function, whereas for the binomial model, I report the latent and link distribution specific variance (Nakagawa et al., 2017).

To compare changes in behaviour between species, I used separate generalised linear-mixed effect models (GLMM) for each context. I used the same variable response (behaviour), and random effect (ID) as for previous models, but I included species, size and repetition, as fixed effects. In the activity context, I excluded *H. cerberea* from the species comparisons. To compare repeatability of behaviours between species, I calculated the mean standardization of each model to determine the magnitude of variation (VI; Dochtermann & Royauté, 2019).

5.4. Results

5.4.1. Within species behavioural variation

Risk-taking behaviour in a predatory context

H. valida showed consistent individual differences in risk taking behaviour over time when individuals received a puff of air (%$R_{(\text{null.model})}$ = 25.360; P = 0.001; Table S5.2a). In contrast, we did not observed repeatability for *H. cerberea* (%$R_{(\text{null.model})}$ = 0. 005; P = 0.987; Table S5.2a), *H. infensa* (%$R_{(\text{null.model})}$ = 0.000; P = 0.996; Table S5.2a), or *A. robustus* (%$R_{(\text{null.model})}$ = 1.000; P = 0.952; Table S5.2a). Size did not have a significant effect on risk taking behaviour in any of the four species (*H. valida*: Z =1.27, SD = 1.11, P = 0.200; *H. cerberea*: Z = 1.26, SD = 0.01, P = 0.209; *H. infensa:* Z = 0.68, SD = 0.00, P = 0.500; *A. robustus*: Z = 0.27, SD = 0.30, P = 0.780).

Aggressiveness in a predatory context

None of the four species (*H. valida:* %$R_{(\text{null.model})}$ = 0.000, P = 0.999; *H. cerberea*: %$R_{(\text{null.model})}$ = 26.700, P = 0.068; *H. infensa*: %$R_{(\text{null.model})}$ = 0.000, P = 0.985; *A. robustus*: %$R_{(\text{null.model})}$ = 0.000, P = 0.987, Table S5.2a) showed consistent individual variation in aggression (frequency of fang movements) over time. Similarly, size did not have a significant effect on aggression in any of the three species (*H. valida*: Z = 0.03, SD = 0.00, P = 0.980; *H. cerberea*: Z = -0.01, SD = 0.04, P = 0.990; *H. infensa:* Z = 0.13, SD = 0.00, P =0.901), with the exception of *A. robustus,* which showed a marginal significance (Z = 2.00, SD = 0.04, P = 0.050).

Aggressiveness in the context of conspecific tolerance

For aggressiveness measured in the context of conspecific tolerance, *H. valida* showed consistent individual variation in aggression (%$R_{(\text{null.model})}$ = 22.482; P = < 0.001; Table S5.2a). However, neither *H. cerberea* (%$R_{(\text{null.model})}$ = 7.101; P = 0.201; Table S5.2a), *H. infensa* (%$R_{(\text{null.model})}$ = 0.000; P = 0.992; Table S5.2a) nor *A. robustus* (%$R_{(\text{null.model})}$ = 3.280; P = > 0.203; Table S5.2a) showed repeatability in aggression. In addition, size did not have a significant effect on aggressiveness in the context of conspecific tolerance in any species (*H.*

valida: Z = -1.22, SD = 1.09, $P = 0.220$; *H. cerberea*: Z = -0.59, SD = 0.56, $P = 0.560$; *H. infensa:* Z = -0.04, SD = 0.00, $P = 0.970$; *A. robustus*: Z = -0.41, SD = 0.55, $P = 0.160$).

Activity in the context of exploration of a new territory

H. valida ($\%R_{\text{(null.model)}} = 37.401$; $P < 0.001$; Table S5.2a) and *A. robustus* ($\%R_{\text{(null.model)}} = 40.489$; $P < 0.001$; Table S5.2a) showed repeatability for activity over time. Both species showed close average values in activity (*H. valida:* $\overline{x} = 131.507$; *A. robustus*: $\overline{x} = 91.857$), and similar repeatability values and magnitudes of among-individual variance relative to their means (*H. valida*: $I_{\text{I}} = 31.649$; *A. robustus*: $I_{\text{I}} = 34.129$). For *H. infensa*, I did not observe repeatability in activity ($\%R_{\text{(null.model)}} = 0.000$; $P = 0.068$; Table S5.2a). There was no significant effect of size on activity in the context of exploration of a new territory in the three species (*H. valida*: Z = 0.05, SD = 1.90, $P = 0.960$; *H. infensa:* Z = 0.31, SD = 0.01, $P = 0.750$; *A. robustus*: Z = 0.66, SD = 1.80, $P = 0.510$).

5.4.2. Between species behavioural variation

Risk taking behaviour in a predatory context

When I compared huddling behaviour between species, I found significant differences between all species (Table S5.2b, Figure 5.1). In general, *A. robustus* resumed their movements faster in response to a puff of air (antipredator stimulus), followed by *H. cerberea*, *H. valida*, and *H. infensa* (Table S5.2b, Figure 5.1). Repetition and size did not have a significant effect on huddling behaviour (Table S5.2b).

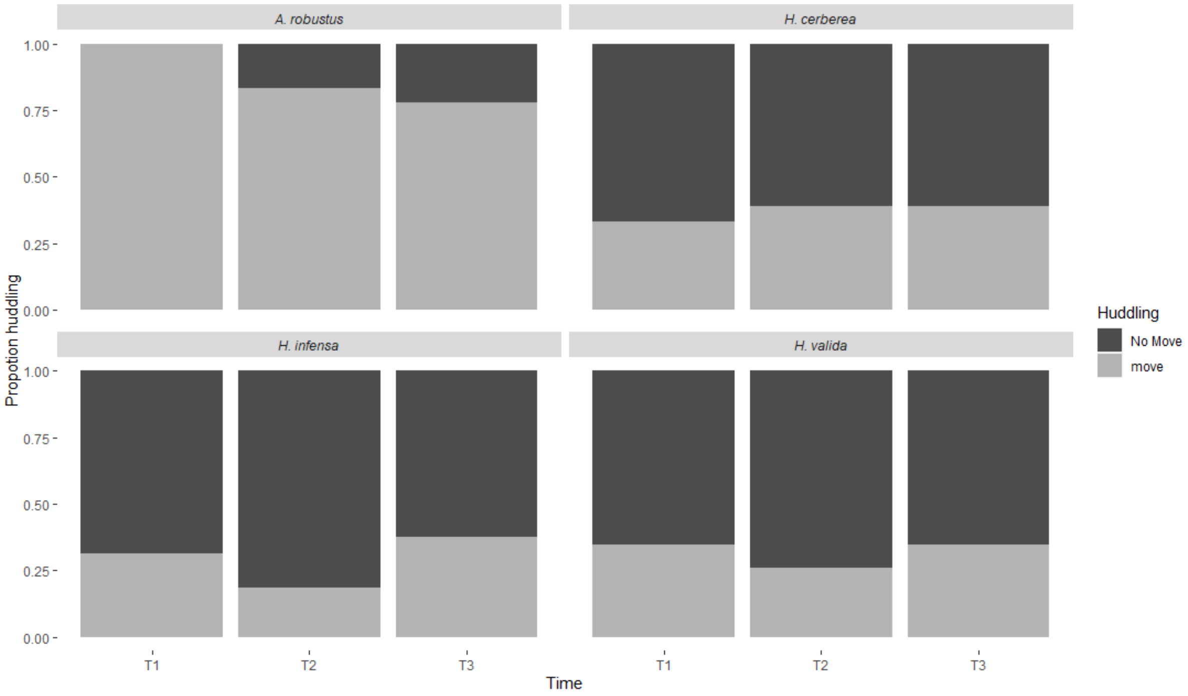


Figure 5.1. Proportion of individuals huddling when a puff of air was applied to the prosoma for each of four Australian funnel-web spider species over three time periods (T1: repetition one, T2: repetition two; T3: repetition three). The dark grey bars represent the proportion of spiders that moved, while the light grey bars represent the proportion of spiders that did not move.

Defensive behaviour in a predatory context

For frequency of fang movements in a predatory context, I found a significant difference between repetitions (Table S5.2b), with an overall increase in the frequency of fang movements noted from T1 to T3, irrespective of spider species or size (Figure 5.2). No other factors had a significant effect on aggressiveness (Table S5.2b).

Defensive behaviour in the context of conspecific tolerance

For frequency of climbing in conspecific tolerance between species, I found no significant differences between species. Similarly, repetition and size did not have a significant effect on the frequency of climbing (Table S5.2b).

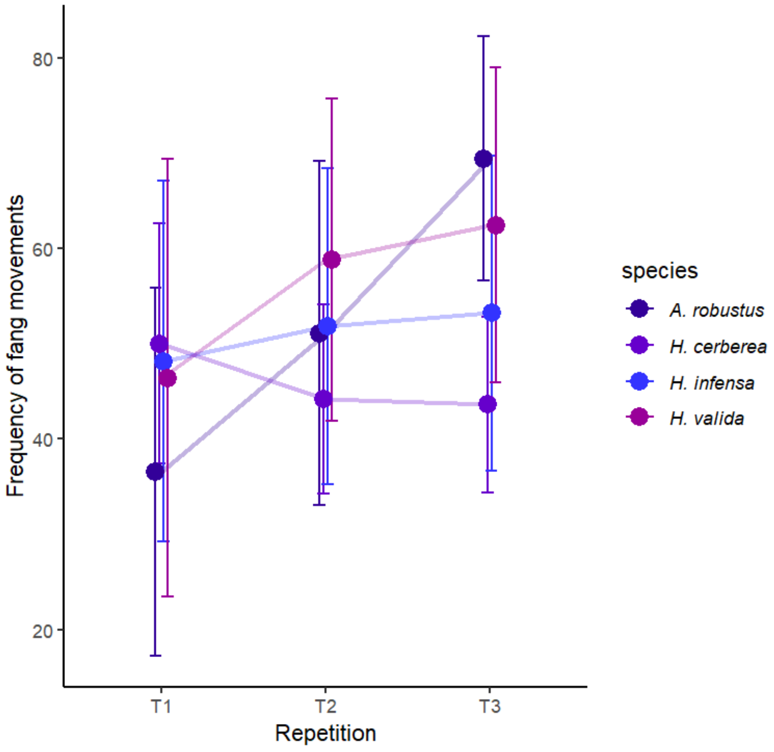


Figure 5.2. Frequency of fang movements (defensive behaviour) for each of four Australian funnel-web spider species in response to a prod stimulus over three time periods (T1: repetition one, T2: repetition two; T3: repetition three).

Activity in the context of exploration of a new territory

For time spent moving around the arena, I found no significant differences between species, repetitions or size classes (Table S5.2b).

5.5. Discussion

Different ecological contexts (i.e., foraging, mating, exploration) affect the ways in which animals respond to their environments, either adjusting their behaviour or maintaining consistent behaviour over time (i.e., repeatability, Chang et al., 2019). My study contributes to a broader understanding of intra- and inter-individual variation in Australian funnel-web spiders, which provides insights into the ability of individuals to respond to different environmental conditions, and potentially to use their biological weapons, namely silk and venom (Hernández Duran et al., 2021). Here, I assessed the repeatability of aggressiveness and risk-taking behaviour in different contexts in four species of funnel-web spider over time.

Hadronyche valida showed repeatability in risk-taking behaviour (when individuals were exposed to an antipredator stimulus), aggressiveness (when individuals were exposed to conspecifics), and activity (when individuals were placed in a new territory). These findings suggest that development, and previous experience (Liedtke et al., 2015) in a particular environment (i.e., with predators, conspecifics) or in different contexts (Hernández Duran et al., 2021, 2022) may favour the maintenance of these behaviours over time in this species. In contrast, when I assessed temporal repeatability in *H. infensa*, *H. cerberea* and *A. robustus* across different contexts, only *A. robustus* showed repeatability in activity in the context of exploration of a new territory. The lack of repeatability in aggressiveness and risk-taking behaviour suggests that these three species are not likely constrained in their behavioural responses, rather responding dynamically to different situations (Köhler & Vollrath, 1995). Furthermore, these results also suggest species-specific responses, which is consistent with findings reported in other taxa (e.g., corvids, Vernouillet & Kelly, 2020).

Species often show differences in behavioural responses (Bengston et al., 2014; Vernouillet & Kelly, 2020), which could be a consequence of differences in the ecology and habitat complexity of each species (Bell, 2007). For example, *H. cerberea* is arboreal, whereas the other three species are ground-dwelling (Gray, 2010). The tree crevices where *H. cerberea* live may reduce or constrain the movement of individuals, which could explain why they barely moved when placed into an open environment. Interestingly, while I found repeatability for behaviour in *H. valida*, I did not find the same response for *H. infensa*, even though these species belong to the same species group and experience similar broad ecological conditions. These results suggest that previous experience with the specific microhabitats in which these spiders occurred prior to collection may affect individual behavioural responses for extended periods, even when the spiders were kept under constant laboratory conditions for a period of time. However, the lack of repeatability in *H. infensa* might be affected by sample size (as suggested by the marginal value, see results).

Differences in behaviour between life stages may be a direct consequence of changes in internal developmental processes (Bengston & Jandt, 2014). However, other extrinsic factors, such as experience and/or microhabitat conditions may also feedback to affect intrinsic physiological processes during development, which could explain why spiders of different ages respond behaviourally to different stimuli. I assessed behavioural differences between stages in *H. cerberea* and *A. robustus*, using size as a proxy of stage. I did not find differences in risk-taking behaviour and aggressiveness in any context. The results are consistent with those observed for nymphs and adults of the cockroach, *Diploptera punctata*, where behaviours remain consistent across life stages (Stanley et al., 2017). The lack of difference in behavioural response between stages may be a result of both stages sharing similar environments and foraging strategies (Stanley et al., 2017). However, these similarities in behaviour between juveniles and adults in *H. cerberea* and *A. robustus* may only be temporarily stable during particular parts of the life stage (Parthasarathy et al., 2019). For example, juveniles (third and fourth instar) of the social spider, *Stegodyphus sarasinorum*, showed lower repeatability values in boldness and aggressiveness compared to subadults (Parthasarathy et al., 2019). Additionally, repeatability for both juveniles and adults tended to decline over longer periods of time (Parthasarathy et al., 2019). To determine whether or not funnel-webs show consistency during particular stages will require further research, which will take several years, given their long lifespans and relatively long periods required to reach maturity (Gray, 1992; Levitt, 1961).

In dangerous situations, such as under high predation risk, an animal that adjusts its behaviour in response to the stimulus (Chang et al., 2019) may have a greater chance of survival. I did not find repeatability in aggressiveness in any of the funnel-web species in response to a direct predation threat (i.e., physical prodding, Jackson & Pollard, 1990; Stankowich, 2009), suggesting that spiders dynamically adjust or modulate behaviours in response to this type of stimulus. Funnel-web spiders showed increasing fang movements over time, indicating an increasing aggressive response over time. This flexibility in behaviour is consistent with responses to the prod test in other species of spiders. For example, under laboratory conditions, the black widow spider, *Lactrodectus hesperus*, modulates its defensive behaviour using venom and dry bites depending on the level of threat to which it is exposed (Nelsen et al., 2014). Different types of stimuli may also affect the ways in which spiders respond to a threatening situation. For example, in daddy long-legs spiders, *Pholcus phalangioides*, individuals reacted differently to a mechanical stimulus (i.e., whirled for longer periods) compared to a puff of air (Jackson & Pollard, 1990). In other arachnids, the behavioural response is also affected by the type of stimulus; in the southern unstriped scorpion, *Vaejovis carolinianus*, males and females perceived prods to the prosoma as the most threatening, and increased their stinging response as the threat persisted (Nelsen et al., 2020).

For assessment of variability in behavioural responses between species, I found species differences in huddling behaviour. The proportion of time spent huddling by *A. robustus* was lower than in the other species of funnel-webs. Although funnel-web spiders are generally well known for their defensive behaviours, *A. robustus* typically performs obvious gaping displays, waving the first pair of legs, moving the fangs and expelling venom, even when the stimulus varies (Jackson & Pollard, 1990; Duran Hernández et al., 2022). These highly visual behaviours are possibly a consequence of the human-altered habitat in which *A. robustus* typically occurs (Gray, 1992). Urban populations of different species often show differences in behaviour to their rural counterparts. For example, individuals from urban populations of *Larinoides sclopetarius*, show reduced fear of novel stimuli compared to individuals from rural populations

(Kralj-Fišer et al., 2017). Faster resumption of movements could affect spider fitness, where higher risk-taking behaviour could help spiders obtain more resources or deter predators, although this might also have increased costs associated with a higher risk of predation (Riechert & Hedrick, 1993). *Hadronyche valida* and *H. infensa* showed a similar proportion of huddling, most likely because both species inhabit similar environments and have overlapping distributions (Gray, 2010), although risk-taking behaviour was only repeatable in *H. valida*. For *H. cerberea*, the huddling response is likely affected by their arboreal life style (i.e., living in tree crevices). While birds may be common predators in trees, bark provides a strong fortress in which the spider can hide and defend itself. Conversely, the level of exposure to other predators and threats is likely to be higher for terrestrial species, particularly in fragmented patches (Gray, 1992), where invasive predators may also pose a threat.

For frequency of fang movements in response to the puff of air across species, I observed differences over repetition, but not between species. The largest variation in aggression was observed in repetition three (T3), where *H. valida*, *H. infensa* and *A. robustus* showed heightened defensive behaviour, whereas the opposite was observed in *H. cerberea*. This could indicate that *H. cerberea* was capable of remembering the puff of air stimulus and had learned that the stimulus did not represent a threat. However, this requires additional testing. The levels of defensive behaviour between species indicate that defensiveness (frequency of fang movements) is quite flexible, and can vary depending on the context and level of threat (Nelsen et al., 2014, 2020; Duran Hernández et al., 2022). While it has been suggested that solitary species, such as funnel-webs, may be more defensive than social species, this is not a general rule and other factors may constrain or trigger the level of aggression (Kralj-Fišer et al., 2017; Nelsen et al., 2014). While studying the behaviour of these spiders in their natural habitats poses significant challenges (e.g., females are unlikely to leave their burrows if the burrows are built in favourable environments), it would be interesting to study funnel-webs under natural conditions. Factors such as microhabitat, predation pressure and population density may all play an important role in behavioural changes, providing information related to the ability to colonise, adapt to, and survive in different environments (particularly suburban and urban environments, Kralj-Fišer et al., 2017).

For climbing frequency and activity, I did not observe significant behavioural differences between funnel-web species. These findings suggest that these behavioural traits may be phylogenetically constrained (Riechert, 1993). However, it is important to consider that other factors might be shaping behavioural responses across species, such as geographical variation (Van Dongen et al., 2010), microhabitat structure (Vernouillet & Kelly, 2020), and developmental experience, which can be related to interactions and exploratory behaviours (Hernández Duran et al., 2021; Vernouillet & Kelly, 2020). If funnel-web spiders experience similar selection pressures that drive how they respond to conspecifics and how they interact directly with their environment to gain information, then this could explain why no differences in these behaviours were found.

In conclusion, the strength of selection pressures (i.e., predation, microhabitat, location), as well as intrinsic factors, such as physiology, genetics, and neurohormones, may affect the expression and variability of repeatability of traits (Hernández Duran et al., 2021; Kralj-Fišer et al., 2017; Vernouillet & Kelly, 2020) across different contexts and species. This is the first study to systematically explore behavioural flexibility and repeatability in four species of Australian funnel-web spiders. A comparison of the level of individual variability in behavioural responses within and between species provides important information about how the dynamics of populations (i.e., dispersion, invasion processes; colonisation, Fogarty et al., 2011) might be affected by behaviour. Furthermore, my results provide information about behavioural traits of venomous arthropods that should be considered in ecology and toxinology studies to understand venom production, the costs and benefits of venom use, as well as the evolution and diversification of other traits (Cooper et al., 2015; Schendel et al., 2019). Understanding the link between particular behaviours (i.e., risk-taking behaviour, activity) in different ecological contexts provides the basis for unveil how venomous animals use their defensive weapons (i.e., venom) and how, depending on the behavioural trait (e.g., aggressive/submissive), these traits are displayed during particular situations, as seen in vertebrates (e.g., bolder threespine sticklebacks *Gasterosteus aculeatus* have less armour; De Winter et al., 2016).

Australian funnel-web spiders are short-range endemic species, making them vulnerable to habitat loss due to urbanisation and fragmentation (Gray, 1992; Harvey, 2002). Understanding how different species of funnel-webs respond to different stimuli and contexts will provide greater insights into the ability of these species to cope with changing or challenging conditions, as well as valuable information for the conservation and management of funnel-webs. Conservation of these species is also critical given their medical importance.

5.6. References

Alewood, D., Birinyi-Strachan, L. C., Pallaghy, P. K., Norton, R. S., Nicholson, G. M., & Alewood, P. F. (2003). Synthesis and characterization of δ-Atracotoxin-Ar1a, the lethal neurotoxin from venom of the Sydney funnel-web spider (*Atrax robustus*). *Biochemistry, 42*(44), 12933–12940. https://doi.org/10.1021/bi030091n

Australian code for the care and use of animals for scientific purposes 8th edition. (2013). https://www.nhmrc.gov.au/about-us/publications/australian-code-care-and- use-animals-scientific-purposes/.

Arbuckle, K. (2017). Evolutionary context of venom in animals. In P. Gopalakrishnakone & A. Malhotra (Eds.), *Evolution of Venomous Animals and Their Toxins* (pp. 3–31). Springer Netherlands. https://doi.org/10.1007/978-94-007-6458-3_16

Bell, A. M. (2007). Future directions in behavioural syndromes research. *Proceedings of the Royal Society B: Biological Sciences*, *274*(1611), 755–761. https://doi.org/10.1098/rspb.2006.0199

Bengston, S. E., & Jandt, J. M. (2014). The development of collective personality: The ontogenetic drivers of behavioural variation across groups. *Frontiers in Ecology and Evolution*, *2*(81), 1–13. https://doi.org/10.3389/fevo.2014.00081

Bengston, S. E., Pruitt, J. N., & Riechert, S. E. (2014). Differences in environmental enrichment generate contrasting behavioural syndromes in a basal spider lineage. *Animal Behaviour*, *93*, 105–110. https://doi.org/10.1016/J.ANBEHAV.2014.04.022

Boevé, J. L., Kuhn-Nentwig, L., Keller, S., & Nentwig, W. (1995). Quantity and quality of venom released by a spider (*Cupiennius salei*, Ctenidae). *Toxicon*, *33*(10), 1347–1357. https://doi.org/10.1016/0041-0101(95)00066-U

Bradley, R. A. (1993). Seasonal activity patterns in Sydney funnel-web spiders, *Atrax* spp. (Araneae: Hexathelidae). *Bull. Br. Arachnol. Soc.*, *9*(6), 189–192.

