

Population Divergence in Venom Bioactivities of Elapid Snake *Pseudonaja textilis*: Role of Procoagulant Proteins in Rapid Rodent Prey Incapacitation

Jure Skejić^{1,2*}, Wayne C. Hodgson²

1 Department of Biochemistry and Molecular Biology, BIO21 Institute, University of Melbourne, Victoria, Australia, **2** Monash Venom Group, Department of Pharmacology, Monash University, Clayton, Victoria, Australia

Abstract

This study looked at how toxic proteins in venoms of adult Australian eastern Brown snakes *Pseudonaja textilis* from South Australian and Queensland populations interact with physiological functions of the lab SD rat *Rattus norvegicus*. Circulatory collapse and incoagulable blood occurred instantly after injection of venom under the dorsal skin of anaesthetised and mechanically ventilated rats in an imitation of a *P. textilis* bite. Intravenous injection of purified *P. textilis* (Mackay, QLD) venom prothrombin activator proteins caused instant failure of circulation, testifying of high toxicity of these proteins and suggesting their role in rapid incapacitation of rodent prey. The hypothesis is further supported by circulatory collapse occurring instantly despite artificial respiration in envenomed rats and the finding of extremely high venom procoagulant potency in rat plasma. LC-MS and physiology assays revealed divergent venom composition and biological activity of South Australian (Barossa locality) and Queensland (Mackay locality) populations, which may be driven by selection for different prey. The Queensland venom of *P. textilis* was found to be more procoagulant and to exhibit predominately presynaptic neurotoxicity, while the South Australian venom contained diverse postsynaptic type II and III α -neurotoxins in addition to the presynaptic neurotoxins and caused significantly faster onset of neuromuscular blockade in the rat phrenic nerve-diaphragm preparation. LC-MS analysis found evidence of multiple coagulation factor X-like proteins in *P. textilis* venoms, including a match to *P. textilis* coagulation factor X isoform 2, previously known to be expressed only in the liver.

Citation: Skejić J, Hodgson WC (2013) Population Divergence in Venom Bioactivities of Elapid Snake *Pseudonaja textilis*: Role of Procoagulant Proteins in Rapid Rodent Prey Incapacitation. PLoS ONE 8(5): e63988. doi:10.1371/journal.pone.0063988

Editor: Luis Eduardo M. Quintas, Universidade Federal do Rio de Janeiro, Brazil

Received: December 17, 2012; **Accepted:** April 9, 2013; **Published:** May 14, 2013

Copyright: © 2013 Skejić, Hodgson. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: Jure Skejić is supported by the International Postgraduate Research Scholarship from the Australian Government, Melbourne International Research Scholarship from the University of Melbourne and Studentship from the Department of Biochemistry and Molecular Biology at the University of Melbourne (www.unimelb.edu.au). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: jskejic@student.unimelb.edu.au

Introduction

Several studies have found a correlation between venom lethality and the type of prey consumed, supporting natural selection as one process driving snake venom evolution [1–4]. While lethality studies (e.g. LD₅₀ experiments) indicate that venom is more likely to kill particular prey, they do not reveal how toxic proteins interact with the physiological systems of the prey to cause incapacitation. Studying toxic activities of venom proteins in prey is of crucial importance in understanding how animal venoms evolve as it is the proteins which are directly subjected to natural selection. Furthermore, the relevance of death as the cessation of all biological functions in prey acquisition is questionable because what determines whether or not the predator gets its meal is disablement of the physiological functions of the prey that would assist escape and or defence. Another problem with lethality studies is that they are often done over an extended time period (e.g. 24 hours) which does not take into account the timeframe in which a particular physiological function is affected [5], and at different venom doses administered different toxins may cause death [6].

The aim of this study is to better understand pathologic processes that affect physiological functions of a rodent organism

caused by the venoms of Australian eastern Brown snake *Pseudonaja textilis* populations. This elapid is a prey generalist, feeding on a variety of rodents (including mice and rats), birds, lizards and frogs [7]. During the attack it wraps coils around its prey [8] and injects its extremely toxic venom with small fangs, which are only 2 to 4 mm long, 2.8 mm on average [9]. *P. textilis* venom contains diverse proteins [10], including venom prothrombin activator psetarin-C [11], presynaptic neurotoxin textilotoxin [12,13], type II or ‘long-chain’ [14,15] and type III ‘short-chain’ α -neurotoxins [16,17], Kunitz-type serine protease inhibitors textilins [18], and snake venom phospholipases A₂ [19]. Several of these proteins have been shown to be lethal when injected into the rodent bloodstream, including *P. textilis* venom prothrombin activator [11], pseudonajatoxin b [14] and short-chain neurotoxins [16].

Studies at the population level are necessary to elucidate processes driving divergence in venom composition and biological activity. Snake venom composition has been found to vary intraspecifically with geographic location [20,21] and studies have shown correlation between the geographic variation in venom composition and prey type [22,23]. *Pseudonaja textilis* has a wide distribution in eastern and central Australia and New Guinea, comprising three distinct phylogenetic clades [24,25]. A study on museum specimens by Shine [7] found that *P. textilis* specimens

from Queensland and Northern Territory reach larger body sizes than those from South Australia and Victoria, and that the proportion of endothermic prey (i.e. mammals and birds) increases with body size. Given its wide distribution and variation in body size, *P. textilis* group represents a good model to study processes driving divergence in venom composition and biological activity. Previous studies on geographic variation in composition and bioactivity of *P. textilis* venoms suggested differences in venom prothrombin activator and plasmin inhibitor abundance and activity [26], and differences in textilotoxin D chain and pseudonaja toxin b abundance and post-translational modifications of factor Xa-like heavy chain [10].

In this study, proteomes and biological activities in lab rats (*SD Rattus norvegicus*) of venom samples from Mackay (Queensland) and Barossa (South Australia) *Pseudonaja textilis* specimens are compared. According to the published haplotype clade distribution maps, it can be inferred that Barossa (SA) and Mackay (QLD) populations of *P. textilis* belong to the southeastern and northeastern clades respectively, but this should be verified.

Materials and Methods

Venom Samples and Model Animals

Pooled freeze-dried venoms of adult *Pseudonaja textilis* snakes from the Barossa region in the vicinity of Adelaide (South Australia, Australia) and Mackay (Queensland, Australia), *Oxyur-*

anus scutellatus scutellatus and *O. microlepidotus* were obtained from Venom Supplies, South Australia. *P. textilis* Barossa batches contained venoms pooled from 8 adults. *P. textilis* Mackay batches contained venoms pooled from 2 adults, with venom from 1 additional adult individual obtained and examined subsequently (i.e. a total of 3 adult specimens). All experiments involving rats were done on adult male laboratory Sprague-Dawley rats *Rattus norvegicus*, weighing 300–400 grams. Male 4–10 day old chicks were used for the chick biventer cervicis nerve-muscle preparation.

Rat Phrenic Nerve – Diaphragm Preparation

Rats were killed by carbon dioxide and exsanguination, and the diaphragm with the phrenic nerves was dissected out following Büllbring [27]. A half of the diaphragm muscle with the phrenic nerve was placed in a 50-ml organ bath with physiological salt solution, composed of: 118.4 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄×7 H₂O, 1.2 mM KH₂PO₄, 25.0 mM NaHCO₃, 11.1 mM D-glucose and 2.5 mM CaCl₂, bubbled with a 95% O₂ and 5% CO₂ gas mixture, and maintained at 37°C. The diaphragm muscle was attached to a FT03 Grass Force-displacement transducer, with tension adjusted to 1 g. The phrenic nerve was threaded through an electrode and stimulated every 10 s by pulses of electric current lasting 0.2 ms at the voltage at which maximum contractions occurred (1–2 V). Pulses were delivered using a Grass S88 stimulator. Prior to experiment, the

