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Proteomics and Deep Sequencing Comparison of Seasonally Active Venom Glands in the Platypus Reveals Novel Venom Peptides and Distinct Expression Profiles*[S]

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The platypus is a venomous monotreme. Male platypuses possess a spur on their hind legs that is connected to glands in the pelvic region. They produce venom only during the breeding season, presumably to fight off conspecifics. We have taken advantage of this unique seasonal production of venom to compare the transcriptomes of in- and out-of-season venom glands, in conjunction with proteomic analysis, to identify previously undiscovered venom genes. Comparison of the venom glands revealed distinct gene expression profiles that are consistent with changes in venom gland morphology and venom volumes in and out of the breeding season. Venom proteins were identified through shot-gun sequenced venom proteomes of three animals using RNA-seq-derived transcripts for peptide-spectral matching. 5,157 genes were expressed in the venom glands, 1,821 genes were up-regulated in the in-season gland, and 10 proteins were identified in the venom. New classes of platypus-venom proteins identified included antimicrobials, amide oxidase, serpin protease inhibitor, proteins associated with the mammalian stress response pathway, cytokines, and other immune molecules. Five putative toxins have only been identified in platypus venom: growth differentiation factor 15, nucleobindin-2, CD55, a CXC-chemokine, and corticotropin-releasing factor-binding protein. These novel venom proteins have potential biomedical and therapeutic applications and provide insights into venom evolution. ***Molecular & Cellular Proteomics 11: 10.1074/mcp.M112.017491, 1354–1364, 2012.***

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The platypus inhabits the river systems of eastern Australia and is one of 12 extant species of venomous mammals. Male platypuses bear erectable keratinous spurs on their hind limbs that are each connected, via a duct, to a kidney-shaped venom gland. Males produce venom in significant quantities only during the breeding season (1). The venom glands increase in size during the breeding period from June to August but regress thereafter, accompanied by a loss of secretory granules and shrinkage of secretory tubules (1, 2). Thus, it is believed that platypus venom functions in mate competition and is used against male rivals (1). Envenomated humans report immediate and severe local pain; hyperalgesia (increased sensitivity to pain); fever; significant swelling that persists for up to three months, contributing to long-term loss of function of the envenomated limb; spreading pain and inflammation; cold sweats; and stomach pains (3, 4). Clinical pathology includes an increased erythrocyte sedimentation rate, which is an indication of inflammation, and increased blood pressure (4).

The availability of the platypus genome sequence and recent advances in sequencing technologies have facilitated the identification of putative platypus toxins based on sequencing of a venom-gland transcriptome (5, 6). Prior to this, the contents of platypus venom were relatively poorly studied, as the result of a lack of captive colonies for experimental research, and difficulty in obtaining venom samples. In addition, platypus envenomation is rare, and no human fatalities have been reported. Early experiments on rabbits and guinea pigs revealed extensive edema spreading from the site of venom injection, with higher doses of venom causing widespread intravascular coagulation and death (3, 7). The reported co-

agulation is consistent with anecdotal evidence that little bleeding can be seen from the puncture site after the spur is forcibly removed (4). Lowered blood pressure was also reported in experimental animals and was attributed to vasodilation. *In vitro* studies show conflicting actions; the venom caused smooth muscle relaxation in rat uterus (8) but muscle contraction in guinea pig uterus and rabbit bowel (7). Platypus venom possesses some cytolytic activity (7) and stimulates calcium-dependent current from intracellular calcium release in cultured dorsal root ganglion cells (9). The venom also has protease activity and induces mast cell histamine release that may contribute to pain, vasodilation, and edema (8, 10).

Early proteomics studies identified at least 19 classes of peptides in platypus venom (8, 10–13). Five different types of molecules were identified: hyaluronidase, C-type natriuretic peptides, nerve growth factor (OvNGF), L-to-D-amino acid-residue isomerase, and defensin-like peptides. Only two of these components have been fully sequenced: natriuretic peptides and defensin-like peptides (10, 14, 15). These polypeptides and enzymes likely work together to cause swelling, lowered blood pressure, and pain. C-type natriuretic mRNA is posttranslationally cleaved to produce peptides that form cation channels in lipid bilayer membranes (16), relax smooth muscles, release mast cell histamine (10), and cause calcium influx into neuroblastoma cells (15). The function of venom defensin-like peptides is unclear; although they have a three-dimensional structure similar to those of sea anemone sodium channel neurotoxins and antimicrobial peptides (defensins), they do not modulate sodium channel function or display antimicrobial activity (14, 12).

