SI Appendix

Australian funnel-web spiders evolved human-lethal δ-hexatoxins for defense against vertebrate predators

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Materials and methods

RNA and cDNA libraries for different species were constructed using a variety of methods as summarized in Table 1. Each species library was then sequenced using Sanger sequencing (for older libraries prior to 2010) or 454-pyrosequencing (libraries post 2010).

Table S1: summary of RNA/mRNA extraction and cDNA library construction methods

Species	**Library construction method**	**5' or 3' RACE**	**Sequencing method**
Atrax robustus	*Marathon™ cDNA amplification kit*	Yes	*Sanger*
Atrax sutherlandi	*Smart™ cDNA synthesis kit*	Yes	Sanger
Hadronyche infensa	*Rapid-library prep and emPCR/*	No	454/Sanger
	Marathon™ cDNA amplification kit	Yes	
Hadronyche modesta	*Creator Smart™ cDNA synthesis kit*	No	Sanger
Hadronyche valida	*Marathon™ cDNA amplification kit*	Yes	Sanger
Hadronyche venenata	*Marathon™ cDNA amplification kit*	Yes	Sanger
Hadronyche versuta	*Marathon™ cDNA amplification kit*	Yes	Sanger
Hadronyche formidabilis	*Rapid-library prep and emPCR*	No	454
Hadronyche cerberea	*Rapid-library prep and emPCR*	No	454
Illawara wisharti	*Marathon™ cDNA amplification kit*	Yes	Sanger

mRNA isolation and cDNA library construction using Marathon cDNA amplification kit

Live specimens of *H. infensa*, *H. valida, H. venenata, H. versuta*, *A. robustus, A. sutherlandi* and *I. wisharti* were subdued by cooling at –20˚C (40 min). The venom glands were then dissected and immediately placed in extraction buffer of the Quickprep Micro mRNA purification kit (Pharmacia Biotech). mRNA was isolated following the manufacturer's protocol. The purified mRNA samples were stored at –20℃ and washed with 80% ethanol before drying on a Speedvac (Savant Speedvac DNA 100). 10 µL of RNase/DNAse-free water was used to resuspend the mRNA samples.

First-strand cDNA synthesis was carried out using a *Not*I-d$(T)_{18}$ bifunctional primer (5'-AACTGGAAGAATTCGCGGCCGCAGGAAT$_{18}$) (Pharmacia Biotech, 3 µL of 0.2 µg/µL) extended with Superscript II Reverse Transcriptase (GIBCOBRL, 2 µL of 200 U/µL), 10 x dNTPs (Pharmacia Biotech, 2 µL), dithiothreitol (DTT) (Pharmacia cDNA Synthesis Kit, 1 µL), and 5×

first-strand buffer (GIBCOBRL, 4 μL). Samples were incubated at 37℃ for 50 min, then at 20℃ for 10 min. Alternatively, first-strand synthesis was done using an Echoclonach-2 primer (5'-GGGCAGGT$_{17}$)(40 ng/μL, 2 μL), 10× dNTPs (Pharmacia Biotech, 10 mM, 1 μL) and Milli Q water (9 μL) incubated at 65℃ for 5 min then extended with Superscript III Reverse Transcriptase (1 μL of 200 U/μL), DTT (0.1 M, 1 μL), 5× first-strand buffer (GIBCOBRL, 4 μL) and Milli Q water (1 μL) and incubated at 50℃ for 1 h, then 75℃ for 15 min.

The cDNA library of *A. sutherlandi* was constructed using the Smart™ cDNA synthesis technology (Smart™, Clontech, USA). mRNA (1–2 ug) had a NOT-1 primer (Pharmacia, 40 ng/μL, 5 μL) and LFE-1A primer (5' – AAGCAGTGGTATCAACGCAGAGTGGCCATTATGGCCGGG –3', 40 NG/μL, 5 μL) added and incubated at 72℃ for 2 min and cooled on ice. Extension was performed with Superscript II Reverse Transcriptase (2 μL of 200 U/μL), 10× dNTPs (2 μL), DTT (20 mM, 2 μL), and 5× first-strand buffer (GIBCOBRL, 4 μL) and incubated at 42℃ for 1 h.