Briffa, M., Rundle, S. D., & Fryer, A. (2008). Comparing the strength of behavioural plasticity and consistency across situations: Animal personalities in the hermit crab *Pagurus bernhardus*. *Proceedings of the Royal Society B: Biological Sciences*, *275*(1640), 1305–1311. https://doi.org/10.1098/rspb.2008.0025

Chang, C. C., Klomp, D. A., Norma-Rashid, Y., & Li, D. (2019). Consistency in boldness expression varies with ecological context in a jumping spider. *Ethology*, *125*(10), 724–732. https://doi.org/10.1111/eth.12925

Chang, C. C., Teo, H. Y., Norma-Rashid, Y., & Li, D. (2017). Predator personality and prey behavioural predictability jointly determine foraging performance. *Scientific Reports*, *7*(1), 1–8. https://doi.org/10.1038/srep40734

Chassagnon, I. R., McCarthy, C. A., Chin, Y. K. Y., Pineda, S. S., Keramidas, A., Mobli, M., Pham, V., De Silva, T. M., Lynch, J. W., Widdop, R. E., Rash, L. D., & King, G. F. (2017). Potent neuroprotection after stroke afforded by a double-knot spider-venom peptide that inhibits acid-sensing ion channel 1a. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(14), 3750–3755. https://doi.org/10.1073/pnas.1614728114

Cooper, A. M., Nelsen, D. R., & Hayes, W. K. (2015). The strategic use of venom by spiders. In A. Gopalakrishnakone, P.; Malhotra (Ed.), *Evolution of Venomous Animals and Their Toxins* (pp. 145–163). Springer Netherlands. https://doi.org/DOI 10.1007/978-94-007-6458-3_13

De Winter, G., Martins, H. R., Trovo, R. A., & Chapman, B. B. (2016). Knights in shining armour are not necessarily bold: defensive morphology correlates negatively with boldness, but positively with activity, in wild threespine stickleback, *Gasterosteus aculeatus*. *Evolutionary Ecology Research*, *17*(2), 279–290.

Dingemanse, N. J., & Wright, J. (2020). Criteria for acceptable studies of animal personality and behavioural syndromes. *Ethology*, *126*(9), 865–869. https://doi.org/10.1111/eth.13082

DiRienzo, N., McDermott, D. R., & Pruitt, J. N. (2015). Testing the effects of biogenic amines and alternative topical solvent types on the behavioural repertoire of two web-building spiders. *Ethology*, *121*(8), 801–812. https://doi.org/10.1111/eth.12395

Dutertre, S., Jin, A. H., Vetter, I., Hamilton, B., Sunagar, K., Lavergne, V., Dutertre, V., Fry, B. G., Antunes, A., Venter, D. J., Alewood, P. F., & Lewis, R. J. (2014). Evolution of separate predation-and defence-evoked venoms in carnivorous cone snails. *Nature Communications*, 5(1), 1–9. https://doi.org/10.1038/ncomms4521

Dochtermann, N. A., & Royauté, R. (2019). The mean matters: going beyond repeatability to interpret behavioural variation. In *Animal Behaviour* (Vol. 153, pp. 147–150). Academic

Press. https://doi.org/10.1016/j.anbehav.2019.05.012

Fogarty, S., Cote, J., & Sih, A. (2011). Social personality polymorphism and the spread of invasive species: A model. *American Naturalist, 177*(3), 273–287. https://doi.org/10.1086/658174

Friard, O., & Gamba, M. (2016). BORIS: a free, versatile open-source event-logging software for video/audio coding and live observations. *Methods in Ecology and Evolution, 7*(11), 1325–1330. https://doi.org/10.1111/2041-210X.12584

Gosling, S. D. (2001). From mice to men: What can we learn about personality from animal research? *Psychological Bulletin, 127*(1), 45–86. https://doi.org/10.1037/0033-2909.127.1.45

Gray, M. (1987). Distribution of the Funnel Web Spiders. In J. Covacevich, P. Davie, & J. Pearn (Eds.), *Toxic plants and animals : a guide for Australia* (pp. 313–321). Queensland Museum. https://books.google.com/books/about/Toxic_Plants_Animals.html?id=qIg_AAAAYAAJ

Gray, M. (2010). A revision of the Australian funnel-web spiders (Hexathelidae: Atracinae). *Records of the Australian Museum*, *62*(2–3), 285–392. https://doi.org/10.3853/j.0067-1975.62.2010.1556

Gray, M. R. (1981). Getting to know funnel webs. *Australian Natural History, 20*, 265–270.

Gray, M. R. (1992). Funnel-webs: separating fact from fiction. *Australia's Leading Nature Magazine, 24*(3), 32–39.

Halpin, R. N., & Johnson, J. C. (2014). A continuum of behavioural plasticity in urban and desert black widows. *Ethology*, *120*(12), 1237–1247. https://doi.org/10.1111/eth.12297

Harvey, M. S. (2002). Short-range endemism among the Australian fauna: Some examples from non-marine environments. *Invertebrate Systematics, 16*(4), 555–570.

https://doi.org/10.1071/IS02009

Hedin, M., Derkarabetian, S., Ramírez, M. J., Vink, C., & Bond, J. E. (2018). Phylogenomic reclassification of the world's most venomous spiders (Mygalomorphae, Atracinae), with implications for venom evolution. *Scientific Reports*, *8*(1636), 1–7. https://doi.org/10.1038/s41598-018-19946-2

Hernández Duran, L., Rymer, T. L., & Wilson, D. T. (2020). Variation in venom composition in the Australian funnel-web spiders *Hadronyche valida*. *Toxicon:X*, *8*, 100063. https://doi.org/10.1016/j.toxcx.2020.100063

Hernández Duran, L., Wilson, D. T., Briffa, M., & Rymer, T. L. (2021). Beyond spider personality: The relationships between behavioural, physiological, and environmental factors. *Ecology and Evolution*, *11*(7), 2974–2989. https://doi.org/https://doi.org/10.1002/ece3.7243

Hernández Duran, L., Wilson, D. T., & Rymer, T. L. (2022). Behaviour of the Sydney funnel-web spider *Atrax robustus* over different contexts, time, and stimuli. *Toxicon: X*, *13*(1–7), 100093. https://doi.org/https://doi.org/10.1016/j.toxcx.2022.100093

Herzig, V., & King, G. F. (2015). The cystine knot is responsible for the exceptional stability of the insecticidal spider toxin ω-Hexatoxin-Hv1a. *Toxins*, *7*(10), 4366–4380. https://doi.org/10.3390/toxins7104366

Herzig, V., Sunagar, K., Wilson, D. T. R., Pineda, S. S., Israel, M. R., Dutertre, S., McFarland, B. S., Undheim, E. A. B., Hodgson, W. C., Alewood, P. F., Lewis, R. J., Bosmans, F., Vetter, I., King, G. F., & Fry, B. G. (2020). Australian funnel-web spiders evolved human-lethal δ-hexatoxins for defense against vertebrate predators. *Proceedings of the National Academy of Sciences of the United States of America*, *117*(40), 24920–24928. https://doi.org/10.1073/pnas.2004516117

Ikonomopoulou, M. P., Fernandez-Rojo, M. A., Pineda, S. S., Cabezas-Sainz, P., Winnen, B., Morales, R. A. V., Brust, A., Sánchez, L., Alewood, P. F., Ramm, G. A., Miles, J. J., & King, G. F. (2018). Gomesin inhibits melanoma growth by manipulating key signaling cascades that control cell death and proliferation. *Scientific Reports*, *8*(11519), 1–14. https://doi.org/10.1038/s41598-018-29826-4

Isbister, G. K., Gray, M. R., Balit, C. R., Raven, R. J., Stokes, B. J., Porges, K., Tankel, A. S., Turner, E., White, J., & McD Fisher, M. (2005). Funnel-web spider bite: a systematic review of recorded clinical cases. In *Medical Journal of Australia* (Vol. 182, Issue 8, pp. 407–411). Med J Aust. https://doi.org/10.5694/j.1326-5377.2005.tb06760.x

Isbister, G. K., Sellors, K. V., Beckmann, U., Chiew, A. L., Downes, M. A., & Berling, I. (2015). Catecholamine-induced cardiomyopathy resulting from life-threatening funnel-web spider envenoming. In *Medical Journal of Australia* (Vol. 203, Issue 7, pp. 302–304). https://doi.org/10.5694/mja15.00279

Jackson, R. R., & Pollard, S. D. (1990). Intraspecific interactions and the function of courtship in mygalomorph spiders: a study of *Porrhothele antipodiana* (Araneae: Hexathelidae) and a literature review. *New Zealand Journal of Zoology*, *17*(4), 499–526. https://doi.org/10.1080/03014223.1990.10422949

Keiser, C. N., Lichtenstein, J. L. L., Wright, C. M., Chism, G. T., & Pruitt, J. N. (2018). Personality and behavioural syndromes in insects and spiders. In G.-S. I. Cordoba-Aguilar A, Gonzalez-Tokman D (Ed.), *Insect behaviour: From mechanisms to ecological and evolutionary consequences* (pp. 236–256). Oxford University Press. https://doi.org/10.1093/oso/9780198797500.003.0016

Klint, J. K., Senff, S., Rupasinghe, D. B., Er, S. Y., Herzig, V., Nicholson, G. M., & King, G. F. (2012). Spider-venom peptides that target voltage-gated sodium channels: pharmacological tools and potential therapeutic leads. *Toxicon*, *60*(4), 478–491. https://doi.org/10.1016/j.toxicon.2012.04.337

Köhler, T., & Vollrath, F. (1995). Thread biomechanics in the two orb-weaving spiders *Araneus diadematus* (Araneae, Araneidae) and *Uloborus walckenaerius* (Araneae, Uloboridae). *Journal of Experimental Zoology, 271*, 1–17. https://doi.org/10.1002/jez.1402710102

Kralj-Fišer, S., Hebets, E. A., & Kuntner, M. (2017). Different patterns of behavioural variation across and within species of spiders with differing degrees of urbanization. *Behavioural Ecology and Sociobiology*, *71*(8), 1–15. https://doi.org/10.1007/s00265-017-2353-x

Kralj-Fišer, S., & Schneider, J. M. (2012). Individual behavioural consistency and plasticity in an urban spider. *Animal Behaviour*, *84*(1), 197–204. https://doi.org/10.1016/j.anbehav.2012.04.032

Levitt, V. (1961). The Funnel-web Spider in Captivity. *Proceedings of the Royal Zoological Society of New South Wales, 1958–59*, 80–84. http://www.biodiversitylibrary.org/item/119542

Liedtke, J., Redekop, D., Schneider, J. M., & Schuett, W. (2015). Early environmental conditions shape personality types in a jumping spider. *Frontiers in Ecology and Evolution*, *3*, 134. https://doi.org/10.3389/fevo.2015.00134

Mason, L. D., Bateman, P. W., & Wardell-Johnson, W. (2018). The pitfalls of short-range endemism: high vulnerability to ecological and landscape traps. *PeerJ*, *2018*(5), e4715. https://doi.org/10.7717/PEERJ.4715/SUPP-1

Nakagawa, S., Johnson, P. C. D., & Schielzeth, H. (2017). The coefficient of determination R2 and intra-class correlation coefficient from generalized linear mixed-effects models revisited and expanded. *Journal of the Royal Society Interface*, *14*(134), 20170213. https://doi.org/10.1098/rsif.2017.0213

Nakagawa, S., & Schielzeth, H. (2010). Repeatability for Gaussian and non-Gaussian data: A practical guide for biologists. *Biological Reviews*, *85*(4), 935–956. https://doi.org/10.1111/j.1469-185X.2010.00141.x

Nakagawa, S., & Schielzeth, H. (2013). A general and simple method for obtaining R2 from generalized linear mixed-effects models. *Methods in Ecology and Evolution*, *4*(2), 133–142. https://doi.org/10.1111/j.2041-210x.2012.00261.x

Nakata, K., & Ushimaru, A. (2013). The effect of predation risk on spider's decisions on web-site relocation. *Behaviour*, *150*(1), 103–114. https://doi.org/10.1163/1568539X-00003039

Nekaris, K. A. I., Campera, M., Nijman, V., Birot, H., Rode-Margono, E. J., Fry, B. G., Weldon, A., Wirdateti, W., & Imron, M. A. (2020). Slow lorises use venom as a weapon in intraspecific competition. *Current Biology*, *30*(20), R1252–R1253. https://doi.org/10.1016/j.cub.2020.08.084

Nelsen, D. R., David, E. M., Harty, C. N., Hector, J. B., & Corbit, A. G. (2020). Risk assessment and the effects of refuge availability on the defensive behaviours of the southern unstriped scorpion (*Vaejovis carolinianus*). *Toxins*, *12*(9), 534. https://doi.org/10.3390/toxins12090534

Nelsen, D. R., Kelln, W., & 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. https://doi.org/10.1016/j.anbehav.2013.12.019

Nicholson, G. M., & Graudins. (2002). Invited Paper: animal toxins of Asia and Australia spiders of medical importance in the Asia - Pacific: atracotoxin, latrotoxin, and related spider neurotoxins. *Clinical and Experimental Pharmacology and Physiology*, *29*(9), 785–794.

Nicholson, G. M., Little, M. J., Tyler, M., & Narahashi, T. (1996). Selective alteration of sodium channel gating by Australian funnel-web spider toxins. *Toxicon*, *34*(11), 1443–1453. https://doi.org/10.1016/S0041-0101(96)00089-X

Noguchi, K., Gel, Y. R., Brunner, E., & Konietschke, F. (2012). nparLD : An R Software Package for the Nonparametric Analysis of Longitudinal Data in Factorial Experiments.

Journal of Statistical Software, *50*(12), 1–23. https://doi.org/10.18637/jss.v050.i12

Opatova, V., Hamilton, C. A., Hedin, M., De Oca, L. M., Král, J., Bond, J. E., & Wiegmann, B. (2020). Phylogenetic systematics and evolution of the spider infraorder mygalomorphae using genomic scale data. *Systematic Biology*, *69*(4), 671–707. https://doi.org/10.1093/sysbio/syz064

Padilla, M., Colley, M. A., & Reading, R. P. (2018). Growth rates of laboratory reared Honduran curly hair tarantulas(Brachypelma albopilosum) in response to power feeding. *Arachnology*, *17*(7), 325–327. https://doi.org/10.13156/arac.2017.17.7.325

Parthasarathy, B., Joshi, C. H., Kalyadan, S. S., & Somanathan, H. (2019). Early ontogenic emergence of personality and its long-term persistence in a social spider. *Behavioural Ecology and Sociobiology*, *73*(3). https://doi.org/10.1007/s00265-019-2645-4

Parthasarathy, B., Müller, M., Bilde, T., & Schneider, J. M. (2022). Hunger state and not personality determines task participation in a spider society. *Animal Behaviour*, *190*, 143–152. https://doi.org/10.1016/J.ANBEHAV.2022.06.002

Pinheiro JC, Bates D, 2000. Linear Mixed-Effects Models: basic concepts and examples. In: Pinheiro J, Bates D editors. *Mixed-Effects Models in Sand S-PLUS*. New York: Springer. 3–56.

Réale, D., Reader, S. M., Sol, D., McDougall, P. T., & Dingemanse, N. J. (2007). Integrating animal temperament within ecology and evolution. *Biological Reviews*, *82*(2), 291–318. https://doi.org/10.1111/j.1469-185X.2007.00010.x

Riechert, S. E. (1993). A test for phylogenetic constraints on behavioural adaptation in a spider system. *Behavioural Ecology and Sociobiology*, *32*(5), 343–348. https://doi.org/10.1007/BF00183790

Riechert, S. E., & Hedrick, A. (1990). Levels of predation and genetically based anti-predator behaviour in the spider, *Agelenopsis aperta*. *Animal Behaviour, 40*(4), 679–687. https://doi.org/https://doi.org/10.1016/S0003-3472(05)80697-9

Riechert, S., & Hedrick, A. (1993). A test for correlations among fitness-linked behavioural traits in the spider *Agelenopsis aperta* (Araneae, Agelenidae). *Animal Behaviour, 46*, 669–675. https://doi.org/https://doi.org/10.1006/anbe.1993.1243

Santana, R. C., Perez, D., Dobson, J., Panagides, N., Raven, R. J., Nouwens, A., Jones, A., King, G. F., & 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*(4), 1–13. https://doi.org/10.3390/toxins9040116

Scaccabarozzi, D., Dods, K., Le, T. T., Gummer, J. P. A., Lussu, M., Milne, L., Campbell, T., Wafujian, B. P., & Priddis, C. (2021). Factors driving the compositional diversity of *Apis mellifera* bee venom from a *Corymbia calophylla* (marri) ecosystem, Southwestern Australia. *PLOS ONE, 16*(6), e0253838. https://doi.org/10.1371/JOURNAL.PONE.0253838

Schendel, V., Rash, L. D., Jenner, R. A., & Undheim, E. A. B. (2019). The diversity of venom: the importance of behaviour and venom system morphology in understanding its ecology and evolution. *Toxins, 11*(11), 666. https://doi.org/10.3390/toxins11110666

Schielzeth, H., Dingemanse, N. J., Nakagawa, S., Westneat, D. F., Allegue, H., Teplitsky, C., Réale, D., Dochtermann, N. A., Garamszegi, L. Z., & Araya-Ajoy, Y. G. (2020). Robustness of linear mixed-effects models to violations of distributional assumptions. *Methods in Ecology and Evolution, 11*(9), 1141–1152. https://doi.org/10.1111/2041-210X.13434

Shearer, T. A., & Pruitt, J. N. (2014). Individual differences in boldness positively correlate with heart rate in orb-weaving spiders of genus *Larinioides*. *Current Zoology, 60*(3), 387–391. https://doi.org/10.1093/czoolo/60.3.387

Sih, A., Bell, A., & Johnson, J. . (2004). Behavioural syndromes: An ecological and evolutionary overview. *Trends in Ecology and Evolution*, *19*(7), 372–378. https://doi.org/10.1016/j.tree.2004.04.009

Stankowich, T. (2009). When predators become prey: flight decisions in jumping spiders. *Behavioural Ecology*, *20*(2), 318–327. https://doi.org/10.1093/beheco/arp004

Stanley, C. R., Mettke-Hofmann, C., & Preziosi, R. F. (2017). Personality in the cockroach *Diploptera punctata*: Evidence for stability across developmental stages despite age effects on boldness. *PLoS ONE*, *12*(5), 1–23. https://doi.org/10.1371/journal.pone.0176564

Stoffel, M. A., Nakagawa, S., & Schielzeth, H. (2017). rptR: repeatability estimation and variance decomposition by generalized linear mixed-effects models. *Methods in Ecology and Evolution*, *8*(11), 1639–1644. https://doi.org/10.1111/2041-210X.12797

Stoffel, M. A., Nakagawa, S., & Schielzeth, H. (2019). *An introduction to repeatability estimation with rptR*. 1–33. https://cran.r-project.org/web/packages/rptR/vignettes/rptR.html

Van Dongen, W. F. D., Maldonado, K., Sabat, P., & Vásquez, R. A. (2010). Geographic variation in the repeatability of a personality trait. *Behavioural Ecology*, *21*(6), 1243–1250. https://doi.org/10.1093/BEHECO/ARQ145

Vernouillet, A., & Kelly, D. M. (2020). Individual exploratory responses are not repeatable across time or context for four species of food-storing corvid. *Scientific Reports*, *10*(1), 1–11. https://doi.org/10.1038/s41598-019-56138-y

Wilson, D., & Alewood, P. (2004). Australian funnel-web spider venom analyzed with on-line RP-HPLC techniques. In Aguilar MI. (Ed.), *HPLC of Peptides and Proteins. Methods in Molecular Biology* (Vol. 251, pp. 307–322). Springer, Totowa, NJ. https://doi.org/https://doi.org/10.1385/1-59259-742-4:307

Wilson, D., & Alewood, P. F. (2006). Taxonomy of Australian funnel-web spiders using RP-HPLC/ESI-MS profiling techniques. *Toxicon*, *47*(6), 614–627. https://doi.org/10.1016/j.toxicon.2006.01.014

Windley, M. J., Herzig, V., Dziemborowicz, S. A., Hardy, M. C., King, G. F., & Nicholson, G. M. (2012). Spider-venom peptides as bioinsecticides. *Toxins*, *4*(3), 191–227. https://doi.org/10.3390/toxins4030191

Wolf, M., & Weissing, F. J. (2012). Animal personalities: Consequences for ecology and evolution. *Trends in Ecology and Evolution*, *27*(8), 452–461. https://doi.org/10.1016/j.tree.2012.05.001

Wullschleger, B., & Nentwig, W. (2002). Influence of venom availability on a spider's prey-choice behaviour. *Functional Ecology*, *16*(6), 802–807. https://doi.org/10.1046/j.1365-2435.2002.00686.x

Zhan, Y., Jiang, H., Wu, Q., Zhang, H., Bai, Z., Kuntner, M., & Tu, L. (2019). Comparative morphology refines the conventional model of spider reproduction. *PLoS ONE*, *14*(7). https://doi.org/10.1371/journal.pone.0218486

Zobel-Thropp, P. A., Bulger, E. A., Cordes, M. H. J., Binford, G. J., Gillespie, R. G., & Brewer, M. S. (2018). Sexually dimorphic venom proteins in long-jawed orb-weaving spiders (*Tetragnatha*) comprise novel gene families. *PeerJ*, *2018*(6), e4691. https://doi.org/10.7717/peerj.4691

5.7. Appendix Chapter 5

Table S5.1. Output of rank-based non-parametric analyses for longitudinal data models of *H. cerberea* and *A. robustus* to test differences in behavioural traits and the effects of repetitions and life stage. The * refers to results that are significant at the α = 0.05 level.

ANOVA-Type Statistic (ATS)			
Model	Risk taking behaviour (huddling) ~ Repetition + Stage, subject= factor (ID)		
	H. cerberea		
	Statistic	**df**	**p-value**
Stage	0.680	1	0.421
Repetition	0.112	1.961	0.889
	A. robustus		
Stage	2.093	1	0.147
Repetition	3.518	1.884	0.032 *
Model	Aggressiveness (fang aggression) ~ Repetition + Stage, subject= factor (ID)		
	H. cerberea		
	Statistic	**df**	**p-value**
Stage	3.294	1	0.09
Repetition	0.596	1.877	0.54
	A. robustus		
Stage	2.247	1	0.195
Repetition	26.308	1.989	<0.001 *
Model	Aggression (frequency of climbs) ~ Repetition + Stage, subject= factor (ID)		
	H. cerberea		
	Statistic	**df**	**p-value**
Stage	0.184	1	0.673
Repetition	1.029	1.893	0.354
	A. robustus		
Stage	0.516	1	0.472
Repetition	0.468	1.828	0.607
Model	Activity ~ Repetition + Stage, subject= factor (ID)		
	A. robustus		
	Statistic	**df**	**p-value**
Stage	0.145	1	0.703
Repetition	1.59	1.723	0.207

Table S5.2. (a) Generalized linear mixed-effects models (GLMMs) results behavioural traits across different ecological contexts for each species of funnel-web: *H. valida*, *H. cerberea*, *H. infensa, A. robustus*. **(b)** Generalized linear mixed-effects models (GLMMs) output behavioural traits across different ecological contexts between species of funnel-webs: H. valida, H. cerberea, H. infensa, A. robustus. The * refers to results that are significant at the α = 0.05 level.