Previously, we used the platypus genome sequence and next-generation transcriptome sequencing to identify putative toxins in the platypus venom gland (5). However, to distinguish toxin proteins from the thousands of non-venom-related proteins in a venom gland, we had to rely on sequence similarity to known venom peptides from other species. Given the independent evolution of venom in the platypus and the evolutionary divergence of platypus and other venomous lineages, this prevented the identification of truly novel venom genes. Here, we have combined proteomic analysis on three samples of whole platypus venom with a comparison between the transcriptomes of an in-season and out-of-season venom gland to identify platypus-venom peptides and compare their expression during the breeding cycle. This approach has the advantage of being able to identify completely novel toxins that do not possess any similarity to known toxins and allows good coverage for the largest proteins. The genome assembly was used to quantify gene expression and to ensure specificity in gene identification. This is the first time such an integrated -omics approach has been used to identify venom peptide in a venomous mammal. Based on our proteomic and transcriptomic results, we have amplified and sequenced full-length cDNAs of several novel venom peptides. Herein we examine their evolution and speculate on the potential roles of these peptides in producing the known physiological effects of platypus envenomation.

EXPERIMENTAL PROCEDURES

Transcriptome Sequencing—cDNA libraries were constructed from the venom glands from different platypuses from Tasmania. One gland was collected from a platypus during the spring breeding season (October 2007; "in-season"), and the other was collected outside of the breeding season, in late summer (March 2009; "out-of-season"). The libraries were paired-end sequenced (100 nt) using the Illumina platform. FastQC was used to check the quality of the reads (17). Reads were aligned to the platypus genome (version 5.0) using the BWA aligner after trimming of low quality reads (18). The MarkDuplicate tool in Picard (http://picard.sourceforge.net) was used to filter out PCR duplicates.

Differential Expression—To obtain count data for genes, Ensembl annotated platypus genes (version 64) were used to bin reads using a custom script with an additional 500 bases at both ends of each gene to account for annotation inaccuracies at UTRs. Reads were also binned to genomic coordinates of unannotated genes of proteins identified via mass spectrometry with a 1,000 nt padding at both ends. Counts were both normalized using the TMM method and analyzed for differential expression using Bioconductor package edgeR (20).

Functional Term Enrichment Analysis—Ensembl genes were sorted based on fold change and matched to human genes using Ensembl orthology assignments (version 64). Only one human orthologue per platypus gene was retained in order to avoid enrichment due to lineage-specific gene gains or losses. Functional term enrichment analyses were performed with Ontologizer (21) using human orthologues of platypus Ensembl genes that were expressed in the venom gland. Gene ontology terms with a p value < 0.01 were visualized using Revigo (22).

Mass Spectrometry—

Venom Collection—Venom samples were collected from three different individuals (OA2, OA4, OA6) along the Murrumbidgee River, in Childowla, New South Wales (NSW), under permits from the NSW Parks and Wildlife Services. Collection methods were approved by the University of Melbourne Animal Experimentation Ethics Committees. OA2 and OA4 were caught on February 9, 2008, and AO6 was caught on November 10, 2008. Body weight and length were measured for two animals: OA2, 1.65 kg and 54 cm; OA4, 1.85 kg and 55 cm. Testis weights for the three animals were as follows: OA2—left = 15.957 g, right = 15.383 g; OA4—left (only) = 15.2 g; OA6—left = 10.45 g, right = 11.18 g. A blunt dissection was used to expose the gland and duct. A 21-gauge needle was introduced into the duct midway down the leg, and the venom was gently aspirated from the duct. Gentle pressure was placed on the gland to express any further venom from the collecting ducts and other minor connecting ducts.

Sample Preparation—Aliquots of 10 μg of milked crude venom were processed in the following manner: One aliquot was diluted to a concentration of 200 ng/μl in 1% formic acid (FA).[1] The other aliquots

[1] The abbreviations used are: FA, formic acid; ESI-QqTOF, electrospray ionization quadruple-time of flight system; RACE, ready for rapid amplificatin of cDNA ends; CRF, corticotropin-releasing factor; CRF-BP, corticotropin-releasing factor-binding protein; GDF15, differentiation factor 15; TGFB, transforming growth factor β; CD55, complement decay-accelerating factor; CRISP, cysteine-rich secretory protein; WAP, whey acidic protein; OvNGF, nerve growth factor; *PGLYRP1*, peptidoglycan recognition protein-1; *SERPINA4*, serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 4; *SERPINA11*, serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 11.

were reduced and alkylated in solution as described elsewhere (23). One sample was dried down using a speed-vac and reconstituted to 200 ng/μl in 1% FA, and the other was digested using trypsin spin columns (Sigma, St. Louis, MO) using the manufacturer's protocol. This latter sample was eluted, diluted to 400 ng/μl, and acidified by 1:1 dilution with 2% FA.

