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Unravelling the complex venom landscapes of lethal Australian funnel-web spiders (Hexathelidae: Atracinae) using LC-MALDI-TOF mass spectrometry

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ABSTRACT

Spider venoms represent vast sources of bioactive molecules whose diversity remains largely unknown. Indeed, only a small subset of species have been studied out of the ~43,000 extant spider species. The present study investigated inter- and intra-species venom complexity in 18 samples collected from a variety of lethal Australian funnel-web spiders (Mygalomorphae: Hexathelidae: Atracinae) using C4 reversed-phase separation coupled to offline MALDI-TOF mass spectrometry (LC-MALDI-TOF MS). An in-depth investigation focusing on four atracine venoms (male *Illawarra wisharti*, male and female *Hadronyche cerberea*, and female *Hadronyche infensa* Toowoomba) revealed, on average, ~800 peptides in female venoms while male venoms contained ~400 peptides, distributed across most HPLC fractions. This is significantly higher than previous estimates of peptide expression in mygalomorph venoms. These venoms also showed distinct intersexual as well as intra- and inter-species variation in peptide masses. Construction of both 3D and 2D contour plots revealed that peptide mass distributions in all 18 venoms were centered around the 3200–5400 *m/z* range and to a lesser extent the 6600–8200 *m/z* range, consistent with previously described hexatoxins. These findings highlight the extensive diversity of peptide toxins in Australian funnel-web spider venoms that that can be exploited as novel therapeutic and biopesticide lead molecules.

Biological significance
In the present study we describe the complexity of 18 venoms from lethal Australian funnel-web spiders using LC-MALDI-TOF MS. The study includes an in-depth investigation, focusing on four venoms, that revealed the presence of ~800 peptides in female venoms and ~400 peptides in male venoms. This is significantly higher than previous estimates of peptide expression in spider venoms. By constructing both 3D and 2D contour plots we were also able to reveal the distinct intersexual as well as intra- and inter-species variation in venom peptide

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masses. We show that peptide mass distributions in all 18 venoms were centered around the 3200–5400 *m/z* range and to a lesser extent the 6600–8200 *m/z* range, consistent with the small number of previously described hexatoxins from these spiders. These findings highlight the extensive diversity of peptide toxins in Australian funnel-web spider venoms that that can be exploited as novel therapeutic and biopesticide lead molecules. The present study has greatly expanded our understanding of peptide variety and complexity in these lethal mygalomorph spiders. Specifically it highlights both the utility of LC-MALDI-TOF in spider taxonomy and the massive combinatorial peptide libraries that spider venoms offer the pharmaceutical and agrochemical industry.

1. Introduction

Spiders have, for a long time, held the interest of the research and medical communities mostly due to their deleterious effects on human health. Nevertheless, only a few spiders produce venoms that are lethal to humans. Indeed, there are only four groups of spiders currently known to cause significant clinical syndromes or human fatalities following envenomation. These include the widely distributed comb-footed spiders (*Latrodectus* and *Steatoda* spp.) as well as recluse spiders (*Loxosceles* spp.), Brazilian armed spiders (*Phoneutria* spp.), and Australian funnel-web spiders (*Atrax*, *Hadronyche* and *Illawarra* spp.) [1–3].

Despite the fearsome reputation generated by the potent biological activity of spider venom components, it is only in the past three decades that the potential of spider venom peptides as pharmacological tools, and drug or bioinsecticide leads has begun to be recognised [4–7]. It has become increasingly apparent that spider venoms contain an unexpected diversity of novel ion channel and receptor ligands, and toxins that modulate neurotransmitter release, all with a wide range of pharmacological actions and targets. Thus they have become a valuable resource for the discovery of specific high-affinity ligands that can be used to study the role, localization, and regulation of cellular signalling pathways such as a variety of ion channel subtypes, G-protein coupled receptors or other targets of interest for drug development. As a result, spider venoms have begun to generate much broader attention in both the scientific community as well as the agrochemical and pharmaceutical industries [8–10].

Spiders are the most speciose venomous animals with over 43,200 extant species described to date [11]. This may be an under-representation of their true speciation, with up to four times as many species predicted to exist, but not yet characterised [12]. Since they rely completely on predation as a trophic strategy, spiders have evolved a complex library of enzymes, neurotoxins and cytolytic compounds in their venom glands [13–16]. These venom components fall into three classes delineated by their molecular mass: (i) low molecular mass acylpolyamines and other nonpeptidic molecules (<1 kDa), (ii) disulfide-rich neurotoxins and linear cytolytic peptides (1–8 kDa), and (iii) high molecular mass proteins (>10 kDa) comprising mainly enzymes and neurotoxins. Nevertheless, a number of studies mostly based on mass spectrometry analyses have shown that the majority of spider venoms are dominated by small disulfide-rich peptide neurotoxins that are believed to have evolved via a genetic strategy where a small number of ancestral toxin genes underwent massive gene duplication and diversification, resulting in a pre-optimized combinatorial library of small bioactive peptides [17]. These toxins are the result of structural and pharmacological enhancements over extended (evolutionary) timescales, and have been finely tuned for optimal activity by the process of evolution. Effectively, the process of natural selection has already pre-screened these huge combinatorial libraries for potential bioactive molecules. Consequently, spider venoms are now being probed for novel analgesics, as well as lead compounds for the development of antiarrhythmics, anticonvulsants, antimicrobials, and even diagnostic and insecticidal agents [7–9].

Despite the size and potential of this large pharmacological resource, we have barely begun to explore spider venoms. Currently only ~900 peptides are listed in the ArachnoServer 2.0 Spider Toxin Database (www.arachnoserver.org), a comprehensive curated database containing available information on spider venom peptides and proteins [18,19]. Recently, it has also become clear that spider venoms are significantly more complex than previously anticipated. Various proteomic analyses have revealed that venom from an individual spider can comprise between 40 and 1000 distinct peptides [20,21]. Coupling this information with measures of taxonomic diversity leads to estimates of around 10 million peptides in all spider venoms based on a conservative estimate of ~50,000 species with each spider venom containing ~200 peptides. This dwarfs the estimated chemical diversity of ~100,000 bioactive peptides in the venoms of scorpions [22] and ~500,000 for cone snails [23] as the spider group includes many more species as well as more extensive ecological, taxonomic and biological diversity with 111 recognized families. Thus, spider venoms represent a combinatorial peptide library of truly enormous proportions, unmatched in size and diversity by any other venomous animal group.

There are several reasons why few studies of spider venoms have been undertaken in the past including difficulty in collecting sufficient amounts of venom, the low resolution of older separation techniques, and the poor resolving power of earlier generation mass spectrometers. These issues have made it difficult to undertake an exhaustive investigation of these highly complex mixtures, which often show the presence of multiple peptide isoforms and contain arrays of peptides with very similar physico-chemical properties. These limitations often resulted in purification and characterisation of only the most highly abundant toxins, leaving the underlying complexity of the venom undetected [20,24,25]. The advent of modern separation techniques such as high-pressure liquid chromatography (HPLC), especially reversed-phase (RP-HPLC), and their