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Transcriptomics-guided bottom-up and top-down venomics of neonate and adult specimens of the arboreal rear-fanged Brown Treesnake,Boiga irregularis, from Guam

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shift in toxin abundance, likely driven by dietary variation between the two age classes. Second-generation antivenomics and Western blot analysis using puri ed anti-Brown Treesnake rabbit serum IgGs (anti-BTS IgGs) showed strong immunoreactivity toward B. irregularisvenom. Interestingly, our anti-BTS IgGs did not cross-react with 3FTxs found in several other rear-fanged snake venoms, or against 3FTxs in the venom of the elapid Ophiophagus hannah, indicating that epitopes in these 3FTx molecules are quite distinct.

1. Introduction

The genus Boiga comprises 33–34 recognized species of venomous, opisthoglyphous colubrid snakes commonly known as the Cat-eyed Snakes because of their vertical pupils (http://www.reptile-database.org). The Brown Treesnake, Boiga irregularis (Colubridae, Bechstein 1802) [1] , is a nocturnal, oviparous snake native to coastal areas of Northern Australia and a large number of islands in northwestern Melanesia, from Sulawesi in eastern Indonesia through Papua New Guinea and the Solomon Islands. Although primarily arboreal, the Brown Treesnake occupies a wide variety of habitats at elevations from sea level to 1200 m [2] . In its native range, the Brown Treesnake is a generalist feeder, preying upon birds, lizards (such as geckos and skinks) and their eggs, bats, rats and other small rodents[2,3] . Natural predators include monitor lizards, cobras, owls, and several mammals [4,5] . Adult B. irregularis average 1.4–1.5 m in length, although individuals reaching lengths of 2.3 m (females) and 3.1 m (males) have been reported on Guam [2] . On Guam, B. irregularis

completed following the NEBNext Ultra RNA library prep manufacturer's protocol for Illumina sequencing. The minimum number of recommended PCR cycles (12) was performed and the final AMPure XP bead purified library was assessed on a Bioanalyzer Agilent 2100 system (Agilent Technologies, USA) for proper fragment size selection and quality. This library was then equally pooled after qPCR KAPA library quantification (KAPA Biosystems, USA) with four other uniquely barcoded libraries and sequenced on a single HiSeq 2500 instrument lane at the Genomics and Microarray Core Facility (University of Colorado Anschutz Medical Campus) to obtain 125 bp paired-end reads.

2.3. Transcriptome assembly, annotation and toxin transcript quantification

Illumina reads were assessed using the Java program FastQC (Babraham Institute Bioinformatics, U.K.), and low quality reads (Phred + 33 score < 30) and contaminating adaptor sequences were removed using Trimmomatic with a sliding window of 4 bps [34] . To obtain a comprehensive venom gland transcriptome assembly, contigs from three different assemblies were used. First, a Trinity (release v2014-07-17) de novo assembly of paired-end reads was completed with default parameters (k-mer size 25) [35] . A second de novo assembly was completed with the program Extender (k-mer size 100) [36] . For the Extender assembly, reads were first merged with PEAR (Paired-End read mergeR v0.9.6 [37] ; default parameters) if their 3′ ends overlapped to create longer contiguous sequences, and these merged reads were used as Extender input. The Extender assembly was performed with the same parameters as specified for the venom gland assembly of B. irregularis originating from Indonesia [30] . Lastly, a genome-guided Trinity (release v2014-07-17) assembly was performed using the available Indonesian B. irregularis venom gland assembly as a reference with Bowtie2 [38] used for generating the bam file input. These three approaches were taken to assemble contigs both shared and unique between snakes from Guam and Indonesia, as well as accounting for transcripts that are best assembled using short or long k-mers.