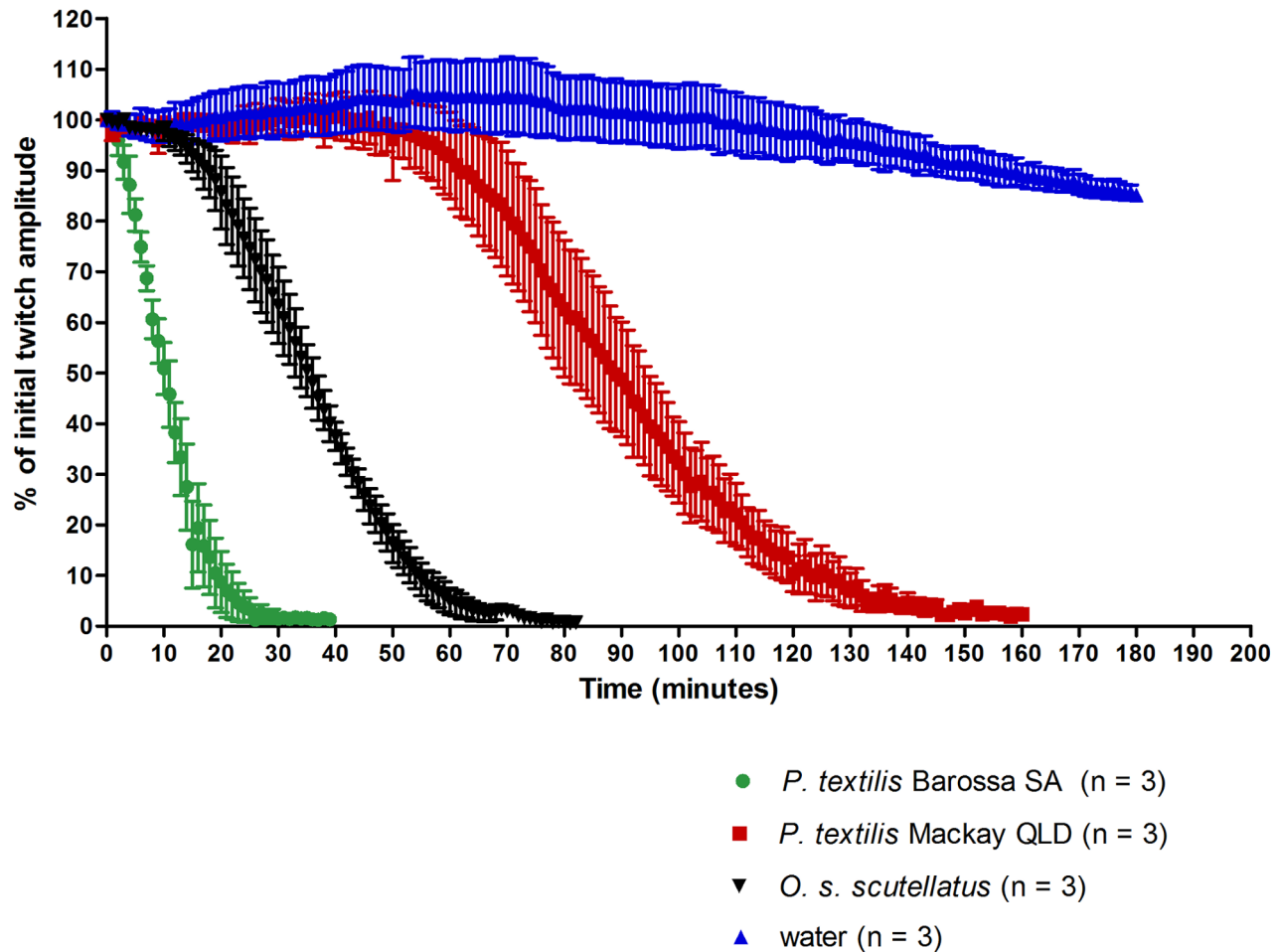


Figure 1. Neurotoxicity in the rat phrenic nerve-diaphragm preparation. 10 µg/ml venom at 37°C (vehicle: water). doi:10.1371/journal.pone.0063988.g001

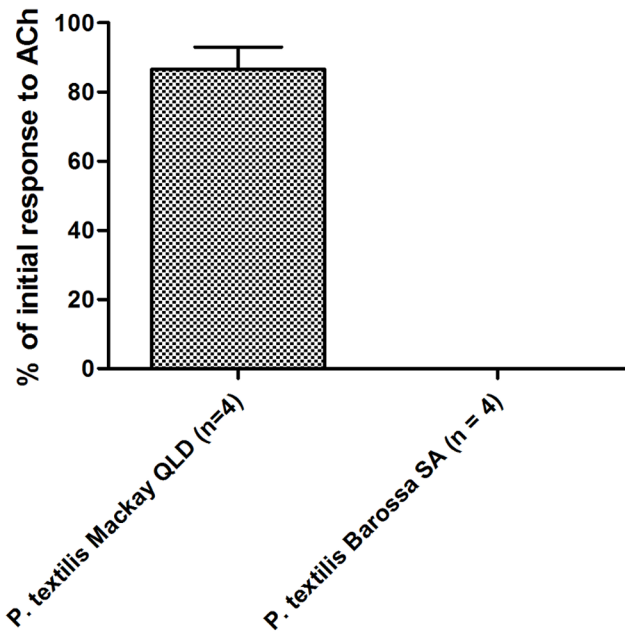


Figure 2. Response to exogenous acetylcholine in the chick biventer cervicis nerve-muscle preparation. 2 hours after exposure to 10 µg/ml venom (vehicle: water). doi:10.1371/journal.pone.0063988.g002

phrenic nerve response was tested by inducing neuromuscular blockade with 2 µM (+)- tubocurarine chloride (Sigma), after which the agent was washed out.

Venom (10 µg/ml) and was added to the organ bath and left in contact with the tissue until contractions were abolished or for a maximum period of 3 h. The contractions were recorded by ADInstruments MacLab software. The contraction amplitude was calculated by using Max-Min function. The t_{95} value was determined by calculating the time point at which the initial (pre-venom) contraction amplitude was reduced by 95%. The t_{95} values were compared with one-way ANOVA, followed by Holm-Sidak’s multiple comparison test. Statistical analyses and figures in the study were made in GraphPad prism.

Chick Biventer Cervicis Muscle – Nerve Preparation

This preparation was made following the methods of Ginsborg and Warriner [28] and Harvey et al [29]. Both biventer cervicis muscles with the nerve-enclosing tendons were dissected and transferred to a 5-ml organ bath containing physiological salt solution. The organ bath was bubbled with 95% O₂ and 5% CO₂ gas mixture and maintained at 40°C. The resting tension on the tissue was adjusted to 1 g and the nerve-surrounding tendon was electrically stimulated every 10 s with 0.2 ms-pulses. Venom (10 µg/ml) was added to the organ bath, and was washed out after a maximum of 2 h if indirect twitches of the preparation were not abolished. Acetylcholine (1 mM for 30 s), carbachol (0.02 mM for 60 s), and KCl (40 mM for 30 s) were added in the absence of electric nerve stimulation before venom addition and after 2 h of the tissue exposure to the venom. The post-venom chick biventer cervicis muscle responses to acetylcholine were compared between the Mackay and Barossa *P. textilis* venoms with a Student’s t test.