Behaviour / Ecological context	***H. valida***			***H. cerberea***		***H. infensa***		***A. robustus***	
Risk taking behaviour-predator cue: huddling model (logit link) binomial binary mixed model	**Model name**	**Null model**	**Full model**	**Null model**	**Full model**	**Null model**	**Full model**	**Null model**	**Full model**
	Fixed effects	*b* [95% CI]	*b* [95% CI]	*b* [95% CI]	*b* [95% CI]	*b* [95% CI]	*b* [95% CI]	*b* [95% CI]	*b* [95% CI]
	intercept	-1.005 [-1.858, -0.152]	5.834 [-13.393, 1.725]	-1.363 [-2.02, -0.700]	-4.837 [-10.374, 0.700]	-0.887 [-1.50 -0.264]	-2.191 [-6.046, 1.664]	-1.995 [-3.403, -0.587]	-1.36 [-6.191, 3.470]
	Size	1.67768E-06	0.558 [-0.299, 1.415]	-	0.425 [-0.238, 1.0888]		0.143 [-0.271, 0.558]		-0.064 [-0.526, 0.397]
	Random effects	δ²	δ²	δ²	δ²	δ²	δ²	δ²	δ²
	id	1.564	1.240	0.0003	0.000	3.46E-07	0.0001	0.247	0.090
	observation-level (distribution-specific)	4.604 (3.289)	4.604 (3.289)	3.289 (4.2882)	3.289 (4.2882)	4.840 (3.298)	4.840 (3.298)	3.289 (8.863)	3.289 (8.863)
	Fixed factors	-	0.201	-	0.184		0.0498		0.0143
	Conditional R² (%)	-	23.846 (30.471)	-	2.911 (5.319)		1.022 (1.496)		1.168 (3.086)
	Marginal R² (%)	-	3.330 (4.256)	-	2.910 (5.317)		1.019 (1.493)		0.160 (0.424)
	ICC (%)	25.356 (32.223)	21.222 (27.38)	0.005 (0.009)	0.001 (0.002)	7.137107e-06 (0.00001)	0.002 (0.004)	1.009 (2.673)	1.009 (2.673)
	AIC	87	87.4	58.6	59	61.9	63.5	45.6	47.6
	Mean standardization (*II*)	189.5014	150.2441	0.070	0.016	4.11E-05	0.0142	68.60328	25.09991
	lmtest repeatability	**<0.001***		0.987		0.996		0.952	
Aggression-predator cue: Fang	**Model name**	**Null model**	**Full model**	**Model name**	**Null model**	**Full model**	**Null model**	**Full model**	**Null model**
	Fixed effects	*b* [95% CI]	*b* [95% CI]	*b* [95% CI]	*b* [95% CI]	*b* [95% CI]	*b* [95% CI]	*b* [95% CI]	*b* [95% CI]

frequency (log-link) negative binomial model	intercept	4.023 [3.928, 4.117]	4.009 [2.990, 5.029]	3.819 [3.740, 3.898]	3.819 [3.740, 3.898],	3.932 [3.819, 4.045]	3.889 [3.215, 4.562]	3.957 [3.797, 4.116]	3.089 [2.236, 3.943]
	Size	-	0.0015 [-0.115, 0.118]		-0.00026 [-0.078, 0.078]		0.005 [-0.068, 0.078]		0.094 [0.001, 0.187]
	Random effects	δ²	δ²	δ²	δ²	δ²	δ²	δ²	δ²
	id	1.25E-07	1.13E-07	0.015	0.015	1.13E-07	1.61E-05	2.07E-07	2.22E-07
	observation-level (distribution-specific)	0.174	0.174		0.0421	0.0421	0.173	0.429	0.387
	Fixed factors	-	1.49E-06		7.12E-08		5.65E-05		0.031
	Conditional R² (%)	-	0.000915		26.695		0.0419		7.415
	Marginal R² (%)	-	0.000851		0.0001		0.0326		7.415
	ICC (%)	7.18E-05	6.45E-05	26.668	26.695	0.000	0.00928	4.81E-05	5.72E-05
	AIC	625.3	627.3	413.6	415.6	429.5	431.5	522.7	520.8
	Mean standardization (*II*)	1.67768E-06	1.5051E-06	0.221	0.221	1.57E-06	0.0002	2.86E-06	3.06E-06
	lrtest repeatability	0.999		0.068		0.985		0.987	
Aggression-territory: climb frequency (log-link) negative binomial model	**Model name**	**Null model**	**Full model**	**Null model**	**Full model**	**Null model**	**Full model**	**Null model**	**Full model**
	Fixed effects	*b* [95% CI]	*b* [95% CI]	*b* [95% CI]	*b* [95% CI]	*b* [95% CI]	*b* [95% CI]	*b* [95% CI]	*b* [95% CI]
	intercept	-0.586 [-1.445,0.273]	4.128 [-3.393, 11.649]	-0.806 [-1.355, -0.255]	0.372 [-3.554, 4.298]	-0.575 [-1.076, -0.074]	-0.522 [-3.497, 2.452]	-0.642 [-1.175, -0.109]	0.805 [-1.1673801 2.778]
	Size	-	-0.533 [-1.386, 0.320]		-0.146 [-0.361, 0.339]		-0.005 [-0.331, 0.319]		-0.161 [-0.385, 0.062]
	Random effects	δ²	δ²	δ²	δ²	δ²	δ²	δ²	δ²
	id	1.605	1.177	0.350	0.317	7.14E-06	6.34E-07	0.371	0.298
	observation-level (distribution-specific)	5.535	6.308	4.588	4.689	3.138	3.139	3.371	3.369
	Fixed factors	-	0.183		0.059		8.34E-05		0.089

	Conditional R^2 (%)	-	17.743		22.993		7.60E-04		10.335
	Marginal R^2 (%)	-	2.398		1.169		7.00E-04		2.384
	ICC (%)	22.482	15.721	7.101	5.326	6.51E-05	5.78E-06	3.280	8.145
	AIC	189.1	189.6	111.3	112.9	103.8	105.8	120.1	120.1
	Mean standardization (*II*)	152.9301	112.1487	47.8557	43.3526	0.0009	8.45E-05	46.868	37.656
	lrtest repeatability	**<0.001***		0.201		0.992		0.203	
Activity-exploration (log-link) gamma mixed model	**Model name**	**Null model**	**Full model**	**Null model**	**Full model**	**Null model**	**Full model**	**Null model**	**Full model**
	Fixed effects	*b* [95% CI]	*b* [95% CI]	*b* [95% CI]	*b* [95% CI]	*b* [95% CI]	*b* [95% CI]	*b* [95% CI]	*b* [95% CI]
	intercept	3.815 [2.898, 4.731]	3.611 [-4.086, 11.308]	-	-	4.751 [4.254, 5.246]	4.275 [1.277, 7.272]	3.54 [2.544, 4.543]	2.006 [-2.675, 6.686]
	Size		0.023 [-0.857, 0.904]	-	-		0.052 [-0.275, 0.380]		0.17 [-0.334, 0.674]
	Random effects	δ²	δ²	δ²	δ²	δ²	δ²	δ²	δ²
	id	3.629	3.623	-	-	0.00009	0.000060	3.271	3.244
	observation-level (distribution-specific)	6.074	6.078	-	-	10.509	10.485	6.198	
	Fixed factors		0.00035	-	-		0.006		0.099
	Conditional R^2 (%)		37.346	-	-		0.063		35.233
	Marginal R^2 (%)		0.004	-	-		0.063		1.045
	ICC (%)	37.401	37.344	-	-	0.0008	0.0005	40.489	34.548
	AIC	664.7	666.7	-	-	490.3	492.2	489.2	490.8
	Mean standardization (*II*)	31.649	19.681	-	-	0.0008	0.0005	34.129	33.847
	lrtest repeatability	**<0.001**		-	-	0.068		**<0.001**	

AICc: Akaike’s Information Criteria corrected for small sample size

Table S5.2b

Risk taking behaviour-predator cue: huddling model (logit link) binomial binary mixed model				**Defensive behaviour-predator cue: Fang frequency (log-link) negative binomial model**				**Defensive behaviour against conspecifics: climbing frequency (log-link) negative binomial model**				**Activity-exploration (log-link) gamma mixed model**			
Model name	**Null model**	**Full model**	**P_value**	**Model name**	**Null model**	**Full model**	**P_value**	**Model name**	**Null model**	**Full model**	**P_value**	**Model name**	**Null model**	**Full model**	**P_value**
Fixed effects	*b* [95% CI]	*b* [95% CI]		*Fixed effects*	*b* [95% CI]	*b* [95% CI]		*Fixed effects*	*b* [95% CI]	*b* [95% CI]		*Fixed effects*	*b* [95% CI]	*b* [95% CI]	
intercept	-0.238 [-0.711, 0.235]	0.199 [-2.499, 2.898]	0.885	intercept	3.943 [3.888, 3.998]	3.57 [3.181,1 3.965]	**<0.001**	intercept	-0.623 [-0.958, -0.288]	0.885 [-0.958, 2.724]	0.345	intercept	3.937 [3.394, 4.479]	3.935 [0.856, 7.013]	0.012
Repetition T2		-0.574 [-1.374, 0.22]	0.16	Repetition T2		0.120 [-0.007, 0.249]	0.0655	Repetition T2		0.203 [-0.324, 0.732]	0.450	Repetition T2		0.373 [-0.378, 1.125]	0.330
Repetition T3		0.34 [-1.147, 0.468]	0.41	Repetition T3		0.215 [0.085, 0.345]	**<0.001****	Repetition T3		-0.004 [-0.552, 0.544]	0.988	Repetition T3		-0.6357 [-1.404, 0.133]	0.105
H. cerberea		-2.599 [-3.865, -1.332]	**<0.001*****	*H. cerberea*		-0.073 [-0.231, 0.083]	0.3594	*H. cerberea*		-0.314 [-1.136, 0.507]	0.454	*H. cerberea*		-	-
H. infensa		3.245 [1.919, 4.570]	**<0.001*****	*H. infensa*		-0.001 [-0.156, 0.153]	0.9835	*H. infensa*		-0.049 [-0.874, 0.775]	0.906	*H. infensa*		0.733 [-0.598, 2.064]	0.281
H. valida		-3.022 [-4.264, -1.779]	**<0.001*****	*H. valida*		0.090 [-0.051, 0.233]	0.212	*H. valida*		0.323 [-0.412, 1.059]	0.388	*H. valida*		0.2583 [-0.962, 1.479]	0.678
Size		0.257 [-0.046, 0.560]	0.097	Size		0.027 [-0.014, 0.0693]	0.1944	Size		-0.183 [-	0.084	Size		-0.0327 [-	0.851

									0.391, 0.024]			0.375, 0.309]
Random effects			*Random effects*				*Random effects*			*Random effects*		
id	2.268	0.644	id	0.221	1.34E-07		id	0.6033	0.507	id	2.834	2.713
observation-level (distribution-specific)	3.289 (4.028)	3.289 (4.028)	observation-level (distribution-specific)	0.17	0.191	-	observation-level (distribution-specific)	2.174	5.624	observation-level (distribution-specific)	2.424	2.331
Fixed factors		1.885	Fixed factors		0.015	-	Fixed factors		0.101	Fixed factors		0.263
Conditional R^2 (%)		38.577 (43.475)	Conditional R^2 (%)		8.099	-	Conditional R^2 (%)		9.385	Conditional R^2 (%)		31.636
Marginal R^2 (%)		28.747 (32.396)	Marginal R^2 (%)		8.099	-	Marginal R^2 (%)		1.623	Marginal R^2 (%)		2.803
AIC	296	265.5	AIC	2012.7	2012.7	-	AIC	522.1	527.9	AIC	1639.1	1639.3

AICc: Akaike’s Information Criteria corrected for small sample size

Chapter 6: Interactions between physiology and behaviour in Australian funnel-web spiders: interspecies comparison

6.1. Abstract

Australian funnel-web spiders are iconic species, characterized as being the most venomous spiders in the world. They are also valued for the therapeutics and natural bioinsecticides potentially hidden in their venom molecules. Although numerous biochemical and molecular structural approaches have tried to determine the factors driving venom complexity, these approaches have failed to consider behaviour, physiology and environmental conditions, which can play a role in the evolution, complexity, and function of venom components in funnel-webs. This study used a novel interdisciplinary approach to understand the relationships between different behaviours (assessed in different ecological contexts) and morphophysiological variables (body condition, hearth rate) that may affect venom composition in four species of Australian funnel-web spiders. I tested defensiveness, huddling behaviour, frequency of climbing, and activity for all species in three ecological contexts: i) predation using both indirect (puff of air) and direct (prodding) stimuli; ii) conspecific tolerance; and iii) exploration of a new territory. I also assessed morphophysiological variables and venom composition of all species. For *H. valida*, the expression of some venom components was associated with heart rate and defensiveness during the predation context. However, I did not find any associations between behavioural traits and morphophysiological variables in the other species, suggests that particular associations may be species-specific. When we assessed differences between species, I found that venom profiles drive the separation between species, while activity and heart rate are likely more affected by individual responses and microhabitat conditions. This study demonstrates how behavioural and morphophysiological traits interact, and contributes to a broader understanding of the function and evolution of venoms in funnel-web spiders.

6.2. Introduction

Behaviour is known as the “evolutionary pacemaker” because animals are able to either change or maintain particular behaviours when exposed to different environmental conditions (Cooper et al., 2015; Schendel et al., 2019). Individual behavioural phenotypes (i.e., aggressive, docile, active) have considerable impact at larger scales, affecting population dynamics, colonisation of new habitats, trophic interactions, distribution and extinction rates (Duckworth, 2009; Sih et al., 2017; Wolf & Weissing, 2012). Therefore, changes in the environment and, correspondingly, behaviour, can mediate the evolution of morphology, life history traits (Duckworth, 2009; Wolf & Weissing, 2012), and physiology, such as venom.

Venom is used across multiple ecological contexts that extend beyond predator-prey interactions (Schendel et al., 2019). Some animals can use venom for communication (Post & Jeanne, 1983), predation (Cooper et al., 2015), defence (Cooper et al., 2015), mating (Sentenská et al., 2017), intraspecific competition (Nekaris et al., 2020; Williams et al., 2013), and social behaviour (Gatchoff & Stein, 2021). The use of venoms can involve different toxins, and various combinations of toxins can result in the ability to perform multiple functions, making venom a unique adaptive biological trait (Cooper et al., 2015; Schendel et al., 2019). In venomous animals, behaviour plays a critical role because the use of venoms is mediated by behavioural responses (Cooper et al., 2015). Venom function and molecular variation are thus constrained by behavioural responses, in which costs of production (high metabolic rates; Morgenstern & King, 2013; Nisani et al., 2012), replenishment, deployment and availability of venom, are moderated by selection (Cooper et al., 2015; Evans et al., 2019).

Multiple extrinsic (environment, diet, density) and intrinsic (genes, and physiology) factors also directly or indirectly affect behaviour, which can in turn affect the use, function and composition of venom (Schendel et al., 2019). To date, seasonality (Wong et al., 2016), sexual dimorphism (Binford, 2001; Herzig, Sunagar, et al., 2020; Wilson, 2016; Zobel-Thropp et al., 2018), age (Arbuckle, 2017), ontogeny (Andrade & Abe, 1999; Santana et al., 2017), geographical origin(Gomes & Palma, 2016), genetics (Wilson & Daly, 2018), and environmental conditions (Zancolli et al., 2019) have been studied independently to understand their effects on the variation in venom composition. However, intrinsic and extrinsic factors interact, affecting physiological and behavioural processes that can vary at a species-specific level (Hernández Duran et al., 2021). Understanding the associations between behaviour, physiology and environment is important because the ecological function of venom depends on how an animal responds to a particular cue (threat level; Nelsen et al., 2014), and the strategies developed by them to deploy the venom (Cooper et al., 2015). For example, physiological traits like body condition (i.e., nutritional state) can affect the performance of animals in a particular context. Individuals in better body condition may take more risks (be more bold) or show more defensive displays (Riechert, 1978), which can then balance the metabolic costs (Lichtenstein et al., 2017) of using and producing venom (Luttbeg & Sih, 2010; Vapenik & Nentwig, 2000).

The costs associated with venom use can also vary depending on ecological context and level of threat (Cooper et al., 2015; Nelsen et al., 2014; Schendel et al., 2019). Defensive and exploratory behaviours usually consume energy, whereas foraging and defending food supplies compensate for costs associated with energy expenditure (Biro & Stamps, 2010). In the case of venomous animals, venom production is energetically expensive (Cooper et al., 2015) and individual nutritional state can increase or decrease the costs of venom expenditure (Cooper et al., 2015; Morgenstern & King, 2013). Venomous animals can compensate for the energetic costs (metabolic rate or its proxy heart rate; Carrel & Heathcote, 1976) of venom expenditure using multiple strategies, such as adjusting bites (Nelsen et al., 2014), using behavioural displays (Gibbons & Dorcas, 2002; Hernández Duran et al., 2022) or controlling the amount of venom expelled (Cooper et al., 2015; Morgenstern & King, 2013; Nelsen et al., 2020).

Australian funnel-web spiders are iconic species endemic to the southern and eastern regions of the continent (Gray, 2010). Funnel-web venoms have diverse pharmacological activities (Pineda et al., 2020), and are the most complex venoms in the natural world (comprising thousands of toxin peptides; Pineda et al., 2020). Funnel-web spiders show inter- and intra-individual differences in venom composition, as well as interspecific differences between closely related species with overlapping distributions (Hernández Duran et al., 2020). Numerous biochemical and molecular structural approaches have explored the factors affecting these complex venoms (Herzig, Sunagar, et al., 2020). However, aspects related to the ecological function of venom, physiological traits, and their interactions with behaviour, have been overlooked. In this study, I used a synergistic approach to assess whether behavioural and morphophysiological traits measured over three ecological contexts (predation, conspecific tolerance, and exploration of a new territory) affect venom composition in four species of Australian funnel-web spiders: *Hadronyche valida*, *H. infensa*, *H. cerberea* and *Atrax robustus*. I expected that the ecological context would affect relationships with venom components and morphophysiological traits, where defensive and more active spiders would have higher heart rates and show expression of specific venom molecules because displaying defensive behaviour and expelling venom are costly traits involving energy expenditure. This study allowed us to identify physiological changes (heart rate and venom components) associated with a particular ecological context and behaviour, which is not only important for understanding the mechanisms underlying the evolution of venoms and toxins, but is also important for understanding how animals use their biological weapons to respond to different environmental conditions.

6.3. Methods

6.3.1. Study species

For this study, 75 spiders from four different Australian funnel-web spider species were used. Adult female *H. valida* (n = 23; collected by manual excavation of burrows in the Currumbin Valley and Mount Tamborine) were purchased from Thargomindah Man Productions in 2019 (Varsity Lakes, QLD, Australia). Adult female *H. infensa* (n = 16) were collected manually in Toowoomba and Ravensbourne in 2019 (collection permit SA 2016/08/55). *H. cerberea* (n = 9 adult females; n = 9 juveniles) were collected across three *Eucalyptus regnans* in Gosford, New South Wales in 2019. *A. robustus* (n = 13 adult females; n = 5 juveniles) were collected from the Gosford/Central Coast region, New South Wales (collection carried out in cooperation with the Australian Reptile Park). I collected any spiders I could find, regardless of size or stage, which led to unbalanced sample sizes. The collection of these animals is particularly difficult as females live in burrows (Gray, 2010), which made it difficult to know whether the spider being collected was an adult or juvenile prior to collection. Adult females were identified by epigyne sclerotisation and the opening of the epigastric furrow (Hernández Duran et al., 2020; Zhan et al., 2019). The spiders were transported alive in plastic containers with damp cotton wool to the laboratory of the Australian Institute of Tropical Health and Medicine (AITHM), James Cook University Nguma-bada (Cairns) campus, Queensland, Australia.

Spiders were kept individually in 5 L plastic containers in a climate-controlled room (temperature: 20 ± 2 °C; relative humidity: 60%) on a reverse light: dark cycle (12L:12D). The spiders were acclimated for one month before commencing behavioural assays (see below). Each spider received one house cricket, *Acheta domestica*, once a week. Two weeks before starting all behavioural tests, food was withheld from the spiders to allow venom regeneration prior to collection. All behaviours were video recorded using a Sony Handycam under red light between 6am and 12pm, and were later analysed using the software BORIS version 7.8.2 (Friard & Gamba, 2016).

6.3.2. Behavioural assays

Multiple behavioural traits (defence, huddling, defence towards conspecifics, and activity) were measured across three ecological contexts namely predation, conspecific tolerance, and exploration of new territory for each species. The methods are described and discussed in Hernández Duran et al., (2020, 2022), which we summarise here briefly. The number of fang movements produced in response to a prod stimulus (using blunt tweezers to touch the first pair of legs) was quantified as a measure of defence. As a measurement of risk-taking behaviour (antipredator behaviour), I used huddling behaviour in response to three rapid puffs of air applied on the prosoma using a camera air blower. Defence towards conspecifics was measured using the number of climbs on a barrier when the spiders were exposed to a conspecific (spiders divided by a mesh barrier in a novel environment to avoid direct agonistic encounters). Finally, as a measure of exploration of new territory, we used the time the spider spent actively moving around a new arena.

The average value of each behavioural trait measured across different ecological contexts and over time (three repetitions with one month between each repetition) were obtained. I previously assessed repeatability and behavioural flexibility in all four species (see Chapter 5), so do not discuss this here. The behaviours from both adults and juveniles for *H. cerberea* and *A. robustus* were analysed together as a longitudinal analysis found no effect of stage differences over time for any of the behavioural traits, heart rate and body condition (see statistical analyses; Supplementary material Table S1).

6.3.3. Morphological and physiological measurements

For each species, one day after I finalised each behavioural assay, I measured body size (cephalothorax width) and body mass (weight) of each spider. I calculated the mean of body size and mass from all repetitions. I tested the normality of each variable using the MVN package(Korkmaz et al., 2014). Then, I calculated body condition using the residual index, in which body mass is regressed against body size (Jakob et al., 1996). I used a linear model (LM) with normal distribution to obtain the residuals.