After the first-strand synthesis, sscDNA was precipitated using 10 μL of 3M ammonium acetate, 10 μL of glycogen (1/20) and 100 μL of 100% ice cold ethanol. The solution was stored at –20℃ for 1 h then centrifuged at 14,000 rpm for 15 min. The pellet was then washed twice with 80% ethanol, or Pellet Paint Co-Precipitant kit (Novagen), and dried. Double-stranded cDNA was synthesized using the following reaction: 2× Reaction Mix (20 μL) (10× DNA Polymerase Buffer, 40 μL (MBI Fermentas); 10× dNTPs, 20 μL; DNAase-1 free BSA, 40 μL of 10 mg/mL (Progen, Australia) and 100 μL of Milli Q water), RNase-H (Gibco-BRL, 1 μL of 4 U/μL), and DNA Polymerase I (MBI Fermentas, 1.6 μL of 15 U/μL). Samples were incubated at 12℃ for 30 min, then at 22℃ for 30 min, followed by heating at 65℃ for 10 min, before cooling on ice and spinning briefly in a Sorvall MC 12V bench centrifuge (Du Pont). dscDNA products were cleaned using a BRESAspin PCR Purification Kit (Bresatec, Australia) and eluted in 50 μL of 1/10 dilution of BRESAspin elution buffer, followed by a further 25 μL elution. The samples were stored overnight at –20℃, and concentrated (~10 μL) for adaptor ligation.

Ligation of the Marathon™ adaptor to the cDNA was performed using 1 μL of the Marathon™ cDNA Amplification adaptor (Clontech Laboratories, USA, ~500 ng), 2 μL of 10× Ligase buffer (MBI Fermentas), 2 μL of 10 mM ATP, 3 μL of Milli Q water, and 2 μL of T4 DNA Ligase (4 U/μL, MBI Fermentas). Ligation reactions were incubated at 12℃ for 3.5 h, then cleaned with

a BRESAspin PCR Purification Kit (Bresatec, Australia). The manufacturer's instructions were followed with the exception of three cleaning washes and elution in two washes of 50 µL each of Milli Q and one 50 µL wash of TE buffer [Tris/EDTA: 10 mM Tris HCl (pH 8.0), 10 mM EDTA (pH 8.0)]. Samples were precipitated using 10 µL of 3 M ammonium acetate, 10 µL of glycogen (1/20) and 100 µL of 100% ice-cold ethanol. The precipitation solution was stored at –20℃ for 1 h, centrifuged at 14,000 rpm for 15 min, then washed twice with 80% ethanol and dried, prior to sample reconstitution in 200 µL TE buffer for template stock.

Primer design for Rapid Amplification of cDNA ENDs (RACE)

5' RACE was performed using a redundant PCR primer (IM4834-B; 5’ – RTCYTCRTTYTTNCCRCACCARTT – 3’, where Y= T + C, R= G + A, and N= A + G + T + C) based on the partial amino acid sequence of the toxin δ-HXTX-Iw1a obtained by Edman degradation, and either the API (5’ – CCATCCTAATACGACTCACTATAGGGC – 3’) or EchoAPI (5’ – CACCCCTAATACGACTCACTATAGG – 3’) primer. The 3' RACE PCR primers IM4834-1A (5’ – TGATCTGCCATAATGAAGGTAATAGC – 3’), IM4834-1B (5’ – AGCCATAATGAAGACTATTGC – 3’) and δ-HXTX-Hv-1a (5’ – GTCTACCATAATGAAAATAACAGC – 3’) were designed from the cDNA leader sequences obtained from 5' RACE of the *I. wisharti* and *H. versuta* cDNA libraries respectively. The IM4834-B, IM4834-1A, IM4834-1B, API and LFE-1A primers were constructed by Pacific Oligos (Australia), while all other primers were purchased from Proligo (Singapore).

PCR was performed using either sscDNA or dscDNA as template and either AmpliTaq Gold DNA polymerase (Perkin Elmer, AmpliTaq Gold with GeneAmp Kit, 0.5 µL) or Thermalace DNA polymerase (Invitrogen, Australia). AmpliTaq Gold PCR followed the following protocol: 1 cycle at 95℃ (5 min), 35 cycles at 95℃ (30 s), 55℃ (60 s), 72℃ (90 s), 1 cycle at 72℃ (10 min), 1 cycle at 30℃ (1 min). The Thermalace DNA Polymerase PCR followed the following protocol: 1 cycle at 96℃ (2 min), 30 cycles at 96℃ (45 s), 50℃ (60 s), 72℃ (3 min), 1 cycle at 72℃ (10 min). The amplified cDNA products were analyzed using agarose gel electrophoresis (1.5% Agarose) run in TAE buffer [Tris Acetate EDTA: 0.8 M Tris base, 0.4 M glacial Acetic acid, 20 mM EDTA, pH 8.0]. Gels were stained with ethidium bromide.