LC-MS/MS—Liquid chromatography and mass spectrometry were performed on an electrospray ionization quadruple-time of flight system (ESI-QqTOF) (AB SCIEX Triple-TOF 5600 system) coupled with a Prominence nanoHPLC system (Shimadzu, Tokyo, Japan). Separation was performed using a Grace-Vydac 150 X 0.075 mm, 300A pore Everest C18 column at a flow rate of 800 nl/min. 1.5 μl of each sample was injected and eluted using a gradient of 1–50% solvent B (90% acetonitrile, 0.1% FA) over 50 min with a spectral acquisition speed of 20 MS/MS per second. ProteinPilot 4.0 (AB SCIEX, Framingham, MA) was used to analyze files using the Paragon Algorithm 4.0.0.0 search method against the Ensembl platypus genebuild (version 64) and an Illumina platypus venom-gland transcriptomic database assembled using Abyss (24) (k = 30) from raw data (5) and translated into 6-frames. Reads containing stop codons were removed. The analysis parameters were set as follows: only trypsin specificity was considered; one missed cleavage was permitted; the search was based on fixed modification of cysteine alkylation by iodoethanol; default settings for variable protein modifications were used; mass tolerance for precursor ions was 0.05 Da $\pm$ 0.0011 Da, and mass tolerance for fragment ions was 0.1 Da $\pm$ 0.01 Da. A stringent detected protein threshold score of 5% false discovery rate (FDR) calculated by decoy searches was used. We also kept proteins with FDR values between 5% and 10% if they had a confidence level above 95% as determined by ProteinPilot. Because of the fragmentation of proteins in the Illumina database, we also considered putative proteins that fell below this cut-off but for which more than one peptide had mapped through BLAST to the same human gene. These database sequences were likely to be partial fragments that could not be fully assembled because of low transcriptomic coverage. We confirmed that these peptides derived from the same protein using cDNA extraction. Matches to platypus keratin were ignored.

cDNA Extraction—RNA was extracted from platypus venom gland tissue using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and treated with Amplification grade DNase I (Sigma-Aldrich, St. Louis, MO) to remove residual DNA. RNA was transformed into cDNA ready for rapid amplification of cDNA ends (RACE) using the Generacer kit with Superscript III RT (Invitrogen, Carlsbad, CA). RNA was dephosphorylated and decapped, and RACE oligo was ligated to the 5′ end. RNA was transcribed into cDNA using Superscript III RT using a 3′ RACE oligo to prime the reaction. Gene specific primers were designed from predicted sequences using Oligo 6 (Molecular Biology Insights, Cascade, CO). Primers were ordered from Sigma-Aldrich. RACE PCRs were performed in 50 μl reactions containing 50 ng RACE ready cDNA, 1x High Fidelity PCR Buffer, 10 pm gene specific primer, 30 pm Generacer primer (Invitrogen), 20 nm $MgSO_4$, 2.5 units Platinum TaqDNA Polymerase High Fidelity (Invitrogen), and 10 nm dNTP. PCR cycling parameters were as follows: samples were heated at 94 °C for 2 min, followed by 5 cycles of 94 °C for 30 s and 72 °C for 1 min, followed by 5 cycles at 94 °C for 30 s and 70 °C for 1 min, followed by a further 30 cycles of 94 °C for 30 s, 64 °C for 30 s, and 68 °C for 1 min, with a final extension step at 68 °C for 10 min. 25 μl of PCR product were run on a 2% agarose gel at 90 V. Bands were cut out and the DNA was extracted using the QIAquick Gel Extraction Kit (Qiagen). For samples that did not generate a clear band at the predicted length, nested PCR was performed. The PCR reaction was the same as for the previous RACE PCRs, except that nested RACE primers and nested gene specific primer were used, and 1 μl of PCR product was used instead of RACE ready cDNA. The nested PCR cycling parameters were as follows: 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 65 °C for 30 s, and 68 °C for 2 min, with a final extension at 68 °C for 10 min. Nested RACE PCR products were run on agarose gel and the DNA was extracted as described above. Extracted DNA was cleaned using ExoSAP-IT digestion (Affymetrix). Samples were sent to the Australian Genome Research Facility for direct sequencing. If samples could not be directly sequenced, they were cloned and sequenced by TOPO TA cloning (Invitrogen), following the manufacturer's specifications. Plasmid DNA was isolated using the Qiaprep Spin Miniprep Kit (Qiagen). Purified plasmid DNA was sequenced.

RESULTS

Fragment ion spectra from mass spectrometry of trypsin-digested whole venom were matched to *de novo* assembled Illumina sequence data from a normalized platypus venom cDNA library (5) (Fig. 1). The assembly contained 87,343 transcripts with an N50 size of 295 nt. High-scoring transcripts were annotated by searching against a public protein database. Full open reading frames of venom genes were amplified from venom-gland cDNA. We then used RNA-seq to compare the expression of venom transcripts in venom glands in and out of breeding season (in-season and out-of-season, respectively) obtained from two opportunistically acquired animals from Tasmania (Fig. 1).