Heart rate

Individual heartbeats have been used as an accurate measure of heart rate in spiders (Carrel & Heathcote, 1976), and heart rate can be used as a standard measurement of metabolic rate in spiders (Carrel & Heathcote, 1976). I measured the heart rate of each individual spider for each species in a resting position (Biro & Stamps, 2010) one day after finishing all the behavioural tests and feeding the spiders (to reduce the effects of any stressful conditions). The heart rate was measured using a non-invasive laser heart rate monitor (Supplementary Material S6.1), following the concept of different bands of frequencies for Infra-red (IR) and red (R) wavelengths (Chu et al., 2017; Gillinov et al., 2017). Spiders were gently extracted from their burrows and left in a resting position for one hour. I placed the heart rate monitor sensor close to the abdomen of the spider, avoiding direct physical contact. The sensor fires harmless light wavelengths towards the spider and reflection from the heart returns the number of beats within a 15 s monitoring period. I measured the heart rate at least 4 times within each measurement period to ensure that the monitor was functioning correctly (all values should be similar). I then calculated the overall mean from all repetitions for each individual.

6.3.4. Venom collection and analysis

For each species, during the defence test, I collected the venom expelled on the tips of the fangs of each spider using a 200μL Gilson P200 pipette with polypropylene micropipette (see Hernandez Hernández Duran et al., 2020, 2022). Each spider was aggravated for 4 min and venom was collected over a 10 min period. After collecting the venom, it was placed in a 1.5 mL microcentrifuge tube with 40 µL of Milli-Q water (Hernández Duran et al., 2020). I repeated this procedure three more times, one month apart, for a total of three venom samples per individual. Venom samples from each individual were analysed using liquid chromatography/electrospray ionisation mass spectrometry (LC/ESI-MS) to generate a venom profile, following the protocol of Hernández Duran et al., (2020).

6.3.5. Matrices

Within species

To analyse the relationship between venom components and both behavioural and morphophysiological variables (heart rate and body condition), I built three matrices: one matrix included the mean of the three repetitions of each behaviour (defence, huddling, defence towards conspecifics, and activity), heart rate and body condition. The second matrix was built from the total of all venom components present across all three repetitions. The low molecular weights obtained from singly charged ions were monoisotopic masses, while those obtained from reconstructed multi-charged ion series were average masses.

The third matrix consisted of a reduced matrix of venom components, which was obtained after performing a Spearman rank correlation to determine whether there was redundancy in the data. I first performed a multivariate analysis using the complete venom matrix, behavioural and morphophysiological variables. I then repeated this analysis using the reduced matrix of venom components, behavioural and morphophysiological variables. I included both complete and reduced venom matrices to determine if the association between traits dramatically changed after reducing the redundancies between venom components. In addition, keeping both matrices allowed me to visualise the association between particular venom molecules with behavioural and morphophysiological traits that might not be present if only one matrix was used.

Between species comparison

To assess interspecific relationships based on venom profiles, behaviours and morphophysiological traits, I followed the same protocol described above, firstly building a matrix that included all behaviours, heart rate and body condition from all species, then building a matrix that included venom profiles from all species. I then reduced the redundancy in venom components (by performing a Spearman rank correlation, cut-off 0.70) and built a reduced venom matrix.

6.3.6. Statistical analyses

All statistical analyses were conducted using R (version 4.1.0, Core R Team 2021). For both adults and juveniles of *H. cerberea* and *A. robustus*, I tested for stage effects on the magnitude of individual behavioural traits (defence, huddling, defence towards conspecifics and activity), heart rate and body condition in each ecological context, and over time, using rank-based non-parametric analyses for longitudinal data (Table S6.1) following Hernández Duran et al., (2022). These analyses offer a robust framework for non-continuous variables, small sample sizes and skewed data (Noguchi et al., 2012). The design used was F1-LD-F1 in the nparLD package (Noguchi et al., 2012). I included the random effect of individual identity as a subject in this model. I did not observe differences between adults and juveniles for either *H. cerberea* or *A. robustus* for behavioural traits, heart rate and body condition (Supplementary material

Table S6.1). Therefore, I did not separate juvenile and adult data for each species when the multivariate analyses were performed.

Within species analysis

To determine the relationship between venom components with behavioural (defence, huddling, climbing and activity) and morphophysiological (heart rate and body condition) variables in each species, I used a multivariate method canonical correspondence analysis (CCA) using the package vegan (Oksanen et al., 2016). The collinearity of explanatory variables (behaviours and morphophysiological variables centered) was measured using the variance inflation factor (VIF) (Borcard et al., 2018). I first ran the CCA using the venom matrix with all venom components, where the Chi-square distance was used by default in the package. Then, I re-ran the analysis using the Bray-Curtis distance, which is more suitable for abundance data (Borcard et al., 2018), to determine which model had the better fit (Supplementary Table S6.2). I repeated this procedure for the reduced venom matrix (Supplementary Table S6.3).

Furthermore, to visualise the correlation structure between venom profiles with behavioural and morphophysiological variables, I used Regularised Canonical Correlation Analysis (rCCA) from the mixOmics package (Lê Cao & Welham, 2021). This package uses omics data and it is particularly suitable when the number of samples (N) is lower than the number of variables provided in two matrices (X and Y) (Lê Cao & Welham, 2021). This method achieves dimension reduction in each data set while maximising similar information within species matrices. The regularisation parameter used in rCCA was cross-validation (Lê Cao & Welham, 2021). The rCCA was performed for the complete matrix of venom components, and then the same analysis was conducted using the reduced matrix of venom components. Finally, using the rCCA results, I built network and cluster image maps (CIM) plot to evaluate the correlation structure between venom components with behavioural and morphophysiological variables (Lê Cao & Welham, 2021).

Between species comparison

To assess how individuals of each species grouped according to venom profiles, I performed nonmetric multidimensional scaling (NMDS) using the package vegan (Oksanen et al., 2016) after reducing the collinearity of venom components shared between species. I used Hellinger transformation and Bray-Curtis dissimilarity distance to separate groups based on the abundancies of venom components (Oksanen et al., 2016). To determine the effect of behaviours and morphophysiological variables on the ordination plot NMDS of all venom profiles, I performed a permutation test (using the envfit package; Borcard et al., 2018).

6.4. Results

6.4.1. Within species: Complete matrix for H. valida

The canonical correspondence analysis of the complete venom matrix (95 venom components, Chi-square distance) for *H. valida* showed that the model as a whole (with all the variables included) was significant (CCA, permutation test, $F_{6,16} = 1.455$, $P = 0.047$, Table 6.1). However, none of the individual canonical axes was significant (Supplementary material Table S6.2a). The amount of constrained variance explained in the venom matrix by the behavioural and morphophysiological variables was 27%. The variables that had the strongest effect on the venom matrix were heart rate (CCA, $F_{1,16} = 2.156$, $P = 0.037$; Table 6.1), and marginally significant climbing (CCA, $F_{1,16} = 1.985$, $P = 0.059$; Table 6.1). Similar results were obtained using the Bray-Curtis distance, where the whole model was significant (CCA, permutation test, $F_{6,16} = 1.455$, $P = 0.044$; Table 6.1), but none of the canonical axes were significant (Supplementary material Table S6.2b). Both heart rate (CCA, $F_{1,16} = 2.156$, $P = 0.056$; Table

6.1) and climbing (CCA, $F_{1,16} = 1.985$, $P = 0.058$; Table 6.1) had marginally significant effects on the venom matrix. No other variables had an effect (Supplementary material Table S2 d,e).

Table 6.1. Results from the ordination analysis CCA of *H. valida* showing the relationship between venom components matrix (complete and reduced), behavioural and morphophysiological variables assessed across different ecological contexts using two different distances (Chi-square and Bray-Curtis).

Model:	**Full venom matrix + Morphophysiological variables (Chi-square)**	**Full venom matrix + Morphophysiological variables (Bray-Curtis)**	**Reduced + Morphophysiological variables (Chi-square)**	**Reduced+ Morphophysiological variables (Bray-Curtis)**
Inertia	0.779	0.779	0.881	0.881
Constrained	0.275	0.278	0.311	0.311
unconstrained	0.504	0.502	0.570	0.570
Accumulated CCA1	0.313	0.313	0.239	0.239
Accumulated CCA2	0.231	0.239	0.225	0.225
ANOVA whole model	**0.047**	**0.044**	**0.012**	**0.016**
Behaviour/ morphophysiological variable (P.value)				
Heart rate	**0.037**	0.056·	**0.031**	**0.040**
Climbing	0.059•	0.058·	0.096•	0.108
Defence	0.100	0.100	**0.013**	**0.024**
Body condition	0.190	0.172	0.096•	0.086·
Activity	0.286	0.268	0.097•	0.083·
Huddle	0.249	0.253	0.205	0.227
R2 adjusted	0.1088	0.1138	0.1129	0.111

Reduced matrix for H. valida

After performing a Spearman rank correlation, the venom matrix was reduced from 95 to 53 venom components. The CCA using the reduced matrix and the Chi-square distance improved the model fit (CCA, permutation test, $F_{6,16} = 1.455$, $P = 0.012$, Table 6.1). Again, none of the canonical axes were significant (Supplementary material Table S6.3a). The behaviour and morphophysiological variables explained 31% of variance in the venom matrix. The morphophysiological variable that had the strongest effect on the venom matrix was again heart rate (CCA, $F_{1,16} = 1.893$, $P = 0.031$; Table 1). However, this time, defence (CCA, $F_{1,16} = 1.940$, $P = 0.013$; Table 6.1), rather than climbing had a larger effect. Climbing (CCA, $F_{1,16} = 1.567$, $P = 0.096$; Table 6.1), body condition (CCA, $F = 1.442$, $P = 0.098$; Table 6.1), and activity (CCA, $F_{1,16} = 1.446$, $P = 0.097$; Table 6.1), while non-significant, were the next most important contributors.

The CCA results using the Bray-Curtis distance were similar to the model using the Chi-square distance (Table 6.1, Figure 6.1). The whole model was significant (CCA, permutation test, $F_{6,16} = 1.455$, $P = 0.016$, Table 6.1, Figure 6.1), but none of the individual canonical axes were significant (Supplementary material Table S6.3b). With regards to the effect of the behavioural and morphophysiological variables, heart rate (CCA, $F_{1,16} = 1.893$, $P = 0.040$; Table 6.1) and defence (CCA, $F_{1,16}= 1.940$, $P = 0.024$; Table 6.1) had significant effects on the venom matrix. Body condition (CCA, $F_{1,16} = 1.442$, $P = 0.086$; Table 6.1, Figure 6.1) and activity (CCA, $F_{1,16} = 1.446$, $P = 0.083$; Table 6.1), while non-significant, were the next most important contributors.

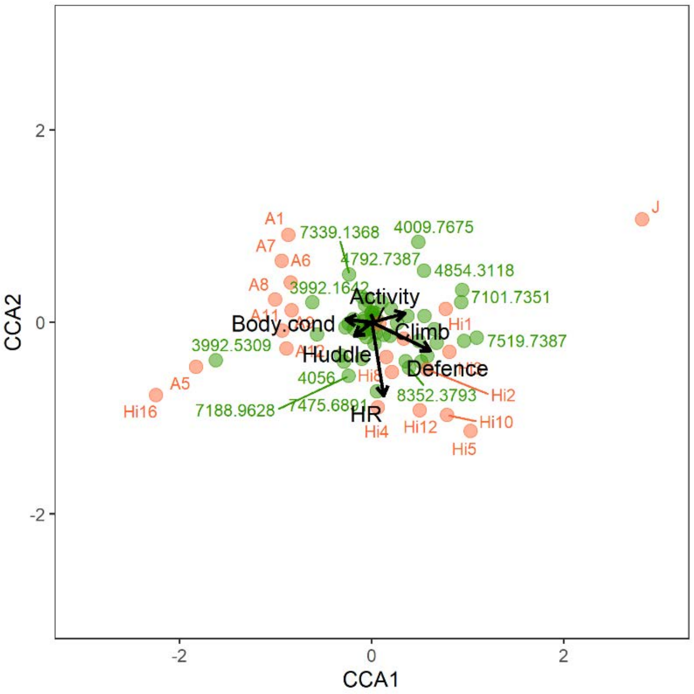


Figure 6. 1. Canonical correspondence analysis (CCA) of the reduced venom profile matrix (Bray-Curtis distance, green letters) constrained by behavioural and morphophysiological (black letters) variables of the individuals (red letters) of *H. valida*.

The network plot showed that heart rate and defence had the strongest correlations with some venom molecules (Figure 6.2), which was supported by the rCCA analysis for both the complete and reduced venom matrices (Supplementary material Figure S6.1, S6.3). In particular, I found that two venom molecules were shared between heart rate and defence for the complete venom matrix (Figure 6.2). The venom molecule 439 was negatively associated, and the molecule 489 was positively associated with defence and heart rate (Figure 6.2). For the reduced venom matrix, the venom molecules 489 and 4397.099 showed a positive association with both heart rate and defence, while venom molecule 4612.546 showed a negative association with these two traits (Supplementary material Figure S6.2). In addition, other specific venom molecules were associated either with defence or heart rate for both the complete venom matrix (Figure 6.2) and the reduced venom matrix (Supplementary material Figure S6.2).

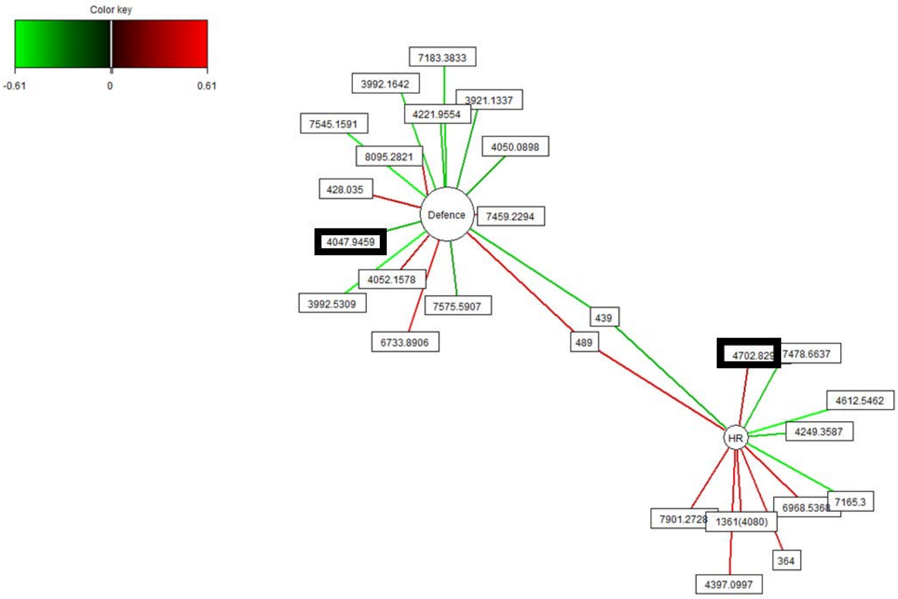


Figure 6.2. Network plot *H. valida* of a regularised canonical correlation analysis (rCCA) showing the structure of the association of venom components (complete venom matrix) with behavioural and morphophysiological variables. The correlation cut-off shown is at 0.40. The colour of each line indicates the nature of the correlation (red: positive association, green: negative association) between venom components defence and heart rate. The black boxes show the venom peptides that have been previously identified, ω-atracotoxin-Hi1b (4047) and δ-atracotoxin-Hva1b (4702). ω-atracotoxin-Hi1b.

The CIM plots showed that both the complete and reduced venom matrices separated into two main clusters (Supplementary material Figure S6.4a, b). In the first cluster, defence and heart rate were grouped together, and showed various associations with specific venom molecules. In the second cluster, climbing, activity, body condition and huddle were clustered together. Both clusters were either positively or negatively associated with a particular venom component (Supplementary material Figure S6.4a, b)

6.4.2. Complete and reduced matrices for the other three species

The CCAs using the complete and reduced venom matrices with the Chi-square distance for *H. infensa, H. cerberea* and *A. robustus* individually were not significant (Table 6.2). Similarly, Bray-Curtis did not improve the model in any species (Table 6.2).

6.4.3. Between species

After running the NMDS in the complete venom matrix, I observed that the analyses were not identifying patterns of variation between species due to the higher correlation between venom molecules between species (a high number of components with only 76 individuals). Thus, I used only the reduced venom matrix for these analyses. After performing the Spearman rank correlation, the venom matrix of all species was reduced from 503 to 136 venom components. The NMDS of venom components obtained from all species showed that each species formed a distinct group according to its venom profiles (Adonis, F.model = 21.891, $R^2 = 0.481$, P = 0.001, Figure 6.3). The venom components that might drive the patterns of species distribution in the plot can be observed in the Supplementary material (Supplementary Table S6.4 a, b). When I projected the behavioural and morphophysiological variables into the NMDS plot of venom components, I found that the variation along the NMDS1 and NMDS2 was positively correlated with heart rate (NMDS1 = 0.033, NMDS2 = 0.999, $r^2 = 0.125$, P = 0.010; Figure 6.3), and negatively correlated with activity (NMDS1 = -0.210, NMDS2 = -0.977, $r^2 = 0.096$, P = 0.023; Figure 6.3; Supplementary Table S6.4 c).

Table 6.2. Results from the ordination analysis CCA showing the relationship between venom components matrix (complete and reduced) with behavioural and morphophysiological variables assessed across different ecological contexts using two different distances (Chi-square and Bray-Curtis) for the species *H. infensa*, *H. cerberea* and *A. robustus*.

H. infensa				
Model:	**Complete venom matrix + Morphophysiological variables (Chi-square)**	**Reduced venom matrix + Morphophysiological variables (Bray-Curtis)**	**Complete venom matrix + Morphophysiological variables (Chi-square)**	**Reduced venom matrix + Morphophysiological variables (Bray-Curtis)**
Inertia	0.693	0.693	0.689	0.689
Constrained	0.260	0.260	0.266	0.266
Unconstrained	0.432	0.432	0.423	0.423
Accumulated CCA1	0.284	0.284	0.289	0.289
Accumulated CCA2	0.190	0.190	0.288	0.288
ANOVA (P.value)	0.717	0.709	0.690	0.675
F	0.903	0.903	0.944	0.944
H. cerberea				
Inertia	0.585	0.585	0.585	0.772
Constrained	0.193	0.193	0.193	0.262
Unconstrained	0.392	0.392	0.392	0.509

Accumulated CCA1	0.285	0.285	0.285	0.305
Accumulated CCA2	0.262	0.262	0.262	0.206
ANOVA (P.value)	0.727	0.690	0.667	0.626
F	0.900	0.906	0.946	0.946
		A. robustus		
Inertia	2.878	2.878	1.644	1.644
Constrained	0.989	0.989	0.498	0.498
Unconstrained	1.889	1.889	1.145	1.145
Accumulated CCA1	0.270	0.270	0.233	0.233
Accumulated CCA2	0.248	0.248	0.196	0.196
ANOVA (P.value)	0.588	0.588	0.998	0.998
F	0.960	0.960	0.798	0.798

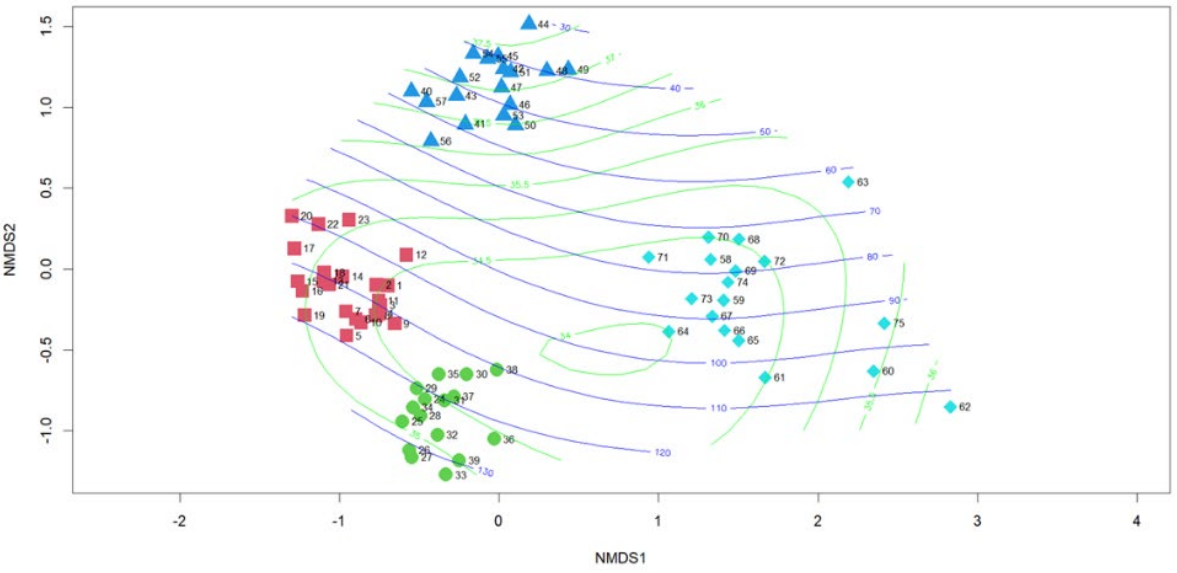


Table 6.3. Ordination plot NMDS (using Bray-Curtis distance) showing the separation of individuals of each species based on venom profiles. Red squares = *H. valida*; green circles = *H. infensa*; dark blue triangles = *H. cerberea*, and light blue diamonds = *A. robustus*. Stress value = 0.148. The lines represent the projection of the significant behavioural (blue: activity) and morphophysiological (heart rate: green) variables on the NMSD plot of venom components. Activity (blue lines) decreases as it projects over *H. cerberea*. In contrast, heart rate (green lines) increases in *H. cerberea*, but shows similar patterns in the other species.

6.5. Discussion

The ecological function of venoms in Australian funnel-web spiders is influenced by different behavioural phenotypes that can also be directly or indirectly affected by physiological and morphological traits. This is the first study to assess the synergistic associations between behavioural, physiological and morphological traits in mygalomorphs spiders (funnel-web spiders) using innovative multivariate statistical techniques. I show for the first time how specific venom components are associated with particular behavioural and physiological variables, and demonstrate that these relationships are context-dependant.

6.5.1. Within species

For *H. valida,* I found significant associations between venom components, defence behaviour and heart rate. Defensive behaviour was positively associated with heart rate frequency (a proxy of metabolic rate; Carrel & Heathcote, 1976) and also with the expression of specific venom components (i.e., 438.980, 488.98 and 4397), which were either positively or negatively associated depending on the ecological context (predation). Although most of the venom molecules in *H. valida* have not yet been characterised, two previously characterised toxins were identified as present: ω-atracotoxin-Hi1b (4047) and δ-atracotoxin-Hva1b (4702). ω-atracotoxin-Hi1b was negatively correlated with defensive behaviour and is also found in the closely related species *H. infensa* (Pineda et al., 2016). This toxin has insecticidal properties and is highly selective for insect voltage-gated calcium channels (Pineda et al., 2016; Wang et al., 2001). Spiders may not produce this toxin during anti-predator defence to reduce energetic costs of costly venom molecule production. δ-atracotoxin-Hva1b was positively associated with heart rate and acts by delaying the inactivation of both vertebrate tetrodotoxin-sensitive voltage-gated sodium (Na_V) channels and insect para-type sodium channels (Alewood et al., 2003; Pineda et al., 2016). If this toxin is costly to produce, spiders with a higher metabolic rate may be able to invest more into its production.