Venom Procoagulant Activity in Citrated Rat Plasma

Frozen (–80°C) citrated SD rat plasma (anticoagulated with 0.109 M (3.2%) buffered sodium citrate) was thawed in a water

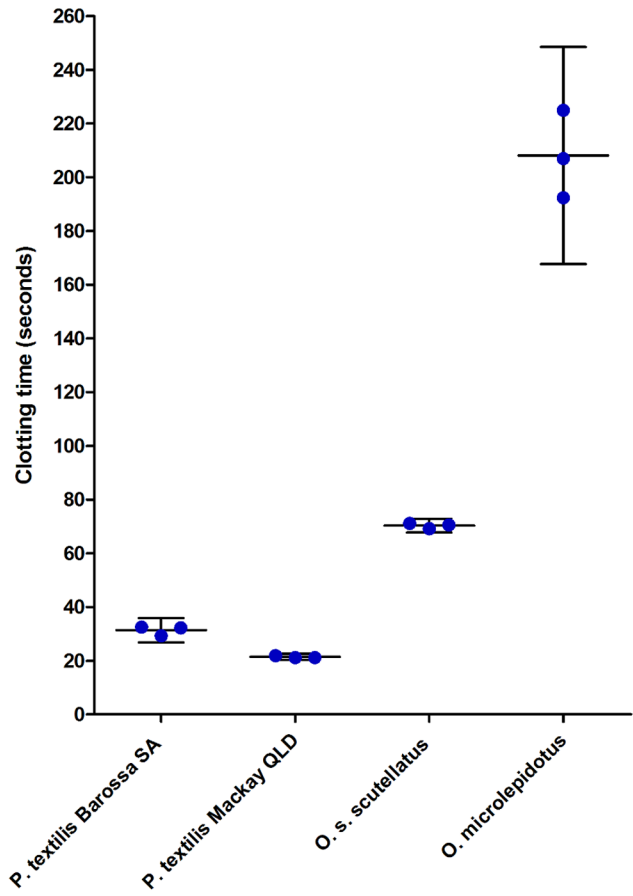


Figure 3. Venom procoagulant activity in rat plasma. 0.5 µg venom (in 50 µl saline) per 100 µl citrated plasma recalcified with 4 µmol CaCl₂ (means and 95% CI shown). doi:10.1371/journal.pone.0063988.g003

bath at 37°C for 5 min. Immediately prior to the assay, thawed plasma (1 ml) was recalcified with CaCl₂ (4 µmol). The clotting assays were done in 96 well polystyrene clear Greiner Bio-One flat-bottom microplates in a Molecular Devices Versamax microplate reader at a temperature of 37°C. Venom (50 µl of 10 µg/ml) in normal saline was added to 100 µl of recalcified rat plasma, and after an automatic 5 s shake step, absorbance was read every 10 s at the wavelength of 340 nm. The clotting time was recorded as the time of onset of a sharp increase in absorbance, indicating fibrin clot formation (for the general turbidimetric method see [30]). The onset times were obtained with SoftMax Pro 5 microplate data acquisition and analysis software with OD set to 0.02. Clotting times of *Pseudonaja textilis* populations and *Oxyuranus* spp. venoms were analysed with repeated measures one-way ANOVA, followed by Holm-Sidak’s multiple comparison test. *P. textilis* population venom effects at different venom concentrations were tested with repeated measures two-way ANOVA.

Venom Effects on Circulation of Anaesthetised Rats

Rats were anaesthetised with pentobarbitone sodium (80 mg/kg, i.p., Troy Laboratories Australia Pty Ltd) and placed on a thermostat-controlled heating mat at 37°C. A cannula was inserted in a surgically exposed left carotid artery and connected to a fluid pressure transducer filled with heparinised saline (25 U/ml). The trachea was cannulated and connected to a 7025 Ugo

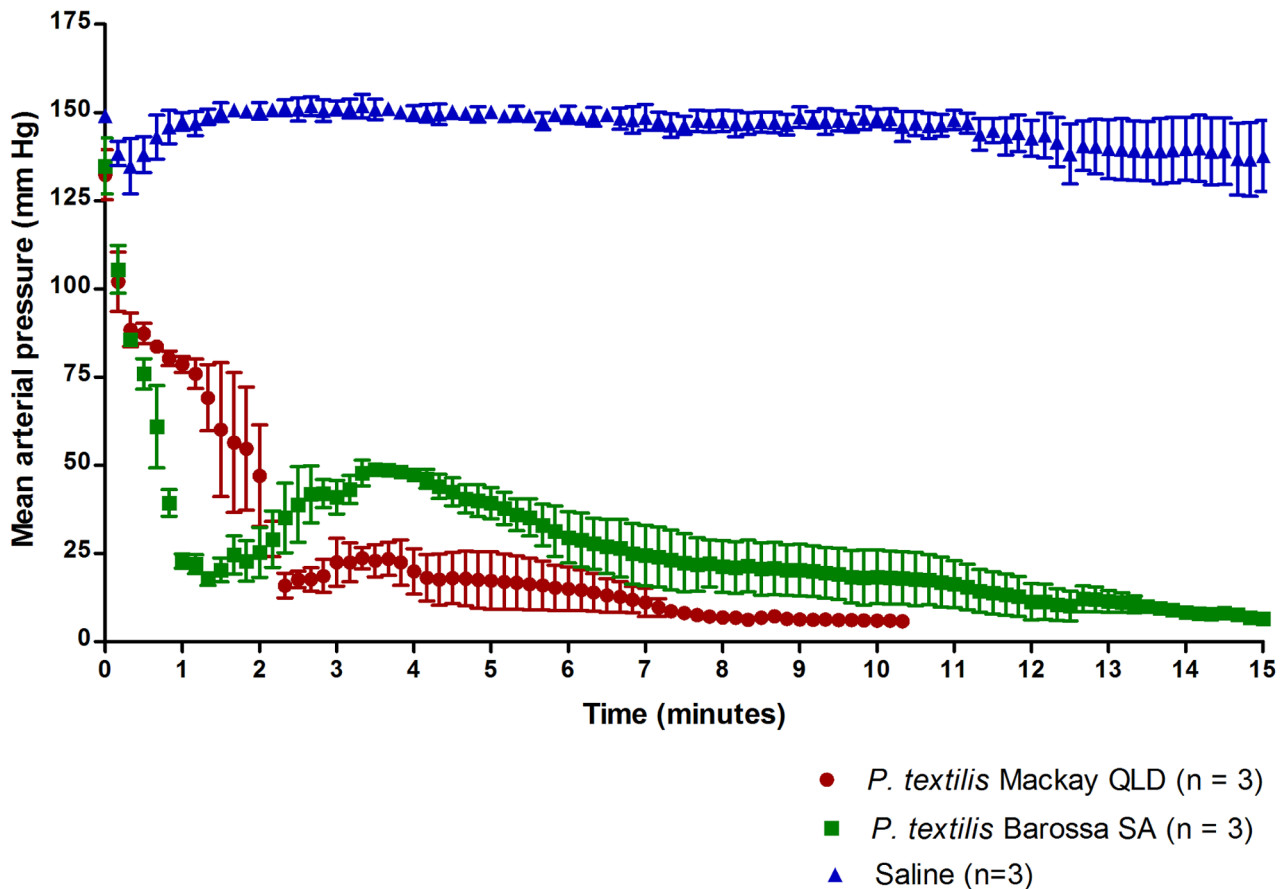


Figure 4. Mean arterial blood pressure in rats injected with 0.5 mg venom under the dorsal skin. Injection volume: 100 μ l, vehicle: saline. Rats anaesthetised with 80 mg/kg ip pentobarbitone sodium. doi:10.1371/journal.pone.0063988.g004

Basile rodent ventilator (approx. 10 ml/kg/stroke, 60 strokes/min). Mechanical ventilation was started before venom or saline injection and maintained throughout the experiment. Freeze-dried venom was dissolved in normal saline, and 0.5 mg in 100 μ l saline was injected under the upper dorsal skin of the rat in an imitation of a snake bite, with 4 mm of the hypodermic needle tip inserted. Control rats were injected with an equivalent volume of normal saline. Systemic blood pressure was monitored on a MacLab system (ADInstruments). The mean arterial pressure (MAP) was approximated from systolic (SP) and diastolic pressure (DP) values for 10-second intervals: $MAP \approx DP + 1/3 (SP - DP)$.

Venom Effects on Blood Coagulation of Anaesthetised Rats

Collapse of the circulatory system was induced in anaesthetised rats by injection of 0.5 mg of venom dissolved in saline (100 μ l) under the dorsal skin, and blood was collected from the surgically exposed vena cava. Blood clotting function was assessed by the 20-minute whole blood clotting test (20WBCT). This all-or-none blood coagulability test has been found to be a reliable indicator of low fibrinogen concentration [31,32]. The test was done by placing approximately 0.5 ml of rat blood in a glass tube (12x75 mm) covered with parafilm and inverting the tube 20 min after. Coagulopathy was indicated if there was no observable clot and the blood fell down on the parafilm.