We performed shotgun proteomics analysis on independent venom samples from three platypuses collected from the Murrumbidgee River (Childowla, NSW) in September and October of 2008. The proteomes of the venom samples from the three animals were similar. A large subset of peptides was routinely detected in venom from different individuals, suggesting that the platypus venom proteome is highly similar among individuals within a population. Up to 20 transcripts were identified from each venom sample (Table I). Many of these transcripts were found in all of the samples tested, but levels fell below our conservative score cut-off in some cases. Because of the fragmentation of the *de novo* assembly, some transcripts were too short to be confidently annotated, and many transcripts were derived from the same protein. All putative toxins were identified using the transcriptomic spectra-matching database. A list of all peptide sequences assigned, including precursor charge and mass/charge, a list of all modifications observed, and scores are in supplemental File 2. All spectra and search results can be found in supplemental Files 3–8 and can be viewed using ProteinPilot.

The in- and out-of-season venom gland libraries were sequenced on an Illumina GAIIx producing 56,614,944 and 63,158,862 reads, respectively (accession: SRP003465). 5,157 platypus Ensembl genes were expressed. After trimming of low quality sequences, the average read length was 41 nt (see supplemental Table S1 for mapping statistics). We did not have biological replicates, but assumed Poisson variation of count data for differential expression analysis using the Bioconductor package edgeR (20). We identified 2,587 genes that were up-regulated in the in-season gland and 1,821 genes that were up-regulated in the out-of-season

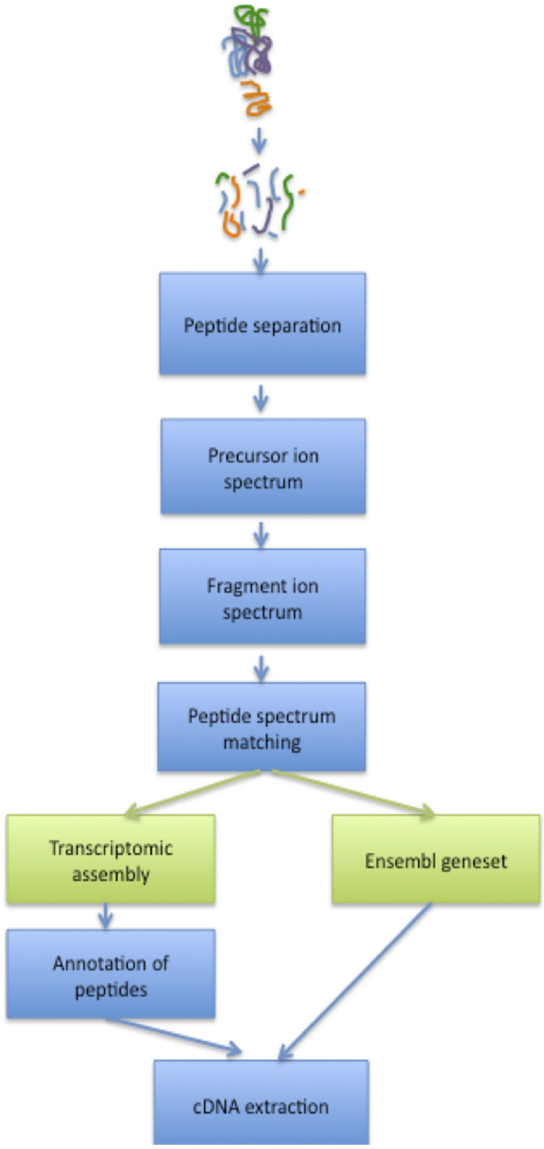

FIG. 1. **Overview of methods used for gene identification.** Whole venom was digested and was separated via liquid chromatography. Eluted peptides were subjected to tandem mass spectrometry. Fragment ions were assigned to protein sequences derived from two sources: an assembled database of transcripts derived from a normalized cDNA library of a venom gland, and a database that contained mostly predicted proteins based on the genome assembly. Annotation of high-scoring transcripts was performed by searching against the UniProt database and matching sequence data back to the platypus genome using BLAT on the UCSC genome browser. Full cDNA sequences were amplified using molecular strategies, and venom genes were checked for differential expression between breeding cycles.

gland (FDR $<$ 0.01). Fold change between in- and out-of-season counts and proteomics data were used to infer the regulation of toxin genes during the breeding cycle (supplemental Table S2 and supplemental Fig. S1). Enriched functional terms from the top 200 genes showing the highest fold change in the in-season and out-of-season glands are visualized in Figs. 2*A* and 2*B*, respectively. These terms have been derived from 3,667 human orthologues of platypus genes expressed in the venom glands.