Contigs from these three assemblies were combined and BLASTx (executed using BLAST+ command line (version 2.6.0); minimum E-value of 10^{-4}) [39] completed against a custom snake protein database, which consisted of all identified squamate venom proteins available in the NCBI databases (

searches were performed at https://blast.ncbi.nlm.nih.gov/Blast.cgi against the non-redundant protein sequences database, using the default parameters of the BLASTP program [42] .

Protein bands of interest were excised from Coomassie Brilliant Blue-stained SDS-PAGE gels and subjected to in-gel reduction (10 mM dithiothreitol, 30 min at 65 °C) and alkylation (50 mM iodacetamide, 2 h in the dark at room temperature), followed by overnight sequencing-grade trypsin digestion (66 ng/ µL in 25 mM ammonium bicarbonate, 10% ACN; 0.25 µg/sample) in an automated processor (using a Genomics Solution ProGest Protein Digestion Workstation) following the manufacturer's instructions. Tryptic digests were dried in a vacuum centrifuge (SPD SpeedVac,® ThermoSavant), redissolved in 15 µL of 5%

neonates were calculated usingt-test and their negative logarithm of 10 was plotted against the logarithm of 2 of the fold change.

2.7. Cladogram of 3FTxs identied in Guam and Indonesian venoms

Multiple sequence alignment of 3FTx sequences identi ed in Guam (this work) and Indonesiam [30] B. irregularis venom proteomes was completed using MEGA version 6.0.6 [51] . A neighbor-joining cladogram was constructed using the same software and default parameters.

2.8. Anti-Brown Treesnake (BTS) venom antibodies and antivenomics

An experimental antiserum was raised in rabbits by subcutaneous injections of adult Guam B. irregularis venom. The rst injection comprised 250 µg venom in 250 µL of isotonic (0.9%) saline solution emulsi ed with an equal volume of Freund's complete adjuvant. Booster injections of the same amount of immunogen emulsi ed in Freund's incomplete adjuvant were administered at 3 week intervals for a period of 2 months. Terminal cardiac blood collection, performed by intracardiac puncture under general anesthesia, was approved by the IBV's Ethics Commission (074/2014-2019) and by the Generalitat Valenciana (2014/VSC/PEA/00060 tipo 2). Collected whole blood was allowed to clot by leaving it undisturbed at room temperature (RT) for 3 h. The clot was removed from serum by centrifugation for 45 min at 2000 × g using a refrigerated centrifuge. The serum pH was adjusted to 5.8 with 120 mM sodium acetate bu er (pH 4.0), and the IgG fraction was puri ed by slowly adding, under vigorous stirring of the serum, caprylic acid to a nal concentration of 6% (v/mrr27a49t210eaoana 3 × g

above. Controls using anti-BTS IgG only were run for each level and subtracted from assay values, and results were reported as % activity remaining (compared to assays with just B. irregularis (Guam) venom). Two individual adult venom samples were assayed in duplicate.

2.11. Data accessibility

Transcriptomic data has been submitted to the NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) (Bioproject ID: PRJNA401953; Biosample accession: SAMN07612293; Sequence Read Archive code: SRR6012492). All transcripts that resulted in complete translated venom proteins were submitted to GenBank with accession numbers MF948008-MF948117. All LC-MS/MS. raw and .mzXML data, as well as search engine outputs and the search database, can be found on the Mass Spectrometry Interactive Virtual Environment (MassIVE) at https://massive.ucsd.edu/(accession number: MSV000081130 at http://massive.ucs d.edu/ProteoSAFe/dataset.jsp?task=931c5819f582493e9bfbd176fc87c616), as well as through ProteomeXchange [56,57] at http://www.proteomexchange.org /(accession number PXD006636, at http://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PXD006636).