Cloning, transformation, and sequencing

RACE products from *H. infensa H. valida, A. sutherlandi and I. wisharti*, were excised and

extracted. The cDNA was extracted using a BRESAspin Gel Extraction Kit (Bresatec, Australia), following the manufacturer's protocol. Samples were dried to a volume of ~10 µL on a Speedvac. Products were cloned into a pCR 2.1 vector and transformed into host INVαF' cells using an Invitrogen TA Cloning Kit (Bresatec, Australia) following the manufacturer's protocol. Successfully transformed clones were cultured in Terrific Broth (TB) with ampicillin (50 µg/mL). The culture samples were tested for insert size by PCR and gel electrophoresis. Shaken cultures (2.5 µL) were added to 10 µL of Milli Q water and heated at 95℃ for 10 min, before cooling on ice and spinning briefly in a bench centrifuge. A master mix (12.5 µL), incorporating the following reagents; Milli Q water (3.5 µL), 10× buffer (2.5 µL), 25 mM MgCl2 (2.5 µL), dNTPs (2.5 µL), pUC FOW 24 primer (Bresatec, Australia, 0.5 µL at 20 ng/µL), pUC REV 22 primer (Bresatec, Australia, 0.5 µL at 20 ng/µL), and C.S.I.R.O. Long Pocket TAQ Polymerase Enzyme (0.5 µL), was added and PCR performed using the following cycling conditions: 1 cycle at 95℃ (30 s), 30 cycles at 95℃ (20 s), 55℃ (45 s), 72℃ (90 s), 1 cycle at 72℃ (10 min), 1 cycle at 30℃ (2 min).

Samples with the inserts were extracted using a BRESAspin Plasmid Mini Kit (Bresatec, Australia) and subjected to sequencing PCR performed using pUC REV 22 primer (Bresatec, Australia, 1 µL at 20 ng/µL) and Big Dye Terminator (Applied Biosystems, 4 µL), and applying the following cycle profile: 1 cycle at 96℃ (30 s), 25 cycles at 96℃ (15 s), 50℃ (20 s), 60℃ (4 min), 1 cycle at 30℃ (1 min). PCR products were precipitated using 2 M NaOAc (pH 4.5, 10 µL), glycogen (Boehringer Mannheim, 10 µL at 2 mg/mL), and absolute ethanol at –20℃ (320 µL), dried and submitted for sequencing (Australian Genome Research Facility).

Alternatively, RACE products from *H. venenata, H. versuta* and *A. robustus,* were excised and extracted using a Concert Gel Purification Kit (Life Technologies, Australia), according to the manufacturer's protocol. Samples were precipitated using Pellet Paint Co-Precipitant (Novagen) and dried. The samples were reconstituted in Milli Q water (1 µL) and phosphorylated by treatment with kinase solution (10 mM ATP (1 µL), 10× kinase buffer A (1 µL), kinase (1 µL)), heated at 37℃ (30 min) then 75℃ (15 min) and cooled on ice. The samples were cleaned using a Concert PCR Purification Kit (Life Technologies, Australia) following the manufacturer's protocols and eluted in 50 µL TE buffer. Samples were then precipitated using Pellet Paint Co-Precipitant.

Dried samples were reconstituted in Milli Q water (0.5 µL), cloned into the pSMART vector

(Lucigen, USA) and transformed into *E. cloni* 10G electrocompetent cells using the Lucigen CloneSmart Blunt Cloning Kit (Lucigen, USA). Transformations were incubated in TB media at 37℃ (1 h) and plated on LB plates containing 100 µg/mL carbenicillin. Colony forming units (cfu) were harvested from the incubated plates and each cfu was washed into 10 mM Tris (20 µL). An insert-size PCR test was performed on each cfu sample (5 µL) and incorporated the following: MilliQ water (8.45 µL), 10× buffer (2.5 µL), 25 mM $MgCl_2$ (2.5 µL), 10× dNTPs (2.5 µL), AmpL1 primer (Bresatec, Australia, 2.5 µL at 20 ng/µL), AmpR1 primer (Bresatec, Australia, 2.5 µL @ 20 ng/µL), and Red Hot TAQ Polymerase Enzyme (Thermo Scientific, 0.5 µL). PCR was performed using the following protocol: 1 cycle at 96℃ (5 min), 25 cycles at 96℃ (45 s), 55℃ (60 s), 72℃ (2 min), 1 cycle at 72℃ (10 min). The PCR products were examined on a 1% agarose gel. Clones with the correct insert size were plated (2 µL) on LB agar plates containing 100 µg/mL carbenicillin and incubated at 37℃ overnight. Plates were sent to Macrogen Inc. (Korea) for sequencing.