TABLE I

The number of transcripts, the number of distinct peptides, and number of spectra corresponding to each venom sample following LC-MS/MS (detected protein threshold score of 1.3 (95% confidence))

	Number of identified database sequences (from assembled transcriptome database)	Number of distinct peptides identified	Number of identified spectra
Sample 1	5	29	195
Sample 2	20	64	335
Sample 3	12	80	374

We identified 10 venom proteins: two enzymes and eight other polypeptides (Table II; Genbank accessions: JQ350810, JQ350811, JQ350812, JQ350813, JQ350814, JQ350815, JQ350816, JQ350817, JQ350818, JQ350819, JQ350820). Five of these have not been identified in the venom of any other species. We also identified nerve growth factor, C-type natriuretic peptides, and venom defensin-like peptides, which had been characterized previously. As is expected of secreted venom proteins, signal peptides were identified in all sequences. We note that mass spectral analysis of a fraction of protein spots from a two-dimensional gel of the venom confirmed our findings and did not result in additional protein information supporting the comprehensiveness of our shotgun strategy for canvassing the venom proteome (data not shown). Comparisons between in- and out-of-season venom glands showed a pattern of marked up-regulation of venom proteins during the breeding season (supplemental Table S2). Six of the ten proteins—serpin, chemokine, hyaluronidase, amide oxidase, GDF15, and CRFBP—were among the top 200 most highly up-regulated genes during the breeding season. One protein (cysteine-rich secretory protein) was not significantly up- or down-regulated between the glands. We provide details of the identified proteins below and speculate upon their role in venom.

Enzymes—We have sequenced the mRNA of hyaluronidase and amide oxidase in platypus venom. Both these enzymes are also found in snake venoms. Hyaluronidase and amide oxidase are not produced in the ancestral glands of platypuses or snakes. Therefore, independent changes to the regulatory mechanisms of these enzymes have evolved independently in each lineage.

Hyaluronidase has been identified in the venoms of vertebrates, arthropods, and mollusks (26). Platypus hyaluronidase, a 140 kDa disulfide-linked trimeric protein, was first detected in platypus venom by de Plater *et al.* (8). The breakdown of hyaluronic acid, widely distributed in connective, epithelial, and neural tissues, has been postulated to play a role in accelerating the spread of toxins and hemostatic factors, thereby potentiat-

FIG. 2. **Visualization of gene ontology (GO) terms of the top 200 genes based on fold change of genes.** *A*, in-season gland. Enriched GO terms of $p < 0.01$ were used to construct the map, and similar functional terms are summarized using the same color. The size of the box corresponds to the number of terms. *B*, out-of-season gland. Enriched GO terms of $p < 0.01$ were used to construct the map, and similar functional terms are summarized using the same color. The size of the box corresponds to the number of terms.

ing their noxious actions (26). As expected, the expression of venom-gland hyaluronidase expression was five-fold up-regulated during the breeding season.

Amine oxidase, a nonhydrolytic protein, was identified in the venoms of all three animals. The gene is located on contig7479 in the genome assembly. Platypus amine oxidase

TABLE II
Information on identified venom genes

Name (accession)	Ensembl ID	Period in which higher expression was observed	Differentially expressed at adjusted $p < 0.01$	Identified in proteome?	Signal peptide present?
Hyaluonidase (JQ350818)	ENSOANG00000000407	In-season	Yes	Yes	Yes
Amide oxidase (JQ350817)	ENSOANG00000005712	In-season	Yes	Yes	Yes
Whey acidic protein (ABL67638)	ENSOANG00000012145	Out-of-season	Yes	Yes	Yes
Peptidoglycan recognition protein-1 (JQ350813)	—	—	—	Yes	Yes
Serpin (JQ350816)	—	In-season	Yes	Yes	Yes
Kunitz-domain containing serine protease inhibitor (JQ350815)	—	—	—	Yes	Yes
Corticotropin-releasing factor-binding protein (JQ350810)	—	In-season	Yes	Yes	Yes
Nucleobindin (JQ350819)	ENSOANG00000007683	In-season	Yes	Yes	Yes
Differentiation factor-15 (JQ350812)	ENSOANG00000001923	In-season	Yes	Yes	Yes
Complement decay-acceleration factor (JQ350811)	ENSOANG00000020509 (partial)	In-season	Yes	Yes	Yes
CXC-chemokine (JQ350820)	ENSOANG00000006911	In-season	Yes	Yes	Yes
Cysteine-rich secretory protein (JQ350814)	—	In-season	No	Yes	Yes