3. Results and discussion

3.1. Guam B. irregularis transcriptome

For the B. irregularis venom gland library, a total of 53,201,077 paired-end reads were obtained with Illumina sequencing and 40,112,608 of these reads were used for the transcriptome assembly after adapter and quality filtering. Trinity de novo assembly produced 134,496 contigs with an average contig length of 1879 bps. The Trinity assembly, completed using the Indonesian B. irregularis venom gland transcriptome as a reference, resulted in 70,716 contigs with an average contig length of 910 bps. A total of 66% of paired-end reads had sequence overlap (minimum of 10 bps) that could be merged and used as Extender input. With these merged reads, Extender assembled 617 contigs with an average contig length of 1886 bps. Of the three

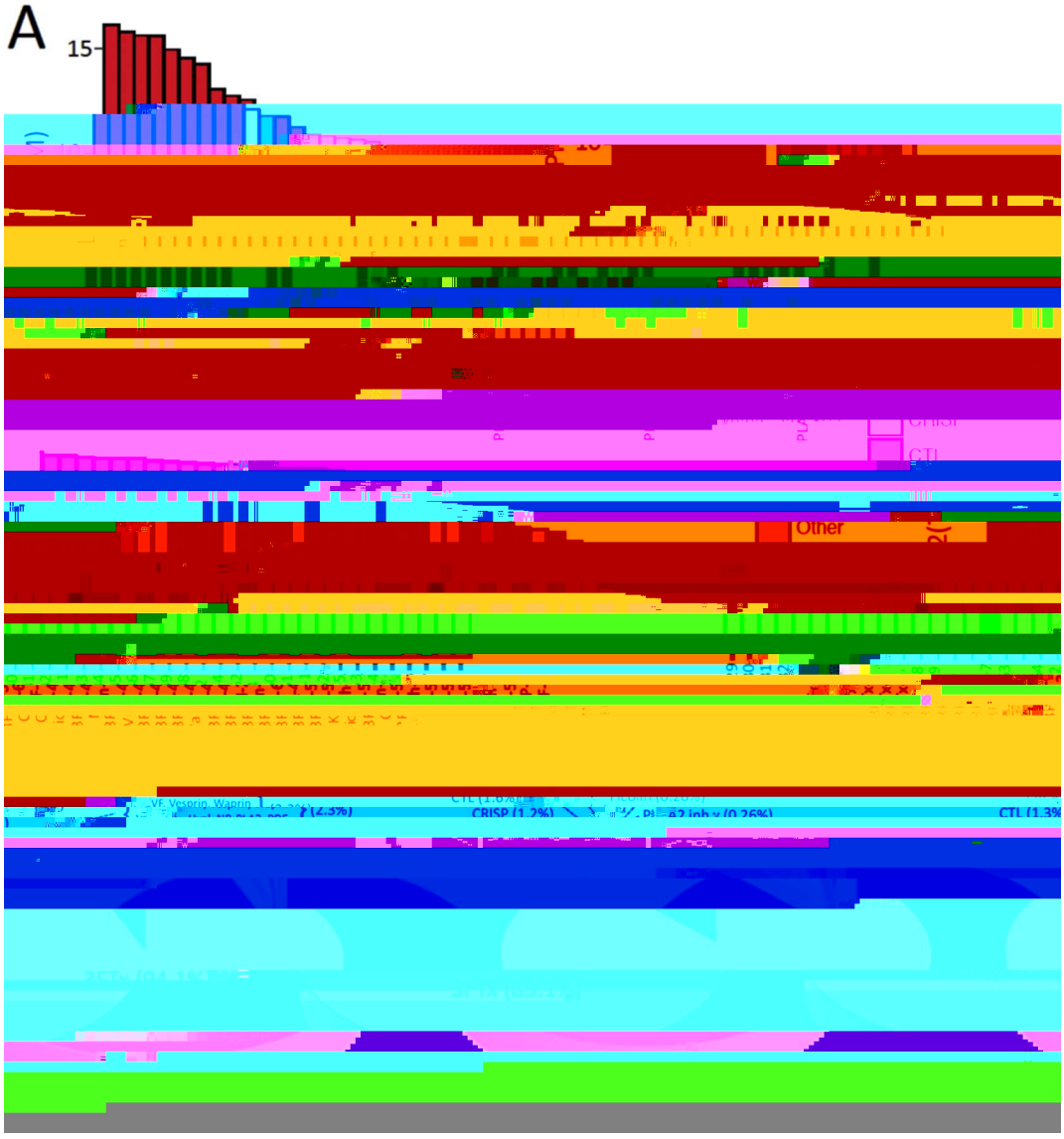

Fig. 1. A) Overview of the 100 most highly transcribed toxin-coding mRNAs in the venom gland of adult Brown Treesnake (B. irregularis) from Guam. B) Pie chart of the relative

assemblies, the Trinity genome-guided and Extender assemblies produced the greatest number of full-length translated venom proteins.

Contigs with the highest Transcripts Per Million (TPM) values were identified as partial or complete venom protein sequences (Fig.1). It was discovered that for the top 1000 expressed contigs, those without a BLASTx result still did have a significant BLASTn hit to a venom protein transcript within the nt database, but they were partial transcripts largely consisting of untranslated regions. This demonstrates the limitation of only using BLASTx to identify venom protein transcripts, and it is most likely the reason that previously examined venom gland transcriptomes report a significant percentage (~40%) of unidentified transcripts [36,58,59] . A high abundance of venom protein transcript expression is commonly observed for snake venom glands[35,57,58] ,

the expression level of venom protein transcript isoforms and venom protein superfamilies has been reported for Crotalus adamanteus from di erent localities [61] . Boiga irregularis also exhibited locality-speci c isoforms, with only a single 3FTx isoform shared (with 100% identity) between the two localities (3FTx_03 from Guam and Birre_3FTx-9d from Indonesia).