Understanding the dynamics of intrinsic and extrinsic factors together with behavioural traits will allow for harvesting/ targeting of particular toxin molecules that may lead to novel drug lead discovery. For example, in *Apis mellifera*, the protein profile and weight of bee venom are affected by behavioural responses (degree of aggression) and ecological factors, such as temperature and geographical location, which can affect the venom harvest (Scaccabarozzi et al., 2021). However, more work is needed to understand these complex relationships in their entirety.

The association between defence behaviour and heart rate has been also reported in the web-building spider, *Larinioides cornutus*, where bolder and more aggressive spiders had higher heart frequencies than shy spiders (Shearer & Pruitt, 2014). These types of correlations are maintained in animals when abundant resources are available and aggressive behaviours are advantageous for securing resources against conspecifics (Shearer & Pruitt, 2014). The relationships between aggressive behaviours, expression of particular venom components, and heart rates in spiders might depend on the individual's body condition, mass, age, reproductive state, food intake and environmental conditions (Biro & Stamps, 2010). In the case of *H. valida*, all the individuals were adult females, showed similar body mass, were under the same environmental conditions, and had the same diet, so these factors are an unlikely explanation for the pattern observed. However, I do not know the age of the females (other than they were all adults), their previous experience in the wild or their genetic relationships, all of which could contribute to the particular relationships we observed.

A relationship between aggression and particular venom components could also be explained by the type of stimulus (Hernández Duran et al., 2022; Nelsen et al., 2014). Spiders can modulate their defensive behaviours according to the type of stimulus or threat to which they are exposed (Nelsen et al., 2014). For example, *A. robustus* individuals that were exposed to a prod stimulus showed an increase in the number of fang movements (Chapter 4) over time, and this behaviour is considered a defensive behaviour against predators. Additionally, juveniles and adults responded differently depending on the type of stimulus (puff of air compared to a prod stimulus; Chapter 4). A direct stimulus, such as prodding or poking, involves physical contact, which can trigger a cascade of multiple behavioural and physiological changes in individuals, such as increasing the heart rate and/or neurohormonal changes (Jones et al., 2011), increasing defensiveness (Cooper et al., 2015) (Nelsen et al., 2014), and expressing or expelling venom on their fangs (Hernández Duran et al., 2022; Kardong, 1986; Nelsen et al., 2014). In contrast, an indirect stimulus, such as a puff of air, could elicit a suite of different responses such as huddling to reduce conspicuousness or fleeing to avoid a predator (Hernández Duran et al., 2022; Jackson et al., 1990).

When considering the other three species, it is possible that an association between morphophysiological traits and venom components was masked by differences between adult and juvenile stages in *H. cerberea* and *A. robustus*. It has been shown that inter- and intra-individual variation in venom composition over time between adults and juveniles differs independently of diet and environmental conditions (Hernández Duran et al., 2020). In the case of *H. infensa*, it is possible that other morphophysiological traits might also be associated with the use of venom components, but this needs to be addressed in future studies. Furthermore, the behaviour and venom composition of these species is known to change over time (Chapter 5), so it would be beneficial to explore whether these associations arise at particular time points within ecological contexts.

6.5.2. Between species

Interestingly, when the venom profiles from all four funnel-web species were compared, I found that venom profiles are a reliable chemotaxonomic marker tool (Chapter 3) for identifying spider species, even when inter- and intra-individual variation plays an important role as a source of variation in venom components, as has been reported in *H. valida* (Hernández Duran et al., 2020). The use of multivariate statistical approaches thus appears to provide a useful tool for assessing taxonomic relationships. However, as other factors, such as genetic structure, ecology, behaviours, geographical variation and environmental conditions can affect the variability of venom components (Zancolli et al., 2019), these should all be considered when determining the identification of venomous species.

Although I did not identify specific relationships between venom components with behavioural and morphophysiological variables in some species, I did observe a significant effect between heart rate and activity with venom profiles from all species when morphophysiological variables were projected on the ordination plot of venoms (NMDS). The correlation between activity and heart rate has also been associated with aggression (Biro & Stamps, 2010; Shearer & Pruitt, 2014), where animals with high metabolic rates are expected to be aggressive, active and bolder to obtain resources to maintain their metabolism (Shearer & Pruitt, 2014). In spiders, venom plays an important role in predation and predatory deterrence, and the behaviours associated with these functions will determine the success or death of the individual (Cooper et al., 2015). However, the use of venom and the display of aggressive behaviours can lead to metabolic costs (Biro & Stamps, 2010; Cooper et al., 2015; Morgenstern & King, 2013). As a result, spiders might use different behavioural strategies (Schendel et al., 2019) to compensate for these costs. In my results, activity was negatively correlated with venom compounds and positively correlated with heart rate. This suggests that spiders might increase their metabolic rate when they use venoms, and reduce their movement when facing a threat. Other strategies to reduce costs associated with the use of venom include adjusting the number of bites (Nelsen et al., 2014), modulating venom deployment and quantity (Cooper et al., 2015), displaying aggressive behaviours without expelling venom (Nelsen et al., 2014), and potentially using particular venom molecules when they are exposed to different types of threats, such as predators (Cooper et al., 2015) and conspecifics (Hernández Duran et al., 2022). For example, spiders can estimate the quantity of venom available in their venom glands, which might help them to select prey (Wullschleger & Nentwig, 2002), and they can also modulate venom expenditure under different threat levels (Nelsen et al., 2014). The relationships between morphophysiological and behavioural traits might have an impact on life history, provoking a change in how animals adapt to changing conditions (Biro & Stamps, 2010). The ability of individuals to cope with stressful conditions will determine their ability to survive in different environments (Koolhaas et al., 2007), such as in places where habitat fragmentation and urbanisation are more evident (Main, 2001).

My holistic approach, where multiple traits are included, allowed me to determine the association between particular venom components with behaviours and morphophysiological measurements over different ecological contexts. These results might be considered in antivenom production (Graudins et al., 2002) and the study of bioactive components found in funnel-webs (Herzig, Cristofori-Armstrong, et al., 2020), which have a potential for drug discovery (Chassagnon et al., 2017; Escoubas & Bosmans, 2007; Klint et al., 2012; Saez et al., 2010). Behavioural factors (degree of defence), together with environmental variables, are essential keys to understanding the function and evolution of venoms, as well as their potential as evolutionary models and molecular toolkits in venomous animals.

6.6. References

Alewood, D., Birinyi-Strachan, L. C., Pallaghy, P. K., Norton, R. S., Nicholson, G. M., & Alewood, P. F. (2003). Synthesis and characterization of δ-Atracotoxin-Ar1a, the lethal neurotoxin from venom of the Sydney funnel-web spider (*Atrax robustus*). *Biochemistry*, *42*(44), 12933–12940. https://doi.org/10.1021/bi030091n

Andrade, D. V., & Abe, A. S. (1999). Relationship of venom ontogeny and diet in *Bothrops*. *Herpetologica*, *55*(2), 200–204. https://www.jstor.org/stable/3893080?seq=1#metadata_info_tab_contents

Arbuckle, K. (2017). Evolutionary context of venom in animals. In P. Gopalakrishnakone & A. Malhotra (Eds.), *Evolution of Venomous Animals and Their Toxins* (pp. 3–31). Springer Netherlands. https://doi.org/10.1007/978-94-007-6458-3_16

Binford, G. J. (2001). An analysis of geographic and intersexual chemical variation in venoms of the spider *Tegenaria agrestis* (Agelenidae). *Toxicon, 39*(7), 955–968. https://doi.org/10.1016/S0041-0101(00)00234-8

Biro, P. A., & Stamps, J. A. (2010). Do consistent individual differences in metabolic rate promote consistent individual differences in behaviour? *Trends in Ecology & Evolution, 25*(11), 653–659. https://doi.org/10.1016/J.TREE.2010.08.003

Borcard, D., Gillet, F., & Legendre, P. (2018). Numerical Ecology with R. In *Numerical Ecology with R* (2nd 2018.). Springer International Publishing. https://doi.org/10.1007/978-1-4419-7976-6

Carrel, J. E., & Heathcote, R. D. (1976). Heart rate in spiders: Influence of body size and foraging energetics. *Science, October*, 148–150. https://doi.org/10.1126/science.935864

Chassagnon, I. R., McCarthy, C. A., Chin, Y. K. Y., Pineda, S. S., Keramidas, A., Mobli, M., Pham, V., De Silva, T. M., Lynch, J. W., Widdop, R. E., Rash, L. D., & King, G. F. (2017). Potent neuroprotection after stroke afforded by a double-knot spider-venom peptide that inhibits acid-sensing ion channel 1a. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(14), 3750–3755. https://doi.org/10.1073/pnas.1614728114

Chu, C.-T., Ho, C. C., Chang, C.-H., & Ho, M.-C. (2017). Non-invasive optical heart rate monitor base on one chip integration microcontroller solution. *2017 6th International Symposium on Next Generation Electronics (ISNE)*, 1–4.

Cooper, A. M., Nelsen, D. R., & Hayes, W. K. (2015). The strategic use of venom by spiders. In A. Gopalakrishnakone, P.; Malhotra (Ed.), *Evolution of Venomous Animals and Their Toxins* (pp. 145–163). Springer Netherlands. https://doi.org/DOI 10.1007/978-94-007-6458-3_13

Duckworth, R. A. (2009). The role of behaviour in evolution: a search for mechanism. *Evolutionary Ecology*, *23*(4), 513–531. https://doi.org/10.1007/s10682-008-9252-6

Escoubas, P., & Bosmans, F. (2007). Spider peptide toxins as leads for drug development. *Expert Opinion on Drug Discovery*, *2*(6), 823–835. https://doi.org/10.1517/17460441.2.6.823

Evans, E. R. J., Northfield, T. D., Daly, N. L., & Wilson, D. T. (2019). Venom costs and optimization in scorpions. *Frontiers in Ecology and Evolution*, *7*, 196. https://doi.org/10.3389/fevo.2019.00196

Friard, O., & Gamba, M. (2016). BORIS: a free, versatile open-source event-logging software for video/audio coding and live observations. *Methods in Ecology and Evolution*, *7*(11), 1325–1330. https://doi.org/10.1111/2041-210X.12584

Gatchoff, L., & Stein, L. R. (2021). Venom and social behaviour: the potential of using spiders to evaluate the evolution of sociality under high risk. *Toxins*, *13*(6), 388. https://doi.org/10.3390/toxins13060388

Gibbons, J., & Dorcas, M. (2002). Defensive behaviour of cottonmouths (Agkistrodon piscivorus) toward humans. *Copeia*, *1*, 195–198. https://doi.org/10.1643/0045-8511(2002)002[0195:DBOCAP]2.0.CO;2

Gillinov, S., Etiwy, M., Wang, R., Blackburn, G., Phelan, D., Gillinov, A. M., Houghtaling, P., Javadikasgari, H., & Desai, M. Y. (2017). Variable accuracy of wearable heart rate monitors during aerobic exercise. *Medicine and Science in Sports and Exercise*, *49*(8), 1697–1703. https://doi.org/10.1249/mss.0000000000001284

Gomes, P. C., & Palma, M. S. (2016). The Nonpeptide Low Molecular Mass Toxins from Spider Venoms. In P. Gopalakrishnakone, G. A. Corzo, M. E. de Lima, & E. Diego-García

(Eds.), *Spider Venoms* (pp. 3–19). Springer Netherlands. https://doi.org/10.1007/978-94-007-6389-0_14

Graudins, A., Wilson, D., Alewood, P. F., Broady, K. W., & Nicholson, G. M. (2002). Cross-reactivity of Sydney funnel-web spider antivenom: neutralization of the in vitro toxicity of other Australian funnel-web (*Atrax* and *Hadronyche*) spider venoms. *Toxicon : Official Journal of the International Society on Toxinology*, *40*(3), 259–266. https://doi.org/10.1016/S0041-0101(01)00210-0

Gray, M. (2010). A revision of the Australian funnel-web spiders (Hexathelidae: Atracinae). *Records of the Australian Museum*, *62*(2–3), 285–392. https://doi.org/10.3853/j.0067-1975.62.2010.1556

Hernández Duran, L., Rymer, T. L., & Wilson, D. T. (2020). Variation in venom composition in the Australian funnel-web spiders *Hadronyche valida*. *Toxicon:X*, *8*, 100063. https://doi.org/10.1016/j.toxcx.2020.100063

Hernández Duran, L., Wilson, D. T., Briffa, M., & Rymer, T. L. (2021). Beyond spider personality: The relationships between behavioural, physiological, and environmental factors. *Ecology and Evolution*, *11*(7), 2974–2989. https://doi.org/https://doi.org/10.1002/ece3.7243

Hernández Duran, L., Wilson, D. T., & Rymer, T. L. (2022). Behaviour of the Sydney funnel-web spider *Atrax robustus* over different contexts, time, and stimuli. *Toxicon: X*, *13*(1–7), 100093. https://doi.org/https://doi.org/10.1016/j.toxcx.2022.100093

Herzig, V., Cristofori-Armstrong, B., Israel, M. R., Nixon, S. A., Vetter, I., & King, G. F. (2020). Animal toxins — Nature's evolutionary-refined toolkit for basic research and drug discovery. *Biochemical Pharmacology*, *181*(114096), 1–14. https://doi.org/10.1016/j.bcp.2020.114096

Herzig, V., Sunagar, K., Wilson, D. T. R., Pineda, S. S., Israel, M. R., Dutertre, S., McFarland, B. S., Undheim, E. A. B., Hodgson, W. C., Alewood, P. F., Lewis, R. J., Bosmans, F., Vetter, I., King, G. F., & Fry, B. G. (2020). Australian funnel-web spiders evolved human-lethal δ-hexatoxins for defense against vertebrate predators. *Proceedings of the National Academy of Sciences of the United States of America*, *117*(40), 24920–24928. https://doi.org/10.1073/pnas.2004516117

Jackson, R. R., Brassington, R. J., & Rowe, R. J. (1990). Anti-predator defences of *Pholcus phalangioides* (Araneae, Pholcidae), a web-building and web-invading spider. *Journal of Zoology*, *220*(4), 543–552. https://doi.org/10.1111/j.1469-7998.1990.tb04733.x

Jakob, E. M., Marshall, S. D., & Uetz, G. W. (1996). Estimating fitness: a comparison of body condition indices. *Oikos*, *77*(1), 61. https://doi.org/10.2307/3545585

Jones, T. C., Akoury, T. S., Hauser, C. K., Neblett, M. F., Linville, B. J., Edge, A. A., & Weber, N. O. (2011). Octopamine and serotonin have opposite effects on antipredator behaviour in the orb-weaving spider, *Larinioides cornutus*. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioural Physiology*, *197*(8), 819–825. https://doi.org/10.1007/s00359-011-0644-7

Kardong, K. V. (1986). Predatory strike behaviour of the rattlesnake, Crotalus viridis oreganus.. *Journal of Comparative Psychology*, *100*(3), 304–314. https://doi.org/10.1037/0735-7036.100.3.304

Klint, J. K., Senff, S., Rupasinghe, D. B., Er, S. Y., Herzig, V., Nicholson, G. M., & King, G. F. (2012). Spider-venom peptides that target voltage-gated sodium channels: pharmacological tools and potential therapeutic leads. *Toxicon*, *60*(4), 478–491. https://doi.org/10.1016/j.toxicon.2012.04.337

Koolhaas, J. M., De Boer, S. F., Buwalda, B., & Van Reenen, K. (2007). Individual variation in coping with stress: A multidimensional approach of ultimate and proximate mechanisms. *Brain, Behaviour and Evolution*, *70*(4), 218–226. https://doi.org/10.1159/000105485

Korkmaz, S., Goksuluk, D., & Zararsiz, G. (2014). MVN: An R package for assessing multivariate normality. *R Journal*, *6*(2), 151–162. https://doi.org/10.32614/rj-2014-031

Lê Cao, K.-A., & Welham, Z. M. (2021). Multivariate data integration using R : methods and applications with the mixOmics package. In *Multivariate data integration using R : methods and applications with the mixOmics package*. Chapman & Hall/CRC.

Lichtenstein, J. L. L., Chism, G. T., Kamath, A., & Pruitt, J. N. (2017). Intraindividual behavioural variability predicts foraging outcome in a beach-dwelling jumping spider. *Scientific Reports*, *7*(1), 1–7. https://doi.org/10.1038/s41598-017-18359-x

Luttbeg, B., & Sih, A. (2010). Risk, resources and state-dependent adaptive behavioural syndromes. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *365*(1560), 3977–3990. https://doi.org/10.1098/RSTB.2010.0207

Main, B. Y. (2001). Historical ecology, responses to current ecological changes and conservation of Australian spiders. *Journal of Insect Conservation*, *5*(1), 9–25. https://doi.org/10.1023/A:1011337914457

Martinek, R., Nedoma, J., Fajkus, M., Kahankova, R., Konecny, J., Janku, P., Kepak, S., Bilik, P., & Nazeran, H. (2017). A phonocardiographic-based fiber-optic sensor and adaptive filtering system for noninvasive continuous fetal heart rate monitoring. In *Sensors* (Vol. 17, Issue 4, p. 890). https://doi.org/10.3390/s17040890

Morgenstern, D., & King, G. F. (2013). The venom optimization hypothesis revisited. In *Toxicon* (Vol. 63, Issue 1, pp. 120–128). https://doi.org/10.1016/j.toxicon.2012.11.022

Nekaris, K. A. I., Campera, M., Nijman, V., Birot, H., Rode-Margono, E. J., Fry, B. G., Weldon, A., Wirdateti, W., & Imron, M. A. (2020). Slow lorises use venom as a weapon in intraspecific competition. *Current Biology*, *30*(20), R1252–R1253. https://doi.org/10.1016/j.cub.2020.08.084

Nelsen, D. R., David, E. M., Harty, C. N., Hector, J. B., & Corbit, A. G. (2020). Risk assessment and the effects of refuge availability on the defensive behaviours of the southern unstriped scorpion (*Vaejovis carolinianus*). *Toxins*, *12*(9), 534. https://doi.org/10.3390/toxins12090534

Nelsen, D. R., Kelln, W., & 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. https://doi.org/10.1016/j.anbehav.2013.12.019

Nisani, Z., Boskovic, D. S., Dunbar, S. G., Kelln, W., & Hayes, W. K. (2012). Investigating the chemical profile of regenerated scorpion (*Parabuthus transvaalicus*) venom in relation to metabolic cost and toxicity. *Toxicon*, *60*(3), 315–323. https://doi.org/10.1016/j.toxicon.2012.04.343

Noguchi, K., Gel, Y. R., Brunner, E., & Konietschke, F. (2012). nparLD : An R Software Package for the Nonparametric Analysis of Longitudinal Data in Factorial Experiments. *Journal of Statistical Software*, *50*(12), 1–23. https://doi.org/10.18637/jss.v050.i12

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P., O'Hara, R. B., Simpson, G., Solymos, P., Stevens, M. H. H., & Wagner, H. (2016). *Vegan: Community ecology package. R package version 2.3-5.* (p. https://CRAN.R-project.org/packa ge=vegan Pagel,).

Pineda, S., Chaumeil, P., Kunert, A., Kaas, Q., Thang, M., Nuhn, M., Herzig, H., Saez, N., Cristofori-Armstrong, B Anangi, R., Senff, S., Gorse, D., & King, G. (2016).

ArachnoServer 3.0: an online resource for automated discovery, analysis and annotation of spider toxins. *Bioinformatics, 31,* 31–37. https://doi.org/10.1093/bioinformatics/xxxxx

Pineda, S. S., Chin, Y. K.-Y., Undheim, E. A. B., Senff, S., Mobli, M., Dauly, C., Escoubas, P., Nicholson, G. M., Kaas, Q., Guo, S., Herzig, V., Mattick, J. S., & King, G. F. (2020). Structural venomics reveals evolution of a complex venom by duplication and diversification of an ancient peptide-encoding gene. *Proceedings of the National Academy of Sciences*, *117*(21), 11399 LP – 11408. https://doi.org/10.1073/pnas.1914536117

Post, D. C., & Jeanne, R. L. (1983). Venom Source of a sex pheromone in the social wasp *Polistes fuscatus* (Hymenoptera: Vespidae). *Journal of Chemical Ecology 1983 9:2*, *9*(2), 259–266. https://doi.org/10.1007/BF00988043

Riechert, S. E. (1978). Games spiders play: behavioural variability in territorial disputes. *Behavioural Ecology and Sociobiology*, *3*(2), 135–162. https://doi.org/10.1007/BF00294986

Saez, N. J., Senff, S., Jensen, J. E., Er, S. Y., Herzig, V., Rash, L. D., & King, G. F. (2010). Spider-venom peptides as therapeutics. *Toxins*, *2*(12), 2851–2871. https://doi.org/10.3390/toxins2122851

Santana, R. C., Perez, D., Dobson, J., Panagides, N., Raven, R. J., Nouwens, A., Jones, A., King, G. F., & 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*(4), 1–13. https://doi.org/10.3390/toxins9040116

Scaccabarozzi, D., Dods, K., Le, T. T., Gummer, J. P. A., Lussu, M., Milne, L., Campbell, T., Wafujian, B. P., & Priddis, C. (2021). Factors driving the compositional diversity of *Apis mellifera* bee venom from a *Corymbia calophylla* (marri) ecosystem, Southwestern Australia. *PLOS ONE*, *16*(6), e0253838.

https://doi.org/10.1371/JOURNAL.PONE.0253838

Schendel, V., Rash, L. D., Jenner, R. A., & Undheim, E. A. B. (2019). The diversity of venom: the importance of behaviour and venom system morphology in understanding its ecology and evolution. *Toxins*, *11*(11), 666. https://doi.org/10.3390/toxins11110666

Sentenská, L., Graber, F., Richard, M., & Kropf, C. (2017). Sexual dimorphism in venom gland morphology in a sexually stinging scorpion. *Biological Journal of the Linnean Society*, *122*(2), 429–443. https://doi.org/10.1093/biolinnean/blx067

Shearer, T. A., & Pruitt, J. N. (2014). Individual differences in boldness positively correlate with heart rate in orb-weaving spiders of genus *Larinioides*. *Current Zoology*, *60*(3), 387–391. https://doi.org/10.1093/czoolo/60.3.387

Sih, A., Montiglio, P. O., Wey, T. W., & Fogarty, S. (2017). Altered physical and social conditions produce rapidly reversible mating systems in water striders. *Behavioural Ecology*, *28*(3), 632–639. https://doi.org/10.1093/beheco/arx021

Vapenik, Z., & Nentwig, W. (2000). The influence of hunger and breeding temperature on the venom production of the spider *Cupiennius salei* (Araneae, Ctenidae). *Toxicon*, *38*(2), 293–298. https://doi.org/10.1016/S0041-0101(99)00146-4

Wang, X.-H., Smith, R., Fletcher, J. I., Wilson, H., Wood, C. J., Howden, M. E. H., & King, G. F. (2001). Structure-function studies of ω-atracotoxin, a potent antagonist of insect voltage-gated calcium channels. *European Journal of Biochemistry*, *264*(2), 488–494.