Creator SMART cDNA library construction

Paired venom glands from four *H. modesta* were dissected out and pooled. Total RNA was extracted using the standard TRIzol® Plus method (Invitrogen/Life Technologies) according to the manufacturer's protocol. One microgram of total RNA was used to construct a cDNA library using the CREATOR™ SMART™ cDNA library construction kit (Clontech Laboratories, CA, USA) following the manufacturer's protocol. Briefly, total RNA was reversed transcribed using the SMART™ Moloney Murine Leukemia virus (MMLV) reverse transcriptase. Second-strand synthesis was completed using long distance PCR as follows: 1 min at 95°C followed by 20 cycles of 1 min at 95°C and 6 min at 68°C. Products were then digested, size fractionated using a CHROMA SPIN-400 DEPC-H_2O column (Clontech Laboratories, CA, USA), then ligated into the pDNR-lib donor vector. Recombinant plasmids were electroporated into E-shot™ DHB10™-$T1^R$ electrocompetent cells (Life Technologies, CA, USA). 384 clones were randomly selected and sequenced by capillary electrophoresis on an AB 3730xl DNA analyzer (Applied Biosystems/Life Technologies, CA, USA) at the Brisbane node of the Australian Genome Research Facility (AGRF-Brisbane). Vector and polyA+ tails were clipped using CLC Main Work Bench (CLC-Bio) then the Blast2GO bioinformatic suite (1, 2) was used to provide Gene Ontology, BLAST and domain/Interpro annotation. Signal sequence cleavage sites were predicted using SignalP v3.0 (3).

454 sequencing

Venom glands were dissected from live specimens subdued by cooling at –20℃. Paired venom glands from two *H. cerberea*, five *H. formidabilis* and three *H. infensa* specimens were dissected and pooled for transcriptomic studies. Total RNA extraction was carried out using Trizol® (Invitrogen) following the manufacturer's instructions. Enrichment for mRNA was then performed using a Dynabeads mRNA DIRECT Kit (Life Technologies or using the Oligotex direct mRNA mini kit (Qiagen). RNA quality and concentration were measured using a Bioanalyzer 2100 pico chip (Agilent technologies). 100–400 ng of mRNA was used to construct a cDNA library using the standard cDNA rapid library preparation and emPCR method from Roche. Sequencing was done at the Brisbane node of the Australian Genome Research Facility using a Roche GS-FLX sequencer. For each dataset, the Raw Standard Flowgram File (.SFF) was processed using Cangs software (4), and low-quality sequences discarded using a Phred score cut-off of 25. *De novo* assembly was done using MIRA v3.2 (5) using the following parameters: -GE:not=4 --project=Hinfensa --job=denovo,est,accurate,454 454_SETTINGS -CL:qc=no -AS:mrpc=1 -AL:mrs=99,egp=1. The assembly of each species was done independently and the assembled data set was visualized using TABLET (6) or Geneious (7) software. Consensus sequences from clusters and contigs were submitted to Blast2GO (www.blast2go.com) to acquire BLAST and functional annotations (1, 2). In parallel to the functional annotation analysis, an "in-house" algorithm was used to predict open reading frames (ORFs) that may encode toxin sequences. Once annotated and predicted lists were merged, redundancies were removed prior to further processing. Signal sequences were determined using SignalP v4.0 (3). Putative propeptide cleavage sites were predicted using the results of a sequence logo analysis of all known spider precursors, and more recently implemented in the Tox|Note pipeline (8). After analysing each transcriptomic dataset, precursors encoding the δ-HXTXs were extracted from the main dataset, grouped in order to remove redundancies, using the CD-hit-EST algorithm, using a homology cut off of 80% (9).