Transcript 3FTx_01 was found to be identical to NCBI database three- nger toxin 3 (KU666930), 3FTx_06 was identical to database three- nger toxin 1 (KU666929) and 3FTx_46 was identical to database three- nger toxin 5 (KU666932); these transcripts originated from extracellular mRNA within Guam B. irregularis venom [62] . With the exception of one amino acid within the signal peptide, 3FTx_02 was found to be identical to database three- nger toxin 6 (KU666933), and a three amino acid di erence in the signal peptide between 3FTx_40 and three- nger toxin 4 (KU666931) was also observed. It is likely that these few amino acid di erences within the signal peptides are due to the use of a degenerate PCR primer within this region to obtain the sequences of extracellular mRNA transcripts [60] . Therefore, transcripts obtained from the next-generation sequencing (NGS) venom gland tissue transcriptome were found to be identical to those observed within the venom of B. irregularis, with one of these transcripts even corresponding to the mature protein sequence of Irditoxin B, highlighting the application of using venom extracellular mRNA to obtain venom protein transcripts of both abundant and novel toxins [62] .

As also noted for the Indonesian B. irregularis venom gland transcriptome, transcript sequences identical to Irditoxin A and B were not found. Transcripts that shared the greatest identity (96% to subunit A and 97% to subunit B) exhibited at least three amino acid substitutions within the signal peptide region, which would still produce a mature protein that would be identical to each Irditoxin subunit. This lack of transcripts identical to both Irditoxin subunit transcripts, coupled with the fact that only one identical 3FTx isoforms [ANN23940, 3FTx_4] (Table S2) is shared between Indonesian and Guam Brown Treesnakes, demonstrates the rapid nature of amino acid substitution exhibited by B. irregularis venom proteins, particularly those that are abundantly expressed within venom glands [63] .