Venom Prothrombin Activator Protein-induced Pathology in Anaesthetised Rats

In vivo setup was as in the whole venom experiment, with an additional cannula inserted in the right jugular vein and no mechanical ventilation. 20 μ g of the size-exclusion HPLC fraction containing the venom prothrombin activator proteins in 100 μ l of saline was injected into the jugular vein. Blood was withdrawn from the jugular vein and tested with 20-WBCT.

Size-exclusion HPLC Venom Profiling

Venom (500 μ l of 1 mg/ml) was separated by size-exclusion chromatography on a Superdex G-200 column (13 μ m, 10x300 mm, GE Healthcare), with optimum biomolecule separation in the 10–600 kDa range, on a Shimadzu HPLC system (LC-10ATvp pump), and eluted with 0.1 M ammonium acetate buffer (pH adjusted to 6.8), at the flow rate of 0.3 ml/min and protein detection at 280 nm. Manually collected fractions were stored at -80°C , and then freeze-dried. The surface areas under the peaks on the HPLC profile were calculated using GraphPad Prism.

Isolation of Venom Prothrombin Activator Proteins

Venom prothrombin activator proteins were obtained by collecting the first eluting size-exclusion HPLC fraction without the shoulder containing textilotoxin, using a Superdex G-75 column (GE Healthcare). The flow rate was 0.3 ml/min, elution

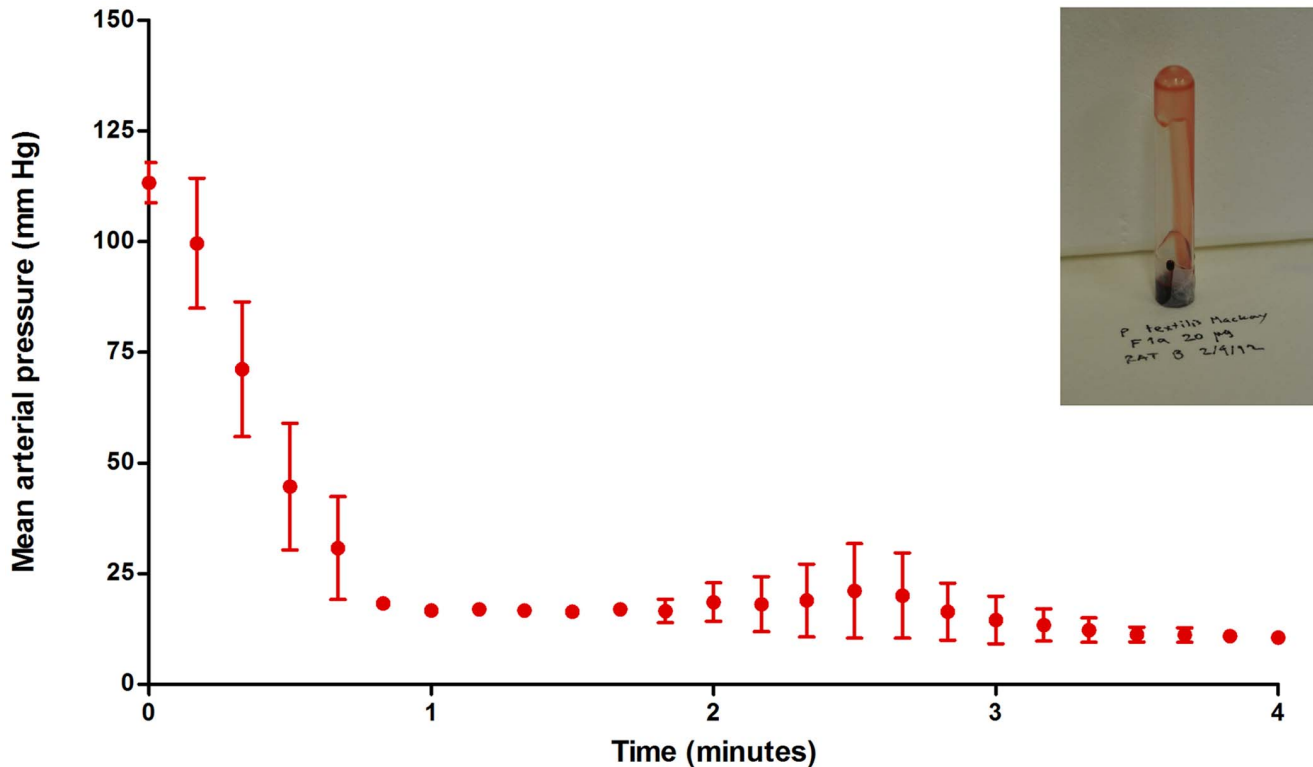


Figure 5. Mean arterial blood pressure in rats injected intravenously *P. textilis* (Mackay) venom prothrombin activator proteins. 20 µg of the HPLC size-exclusion fraction containing proteins matched by LC-MS to Q75ZN0, Q56VR3 and Q1L658 was injected in the jugular vein of pentobarbitone-anaesthetised rats. 20-minute whole blood clotting test confirmed coagulopathic activity of the toxins. Injection volume: 100 µl, vehicle: saline. doi:10.1371/journal.pone.0063988.g005

buffer 0.01 M ammonium acetate (pH = 6.8) and protein detection at 280 nm. The toxins were frozen at -80°C and freeze-dried. The toxin purity was confirmed by LC-MS analysis.

Mass Spectrometry-liquid Chromatography of Venoms

Venom samples were reconstituted in 100 µl of 20 mM ammonium bicarbonate (NH_4HCO_3). 20 µl of each sample was used for digestion with 5 µl of trypsin solution (100 ng trypsin per µl of 1% acetic acid). Acetonitrile was added to 10% v/v and the samples were incubated at 37°C . Tryptic digests were analysed by LC-MS/MS using the QExactive mass spectrometer (Thermo Scientific, Bremen, Germany) coupled online with a RSLC nano HPLC (Ultimate 3000, Thermo Scientific, Bremen, Germany). Samples injected onto a Thermo RSLC pepmap100, 75 µm id, 100 Å pore size, reversed phase nano column with 95% buffer A (0.1% Formic acid) at a flow rate of 300 nl/minute. The peptides were eluted over a 30 min gradient to 70% B (80% acetonitrile, 0.1% formic acid). The eluant was nebulised and ionised using the Thermo nano electrospray source stainless steel emitter with a capillary voltage of 2000 V. Peptides were selected for MS/MS analysis in Full MS/dd-MS² (TopN) mode with the following parameter settings: TopN 10, MSMS AGC target 2×10^5 , 80 ms Max IT, NCE 28 and 2 m/z isolation window. Dynamic exclusion was set to 30 seconds. Data from LC-MS/MS run were exported in Mascot generic file format (*.mgf) using proteowizard 3.0.3631 (open source software, <http://proteowizard.sourceforge.net>).

Proteomic Analysis

The mgf. files were analysed in Mascot MS/MS Ion Search using the following parameters: *Enzyme*: Trypsin; *Missed cleavages*: 1; *Taxonomy*: Chordata (Vertebrates and relatives); *Modifications*: none; *Peptide mass tolerance*: ± 10 ppm; *Peptide fragment mass tolerance*: ± 0.05 Da; *Peptide charge*: +2, +3, +4; *Database*: UniProtKB/Swiss-Prot. The significance threshold was $p < 0.05$. The results were filtered with ion score cut-off set to 0.05 to exclude non-significant matches. Subset proteins were not included in the venom component list since there is no conclusive evidence for their presence.

Animal Ethics Statement

Experimental procedures were approved by the Monash University Animal Ethics Committee (permits: 2008/05, 2010/35, 2012/008). *In vivo* experiments were done under pentobarbitone sodium anaesthesia. Every effort was made to minimise distress.