Genes without Ensembl annotation are denoted by "—." The Kunitz-domain protein was not identified in the genome, and thus expression information is not available (denoted by "—"). Peptidoglycan recognition protein-1 was partially identified in the genome, which might account for its low counts (less than 20). As a result of the low values, its expression was not analyzed and is also denoted by "—."

is more similar to copper-containing amine oxidase than the flavin-containing L-amino acid oxidases found in snake venoms (27). Copper-containing amine oxidases degrade primary amines such as histidine to aldehydes, releasing ammonia and hydrogen peroxide. In viperid, crotalid, and elapid snakes, amide oxidases have high enzymatic activities that are believed to contribute to toxicity upon envenomation through the production of hydrogen peroxide (27). They have been shown to induce human platelet aggregation (28), assist in hemorrhage (27) and apoptosis (29), and cause edema and hemolysis (30). We predict that the platypus protein would cause similar symptoms. Consistent with a toxin role, expression of amine oxidase was 21-fold up-regulated in the breeding-season venom gland.

Polypeptides—

Antimicrobials—

Peptidoglycan Recognition Protein-1—A putative antibacterial peptidoglycan recognition protein-1 peptide was found to be expressed in all three platypus venoms studied. This is the first time a member of the peptidoglycan family has been identified in vertebrate venom. Platypus peptidoglycan recognition protein-1 (*PGLYRP1*) is partially located on contig17172, with the 5′ end of the gene not detected in the genome assembly. Given the short length of the sequence that aligned to the genome (~1 kb), and also perhaps because of comparatively low expression in the venom gland, we were unable to detect changes in the level of expression of the gene in and out of the breeding season. *PGLYRP1* is a leukocyte-secreted disulfide-linked pattern receptor that hydrolyzes bacterial cell wall peptidoglycan and exerts direct bactericidal activity against both Gram-positive and Gram-negative bacteria. *PGLYRP1* expression has also been detected in another modified sweat gland, the mammary gland, suggesting *PGLYRP1* was a component of the common ancestral sweat gland (31). Peptidoglycan recognition family proteins are known to play a role in wasp venoms. However, it is likely that the wasp and platypus proteins target different pathways and exert distinct physiological effects as insect PGYLRPs are not bactericidal-it is believed they are used to evade the host immune response through suppression of the phenoloxidase cascade, a major insect host defense pathway.

WAP—Notably, a WAP peptide, WFDC2 (*WAP* four-disulfide core domain 2), identical to a WAP peptide expressed in the platypus lactating mammary gland (EMBL:ABL67638) (32) was identified in the venom using the Ensembl database. WAP-domain-containing peptides have diverse functions, including protease inhibitory and antimicrobial activities (33). Two snake venom WAP peptides, nawaprin and omwaprin, have been isolated (34, 35). The biological function of nawaprin is unknown, but omwaprin has been found to exhibit antimicrobial activity against Gram-positive bacteria with no protease inhibitory activity or *in vivo* toxicity in mice (34). The convergent evolution of WAP domain peptides in the venom of platypuses and snakes suggests that the platypus peptide might perform a toxin-related role, but its biological significance is unclear. Antimicrobial peptides have been identified in both digestive glands and sweat glands, which gave rise to the venom glands of snakes and platypuses, respectively (1, 37, 38, 25). Thus, the platypus and snake WAP-domain venom peptides might be remnants of the respective venom glands' ancestral functions, and might also function in protecting the venom glands themselves. Consistent with this, the gene was five times as highly expressed in the out-of-season venom gland.

Protease Inhibitors—

Serpin—We amplified a kallistatin-like venom mRNA using a primer designed from a 31-residue fragment identified using mass spectrometry. The 5′ end of the gene aligned to the middle of the scaffold Ultra378 (1,065,020–1,065,532) in the genome assembly, whereas the 3′ end aligned to contig14450 in the genome assembly, indicative of misassembly of this region. The gene was one of the most highly up-regulated genes during the breeding season (7,642-fold), suggesting a key role in venom function. The predicted peptides are most similar to therian serine protease inhibitors *SERPINA4* and *SERPINA11*. Serine protease inhibitors are found in the venoms of snakes, where they play key roles in blood coagulation (39, 40). They have also been identified in sea anemone (41) and wasp (42) venoms. The platypus serpin does not belong to the Kunitz family of serine protease inhibitors found in snake and anemone venoms, but instead is a member of MEROPS inhibitor family 14, clan ID. These proteins function in proteolytic cascades, including blood clotting and inflammation (43), and cause irreversible "suicide inhibition" when they bind to their protease substrate (44).