3.2. The venom proteomes of neonate and adult B. irregularis from Guam

The venom toxin compositions of neonate and adult Guam Brown Treesnakes were initially assessed through a bottom-up protocol. Venoms were fractionated by reverse-phase (RP) HPLC (Fig. 2, panels A and B), the individual peaks quanti ed spectrophotometrically (Fig. 2, panels C and D), the RP fractions analyzed by SDS-PAGE, and the [illegible] (Fig. 3) [illegible] (Fig. 4) [illegible] (Table 1) [illegible] (Table 2) [illegible] (Table 3) [illegible] (Table 4) [illegible] (Table 5) [illegible] (Table 6) [illegible] (Table 7) [illegible] (Table 8) [illegible]

in their overall toxin compositional patterns and, strikingly, also in their CRISP content (compare Fig. 1A and panels C and D of Fig. 2). The reasons for these discrepancies remain elusive, but have been postulated to include i) transient/individual/temporal expression patterns of mRNA expression; ii) post-genomic regulation [64–66]; iii) a "hidden repertoire" of readily translatable transcripts for functional venom adaptation; and iv) methodological or statistical issues [67,68].

The poor resolution of the RP chromatogram section containing 3FTxs (elution time between 9 and 13 min in panels A and B of Fig. 2) did not allow separation of these venom components. High performance liquid chromatography (HPLC) (Fig. 3A) in conjunction with high-resolution top-down MS analysis has the potential for achieving full individual protein characterization [69–72]. This approach enabled the identi cation of 30 and 25 full-length 3FTxs in the venom proteome of neonate (Table S4) and adult (Table S5) Brown Treesnakes, respectively. In both age classes, these venom toxins are proteo-2972w8 (stml66hg297epanwg/T1_1 1 Tf 1.5435 0m(thoth10repanc9th)-299.6m(th5le)-596.8 (tran4

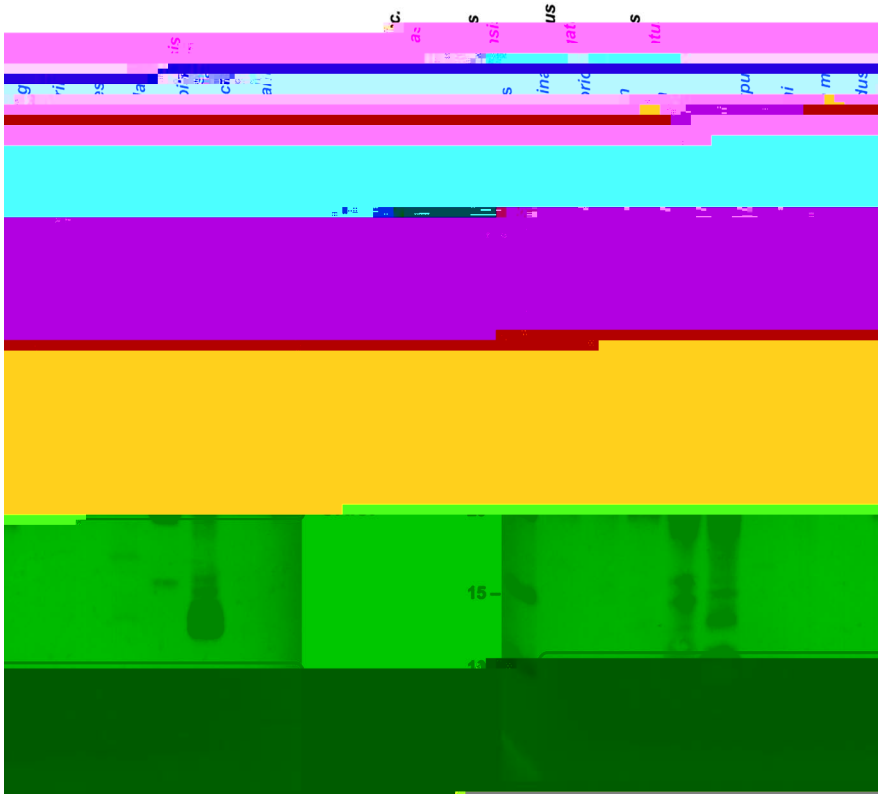

Figure 6. Reactivity of various "colubrid " venoms with Boiga venom antibodies. 8 µg venom were run in each lane. Blots were incubated with 40 µL (10 mg/mL) anti-BTS IgGs in 15 mL PBS overnight at room temperature. Blots were developed with goat anti-rabbit labeled with alkaline phosphatase and NBT/BCIP substrate.