Results

Rat Diaphragm was Paralysed by the Neurotoxic Effects of *P. textilis* Venoms, but with Large Population Differences in the Paralysis Onset Time

Venom of the Barossa *P. textilis* population induced neuromuscular blockade in the phrenic nerve-diaphragm preparation significantly faster than venom from the Mackay population (Fig. 1). The mean time (± 1 standard error of the mean) to abolish the initial diaphragm contraction height by 95% (t_{95}) at a venom

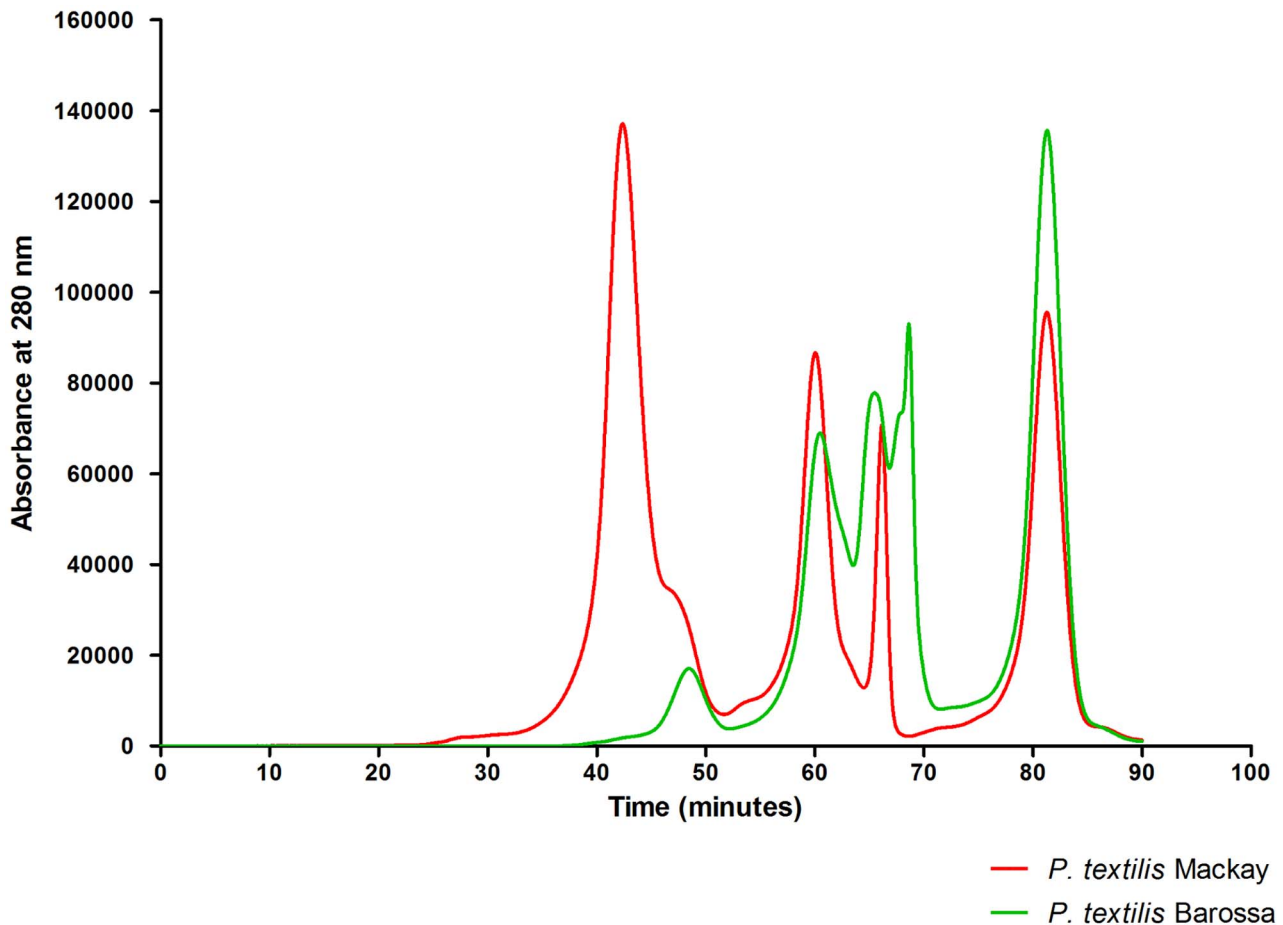


Figure 6. Size-exclusion chromatograms of *Pseudonaja textilis* population venoms. GE Healthcare Superdex G-200 column, 500 μ l of 1 mg/ml venom, 0.1 M pH=6.8 ammonium acetate buffer, 0.3 ml/min. doi:10.1371/journal.pone.0063988.g006

concentration of 10 μ g/ml was 21.7 \pm 3.5 min by the Barossa/Adelaide (n = 3) and 132.7 \pm 6.8 min by the Mackay (n = 3) venom. Interestingly, the difference in neurotoxic activity between the studied populations of *P. textilis* was larger than the difference between *P. textilis* and Australian coastal taipan *Oxyuramus s. scutellatus*, which abolished the diaphragm contractions by 95% in 60.0 \pm 3.5 min (n = 3). One-way ANOVA on t_{95} values produced a statistically significant result (p<0.0001), and Holm-Sidak’s multiple comparison test was significant for all comparisons at the significance level (alpha) of 0.05.

The Population Venoms Differed with Respect to the Contractile Response of the Chick Biventer Cervicis Muscle to Exogenous Acetylcholine

The contractile response of the chick biventer cervicis muscle to exogenously administered acetylcholine after *P. textilis* Mackay venom addition was largely preserved, with the mean (\pm SEM) percentage of initial contractile response of 86.7 \pm 6.4%. In contrast, the response after *P. textilis* Barossa venom addition (n = 4) was completely absent (0%) (Fig. 2). The Student’s t test comparing the post-venom chick biventer cervicis muscle responses to acetylcholine between the Mackay and Barossa *P. textilis* population venoms found a statistically significant difference (p<0.0001).

Both *P. textilis* Population Venoms were Found to be Extremely Procoagulant in Rat Plasma, Mackay (QLD) Population Venom having Higher Potency than that of Barossa (SA)

Venoms of both *P. textilis* populations were found to induce rapid clotting of citrated rat plasma, even at an extremely low dose of 5 μ g/100 μ l of the plasma, at which the mean time (\pm SEM) to the onset of clotting was 21.4 \pm 0.3 s for the Mackay sample (n = 3) and 31.4 \pm 1.0 s for the Barossa (n = 3) population sample. In comparison, venoms of *Oxyuramus* species had lower procoagulant activity than *P. textilis*, with a clotting time of 70.3 \pm 0.6 s for *O. scutellatus* (n = 3) and 208.1 \pm 9.4 s for *O. microlepidotus* (n = 3) (Fig. 3). A repeated measures one-way ANOVA produced a statistically significant result (p = 0.0026), and a Holm-Sidak multiple comparison test was statistically significant for all species comparisons at the significance level of 0.05. The Mackay *P. textilis* population sample was consistently more potent than the Barossa sample at all concentrations tested: At 50 ng/100 μ l of plasma the clotting times were 100.0 \pm 4.1 s (n = 3) for venom from the Mackay population and 148.3 \pm 4.0 s (n = 3) for the Barossa population, and at 25 ng/100 μ l plasma 174.4 \pm 9.1 s (n = 3) for Mackay and 263.8 \pm 12.3 s (n = 3) for Barossa samples. Repeated measures two-way ANOVA on *P. textilis* population venom effects at different venom concentrations found the population differences statistically significant, with p<0.0001.

Table 1. LC-MS/MS profile of *Pseudonaja textilis* population venom proteomes.