Phylogenetics and selection analyses

Sequence datasets were compiled using BLAST searches (10) against the National Center for Biotechnology Information's (NCBI) nucleotide database. An alignment of δ-HXTX sequences was generated in MEGA X (11) using MUSCLE v.3.8.31 (12) by first aligning amino acids and then performing a back-translation to obtain an aligned nucleotide dataset. Phylogenetic reconstructions using maximum likelihood inference was implemented in PhyML 3.0 (13) on the

ATGC webserver (14), and node support was evaluated with 100 bootstrapping replicates. The built-in Smart Model Selection (SMS) tool (15) was used to determine the best model of nucleotide substitution for phylogenetic reconstructions, and this was identified as the Hasegawa-Kishino-Yano, 85 + Gamma + Inverse (HKY85 +G +I) model. The phylogenetic tree was midpoint rooted.

The rate of evolution of the signal peptide, propeptide, and mature peptide domains was evaluated using maximum likelihood models (16) implemented in Codeml of the PAML package 4.9i (17). We used site-specific models (M7 versus M8) to identify positive selection as a non-synonymous-to-synonymous substitution rate ratio (ω) statistically greater than 1, and the Bayes empirical Bayes (BEB) approach (18) of M8 for identifying codon sites under selection. We further utilized FUBAR (19), implemented on the datamonkey webserver (20, 21), to detect sites evolving under the pervasive influence of diversifying and purifying selection, while the Mixed Effects Model Evolution (MEME) was employed to identify sites that episodically experience diversifying selection (22). The evolutionary variability of residues in δ-HXTXs, which was determined using the Consurf webserver (23), was mapped onto the model of δ-HXTXs built using the Phyre 2 webserver (24). We used Pymol 1.3 (25) for visualization and generation of images.

Purification of δ-HXTX-Ar1a using HPLC

Male *A. robustus* venom was loaded onto an analytical C_{18} reversed-phased HPLC column (Agilent Zorbax Eclipse Plus, 4.6 × 100 mm, 95 Å pore size, 3.5 μm particle size). Venom components were eluted in 1-min fractions using a linear gradient from 5% to 80% Buffer B (Buffer A = 0.05% TFA; Buffer B = 90% acetonitrile/0.045% TFA) over 75 min at a flow rate of 1 mL/min governed by an Agilent 1260 Infinity system. Peptide elution was detected from UV absorbance at 214 nm, and eluted components were collected in polyethylene 1.1 mL deep 96-well plates (Axygen). Collected components were lyophilised and stored at –20°C for further analysis.

The fraction containing δ-HXTX-Ar1a (Ar1a) was identified based on matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) using a Model 4700 Proteomics Analyzer (Applied Biosystems). HPLC fractions were spotted with α-cyano-4-hydroxycinnamic acid (CHCA) (6 mg/ml in 50% acetonitrile). Further purification was performed using a hydrophobic interaction column (Grace VisionHT HILIC, 5 μm particle size, 150 × 4.6

mm) on a Shimadzu Prominence HPLC system. Solvent A was 0.05% TFA, solvent B was 90% acetonitrile in 0.043% TFA, and the flow rate was 1 ml/min. After 3 min at 95% solvent B, a linear gradient of 95–50% B was run over 45 min. The UV absorbance was monitored at 214 nm and eluted components were collected, lyophilised and stored at –20°C for further analysis. The fraction containing pure Ar1a was identified based on MALDI-TOF MS.

Selectivity profiling of Ar1a on Na_V channel subtypes

HEK293 cells stably expressing the human Na_V β1 subunit and one of the human Na_V channel isoforms $Na_V1.1$–$Na_V1.8$ (Scottish Biomedical, Glasgow, Scotland) or rat $Na_V1.6$ were routinely maintained in minimal essential medium (Sigma-Aldrich, Australia) supplemented with L-glutamine, 10% foetal bovine serum (Bovogen Biologicals, France) and appropriate selection antibiotics (blasticidin and G418 for $Na_V1.1$, 1.2, 1.3, 1.5, 1.7 and 1.8; blasticidin, G418 and zeocin for $Na_V1.4$). Cell lines were split every 3–6 days in a ratio of 1:6 using TrypLE Express TM (Life Technologies). Cells were plated on 384-well black-walled imaging plates (Corning) at a density of 10,000–15,000 cells per well 48 h before loading with 15 uL of red membrane potential dye (proprietary formulation; Molecular Devices, Sunnyvale, CA) in physiological salt solution with the following composition (in mM): NaCl (140), glucose (11.5), KCl (5.9), $MgCl_2$ (1.4), NaH_2PO_4 (1.2), $NaHCO_3$ (5), $CaCl_2$ (1.8), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (10), pH 7.4). Cells were incubated with membrane potential dye for 30 min at 37ºC before subsequent additions of δ-HXTXs and veratridine by the $FLIPR^{TETRA}$ system. Changes in fluorescence (excitation 510–545 nm, emission 565–625 nm) as a result of addition δ-HXTXs and EC_{10} concentrations of veratridine (5–20 μM), a Na_V channel activator, were assessed for 300 s after each addition. Changes in fluorescence after addition of δ-HXTXs and veratridine were analysed using Screenworks 3.1.1.4 (Molecular Devices) and determined in at least 3-4 independent experiments. A 4-parameter Hill equation with variable slope was fitted to the data using Prism v.4.00 (GraphPad, San Diego, CA, USA). All data, unless otherwise stated, are expressed as mean ± standard error of the mean (SEM).