Williams, G. A., Serena, M., & Grant, T. R. (2013). Age-related change in spurs and spur sheaths of the platypus (*Ornithorhynchus anatinus*). *Australian Mammalogy*, *35*(1), 107–114. https://doi.org/10.1071/AM12011

Wilson, D. (2016). The Venom of Australian Spiders. In & E. D.-G. P. Gopalakrishnakone, G.

Corzo, M. E. de Lima (Ed.), *Spider Venoms* (pp. 21–46). Springer Netherlands. https://doi.org/10.1007/978-94-007-6389-0_21

Wilson, D., & Daly, N. (2018). Venomics: a mini-review. *High-Throughput*, *7*(3), 19. https://doi.org/10.3390/ht7030019

Wolf, M., & Weissing, F. J. (2012). Animal personalities: Consequences for ecology and evolution. *Trends in Ecology and Evolution*, *27*(8), 452–461. https://doi.org/10.1016/j.tree.2012.05.001

Wong, M. K. L., Woodman, J. D., & Rowell, D. M. (2016). Temporal variation in venom yield of the Australian funnel-web spider *Atrax sutherlandi* (Hexathelidae: Atracinae). *Arachnology*, *17*(1), 7–9. https://doi.org/10.13156/arac.2006.17.1.7

Wullschleger, B., & Nentwig, W. (2002). Influence of venom availability on a spider's prey-choice behaviour. *Functional Ecology*, *16*(6), 802–807. https://doi.org/10.1046/j.1365-2435.2002.00686.x

Zancolli, G., Calvete, J. J., Cardwell, M. D., Greene, H. W., Hayes, W. K., Hegarty, M. J., Herrmann, H. W., Holycross, A. T., Lannutti, D. I., Mulley, J. F., Sanz, L., Travis, Z. D., Whorley, J. R., Wüster, C. E., & Wüster, W. (2019). When one phenotype is not enough: Divergent evolutionary trajectories govern venom variation in a widespread rattlesnake species. *Proceedings of the Royal Society B: Biological Sciences*, *286*(1898), 20182735. https://doi.org/10.1098/rspb.2018.2735

Zhan, Y., Jiang, H., Wu, Q., Zhang, H., Bai, Z., Kuntner, M., & Tu, L. (2019). Comparative morphology refines the conventional model of spider reproduction. *PLoS ONE*, *14*(7). https://doi.org/10.1371/journal.pone.0218486

Zobel-Thropp, P. A., Bulger, E. A., Cordes, M. H. J., Binford, G. J., Gillespie, R. G., & Brewer,

M. S. (2018). Sexually dimorphic venom proteins in long-jawed orb-weaving spiders (*Tetragnatha*) comprise novel gene families. *PeerJ, 2018*(6), e4691. https://doi.org/10.7717/peerj.4691

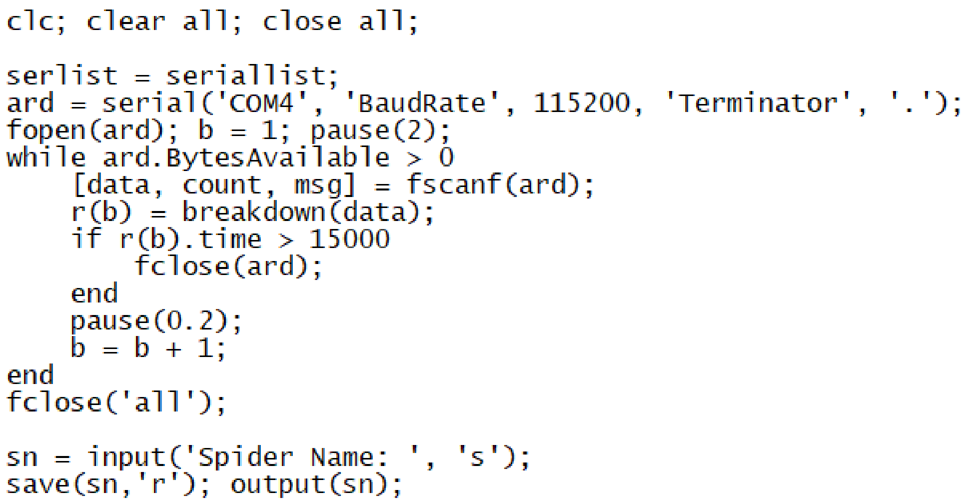
6.7. Appendix Chapter 6

Appendix 6. 1. Heart rate monitor- description.

The signal of the sensor was digitised to facilitate analysis and graphing using MATLAB (model: R2018a, version: 9.4.0.813654). The step of data acquisition implements FIFO (First in First out), where a rapid read of the data is implemented to avoid losing data. A micro-controller was connected to ensure both, the computer and the sensor, were always connected and synchronised to ensure that a clear track for data were present (Chu et al., 2017). The only power for the sensor to switch on is 2-wire, Serial Data (SDA) and Serial Clock (SCL). Serial Data are data read through a serial connection, while the Serial Clock gives each data section on the SDA line a specific timing for it to be transferred. This facilitates no overlapping or loss of data (Martinek et al., 2017). After acquiring the data and securing its integrity, the data were converted into presentable information associated with each spider.

The scrip used to run registered the heart rate monitor using MATAB is described below:

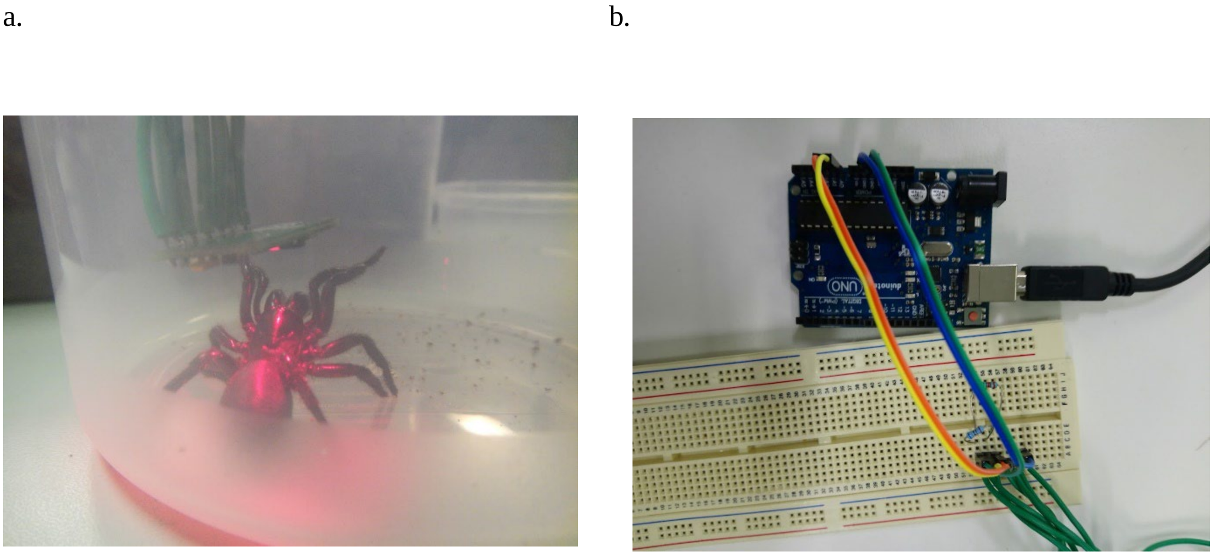
Open and run



```
clc; clear all; close all;

serlist = seriallist;
ard = serial('COM4', 'BaudRate', 115200, 'Terminator', '.');
fopen(ard); b = 1; pause(2);
while ard.BytesAvailable > 0
    [data, count, msg] = fscanf(ard);
    r(b) = breakdown(data);
    if r(b).time > 15000
        fclose(ard);
    end
    pause(0.2);
    b = b + 1;
end
fclose('all');

sn = input('Spider Name: ', 's');
save(sn,'r'); output(sn);
```



The picture shows the heart rate monitor measuring spider heart beats (a). The heart rate monitor was built using a protoboard to build the circuit (b), which allowed a direct connection with the laptop to register the heart rate frequency using the software MATLAB.

Table S6.1. Output of rank-based non-parametric analyses for longitudinal data models of *H. cerberea* and *A. robustus* to test differences in behavioural traits, heart rate and body condition, and the effects of repetitions and life stage. The * refers to results that are significant at the α = 0.05 level.

ANOVA-Type Statistic (ATS)			
Model	Huddling ~ Repetition + Stage, subject= factor (ID)		
	H. cerberea		
	Statistic	**df**	**p-value**
Stage	0.680	1	0.421
Repetition	0.112	1.961	0.889
	A. robustus		
Stage	2.093	1	0.147
Repetition	3.518	1.884	0.032 *
Model	Defence (fang frequency) ~ Repetition + Stage, subject= factor (ID)		
	H. cerberea		
	Statistic	**df**	**p-value**
Stage	3.294	1	0.09
Repetition	0.596	1.877	0.54
	A. robustus		
Stage	2.247	1	0.195
Repetition	26.308	1.989	<0.001 *
Model	Defence (frequency of climbing) ~ Repetition + Stage, subject= factor (ID)		
	H. cerberea		
	Statistic	**df**	**p-value**
Stage	0.184	1	0.673
Repetition	1.029	1.893	0.354

	A. robustus		
Stage	0.516	1	0.472
Repetition	0.468	1.828	0.607
Model	Activity ~ Repetition + Stage, subject= factor (ID)		
	A. robustus		
	Statistic	**df**	**p-value**
Stage	0.145	1	0.703
Repetition	1.59	1.723	0.207
Model	Heart rate ~ Repetition + Stage, subject= factor (ID)		
	H. cerberea		
	Statistic	**df**	**p-value**
Stage	0.006	1	0.940
Repetition	1.094	1.9	0.332
	A. robustus		
Stage	0.042	1	0.839
Repetition	0.300	1.564	0.685
Model	Body condition ~ Repetition + Stage, subject= factor (ID)		
	H. cerberea		
	Statistic	**df**	**p-value**
Stage	2.131	1	0.1636
Repetition	16.456	1.827	<0.001 *
	A. robustus		
Stage	0.764	1	0.410
Repetition	2.425	1.252	0.111

Table S6.2. (a) Output axes canonical correspondence analysis (CCA) of complete venom matrix using Chi-square distance Vs behaviour and morphophysiological variables. **(b)** Output axes canonical correspondence analysis (CCA) of complete venom matrix using Bray-Curtis distance Vs behaviour and morphophysiological variables. **(c)** Canonical eigenvalues results are the same for the model with both distances. **(d)** Coefficients CCA using Chi-square distance. **(e)** Coefficients CCA using Bray-Curtis.

a.

Model	**Venom matrix ~ Heart rate + body condition + defence + climbing + activity + huddle, method = Chi-square**			
	Df	Chi-square	F	Pr(>F)
CCA1	1	0.0863	2.7375	0.408
CCA2	1	0.06361	2.0177	0.530
CCA3	1	0.04743	1.5045	0.697
CCA4	1	0.034	1.0785	0.856
CCA5	1	0.02794	0.8862	0.817
CCA6	1	0.01608	0.51	0.903
Residual	16	0.5044		

b.

Model:	**Venom matrix ~ heart rate + body condition + defence + climbing + activity + huddle, method = Bray-Curtis**			
	Df	Bray-Curtis	F	Pr(>F)
CCA1	1	0.0863	2.7375	0.365
CCA2	1	0.06361	2.0177	0.464
CCA3	1	0.04743	1.5045	0.642
CCA4	1	0.034	1.0785	0.83
CCA5	1	0.02794	0.8862	0.8
CCA6	1	0.01608	0.51	0.903
Residual	16	0.5044		

c.

	CCA1	CCA2	CCA3	CCA4	CCA5	CCA6	CA1	CA2	CA3
Eigenvalue	0.086	0.064	0.047	0.034	0.028	0.016	0.181	0.079	0.044
Proportion explained	0.111	0.082	0.061	0.044	0.036	0.021	0.232	0.101	0.056
Cumulative proportion	0.111	0.192	0.253	0.297	0.333	0.353	0.585	0.686	0.743

	CA4	CA5	CA6	CA7	CA8	CA9	CA10	CA11	CA12
Eigenvalue	0.039	0.030	0.021	0.019	0.017	0.015	0.013	0.012	0.011
Proportion explained	0.050	0.038	0.028	0.024	0.022	0.019	0.017	0.016	0.014
Cumulative proportion	0.793	0.831	0.859	0.883	0.904	0.923	0.940	0.956	0.970

	CA13	CA14	CA15	CA16
Eigenvalue	0.008	0.007	0.004	0.003
Proportion explained	0.011	0.009	0.006	0.004
Cumulative proportion	0.981	0.990	0.996	1

d.

	CCA1	CCA2	CCA3	CCA4	CCA5	CCA6
Heart rate	-0.437	-0.022	0.311	0.008	0.120	0.134
Body condition	-0.102	-2.584	0.434	-3.665	1.402	0.042
Defence	0.003	0.077	-0.032	-0.048	-0.024	-0.020
Climbing	0.445	0.236	0.300	-0.004	0.312	0.009
Activity	-0.002	0.002	0.003	-0.004	-0.007	0.000
Huddle	-1.527	-0.564	1.158	1.304	0.152	-2.509

e.

	CCA1	**CCA2**	**CCA3**	**CCA4**	**CCA5**	**CCA6**
Body condition	-0.102	-2.584	0.434	-3.665	1.402	0.042
Heart rate	-0.437	-0.022	0.311	0.008	0.120	0.134
Defence	0.003	0.077	-0.032	-0.048	-0.024	-0.020
Climbing	0.445	0.236	0.300	-0.004	0.312	0.009
Activity	-0.002	0.002	0.003	-0.004	-0.007	0.000
Huddle	-1.527	-0.564	1.158	1.304	0.152	-2.509

Table S6.3. (a) Output axes canonical correspondence analysis (CCA) of reduced venom matrix using Chi-square distance Vs morphophysiological variables. **(b)** Output axes canonical correspondence analysis (CCA) of reduced venom matrix using bray-Curtis distance Vs morphophysiological variables. **(c)** Coefficients CCA using Chi-square distance. **(d)** Coefficients CCA using bray-Curtis.

a.

Model	**Reduced venom matrix ~ heart rate + body condition + Defence + climbing + activity + huddle**			
	Df	Chi-square	F	Pr(>F)
CCA1	1	0.075	2.091	0.418
CCA2	1	0.070	1.972	0.374
CCA3	1	0.061	1.710	0.433
CCA4	1	0.048	1.340	0.616
CCA5	1	0.041	1.161	0.572
CCA6	1	0.016	0.456	0.977
Residual	16	0.570		

b.

model:	**Reduced venom matrix ~ heart rate + body condition + defence + climbing + activity + huddle, model = bray**			
	Df	Chi-square	F	Pr(>F)
CCA1	1	0.075	2.091	0.412
CCA2	1	0.070	1.972	0.371
CCA3	1	0.061	1.710	0.400
CCA4	1	0.048	1.340	0.596
CCA5	1	0.041	1.161	0.578
CCA6	1	0.016	0.456	0.977
Residual	16	0.570		

c.

	CCA1	**CCA2**	**CCA3**	**CCA4**	**CCA5**	**CCA6**
Body condition	-1.639	-0.245	2.167	2.865	2.633	0.505
Heart rate	-0.159	-0.543	-0.084	-0.011	0.127	-0.102
Defence	0.088	-0.007	0.038	0.010	-0.017	0.019
Climbing	0.290	0.178	-0.420	0.094	0.416	0.093
Huddle	-1.369	-1.634	-1.166	-0.219	-1.014	2.262
Activity	0.003	-0.004	0.001	0.005	-0.004	-0.002

d.

	CCA1	CCA2	CCA3	CCA4	CCA5	CCA6
Body condition	-1.639	-0.245	2.167	2.865	2.633	0.505
Heart rate	-0.159	-0.543	-0.084	-0.011	0.127	-0.102
Defence	0.088	-0.007	0.038	0.010	-0.017	0.019
Climbing	0.290	0.178	-0.420	0.094	0.416	0.093
Huddle	-1.369	-1.634	-1.166	-0.219	-1.014	2.262
Activity	0.003	-0.004	0.001	0.005	-0.004	-0.002

Table S6.4. (a) Envfit analysis showing the venom components driving the species distribution pattern. The * refers to results that are significant at the α = 0.05 level. **(b)** Output Anova permutation test showing different variances between species. The * refers to results that are significant at the α = 0.05 level. **(c)** Envfit analysis showing the correlation between behavioural and morphophysiological variables with venom components. The * refers to results that are significant at the α = 0.05 level.

a.

Venom components	NMDS1	NMDS2	r2	P. value	
488.999	-0.993	-0.117	0.131	0.010	**
268.073	-0.984	0.176	0.423	0.001	***
279.006	-0.995	0.104	0.361	0.001	***
3863.399	-0.967	-0.256	0.183	0.004	**
7843.648	-0.992	-0.130	0.058	0.098	.

3992.53	-0.708	-0.707	0.063	0.086	.
3921.133	-0.492	-0.870	0.448	0.001	***
4035.537	-0.959	-0.284	0.050	0.153	
3950.169	-0.997	-0.078	0.116	0.01	**
4056.479	-0.977	-0.213	0.038	0.22	
7082.815	-0.998	0.058	0.126	0.005	**
7066.596	-0.983	-0.182	0.230	0.001	***
7049.498	-0.960	-0.278	0.239	0.002	**
4854.311	-0.991	-0.136	0.019	0.574	
4608.323	-0.992	-0.127	0.165	0.004	**
7545.159	-0.905	-0.426	0.044	0.171	
4840.04	-0.970	-0.241	0.014	0.719	
1553.626	-0.995	-0.100	0.044	0.194	
4011.038	-0.892	-0.453	0.073	0.061	.
4612.546	-0.873	-0.488	0.008	0.925	
8216.286	-0.996	0.087	0.140	0.007	**
4792.738	-0.940	-0.341	0.083	0.037	*
7188.962	-0.980	-0.198	0.160	0.001	***
7516.366	-0.995	-0.098	0.215	0.002	**
4163.917	-0.994	-0.106	0.124	0.008	**
7459.229	-0.984	-0.181	0.104	0.01	**
7512.834	-0.964	-0.265	0.007	0.983	
7475.689	-0.997	-0.071	0.048	0.134	
8352.379	-0.996	0.093	0.092	0.029	*
7339.136	-0.945	-0.327	0.062	0.084	.
8420.294	-0.615	-0.789	0.598	0.001	***
5216.099	-0.285	-0.959	0.116	0.01	**
500.381	-0.214	-0.977	0.173	0.002	**
4052.492	-0.265	-0.964	0.046	0.165	
7843.648	-0.247	-0.969	0.108	0.014	*
7798.516	-0.236	-0.972	0.037	0.262	

3724.232	-0.235	-0.972	0.081	0.043	*
8114.018	-0.113	-0.994	0.035	0.235	
414	-0.284	-0.959	0.088	0.032	*
4217.013	-0.194	-0.981	0.228	0.002	**
7057.681	-0.243	-0.970	0.061	0.085	.
7139.291	-0.177	-0.984	0.105	0.019	*
7043.552	-0.296	-0.955	0.089	0.027	*
8036.034	-0.298	-0.954	0.040	0.18	
4824.59	-0.217	-0.976	0.085	0.036	*
8160.238	-0.297	-0.955	0.019	0.654	
7564.274	-0.220	-0.976	0.018	0.68	
7172.912	-0.182	-0.983	0.165	0.005	**
7429.138	-0.123	-0.992	0.080	0.038	*
4202.898	-0.227	-0.974	0.195	0.001	***
4859.736	-0.244	-0.970	0.169	0.001	***
7295.126	-0.140	-0.990	0.041	0.14	
6793.015	-0.223	-0.975	0.036	0.232	
7175.199	-0.219	-0.976	0.294	0.001	***
1336.104	-0.155	-0.988	0.182	0.001	***
298.085	-0.051	0.999	0.296	0.001	***
261.222	-0.052	0.999	0.312	0.001	***
373.788	-0.037	0.999	0.413	0.001	***
330.132	-0.064	0.998	0.041	0.174	
394.011	-0.184	0.983	0.068	0.052	.
694.262	-0.097	0.995	0.143	0.005	**
617.485	-0.002	1.000	0.042	0.097	.
2562.365	-0.106	0.994	0.267	0.001	***
428.121	-0.193	0.981	0.078	0.029	*
1469.438	-0.260	0.966	0.033	0.254	
292.796	-0.241	0.971	0.041	0.157	
6670.43	0.108	0.994	0.127	0.006	**

3906.407	-0.279	0.960	0.018	0.696	
3659.836	-0.016	1.000	0.082	0.037	*
4877.404	-0.017	1.000	0.242	0.001	***
8168.618	-0.063	0.998	0.336	0.001	***
7827.028	-0.101	0.995	0.114	0.011	*
3911.605	-0.084	0.996	0.186	0.001	***
7827.041	0.187	0.982	0.039	0.152	
7514.782	-0.053	0.999	0.401	0.001	***
4604.15	-0.024	1.000	0.133	0.007	**
3392.005	-0.041	0.999	0.282	0.001	***
4231.486	0.001	1.000	0.433	0.001	***
4135.891	-0.079	0.997	0.091	0.025	*
4174.31	-0.127	0.992	0.020	0.612	
7085.805	0.058	0.998	0.076	0.035	*
8261.795	-0.058	0.998	0.101	0.016	*
274.999	-0.070	0.998	0.215	0.001	***
4166.554	-0.056	0.998	0.320	0.001	***
337.245	0.995	-0.103	0.027	0.389	
351.258	0.999	-0.045	0.217	0.001	***
298.711	0.998	-0.066	0.349	0.001	***
4840.189	1.000	0.028	0.074	0.044	*
348(2083.05)	0.911	-0.413	0.190	0.002	**
3503.601	0.978	-0.206	0.200	0.001	***
474.945	0.988	-0.157	0.070	0.055	.
282.702	0.901	-0.433	0.282	0.001	***
3881.267	1.000	-0.015	0.029	0.355	
545.381	0.984	-0.180	0.185	0.001	***
4506.91	0.953	-0.304	0.097	0.015	*
3795.305	0.936	-0.351	0.399	0.001	***
6166.258	0.936	-0.352	0.162	0.004	**
4744.134	0.974	0.226	0.122	0.005	**

1561.063	0.910	0.415	0.070	0.043	*
306	0.923	-0.385	0.210	0.002	**
7154.768	0.981	-0.192	0.187	0.002	**
1460.2427(4377.95)	0.892	-0.452	0.156	0.001	***
4827.471	0.973	-0.230	0.063	0.072	.
4826.311	0.972	-0.236	0.145	0.005	**
4547.85	0.963	0.269	0.024	0.481	
7107.62	1.000	0.011	0.127	0.004	**
4588	0.987	-0.160	0.085	0.033	*
7979.266	0.955	-0.297	0.190	0.001	***
7156.183	0.963	-0.268	0.061	0.075	.
4185.327	0.888	-0.459	0.134	0.004	**
8125.906	0.803	-0.597	0.048	0.071	.
7963.781	0.900	-0.436	0.260	0.001	***
7117.4818	1.000	-0.026	0.188	0.001	***
4214.5417	0.859	-0.512	0.119	0.009	**
4547.2444	0.990	-0.143	0.254	0.001	***
7946.7021	0.882	-0.472	0.213	0.001	***
4103.3168	0.895	-0.446	0.030	0.336	
4528.6558	0.952	-0.305	0.269	0.001	***
7229.2298	0.926	-0.379	0.212	0.001	***
4527.9452	0.944	-0.329	0.078	0.048	*
7301.7331	0.927	-0.374	0.026	0.455	
7274.4236	0.974	0.228	0.140	0.004	**
7330.3427	0.986	0.168	0.174	0.001	***
8821.6224	0.944	-0.330	0.165	0.004	**
7299.3702	0.999	0.054	0.037	0.191	
7618.7917	0.900	-0.436	0.208	0.001	***
7235.0821	0.893	-0.449	0.376	0.001	***
4606.597(1448.051)	0.873	-0.489	0.090	0.017	*
7287.73	0.969	-0.248	0.027	0.411	

2961.7804	0.873	-0.487	0.123	0.014	*
4092.106(1347.312)	0.967	-0.254	0.186	0.001	***
1337.3453	0.946	-0.324	0.295	0.001	***
3992.89(1331.195)	0.896	-0.444	0.366	0.001	***
1394.2998	0.878	-0.479	0.034	0.251	
4076.17(1341)	0.990	-0.144	0.319	0.001	***
1384.0017	0.829	-0.559	0.019	0.637	

b.