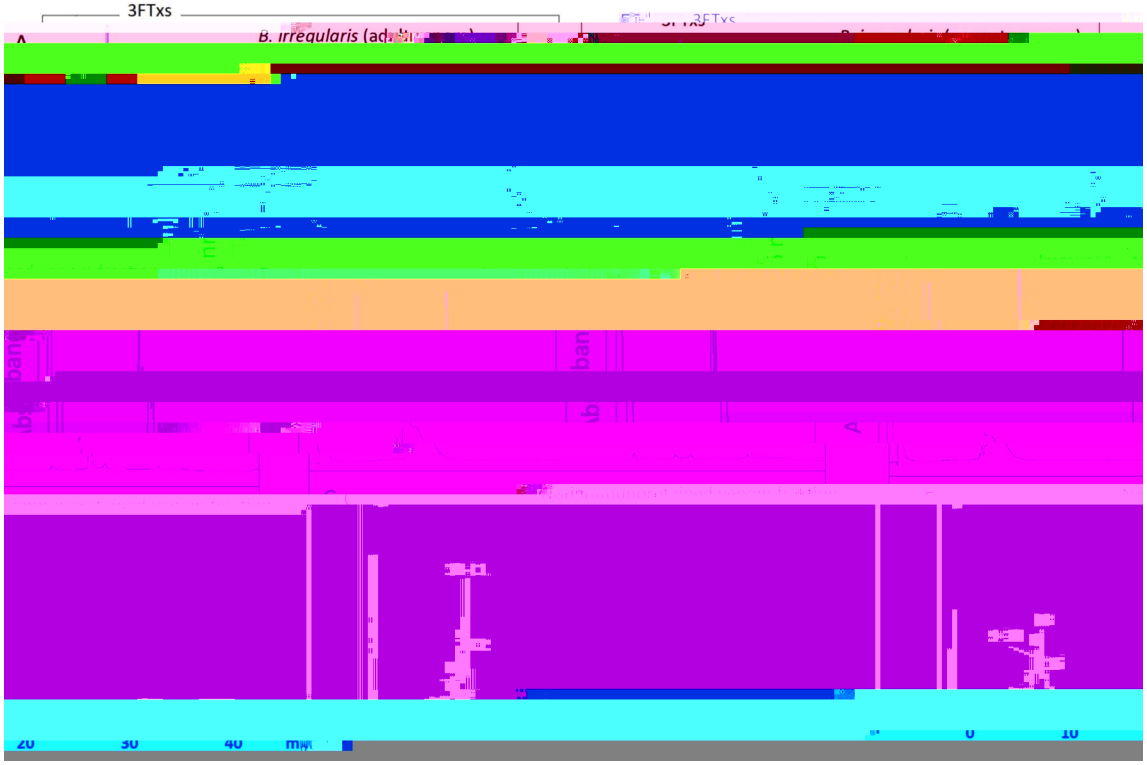

Fig. 7. Immunoa nity antivenomics analysis of adult (A –C) and neonate (D–F) venom against immobilized anti-BTS venom antibodies. Panels A and D, reference RP-HPLC separation of proteins of adult and neonate venoms, respectively. Protein classes identi ed in the di erent chromatographic fractions are highlighted. Panels B and E, and C and F display, respectively, reverse-phase separations of the immunocaptured and the non-bound column fractions recovered after incubation of 100 µg of venom with 300 µL of a nity matrix containing 4.8 mg of immobilized IgGs. Column eluates were monitored at 215 nm and quanti ed by comparing the areas of homologous peaks in the two fractions.

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