Assessment of mammalian nocifensive effects of Ar1a

Male C57BL/6 mice, age 6–8 weeks, were housed in groups of 3 or 4 per cage under 12 h light-dark cycles, with *ad libitum* access to standard rodent chow and water. For assessment of

nocifensive responses to intraplantar administration of Ar1a, animals were anesthetized using 3% isoflurane, then a single dose of Ar1a (100 nM, total volume of 20 µL) was administered by shallow subcutaneous injection into the left hind paw. Animals were allowed to recover from anesthesia and placed individually in a Plexiglas chamber (10 × 10 × 15 cm) for behavioural quantification by a blinded observer. The number of paw flinches, lifts, shakes or licks were counted every 5 min for approximately 45 min by a blinded observer in order to quantify spontaneous pain behaviour.

Insecticidal effects of δ-HXTX-Ar1a in sheep blowflies

Native Ar1a was dissolved in MilliQ water and injected into the ventro-lateral thoracic region of sheep blowflies (*L. cuprina*; mass 25.3–30.1 mg) as previously described (26). A 1.0 ml Terumo Insulin syringe (B-D Ultra-Fine, Terumo Medical Corporation, Maryland, USA) with a fixed 29G needle was used and fitted to an Arnold hand micro-applicator (Burkard Manufacturing Co. Ltd., UK), applying a maximum volume of 2 µl per fly. Thereafter, flies were individually housed in 2 ml tubes and the paralytic activity was determined 1 h and 24 h post-injection. A total of three tests were carried out and for each test six doses of Ar1a (n = 10 flies per dose) and the appropriate control (MilliQ water; n = 20 flies each) were used. PD_{50} values were calculated as described previously (26, 27).

Activity of Ar1a on BgNa$_V$1 measured using two-electrode voltage-clamp electrophysiology

The DNA sequence of BgNa$_V$1 (28) was confirmed by automated DNA sequencing and cRNA was synthesized using T7 polymerase (mMessage mMachine kit, Life Technologies, USA) after linearizing the DNA with an appropriate restriction enzyme. BgNa$_V$1 was expressed in *Xenopus* oocytes together with the TipE subunit (29) (1:5 molar ratio), and studied following 1–2 days incubation after cRNA injection (incubated at 17 °C in 96 mM NaCl, 2 mM KCl, 5 mM HEPES, 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$, 50 µg ml^{-1} gentamycin, pH 7.6 with NaOH) using two-electrode voltage-clamp recording techniques (OC-725C, Warner Instruments, USA) with a 150-µl recording chamber. Data were filtered at 4 kHz and digitized at 20 kHz using pClamp software (Molecular Devices, USA). Microelectrode resistances were 0.5–1 MΩ when filled with 3 M KCl. The external recording solution contained (in mM): 100 NaCl, 5 HEPES, 1 $MgCl_2$ and 1.8 $CaCl_2$, pH 7.6 with NaOH. All experiments were performed at room temperature (~22 °C). Leak and background conductances, identified by blocking the channel with tetrodotoxin (Alomone labs, Israel), were

subtracted for the BgNa$_V$1 currents shown. Voltage–activation relationships were obtained by measuring steady-state currents and calculating conductance (G) for Na$_V$ channels. After addition of Ar1a to the recording chamber, the equilibration between toxin and channel was monitored using weak depolarizations elicited at 5-s intervals. Off-line data analysis was performed using Clampfit 10 (Molecular Devices, USA), and Origin 8.0 (Originlab).