Analysis of variance

	Df	Sum Sq	Mean Sq	F value	P. value
Groups	3	0.23529	0.07843	17.912	< 0.001
Residuals	71	0.31088	0.004379		

Pairwise comparisons between species

	A. robustus	*H. cerberea*	*H. infensa*	*H. valida*
A. robustus		1.00E-03	1.00E-03	0.001
H. cerberea	9.26E-06		5.32E-01	0.038
H. infensa	7.18E-07	5.48E-01		0.136
H. valida	3.10E-08	4.14E-02	1.33E-01	

c.

Behaviour	**NMDS1**	**NMDS2**	**r2**	**P. value**	
Heart rate	0.03338	0.99944	0.1256	0.01	**
Defence	-0.30979	-0.95081	0.0439	0.163	
Climbing	-0.82594	-0.56376	0.0251	0.434	
Activity	-0.21072	-0.97755	0.0965	0.016	*
Huddling	-0.21746	0.97607	0.0426	0.219	
Body condition	-0.97363	-0.22811	0.0047	0.855	

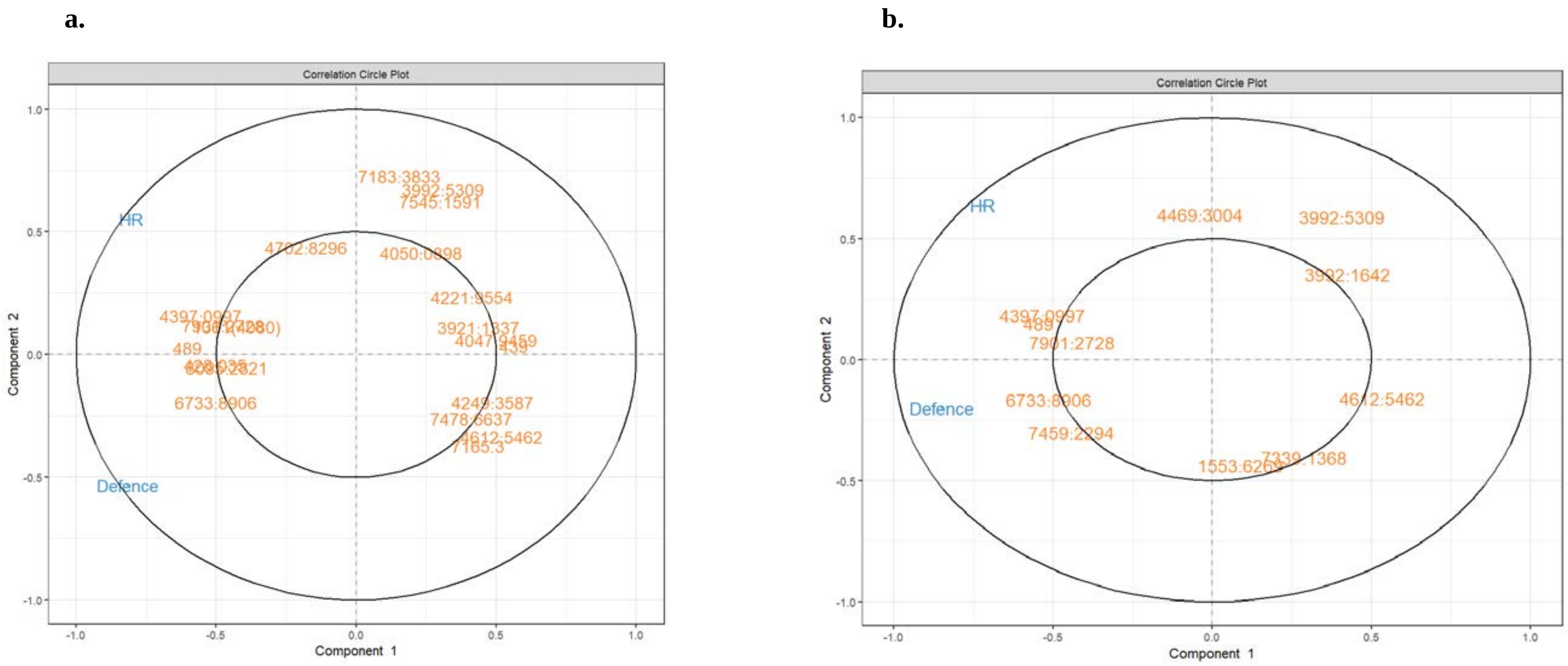


Figure S6.1. a) Correlation circle plots showing the relationship between venom components (complete venom matrix) with behavioural and morphophysiological variables. The variables and venom components outside of the circle show a strong correlation; the variables inside the circle show a weak correlation. b) correlation plot reduced venom matrix Vs venom components. cut-off 0.40.

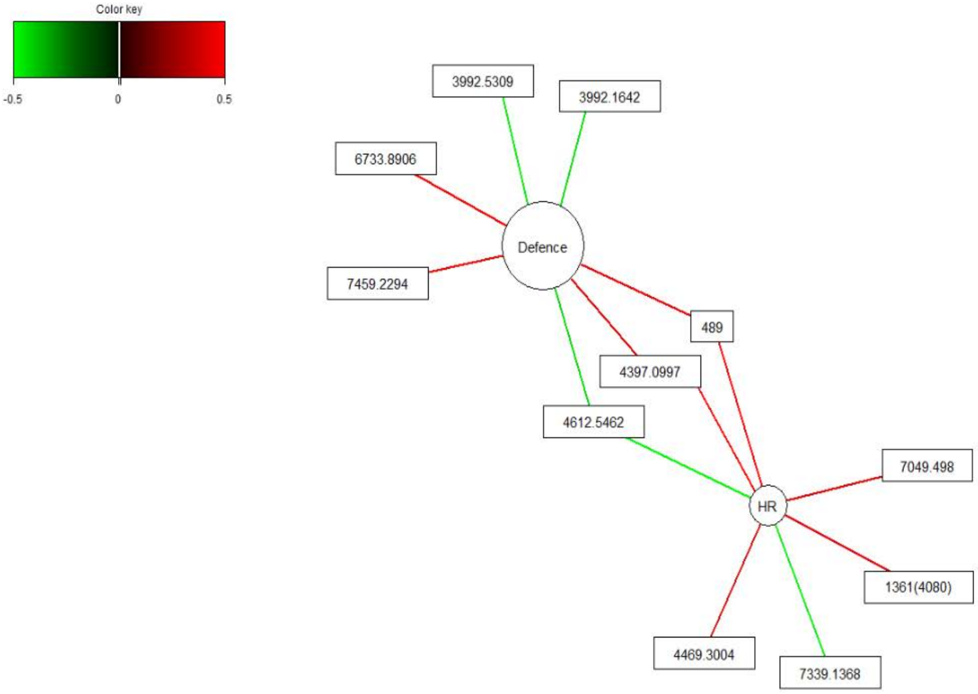
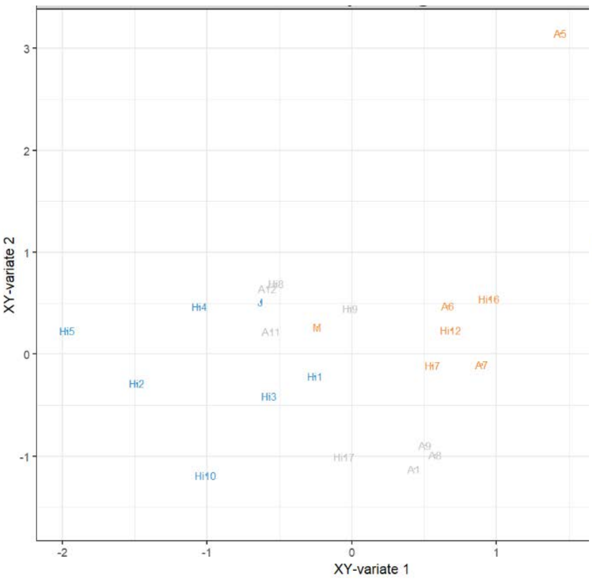


Figure S6.2. Network plot of a regularised canonical correlation analysis (rCCA) showing the structure of the association between venom components (reduced venom component matrix) and morphophysiological variables. The correlation cut-off showed is at 0.40. The colour of each line (red: positive, green: negative association) indicates the nature of the correlation.

a.


XY-variate 2
XY-variate 1
3
2
1
0
-1
-2
-1
0
1
A5
Hi8
A12
Hi4
J
Hi9
A6
Hi16
Hi5
A11
M
Hi12
Hi2
Hi1
Hi7
A7
Hi3
A9
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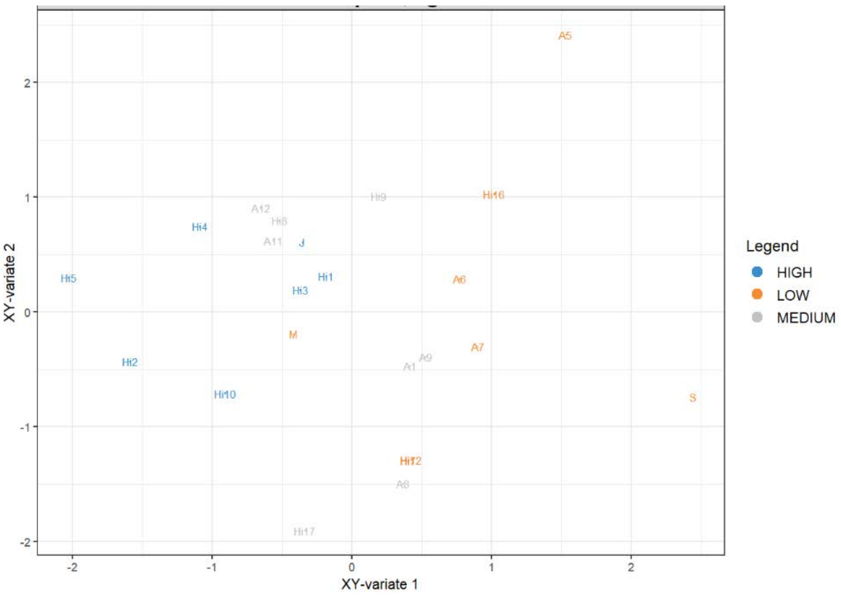
b.


XY-variate 2
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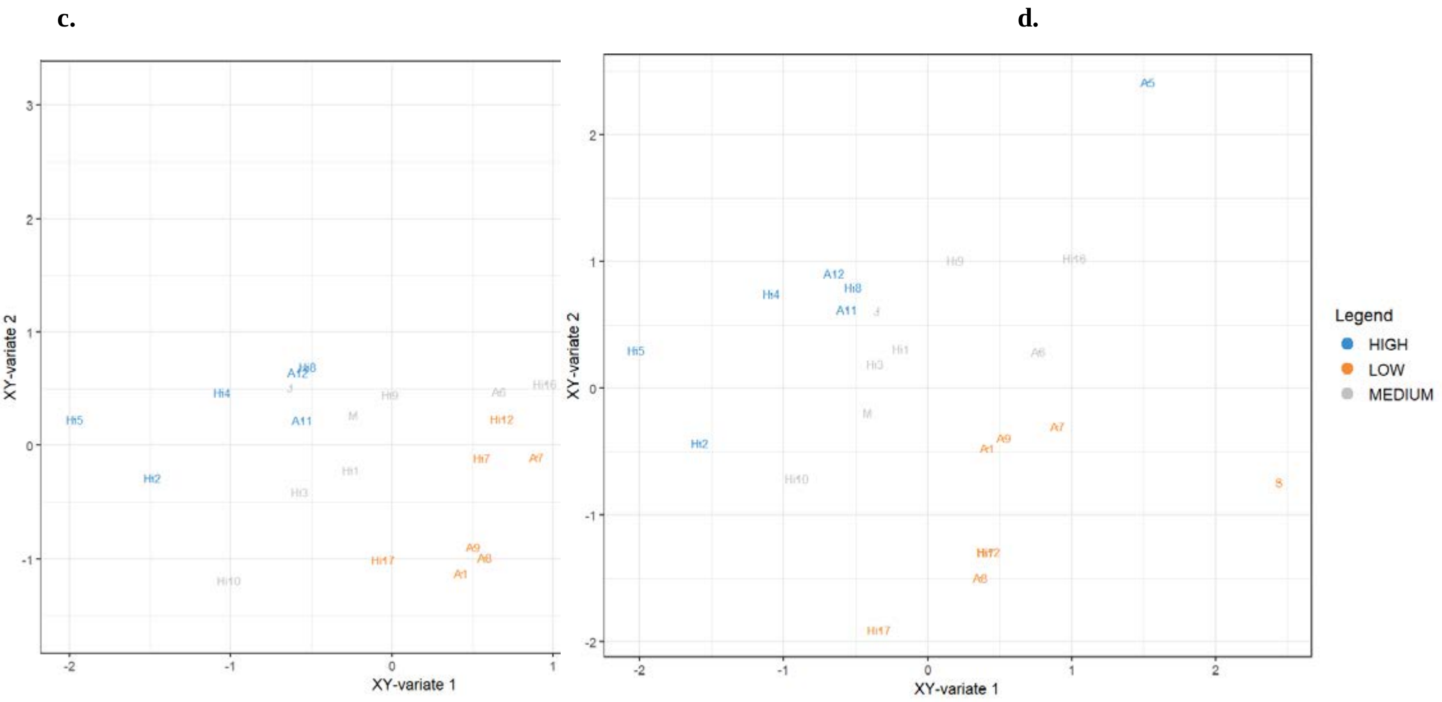
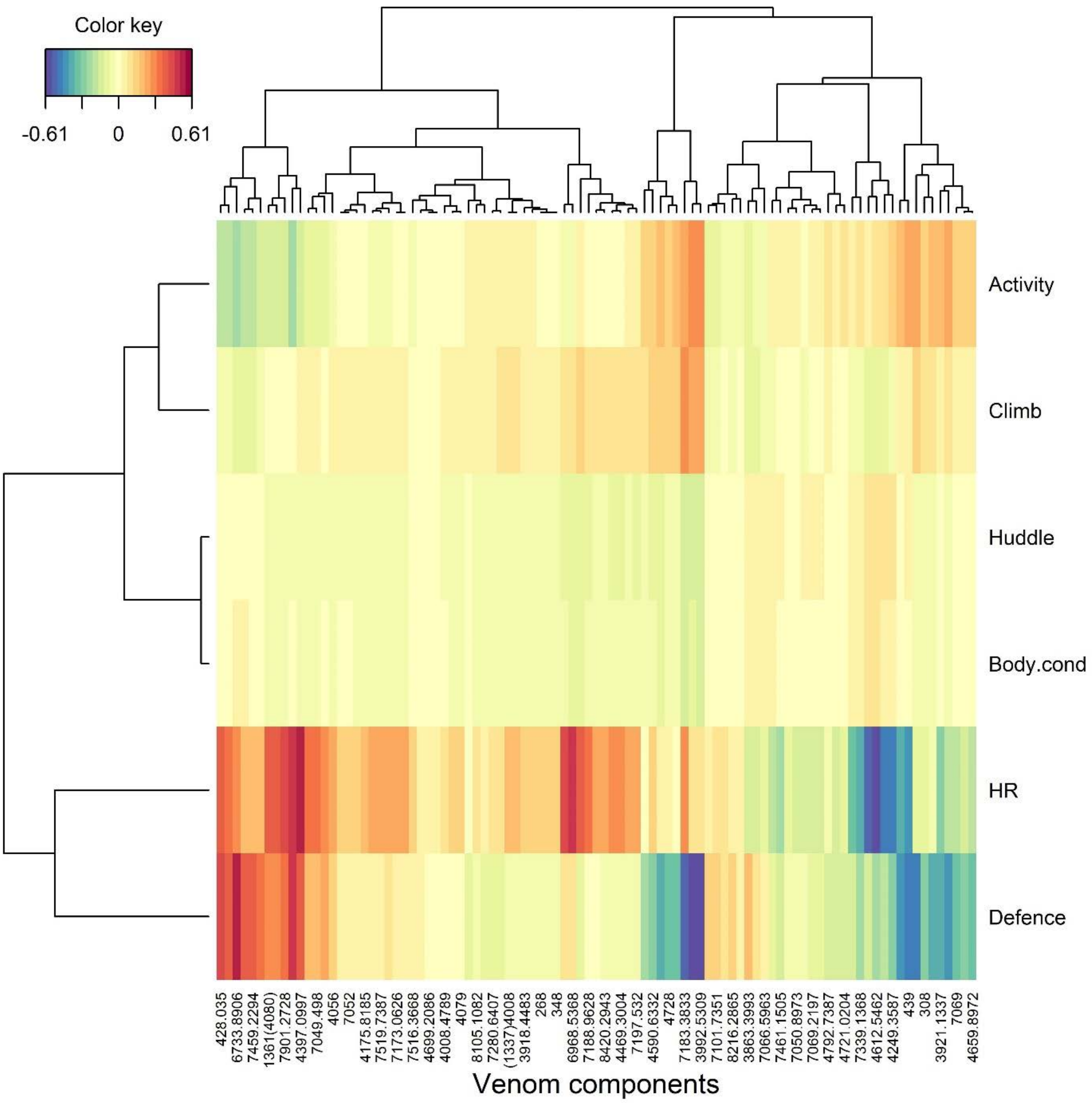


Figure S6.3. Canonical variates corresponding to the relationship between venom components (complete venom matrix) with behavioural and morphophysiological variables. Individuals are projected into the space spanned by the averaged canonical variates and coloured according to the behavioural and morphophysiological information. a) Defensiveness, complete venom matrix; b) Defence, reduced venom matrix; c) Heart rate, complete venom matrix; d) Heart rate, reduced venom matrix

a.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348
6968.5368
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4469.3004
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4728
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3863.3993
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7461.1505
7050.8973
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4792.7387
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7069
4659.8972
Venom components

b.

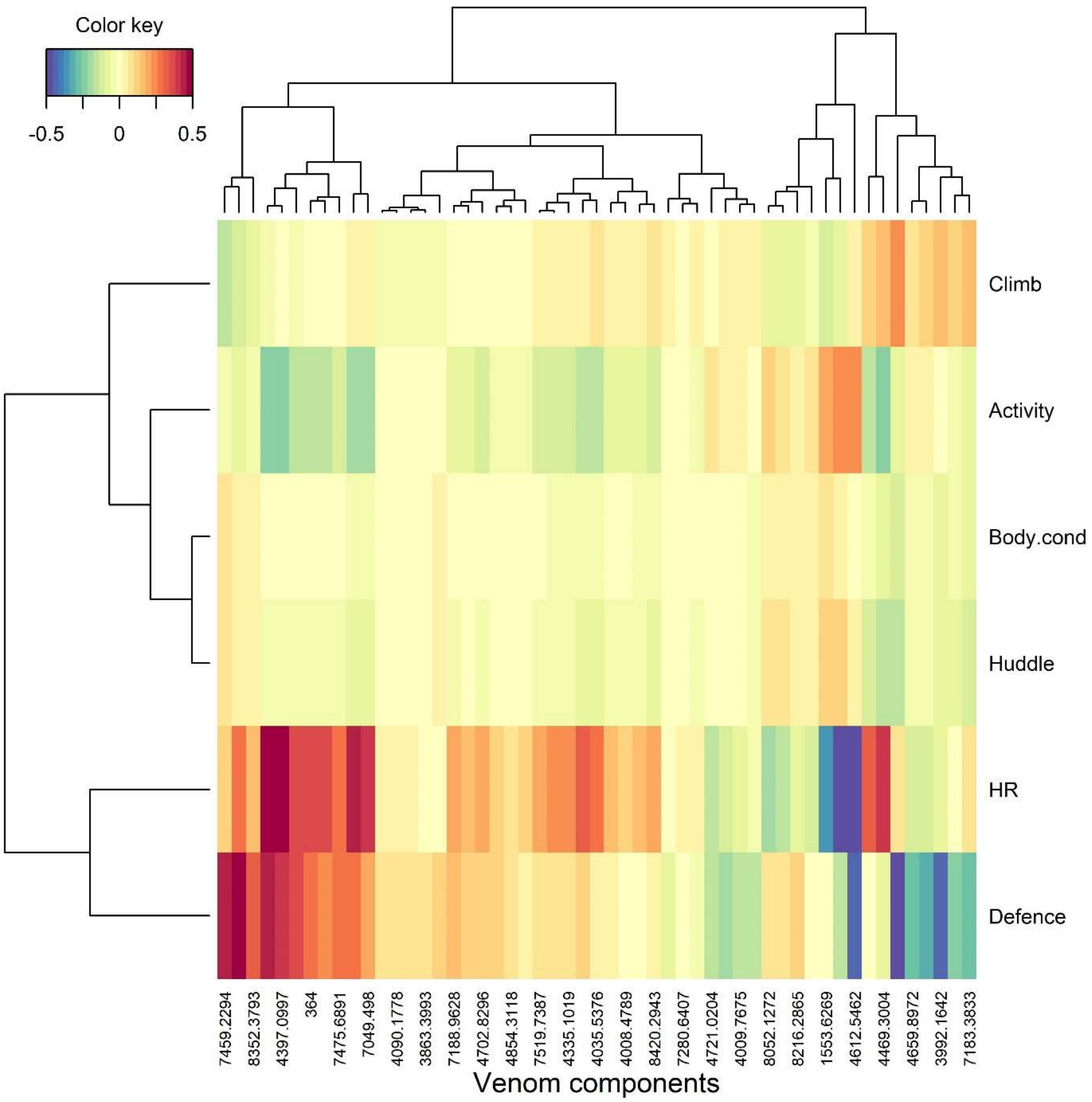

Figure S6.4. Cluster Image Map from regularised canonical correlation analysis (rCCA) showing the correlation structure of venom components. Complete matrix (a) and reduced matrix (b) with behavioural and morphophysiological variables. The first two dimensions from the CCA are display in the cluster.