The function of platypus serpin is unclear. Its apparent homology to eutherian *SERPINA4* and *SERPINA11* suggests that it might induce hypertension via the inhibition of kallikreins that cause blood vessel dilation (45). As such, the protein might contribute to increased blood pressure following envenomation (4). Serpins share sequence homology with snake neurotoxins that modulate ion channel function (40, 46), but we do not know whether the platypus serpin has this activity. The irreversible termination of proteolytic function attributed to *SERPINA4* suggests that this platypus serpin is unlikely to function in a protective capacity in the venom gland to allow storage of the protease component of venom, as suggested for other types of protease inhibitors (5).

Kunitz-domain-containing Serine Protease Inhibitor—We have identified a Kunitz-type protease inhibitor in platypus venom that does not align to the genome assembly, suggesting that it was not sequenced in the genome. Other Kunitz-type protease inhibitors were previously identified in the platypus venom gland (5). The ancestral function of these proteins is the inhibition of serine proteases, such as those involved in hemostasis, but some venom Kunitz-domain proteins have evolved ion-channel blocking activity (41, 47, 48). These toxin proteins are well documented in snakes but are also found in the venoms of spiders, cone snails, and sea anemones (41, 47, 48).

Stress Response—

Corticotropin-releasing Factor-binding Protein—Corticotropin-releasing factor-binding protein (CRF-BP) competitively inhibits corticotropin-releasing factor (CRF), a neuropeptide secreted in response to stress (49). The gene was up-regulated 44-fold in the in-season venom gland, consistent with a toxin role. CRF-BP expression in mammals has been linked to changes in psychological and behavioral states. Exposure of rats to a predator increases CRF-BP expression in the amygdala, which correlates with behavioral inhibition and submissive posturing (50). Platypuses are purported to use venom during intraspecific competition for females, and as such, increased submissive behavior in the spurred individual might increase the reproductive success of the spurrer. CRF-BP can also trigger synaptic transmission when coupled with CRF in mice, suggesting that it might also function as a neurotoxin (51). Furthermore, CRF-BP might have a proinflammatory effect by limiting CRF. CRF stimulates the release of anti-inflammatory glucocorticoids through adrenocorticotropic hormone secretion. Indeed, administration of corticosteroids to patients following platypus envenomation has led to decreased pain and swelling (4).

Nucleobindin—Platypus nucleobindin 2 (ENSOANP00000012244) is the first nucleobindin molecule identified in any animal venom. Nucleobindin 2 is the precursor of a secreted calcium-binding protein, nesfatin-1, that influences the excitability of neurons in a part of the brain associated with stress and physiological changes (52). It stimulates autonomic nervous system activity, increases blood pressure (53), and has been associated with a range of brain functions, including the regulation of feeding. Nucleobindin induces fear and anxiety in rats (54). Nesfatin-1 also elevates the intracellular concentration of Ca^{2+} in mouse neurons and causes calcium signaling via calcium influx through $Ca_V2.2$ calcium channels (55). Although platypus venom also elicits calcium signaling, this is believed to be due to the release of calcium from intracellular stores rather than the activation of calcium channels on the plasma membrane (9). Nesfatin-1 and CRF-BP might act synergistically. Nesfatin-1 activates CRF-responsive neurons in rats (52). Induction of CRF expression by venom nesfatin-1 in envenomated animals could result in the downstream interaction of CRF and venom CRF-BP. Thus, nucleobindin 2/nesfatin-1 might function to potentiate the effects of CRF-BP. As expected, nucleobindin-2 was up-regulated six-fold during the breeding season.

Other Venom Proteins—

Differentiation Factor 15—Differentiation factor 15 (GDF15) (ENSOANG00000001923) was identified in the venom proteome and up-regulated 48-fold during the breeding season. This was the first time GDF15 had been identified in venom. The platypus peptide contains the seven conserved cysteine residues in the C-terminal region that are required for the formation of a cysteine knot (56). GDF15 is a growth differentiation factor that is a divergent member of the transforming growth factor β (TGFB) superfamily of immune molecules. GDF15 is a regulator of the inflammatory response and is required for apoptosis (57); it is triggered in response to injury and stress conditions in humans and mice (56).

Platypus venom triggers nociceptor excitation via the activation of a serine or tyrosine kinase (9). Because GDF15, like other TGFB superfamily ligands, binds to and activates a serine/threonine receptor kinase as a first step in the signaling

cascade (58), we suggest that GD15 might function in platypus venom to cause pain and hyperalgesia. Based on accounts of spreading pain in envenomated patients (4), it is possible that the protein might exert both systemic and local effects on nerve cells at the site of envenomation.