Chick biventer nerve-muscle preparation

The vertebrate neurotoxicity of male *H. modesta* venom was determined according to methods previously described (27). Briefly, biventer cervicis muscles were removed from male chicks (8–15 days old), mounted in 5 ml organ baths and maintained at 34ºC under 1 g resting tension in a physiological saline solution. Isometric contractions were measured via a Grass transducer (FTO3) connected via a Quad Bridge (Model ML118, ADInstruments, Australia) to a Powerlab 4/20 system (Model ML840, ADInstruments, Australia). Twitches were evoked by stimulating the motor nerve (supramaximal voltage, duration 0.2 ms, 0.1 Hz) via silver electrodes connected to a Grass S88 stimulator. Nerve- mediated (indirectly evoked) twitches were confirmed by the addition of D-tubocurarine (10 μM). In the absence of electrical stimulation, responses to exogenous acetylcholine (ACh, 1 mM, 30 s), carbachol (CCh, 0.02 mM, 60 s) and KCl (40 mM, 30 s) were obtained prior and after the addition of venom. Preparations were allowed to equilibrate for at least 20 min with continuous stimulation before addition of venom. Venom was added in incremental aliquots until a response was elicited. Funnel-web spider antivenom (CSL Ltd., Parkville, Australia) was either added after male *H. modesta* venom to assess its potential in reversing the venom effects or before the venom to determine if the antivenom can neutralize the venom effects.

Results and discussion

Chick biventer assay

Given the continuous expansion of human population centers into funnel-web spider habitats, we examined whether the venom of *H. modesta*, which is found in the in the Dandenong Ranges on the populated outskirts of Melbourne, is capable of causing serious envenomations and whether those could be treated with the marketed funnel-web spider antivenom, we found a notable difference in the amounts of venom required to elicit an effect in the chick biventer cervicis nerve-muscle preparation, ranging from 10 to 33.6 μg/ml of male *H. modesta* venom (mean 20.9 ± 9.5

μg/ml, n = 7). Addition of the venom was found to induce increased resting muscle tension and muscle fasciculations (Fig S1A), which is typical for other funnel-web spider venoms (30). The effect of the venom was reversed following addition of funnel-web spider antivenom after the venom (Fig. S1B), whereas pretreatment of the muscle with funnel-web spider antivenom did not neutralize the muscle contracture and fasciculations caused by male *H. modesta* venom (Fig. S1C).

Severe envenomations have so far only been reported from six species of funnel-web spiders, namely *H. cerberea, H. formidabilis, H. macquariensis* (previously *H. sp. 14;* (31)), *H. infensa, H. versuta* and *A. robustus* (32), and from the actinopodid spider *M. bradleyi* (33). We found that despite their small size, male *H. modesta* venom caused potent effects that resemble those effects that are typically induced by δ-HXTXs in the chick biventer assay. However, the average venom dose required to elicit those effects was 2.4-times higher than for any other male funnel-web spider (30). This might explain why no serious envenomation incidents have been reported from *H. modesta*, despite it being found in the outskirts of the city of Melbourne. So far, two envenomations with *H. modesta* have been recorded, both resulting in local effects only (32). We found that, as for all other funnel-web spider venoms tested previously (30), commercial antivenom reversed the effects caused by male *H. modesta* venom. However, the twitch tension did not completely return to previous levels, in line with previous observations for other funnel-web spider venoms and may reflect incomplete reversal of venom effects on muscle fibres (30). In contrast to this partial reversal of effects, the antivenom when applied prior to venom failed to neutralize the effects of male *H. modesta* venom in the present study, which is analogous to previous results for male *A. robustus*, *H. cerberea*, *H. infensa*, and *H. valida* venoms (30). This may have been due to an insufficient dose of antivenom, but unfortunately there was not enough venom left to test whether increased doses of funnel-web spider antivenom could neutralize the effect of male *H. modesta* venom. In summary, our results suggest that, based on the venom yield and relative potency of male Dandenong Ranges funnel-web spiders, they are less likely than other funnel-web spiders to cause severe human envenomations. However, the overall effect of male *H. modesta* venom in the chick biventer was similar to other medically important funnel-web spider venoms. In combination with potential intraspecific variability in the expression levels of δ-HXTXs and its incomplete reversal and very poor neutralization by funnel-web spider antivenom, we cannot completely rule out the possibility of serious envenomations caused by *H. modesta*.