Chapter 7: General discussion

This thesis provides a framework for understanding the ecological role of venoms in Australian funnel-web spiders. For the first time, I included new factors (behaviour, physiology and ecology) that have not been considered before in the study of funnel-web venoms, and demonstrate how these factors can also be incorporated in the study of other venomous animals. Furthermore, by highlighting the relevance of each factor independently, I then demonstrate how understanding the synergistic interactions between them contribute to determining the ecological role, evolution and diversification of funnel-web venoms. Each chapter of the thesis attempted to close the gap between multiple disciplines (toxinology, behaviour, physiology and ecology) by integrating multiple methodologies and statistical tools not used before in the study of venom.

Individual variation at different levels

To understand changes in phenotypic traits, such as venom and behaviour, it is necessary to consider the variability at the inter- and intra-individual levels. Intra-individual variation promotes changes in population dynamics, affects interspecific interactions, and constrains or promotes predator defences and the ability to compete and thrive in changing conditions (Gibert & DeLong, 2015). While the variability in venom composition between individuals has been studied previously in some spider species (e.g., *Phlogius crassipes*; Santana et al., 2017), two of the novelties of this thesis (Chapter 3) include the study of intra-individual variation in venom composition and inter-individual variation in venom composition over time. While other studies have assessed repeatability of behaviour over time in other species, such as *L. sclopetarius*, *Zygiella x-notata*, *Nuctenea umbratica* (Kralj-Fišer et al., 2017; Kralj-Fišer & Schneider, 2012; Shearer & Pruitt, 2014), *Marpissa muscosa* (Liedtke & Schneider, 2017), this thesis is the first to explore how different behaviours of funnel-webs differs within and between individuals over both time and context (Chapters 4 and 5).

The results obtained in Chapter 3 highlight how venom components within and between individuals varied between stages and over time. I found considerable intra-individual variation over time, indicating that funnel-web venoms are dynamic and fluctuate over time, irrespective of diet and environmental conditions (principally temperature and humidity). Furthermore, the variability observed between individuals in *H. valida* suggests variability in ontogenetic factors (Gibbs et al., 2011; Herzig, 2010; Santana et al., 2017), different selection pressures experienced by spiders in their original microhabitats, as seen in the Egyptian scorpion, *Scorpio maurus palmatus* (Abdel-Rahman et al., 2009), or seasonal variation, as seen in the funnel-web spider *Atrax sutherlandi* (Wong et al., 2016). Although further research on funnel-web venoms is ongoing, given their potential for biodiscovery of drug lead molecules (Redd et al., 2021), it is important to consider both inter- and intra-individual variation when targeting specific molecules, as these might only be present at particular times or present in particular individuals.

The factors that promote and maintain phenotypic variation should not be studied independently, as the results from Chapters 3 and 4 show. Behaviour can affect the activities performed by individuals (e.g., foraging rates), the intensity of response to stimuli/threats, and the ability to cope with changing conditions (Gibert & DeLong, 2015; Nelsen et al., 2014). This is the reason behind my exploration of the role of behaviour in funnel-webs, using multiple ecological contexts and assessing how behaviour changes over time. In Chapter 4, I provided a framework to explore behaviour in funnel-web spiders through the validation of methodologies, and also to compare differences between life stages and types of stimuli over time. The results of this chapter provide insights into understanding the behavioural traits that may be relevant for consideration in future studies focused on in funnel-web antivenom production. *A. robustus* has been the main focus of research due to the threat that δ-atracotoxins present to humans (Graudins et al., 2002; Isbister et al., 2015). Incorporating different behaviours and types of stimuli could provide information about the time spiders take to regenerate these toxins, the ecological role of these toxins, and the optimal conditions for harvesting these molecules, as has been reported in the bee *Apis mellifera* (Scaccabarozzi et al., 2021), where ecological factors and behavioural responses play an important role in bee venom harvesting. This information will be valuable for antivenom production and management of this iconic spider species. Furthermore, consideration of the level of individual variability and responses to different stimuli/threats contributes to a broader understanding of variability at the population level, and how these spiders respond to environmental challenges. Although I did not include males in this thesis, the behavioural framework provided by Chapter 4 will allow future studies to incorporate males, and to consider other factors such as development stage, genetics and type of habitat, to increase the number of potential target molecules.

Variability in venom and behaviour within and between species

While *A. robustus* has been the primary focus of most studies, other funnel-web species have recently gained attention due to their potential for drug discovery, as is the case for *H. infensa* (Redd et al., 2021). However, the factors underlying the variability and production of venoms can be constrained by selection pressures that each species experience in its habitat (Abdel-Rahman et al., 2009; Vernouillet & Kelly, 2020). This has not been considered before in ecological or toxinology studies of funnel-webs. The results obtained in Chapter 3 show that for *H. valida*, season or timing of collection could affect spider venom composition, while geographical origin or simply species-specific characteristics could affect venom composition between species. Despite the considerable within-individual variation that I observed, I still demonstrated that venom composition is a reliable chemotaxonomic marker for species delineation, which has significant relevance for providing taxonomic certainty when attempting to identify species with overlapping distributions.

Furthermore, behavioural traits vary considerably within and between species. As the results obtained in Chapter 5 suggest, the consistency of behaviours within species varies with ecological context (e.g., predation, territory defence and exploration of a new territory). Some species did not show consistency in any behaviours in any contexts (e.g., *H. infensa* and *H. cerberea)*, suggesting that these traits are more flexible or, alternatively, that other traits not considered here, such as developmental experience, food abundance (DiRienzo & Montiglio, 2016), reproductive status (recently moulted females compared to gravid females; Mooney & Haloin, 2006), diet and ecological conditions (studies in the field), could affect the strength, stability and consistency of behaviours in those particular contexts and time periods for each species (Watts et al., 2015).

Between species comparisons are important for understanding species dynamics and how each species modulates their behaviours when they are facing possible threats. These findings are relevant because the level of within species variation can impact species resilience, the ability to colonise new habitats, and exploitation of new resources (Kralj-Fišer & Schneider, 2012; Gibert & DeLong, 2015; Kralj-Fišer et al., 2017). As funnel-web are short-range endemic species vulnerable to disturbance (Mason et al., 2018), understanding how each species responds and adapts to different conditions provides relevant information for the management and conservation of these spiders. Future studies should compare the variability within and between populations from both urban and rural areas to gain insights into the ability of spiders to survive or adapt to disturbed environments, considering that urbanisation and the intensity of other disturbances are increasing (e.g., fires and floods; Main, 2001; Wright et al., 2010; Mason et al., 2019).

An holistic view

Schendel et al (2019) noted that it is impossible to understand the ecological role of venoms if the interactions between multiple factors are not considered. Continuing to independently study the factors that affect the variability of venoms will also limit the discovery of toxin molecules with pharmacological potential. The results obtained in Chapter 6 represent the first approach to understanding holistically how behavioural traits, and their association with morphophysiological traits, work in concert within venomous animals to affect their biochemical arsenal – the cocktails of neurotoxins comprising the venom. This chapter, as well as previous chapters, further highlights the importance of considering ecological contexts and types of stimuli to which individuals are exposed.

The results for *H. valida* showed that defensive behaviours could covary with antipredator defences (e.g., expression of particular venom molecules), suggesting that individuals that showed a higher level of aggression against predators (prod stimulus) could afford to face the costs (higher heart rate) of displaying defensive behaviours and expelling venom. The metabolic costs of venom use and their relationship with behavioural traits have not been studied before in spiders. Here, I found that spiders that expelled particular venom molecules and performed defensive behaviours had higher heart rates (proxy of metabolic rate), meaning that the use of those traits likely leads to costs of production (energy consumption) and regeneration of venom. My findings are consistent with Shearer & Pruitt, (2014), where individuals that had higher heart rate frequencies tended to exhibit defensive behaviours in order to secure resources to maintain their metabolism. This can be advantageous when resources are abundant because aggressive spiders can better protect their territories and deter predators (Luttbeg & Sih, 2010; Shearer & Pruitt, 2014). It is important to note that those behaviours will depend on the prevailing habitat conditions, species, sexes and selection pressures (i.e., individuals under lower predation pressure might exhibit riskier behaviours; Zamora-Camacho, 2022) to which individuals are exposed.

The integration of variables should be considered on a species basis due to differences in selection pressures (e.g., prey type and predators) and habitat types experienced by different species, as the results from Chapter 6 highlight. The differences in microhabitat conditions between *A. robustus*, *H. cerberea* and *H. infensa* could have concealed an association between venom components with behavioural and morphophysiological variables. Other behavioural traits, or a combination of them, could play a different role in a particular species in different contexts (Vernouillet & Kelly, 2020; Zamora-Camacho, 2022). Furthermore, the variability at the individual level in behaviour and venom components accounted for by juveniles and adults in *A. robustus* and *H. cerberea* could have affected the association between traits (Chapter 5), even though the effects between stages were considered in the analyses. It has been observed that juvenile and adults differ in venom profiles (Hernández Duran et al., 2020; Santana et al., 2017) and behavioural responses when they are exposed to different contexts, such as antipredator behaviour (Jackson et al., 1990; Llewelyn et al., 2012). Future studies should consider juveniles and adults separately (or should track individuals over the course of their lifetimes) and other behavioural traits that may have specific relevance for each stage (Langenhof & Komdeur, 2018). For example, in the spider, *Pholcus phalangioide,* juveniles and adults showed differences in behavioural responses when they were exposed to a threatening stimulus (Jackson et al., 1990).

The association between behavioural traits and morphophysiological variables between species in Chapter 6 further highlights the relevance of venom components for species identification. I demonstrated again that venom profiles are reliable chemotaxonomic markers for delineating species, despite the variability in venom composition observed at both inter- and intra-individual levels. Although many recent taxonomic tools are based on genetic markers, my findings demonstrate that incorporating venom profiles in taxonomy and systematics will provide considerable support for species delimitation. The association between behaviours and morphophysiological variables between species is a step forward to understanding how multiple drivers of phenotypic variation (e.g., venom variability, behavioural traits expression) interact. My findings that activity and heart rate are associated with specific venom compounds in all species suggests that these patterns may be driven by individual variability and microhabitat conditions, rather than species-specific responses. For example, while less active individuals were all *H. cerberea*, a tree-dwelling species characterised by its restricted mobility (Gray, 2010), some individuals from *H. valida, A. robustus* and *H. infensa* showed similar activity patterns, as all are ground-dwelling species that have a more active life style compared to the tree-dwelling individuals. Furthermore, heart rate was positively associated with particular venom components and showed similar patterns to activity, where individuals of *H. cerberea* showed the highest heart rate frequencies, while individuals of *H. valida, H. infensa* and *A. robustus* were more similar, showing lower heart rate frequencies. While it might be tempting to suggest that an inactive spider with a high heart rate could be identified as *H. cerberea*, my findings stress the importance of understanding behaviour and morphophysiological characteristics within the ecological context, and demonstrate that venom components are differentially associated with these characteristics, despite species type.

Contribution and future directions

The findings from this thesis demonstrate that considering multiple factors such as context, stimulus, behavioural traits and morphophysiological variables, as well as their interactions, contribute to a greater understanding of underlying processes driving the ecological role, diversification and evolution of funnel-web venoms. Understanding how behavioural responses trigger physiological processes, involving changes in metabolic consumption, venom variability and functionality, may significantly increase the number of potential venom molecules for drug discovery. For example, to target toxins related with bioinsecticidal properties, it is necessary to consider a foraging context, where the spiders likely will use their venom for predation. Conversely, to target δ-atracotoxins, an anti-predatory context should be considered, where the spiders are facing a threat, leading to a higher likelihood that these toxins will be expressed (Herzig et al., 2020). This may also contribute to improving antivenom production through the optimisation of methods for harvesting animals. Additionally, the results of this thesis also contribute to understanding what aspects may be considered for the management and conservation of these iconic spiders. Knowing how consistent or flexible some behavioural traits are in particular contexts will provide information about how resilient or vulnerable funnel-webs are to urbanisation and the intensification of natural disturbances.

The experiments and results of this thesis fill several gaps in understanding the factors that drive the variability and evolution of funnel-web venoms. However, multiple questions have also arisen from this thesis. For example, how do other factors, such as diet or substrate type, affect variability in behaviour and venom composition? Experiments with diet variation maybe provide insights into venom changes when the spiders are fed with different invertebrates, also the substrate differs in each habitat which can affect physiology. How do males respond to different stimuli and contexts? Running behavioural experiments on males, as well as including other factors such as environmental variables, could provide information about when and how particular toxins are expressed. What other physiological changes, besides heart rate and venom composition, interact with behavioural responses? Environmental conditions and experiments *in situ* will reveal questions related to variation in venom composition and the adaptations that each species have gone through each selection pressure in their own habitat. There is still much to learn about funnel-web spider biology more broadly, but the experiments and methodologies in this thesis provide a framework from which future research can continue to build our understanding of the drivers of phenotypic variation in venomous animals.

7.1. References

Abdel-Rahman, M. A., Omran, M. A. A., Abdel-Nabi, I. M., Ueda, H., & McVean, A. (2009). Intraspecific variation in the Egyptian scorpion *Scorpio maurus palmatus* venom collected from different biotopes. *Toxicon*, *53*(3), 349–359. https://doi.org/10.1016/j.toxicon.2008.12.007

DiRienzo, N., & Montiglio, P. O. (2016). The contribution of developmental experience vs. condition to life history, trait variation and individual differences. *Journal of Animal Ecology*, *85*(4), 915–926. https://doi.org/10.1111/1365-2656.12512

Gibbs, H. L., Sanz, L., Chiucchi, J. E., Farrell, T. M., & 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*(10), 2169–2179. https://doi.org/10.1016/j.jprot.2011.06.013

Gibert, J. P., & DeLong, J. P. (2015). Individual variation decreases interference competition but increases species persistence. In *Advances in Ecological Research* (Vol. 52, pp. 45–64). Academic Press. https://doi.org/10.1016/bs.aecr.2015.01.002

Graudins, A., Wilson, D., Alewood, P. F., Broady, K. W., & Nicholson, G. M. (2002). Cross-reactivity of Sydney funnel-web spider antivenom: neutralization of the in vitro toxicity of other Australian funnel-web (*Atrax* and *Hadronyche*) spider venoms. *Toxicon : Official*

Journal of the International Society on Toxinology, *40*(3), 259–266. https://doi.org/10.1016/S0041-0101(01)00210-0

Gray, M. (2010). A revision of the Australian funnel-web spiders (Hexathelidae: Atracinae). *Records of the Australian Museum*, *62*(2–3), 285–392. https://doi.org/10.3853/j.0067-1975.62.2010.1556

Hernández Duran, L., Rymer, T. L., & Wilson, D. T. (2020). Variation in venom composition in the Australian funnel-web spiders *Hadronyche valida*. *Toxicon:X, 8,* 100063. https://doi.org/10.1016/j.toxcx.2020.100063

Herzig, V. (2010). Ontogenesis, gender, and molting influence the venom yield in the spider *Coremiocnemis tropix* (Araneae, Theraphosidae). *Journal of Venom Research, 1,* 76–83. http://www.ncbi.nlm.nih.gov/pubmed/21544186

Herzig, V., Sunagar, K., Wilson, D. T. R., Pineda, S. S., Israel, M. R., Dutertre, S., McFarland, B. S., Undheim, E. A. B., Hodgson, W. C., Alewood, P. F., Lewis, R. J., Bosmans, F., Vetter, I., King, G. F., & Fry, B. G. (2020). Australian funnel-web spiders evolved human-lethal δ-hexatoxins for defence against vertebrate predators. *Proceedings of the National Academy of Sciences of the United States of America, 117*(40), 24920–24928. https://doi.org/10.1073/pnas.2004516117

Isbister, G. K., Sellors, K. V., Beckmann, U., Chiew, A. L., Downes, M. A., & Berling, I. (2015). Catecholamine-induced cardiomyopathy resulting from life-threatening funnel-web spider envenoming. In *Medical Journal of Australia* (Vol. 203, Issue 7, pp. 302–304). https://doi.org/10.5694/mja15.00279

Jackson, R. R., Brassington, R. J., & Rowe, R. J. (1990). Anti-predator defences of *Pholcus phalangioides* (Araneae, Pholcidae), a web-building and web-invading spider. *Journal of Zoology*, *220*(4), 543–552. https://doi.org/10.1111/j.1469-7998.1990.tb04733.x

Kralj-Fišer, S., Hebets, E. A., & Kuntner, M. (2017). Different patterns of behavioural variation across and within species of spiders with differing degrees of urbanization. *Behavioural Ecology and Sociobiology*, *71*(8), 1–15. https://doi.org/10.1007/s00265-017-2353-x

Kralj-Fišer, S., & Schneider, J. M. (2012). Individual behavioural consistency and plasticity in an urban spider. *Animal Behaviour*, *84*(1), 197–204. https://doi.org/10.1016/j.anbehav.2012.04.032

Langenhof, M. R., & Komdeur, J. (2018). Why and how the early-life environment affects development of coping behaviours. *Behavioural Ecology and Sociobiology*, *72*(3), 2–23. https://doi.org/10.1007/s00265-018-2452-3

Liedtke, J., & Schneider, J. M. (2017). Social makes smart: rearing conditions affect learning and social behaviour in jumping spiders. *Animal Cognition*, *20*(6), 1093–1106. https://doi.org/10.1007/s10071-017-1125-3

Llewelyn, J., Bell, K., Schwarzkopf, L., Alford, R. A., & Shine, R. (2012). Ontogenetic shifts in a prey’s chemical defences influence feeding responses of a snake predator. *Oecologia*, *169*(4), 965–973. https://doi.org/10.1007/S00442-012-2268-1

Luttbeg, B., & Sih, A. (2010). Risk, resources and state-dependent adaptive behavioural syndromes. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *365*(1560), 3977–3990. https://doi.org/10.1098/RSTB.2010.0207

Main, B. Y. (2001). Historical ecology, responses to current ecological changes and conservation of Australian spiders. *Journal of Insect Conservation*, *5*(1), 9–25. https://doi.org/10.1023/A:1011337914457

Mason, L., Bateman, P. W., Miller, B. P., & Wardell-Johnson, G. W. (2019). Ashes to ashes: Intense fires extinguish populations of urban short-range endemics. *Austral Ecology*, *44*(3), 514–522. https://doi.org/10.1111/AEC.12685

Mason, L. D., Bateman, P. W., & Wardell-Johnson, W. (2018). The pitfalls of short-range endemism: high vulnerability to ecological and landscape traps. *PeerJ*, *2018*(5), e4715. https://doi.org/10.7717/PEERJ.4715/SUPP-1

Mooney, K. A., & Haloin, J. R. (2006). Spider size and guarding of offspring affect *Paraphidippus aurantius* (Araneae, Salticidae) response to predation threat. *The Journal of Arachnology*, *34*, 34–98. https://doi.org/10.1636/S02-64.1

Nelsen, D. R., Kelln, W., & 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. https://doi.org/10.1016/j.anbehav.2013.12.019

Redd, M. A., Scheuer, S. E., Saez, N. J., Yoshikawa, Y., Chiu, H. S., Gao, L., Hicks, M., Villanueva, J. E., Joshi, Y., Chow, C. Y., Cuellar-Partida, G., Peart, J. N., See Hoe, L. E., Chen, X., Sun, Y., Suen, J. Y., Hatch, R. J., Rollo, B., Xia, D., … Palpant, N. J. (2021). Therapeutic inhibition of acid-sensing ion channel 1a recovers heart function after ischemia–reperfusion injury. *Circulation*, *144*(12), 947–960. https://doi.org/10.1161/CIRCULATIONAHA.121.054360

Santana, R. C., Perez, D., Dobson, J., Panagides, N., Raven, R. J., Nouwens, A., Jones, A., King, G. F., & 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*(4), 1–13. https://doi.org/10.3390/toxins9040116

Scaccabarozzi, D., Dods, K., Le, T. T., Gummer, J. P. A., Lussu, M., Milne, L., Campbell, T., Wafujian, B. P., & Priddis, C. (2021). Factors driving the compositional diversity of *Apis mellifera* bee venom from a *Corymbia calophylla* (marri) ecosystem, Southwestern Australia. *PLOS ONE, 16*(6), e0253838. https://doi.org/10.1371/JOURNAL.PONE.0253838

Shearer, T. A., & Pruitt, J. N. (2014). Individual differences in boldness positively correlate with heart rate in orb-weaving spiders of genus *Larinioides*. *Current Zoology, 60*(3), 387–391. https://doi.org/10.1093/czoolo/60.3.387

Sih, A., Cote, J., Evans, M., Fogarty, S., & Pruitt, J. (2012). Ecological implications of behavioural syndromes. *Ecology Letters, 15*(3), 278–289. https://doi.org/10.1111/j.1461-0248.2011.01731.x

Vernouillet, A., & Kelly, D. M. (2020). Individual exploratory responses are not repeatable across time or context for four species of food-storing corvid. *Scientific Reports, 10*(1), 1–11. https://doi.org/10.1038/s41598-019-56138-y

Watts, J. C., Ross, C. R., & Jones, T. C. (2015). Diel and life-history characteristics of personality: Consistency versus flexibility in relation to ecological change. *Animal Behaviour, 101*, 43–49. https://doi.org/10.1016/j.anbehav.2014.12.020

Wong, M. K. L., Woodman, J. D., & Rowell, D. M. (2016). Temporal variation in venom yield of the Australian funnel-web spider *Atrax sutherlandi* (Hexathelidae: Atracinae). *Arachnology, 17*(1), 7–9. https://doi.org/10.13156/arac.2006.17.1.7

Wright, T. F., Eberhard, J. R., Hobson, E. A., Avery, M. L., & Russello, M. A. (2010). Behavioural flexibility and species invasions: The adaptive flexibility hypothesis. *Ethology Ecology and Evolution, 22*(4), 393–404. https://doi.org/10.1080/03949370.2010.505580

Zamora-Camacho, F. J. (2022). The relationships between toad behaviour, antipredator defences, and spatial and sexual variation in predation pressure. *PeerJ, 10*, e12985. https://doi.org/10.7717/peerj.12985