PROTEIN	ACCESSION No.	MASS (Da)	Mackay QLD <i>P. textilis</i>				Barossa SA <i>P. textilis</i>			
			Score	Pep	Seq	Cov (%)	Score	Pep	Seq	Cov (%)
Venom prothrombin activator Pseutarin-C non-catalytic subunit <i>Pseudonaja textilis</i>	Q7SZN0	165828	31059	981	80	66%	21020	654	65	64%
Venom prothrombin activator Pseutarin-C catalytic subunit <i>P. textilis</i>	Q56VR3	52181	3037	85	8	24%	2827	82	7	22%
Venom prothrombin activator Oscutarin-C catalytic subunit <i>Oxyuranus scutellatus</i>	Q58L96	52420					2015	52	5	18%
Coagulation factor X isoform 2 <i>P. textilis</i>	Q1L658	51626	1455	56	10	26%	384	13	4	15%
Venom prothrombin activator Nomenclature D1 <i>Notechis scutatus</i>	P82807	51430					139	12	2	7%
Protease inhibitor textilinin-1 <i>P. textilis</i>	Q90WA1	9167					2471	36	6	59%
Protease inhibitor textilinin-2 <i>P. textilis</i>	Q90WA0	9173	1818	91	3	39%	2468	34	4	59%
Protease inhibitor textilinin-4 <i>P. textilis</i>	Q90W98	8977					88	3	1	16%
Protease inhibitor textilinin-5 <i>P. textilis</i>	Q90W97	9025	32	3	1	24%				
Protease inhibitor textilinin-6 <i>P. textilis</i>	Q90W96	9054					420	15	1	27%
Phospholipase A ₂ 1 <i>P. textilis</i>	Q9W7J4	16833	991	18	2	30%	3046	58	4	58%
Phospholipase A ₂ 2 <i>P. textilis</i>	Q9W7J3	17128	1435	55	3	20%	889	40	3	27%
Phospholipase A ₂ textilotoxin A chain <i>P. textilis</i>	P23026	13839	63	1	1	8%	44	1	1	8%
Phospholipase A ₂ textilotoxin B chain <i>P. textilis</i>	P23027	13789	138	10	3	26%	95	2	2	22%
Phospholipase A ₂ textilotoxin D chain <i>P. textilis</i>	P23028	14912	279	10	3	18%	50	2	2	15%
Pseudonajatoxin b homolog <i>P. textilis</i>	Q9W7J5	11262					5276	174	7	66%
Pseudonajatoxin b <i>P. textilis</i>	P13495	7756					2943	84	4	66%
Long neurotoxin 1 <i>P. textilis</i>	A8HDK6	9875					130	4	1	14%
Short neurotoxin 1/5 <i>P. textilis</i>	Q9W7K2	8700					75	3	1	39%
Short neurotoxin 2 <i>P. textilis</i>	Q9W7K1	8590					8590	77	3	67%
Short neurotoxin 3 <i>P. textilis</i>	Q9W7K0	8576					259	8	3	67%
Short neurotoxin 6 <i>P. textilis</i>	Q9W7J7	8564					451	24	1	21%
Short neurotoxin 7 <i>P. textilis</i>	Q9W7J6	8880	89	2	1	17%	586	26	2	37%
Pseudechotoxin-like protein <i>P. textilis</i>	Q3SB05	26412					228	5	3	22%
C-type lectin BFL-1 <i>Bungarus fasciatus</i>	Q90W18	18626					58	3	2	9%

Notes: Mascot protein family search results for each population venom: score, peptide matches (Pep), sequences (Seq) and percent sequence coverage (Cov%). Accession no. = UniProtKB/Swiss-Prot database entry. doi:10.1371/journal.pone.0063988.t001

Collapse of the Circulatory System Occurred Instantly Following *P. textilis* Venom Injection in the Rat Dorsum Despite Artificial Respiration

Injection of 0.5 mg of *P. textilis* venom under the dorsal skin of the rat resulted in rapid failure of circulation in 100% of the rats injected (Fig. 4), at sudden drop in the mean arterial pressure below 20 mm Hg in 126.7±13.3 s in Mackay (n = 3) and 76.7±3.3 s in Barossa (n = 3) venom-injected rats. As expected, circulation was unaffected in rats injected with saline only (n = 3).

Severe Coagulopathy Develops Rapidly Following Injection of *P. textilis* Venom under the Rat Dorsal Skin

Venous blood of rats taken immediately following circulatory collapse induced by venom injection of 0.5 mg of Mackay (n = 3)

or Barossa (n = 3) population venoms under the dorsal rat skin was found to be completely incoagulable, whereas that from saline-injected rats (n = 3) clotted normally.

Injection of *P. textilis* Venom Prothrombin Activator Proteins into the Rat Bloodstream Results in Extremely Rapid Circulatory Failure and Incoagulable Blood

Injection of 20 µg of the size-exclusion fraction containing the Mackay *P. textilis* venom proteins matched to the *P. textilis* prothrombin activator pseutarin-C and coagulation factor X isoform 2 (Q7SZN0, Q56VR3, Q1L658) into the jugular vein of the rat caused complete circulatory failure (Fig. 5), with the mean arterial pressure falling below 20 mmHg in 46.7±3.3 s. 20-minute whole blood clotting test confirmed incoagulable blood *in vivo*.

SE HPLC Revealed Large Differences in the Proportion of the First-eluting Fraction between the *P. textilis* Mackay and Barossa Population Venoms

The highest molecular weight fraction of the Mackay venom comprised 46.6% of the size-exclusion chromatogram. In contrast, this peak comprised only 6.1% in the Barossa sample. Lower molecular weight toxins were more abundant in the venom from the Barossa population (Fig. 6). Ion trap LC-MS analysis of the chromatographic fractions determined that the first eluting fraction contained the venom prothrombin activator proteins and the presynaptic neurotoxin textilotoxin.

Proteomic Analysis Revealed the Presence of Multiple Coagulation Factor X-like Proteins in the Venoms of *Pseudonaja textilis*

Orbitrap liquid chromatography-mass spectrometry provided a fascinating insight into the venom proteomes of the Australian eastern brown snake populations. Significant protein hits in Mascot are shown in Table 1.

Venom prothrombin activator pseutarin-C, with its constituent catalytic (Q56VR3) and non-catalytic (Q7SZN0) subunits, was found in the venom samples of both Barossa and Mackay *P. textilis* populations. Interestingly, the presence of two distinct protein members of the coagulation factor X-like protein family in the venom sample of Mackay *P. textilis* population was revealed. The protein matched to the catalytic subunit of the venom prothrombin activator complex pseutarin-C (*Pseudonaja textilis*) had 6 significant unique peptide sequences, whereas the other protein matching to the coagulation factor X isoform 2 (Q1L658) of *Pseudonaja textilis* had 8 significant unique peptide sequences. Only 2 of the detected peptide sequences were shared by the two proteins, providing strong support for the existence of at least two coagulation factor-X like proteins in the venom of this population (Table S1). The match to *P. textilis* coagulation factor X isoform 2, known to be produced in the liver tissue, is strongly supported, which suggests that the gene coding for this protein or its close homologue may also be expressed in the venom gland.

In the venom sample from the Barossa population, 4 significant peptide sequences were unique to the protein matched to venom prothrombin activator pseutarin-C catalytic subunit, 2 sequences unique to the coagulation factor X isoform 2 of *P. textilis*, and a single significant sequence each unique to venom prothrombin activator oscutarin-C catalytic subunit of *O. scutellatus* (Q58L96) and notecarin-D1 of *N. scutatus* (P82807), confirming presence of multiple coagulation factor X-like proteins in the venoms of this population (Table S2).

Numerous Postsynaptic α -neurotoxins were Found in *P. textilis* Barossa (SA) Venom Whereas they were Largely Absent in the Venom Samples from Mackay (QLD)

LC-MS analysis of the venom sample from Barossa population of *P. textilis* revealed a diverse assemblage of postsynaptic neurotoxins, including 'long chain' of the type II (Q9W7J5, P13495 and A8HDK6) and type III (Q9W7K2, Q9W7K1, Q9W7J7, Q9W7J6) alpha three-finger neurotoxins. Interestingly, only a single postsynaptic neurotoxin (Q9W7J6) from the α -neurotoxin type III group was found in Mackay venom sample.

Phospholipase A₂ subunits of the presynaptic neurotoxin textilotoxin (P23026, P23027, P23028) were found in both populations.

Other noted differences include detection of Pseudechetoxin-like protein (Q3SB05) and a protein with sequence similarity to C-type lectin BFL1 of *Bungarus fasciatus* (Q90WI8) solely in Barossa *P.*

textilis venom sample. Different forms of protease inhibitors textililins were found in the venoms of both populations (Table 1).