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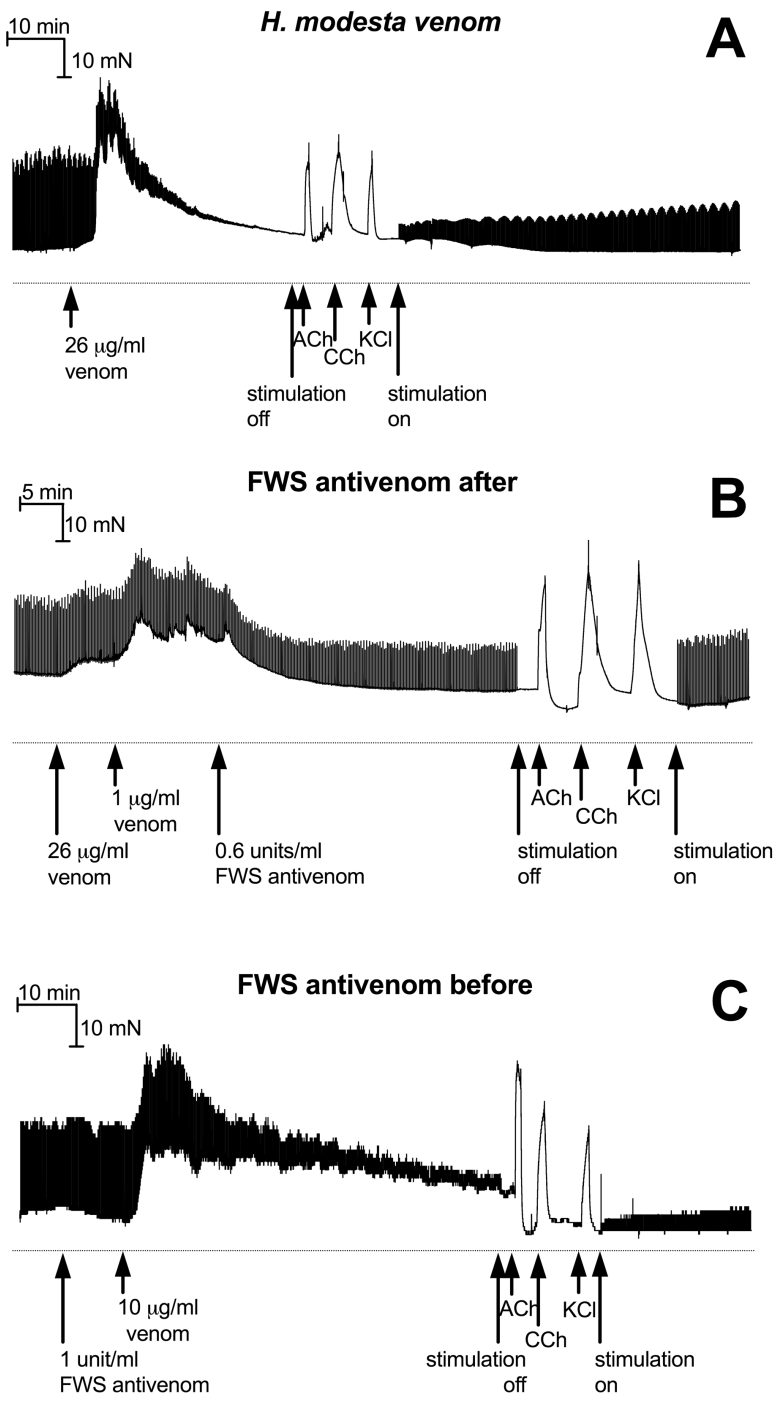

Fig. S1: Representative responses of the chick biventer cervicis nerve-muscle preparation to male *H. modesta* venom and funnel-web spider antivenom. (**A**) Male *H. modesta* venom induced muscle contracture and fasciculations, but had no effect on the response to ACh, CCh and KCl compared to the responses to those drugs determined prior to addition of the venom. After washing the organ bath, the twitches recovered slowly but not completely. (**B**) Addition of 0.6 units/ml of commercial funnel-web spider (FWS) antivenom after addition of male *H. modesta* venom reversed the venom effect. (**C**) Addition of 1 unit/ml of FWS antivenom before addition of 10 μg/ml of male *H. modesta* venom failed to neutralize the effect of the venom.