Discussion

Venoms from the South Australian and Queensland *P. textilis* populations injected in live rats were found to cause rapid collapse of the circulatory system. Several findings in this study support the role of procoagulant toxins in rapid rodent prey incapacitation: (i) circulatory collapse resulting from intravenous injection of *P. textilis* (Mackay) purified venom prothrombin activators in the rat, including proteins matched to pseutarin-C non-catalytic and catalytic subunits and coagulation factor X isoform 2, (ii) high procoagulant venom activity in rat plasma, and (iii) instant collapse of the circulatory system following injection of venom under the dorsal skin despite artificial respiration. It is remarkable that these proteins have evolved such toxicity to incapacitate and kill endothermic prey as large as a rat in an instant. High procoagulant *P. textilis* venom activity in rat plasma found in this study and histological sections of lung tissue of mice injected i.v. with *Oxyuranus scutellatus* venom showing the presence of thrombi in the study by Herrera et al [6] suggest that thrombosis should be examined as a possible mechanism responsible for rapid collapse of the rodent circulatory system in envenomations with procoagulant venoms of *Pseudonaja* and *Oxyuranus* species.

Venom proteins diversify by gene duplication and subsequent divergence in structure and function [33], which is consistent with multiple coagulation factor X-like proteins found in the venoms of *P. textilis* populations. In addition to venom prothrombin activator pseutarin-C, a significant protein match is to the *P. textilis* coagulation factor X isoform 2, which is particularly well supported for Mackay *P. textilis* venom. Interestingly, the real-time polymerase chain reaction (PCR) study of tissue-specific expression by Reza et al [34] found *P. textilis* coagulation factor X isoform 2 gene to be expressed in the liver, but not in the venom gland.

Venoms of both populations were neurotoxic, causing complete inhibition of the rat diaphragm contractions in the isolated phrenic nerve-diaphragm preparation, but the times to blockade differed significantly. A quicker paralytic effect of Barossa *P. textilis* venom may likely result from the activity of diverse postsynaptic α -neurotoxins, which, with exception of one type III α -neurotoxin, were not found in venom samples from the Mackay population. This hypothesis is supported by the studies in nerve-muscle preparations which showed that the isolated presynaptic PLA₂ neurotoxins of *P. textilis* (textilotoxin) and *O. microlepidotus* (paradoxin) induce significantly slower onset of neuromuscular blockade than the whole venoms of these species, even at high concentrations of the presynaptic neurotoxins [35,36]. A study of abundance and bioactivity of the presynaptic PLA₂ neurotoxins in each population should be carried out to exclude any possibility of the quicker effect observed being due to the abundance or population-specific activity of these neurotoxins. There was no response to exogenously administered acetylcholine in the chick biventer cervicis muscle exposed to the Barossa *P. textilis* venom, which could result from postjunctional blockade of acetylcholine receptors by the postsynaptic neurotoxins (see [29]), but myotoxicity should be assessed at the population level as well. Interestingly, the venom proteome of the Barossa population contains diverse postsynaptic α -neurotoxins, which are mostly lacking in venom samples from the Mackay population. The adaptive roles of these neurotoxins in prey incapacitation are unknown, but due to skeletal muscle paralytic activity, they could potentially be advantageous in acquisition of ectothermic prey.

Venoms of both Mackay and Barossa populations of *P. textilis* have extremely potent procoagulant venom activity in rat plasma, but the Queensland venom appears to have a larger concentration of venom prothrombin activator proteins and is more procoagulant. The procoagulant activities of *O. scutellatus* and *O. microlepidotus* venoms in rat plasma were significantly lower than those of *P. textilis* populations. This finding is consistent with the SDS-PAGE profile of *Pseudonaja* spp. and *Oxyuranus* spp. venoms, with *P. textilis* showing a much denser band than those of *O. scutellatus* and *O. microlepidotus*, indicating a higher concentration of venom prothrombin activator [11]. Despite the venom being less procoagulant than that of *P. textilis* in rodent plasma, *O. scutellatus* is known to deliver high yields of venom in multiple quick bites, and thus large amounts of the venom prothrombin activator and neurotoxic proteins. Different venom yield potentials of *P. textilis* populations and *Oxyuranus* species are demonstrated in the study by Mirtschin et al [37]. A recent study [6] reports that mice injected i.v. with a high dose of whole venom of *O. scutellatus* died sooner than those injected with the same dose of the purified presynaptic PLA₂ neurotoxin (taipoxin), suggesting that a role of procoagulant toxins in rapid rodent prey incapacitation should be considered for this species.

Physiological responses of the readily available lab SD (Sprague-Dawley) rat *Rattus norvegicus* to injected venom proteins can give us a good insight into pathological processes that occur in envenomed rodent prey, but because susceptibility to venom proteins may differ, when feasible, studies of venom toxicity should be conducted on wild prey. The number of Mackay individuals from which the venom was pooled was small, but the venom from the recently captured third individual showed the same bioactivity and proteomic profile as the venom samples pooled from the two previously collected individuals, supporting the finding of population differences in venom composition and bioactivities in *P. textilis*. Further support is from observations of the venom sample from another Queensland population of *P. textilis* from the Gold Coast lacking significant postsynaptic neurotoxicity as well (unpublished data). Flight et al [26] reported that the venom of the Gold Coast population has higher procoagulant activity in human plasma and a higher first size-exclusion chromatographic peak than that of the South Australian population. This suggests that the venom and bioactivity profile of the Mackay *P. textilis* venom described in this study is likely widespread in populations of the northeastern clade of *P. textilis*. The study should be expanded to include a larger number of individuals and sampling from more localities. Importantly, additional mass spectrometry techniques should be used to study the venom proteomes and potentially

detect toxins which may not have been detected with the single LC-MS technique used in this study. In conclusion, high divergence in venom composition and bioactivity of South Australian and Queensland populations of *P. textilis* is revealed, and multiple experiments support the hypothesis of procoagulant toxins causing rapid collapse of the circulatory system in envenomed rodent prey.

Supporting Information

Table S1 Coagulation factor X-like protein family in *P. textilis* Mackay (Queensland, Australia) venom sample.

Software: Mascot; Database: UniProtKB/Swiss-Prot. Proteins are denoted with Roman numerals: I = venom prothrombin activator psetarin-C catalytic subunit *Pseudonaja textilis* (Q56VR3); II = coagulation factor X isoform 2 *P. textilis* (Q1L658). X denotes presence of a peptide in a particular protein. (PDF)

Table S2 Coagulation factor X-like protein family in *P. textilis* Barossa (South Australia, Australia) venom sample.

Software: Mascot; Database: UniProtKB/Swiss-Prot. Proteins are denoted with Roman numerals: I = venom prothrombin activator psetarin-C catalytic subunit *Pseudonaja textilis* (Q56VR3); II = venom prothrombin activator oscutarin-C catalytic subunit *Oxyuranus scutellatus* (Q58L96); III = coagulation factor X isoform 2 *P. textilis* (Q1L658); IV = venom prothrombin activator notecarin-D1 *Notechis scutatus* (P82807). X denotes presence of a peptide in a particular protein. (PDF)

Acknowledgments

Nathan Dunstan's acquisition of Mackay *P. textilis* venom was very helpful for the study. LC-MS/MS runs were done by Shane Reeve at the Monash Biomedical Proteomics Facility. Muhamad Rusdi Ahmad Rusmili and David Steer gave helpful advice in proteomic techniques. Thanks to colleagues in the Department of Pharmacology at Monash University for help and advice, particularly Andrew Hart, Carmel Barber and Rachelle Kornhauser. Comments from the reviewers helped improve the manuscript.

Author Contributions

Conceived and designed the experiments: JS WCH. Performed the experiments: JS. Analyzed the data: JS. Contributed reagents/materials/analysis tools: WCH. Wrote the paper: JS. Reviewed the manuscript: WCH.

References

- Jorge da Silva N, Jr., Aird SD (2001) Prey specificity, comparative lethality and compositional differences of coral snake venoms. *Comp Biochem Physiol C Toxicol Pharmacol* 128: 425–456.
- Starkov VG, Osipov AV, Utkin YN (2007) Toxicity of venoms from vipers of the Peliass group to crickets *Gryllus assimilis* and its relation to snake entomophagy. *Toxicon* 49: 995–1001.
- Barlow A, Pook CE, Harrison RA, Wuster W (2009) Coevolution of diet and prey-specific venom activity supports the role of selection in snake venom evolution. *Proc Biol Sci* 276: 2443–2449.
- Gibbs HL, Mackessy SP (2009) Functional basis of a molecular adaptation: prey-specific toxic effects of venom from *Sistrurus rattlesnakes*. *Toxicon* 53: 672–679.
- Hodgson WC, Wickramaratna JC (2002) In vitro neuromuscular activity of snake venoms. *Clin Exp Pharmacol Physiol* 29: 807–814.
- Herrera M, Fernandez J, Vargas M, Villalta M, Segura A, et al. (2012) Comparative proteomic analysis of the venom of the taipan snake, *Oxyuranus scutellatus*, from Papua New Guinea and Australia: role of neurotoxic and procoagulant effects in venom toxicity. *J Proteomics* 75: 2128–2140.
- Shine R (1989) Constraints, allometry, and adaptation: Food habits and reproductive biology of Australian Brownsnakes (*Pseudonaja*: Elapidae). *Herpetologica* 45: 195–207.
- Fleay D (1943) The Brown snake - a dangerous fellow. *Victorian Naturalist* 59: 147–152.
- Fairley NH (1929) The dentition and biting mechanism of Australian snakes. *Medical Journal of Australia* 1: 313–327.
- Birrell GW, Earl S, Masci PP, de Jersey J, Wallis TP, et al. (2006) Molecular diversity in venom from the Australian Brown snake, *Pseudonaja textilis*. *Mol Cell Proteomics* 5: 379–389.
- Masci PP, Mirtschin PJ, Nias TN, Turnbull RK, Kuchel TR, et al. (1998) Brown snakes (*Pseudonaja* genus): venom yields, prothrombin activator neutralization and implications affecting antivenom usage. *Anaesth Intensive Care* 26: 276–281.
- Coulter AR, Broad AJ, Sutherland SK (1979) Isolation and properties of a high molecular weight neurotoxin from the eastern Brown snake (*Pseudonaja textilis*). In: Chubb IW, Geffen LB, editors. *Neurotoxins, Fundamental and Clinical Advances*: Adelaide University Union Press. pp.260.
- Su MJ, Coulter AR, Sutherland SK, Chang CC (1983) The presynaptic neuromuscular blocking effect and phospholipase A2 activity of textilotoxin, a potent toxin isolated from the venom of the Australian brown snake, *Pseudonaja textilis*. *Toxicon* 21: 143–151.

14. Tyler MI, Spence I, Barnett D, Howden ME (1987) Pseudonajatoxin b: unusual amino acid sequence of a lethal neurotoxin from the venom of the Australian common brown snake, *Pseudonaja textilis*. *Eur J Biochem* 166: 139–143.
15. Gong N, Armugam A, Mirtschin P, Jeyaseelan K (2001) Cloning and characterization of the pseudonajatoxin b precursor. *Biochem J* 358: 647–656.
16. Gong N, Armugam A, Jeyaseelan K (1999) Postsynaptic short-chain neurotoxins from *Pseudonaja textilis*. cDNA cloning, expression and protein characterization. *Eur J Biochem* 265: 982–989.
17. Fry BG, Wuster W, Kini RM, Brusic V, Khan A, et al. (2003) Molecular evolution and phylogeny of elapid snake venom three-finger toxins. *J Mol Evol* 57: 110–129.
18. Masci PP, Whitaker AN, Sparrow LG, de Jersey J, Winzor DJ, et al. (2000) Textilins from *Pseudonaja textilis textilis*. Characterization of two plasmin inhibitors that reduce bleeding in an animal model. *Blood Coagul Fibrinolysis* 11: 385–393.
19. Armugam A, Gong N, Li X, Siew PY, Chai SC, et al. (2004) Group IB phospholipase A2 from *Pseudonaja textilis*. *Arch Biochem Biophys* 421: 10–20.
20. Jayanthi GP, Gowda TV (1988) Geographical variation in India in the composition and lethal potency of Russell's viper (*Vipera russelli*) venom. *Toxicon* 26: 257–264.
21. Calvete JJ, Sanz L, Perez A, Borges A, Vargas AM, et al. (2011) Snake population venomomics and antivenomics of *Bothrops atrox*: Paedomorphism along its transamazonian dispersal and implications of geographic venom variability on snakebite management. *J Proteomics* 74: 510–527.
22. Daltry JC, Wuster W, Thorpe RS (1996) Diet and snake venom evolution. *Nature* 379: 537–540.
23. Daltry JC, Wuster W, Thorpe RS (1997) The role of ecology in determining venom variation in the Malayan pit viper. In: Thorpe RS, Wuster W, Malhotra A, editors. *Venomous snakes: Ecology, Evolution and Snakebite*, Symposia of the Zoological Society of London, No 70: Clarendon Press Oxford. pp.155–171.
24. Skinner A, Donnellan SC, Hutchinson MN, Hutchinson RG (2005) A phylogenetic analysis of *Pseudonaja* (Hydrophiinae, Elapidae, Serpentes) based on mitochondrial DNA sequences. *Mol Phylogenet Evol* 37: 558–571.
25. Williams DJ, O'Shea M, Daguerre RL, Pook CE, Wuster W, et al. (2008) Origin of the eastern brownsnake, *Pseudonaja textilis* (Dumeril, Bibron and Dumeril) (Serpentes : Elapidae : Hydrophiinae) in New Guinea: evidence of multiple dispersals from Australia, and comments on the status of *Pseudonaja textilis* pughi Hoser 2003. *Zootaxa*: 47–61.
26. Flight S, Mirtschin P, Masci PP (2006) Comparison of active venom components between Eastern brown snakes collected from South Australia and Queensland. *Ecotoxicology* 15: 133–141.
27. Bulbring E (1946) Observations on the isolated phrenic nerve diaphragm preparation of the rat. *Br J Pharmacol Chemother* 1: 38–61.
28. Ginsborg BL, Warriner J (1960) The isolated chick biventer cervicis nerve-muscle preparation. *Br J Pharmacol Chemother* 15: 410–411.
29. Harvey AL, Barfaraz A, Thomson E, Faiz A, Preston S, et al. (1994) Screening of snake venoms for neurotoxic and myotoxic effects using simple in vitro preparations from rodents and chicks. *Toxicon* 32: 257–265.
30. O'Leary MA, Isbister GK (2010) A turbidimetric assay for the measurement of clotting times of procoagulant venoms in plasma. *J Pharmacol Toxicol Methods* 61: 27–31.
31. Warrell DA, Davidson N, Greenwood BM, Ormerod LD, Pope HM, et al. (1977) Poisoning by bites of the saw-scaled or carpet viper (*Echis carinatus*) in Nigeria. *Q J Med* 46: 33–62.
32. Sano-Martins IS, Fan HW, Castro SC, Tomy SC, Franca FO, et al. (1994) Reliability of the simple 20 minute whole blood clotting test (WBCT20) as an indicator of low plasma fibrinogen concentration in patients envenomed by *Bothrops* snakes. Butantan Institute Antivenom Study Group. *Toxicon* 32: 1045–1050.
33. Kordis D, Gubensek F (2000) Adaptive evolution of animal toxin multigene families. *Gene* 261: 43–52.
34. Reza MA, Minh Le TN, Swarup S, Manjunatha Kini R (2006) Molecular evolution caught in action: gene duplication and evolution of molecular isoforms of prothrombin activators in *Pseudonaja textilis* (brown snake). *J Thromb Haemost* 4: 1346–1353.
35. Barber CM, Isbister GK, Hodgson WC (2012) Solving the 'Brown snake paradox': in vitro characterisation of Australasian snake presynaptic neurotoxin activity. *Toxicol Lett* 210: 318–323.
36. Hodgson WC, Dal Belo CA, Rowan EG (2007) The neuromuscular activity of paradoxin: a presynaptic neurotoxin from the venom of the inland taipan (*Oxyuranus microlepidotus*). *Neuropharmacology* 52: 1229–1236.
37. Mirtschin PJ, Dunstan N, Hough B, Hamilton E, Klein S, et al. (2006) Venom yields from Australian and some other species of snakes. *Ecotoxicology* 15: 531–538.