CD55—Complement decay-accelerating factor (CD55) was sequenced from venom-gland cDNA based on the identification of the peptide in venom. CD55, along with CD59 (protectin), is a potent inhibitor of complement-mediated lysis in innate immunity. This was the first time CD55 was identified in the venom of any species, and its putative function is uncertain. Interestingly, CD59, a protein with an analogous function, is structurally related to snake venom neurotoxins, suggesting that toxin CD55 might have a similar neurotoxic role in the platypus (59). Consistent with this, CD55 was ~0.3-fold up-regulated during the breeding season.

CRISP—A cysteine-rich secretory protein (CRISP) peptide, corresponding to a gene that was previously identified in the venom-gland transcriptome (5), was also identified and sequenced. CRISPs contain an SCP domain (an extracellular domain also found in plant defense and mammalian testis-specific proteins) and a C-terminal cysteine-rich region. CRISPs are found in snake and reptile venoms, where they act as ion channel neurotoxins (60) or myotoxins (61); and in cone snail venoms, where they have proteolytic activity (62). They are also found in the venoms of hymenopterans, arachnids, and cephalopods and in the feeding secretions of various hematophagous taxa (26, 63). Thus, platypus CRISP might play a role in producing the symptoms of muscle atrophy seen in envenomated patients through toxic action on muscle and nerve tissues (5). It might also form the protease component of platypus venom (8). Platypus CRISP shared the highest sequence similarity (75%, based upon an alignment using the BLOSUM62 scoring matrix) with an Anguimorpha lizard venom CRISP (*Gerrhonotus infernalis*), and it is also similar to helothermine, a toxin from the Mexican beaded lizard that blocks ryanodine receptors, intracellular calcium channels found in some excitable tissues (64). Yet, despite its sequence similarity to known toxins, CRISP is expressed at similar levels in both in- and out-of-season venom glands (0.1-fold difference). It is possible that the protein possesses both toxic and nontoxic roles in venom. Nontoxic functions of eutherian CRISP proteins include antimicrobial activity, sperm maturation, and gamete fusion.

Chemokine—A CXC chemokine was identified in the venom proteome (ENSOANG00000006911) and was three-fold up-regulated during the breeding season. Chemokines in this group are chemotactic, mediate cell growth, and trigger an inflammatory response. The platypus chemokine is a lineage-specific gene duplicate (122 residues) that is most similar to mammalian macrophage inflammatory protein-2. Cytokines have important roles in the regulation of the immune system, and platypus venom chemokine might serve to disrupt immune homeostasis in envenomated animals.

DISCUSSION

Using an integrated genomic, transcriptomic, and proteomic approach, we have uncovered ten putative toxins in the venom of the platypus. The use of this -omics approach has the benefit of detecting toxins that share little or no homology with known toxins. Accordingly, five of the venom proteins we identified are novel and had not been found in any other animal venom prior to this work. It is important to note that the sensitivity of protein identification is affected by protein stability, ionization biases, and abundance (65), and is also dependent on the sequence coverage of the transcriptome. However, we note that the transcriptomic database used for spectral searching was comprehensive enough to contain 1.33 Gb that matched over 7,600 total platypus Ensembl genes at 20 counts and above. In addition, we also used the Ensembl platypus genebuild, which contains 22,369 genes for peptide-spectra identification. Of course, a venom-gland transcriptome sequenced at higher depth will produce longer contigs that will lead to greater sensitivity in peptide-spectral matching with better resolution of less abundant proteins and alternative isoforms. The availability of a sequenced genome allows for resolution between alleles, gene duplicates, and alternative transcripts, which can be difficult to distinguish because of similarities in the sequences of venom transcripts.

Despite being independently derived in each lineage, the venom of the platypus is most similar to those of snakes and other reptiles in that it contains amide oxidase, WAP, protease inhibitors, and CRISP. The similarities might reflect the two taxa's more recent common ancestry compared with other well-studied venomous taxa.

A large number of immune-related genes were identified in platypus venom, including *CD55*, a chemokine, *PGLYRP1*, and *GDF15*. Immune genes might be suitable candidates for venom activity because of their role in coordinating fast-acting local and systemic responses. Genes that function in rapid physiological processes are likely to be co-opted to venom function (26). Their rapid rate of gene evolution might also render them favorable for co-option to novel functions (neofunctionalization) (6). It is notable that one class of antimicrobials, the β-defensins, gave rise to a major component of platypus venom, the venom defensin-like peptides (66).

CRISP and *WAP* were not up-regulated during the mating season. Our results indicate that *CRISP* is constitutively expressed throughout the year, with *WAP* being up-regulated outside of the mating season. This suggests that these genes might not function as toxins and instead might play a role in up-keep and protection of the venom gland. It is also possible that some genes—particularly *CRISP*, given its similarity to lizard toxins—possess both toxin and nontoxin roles. It is also worth noting that all other venom genes were also expressed outside of the breeding season, albeit at much lower levels, suggesting that venom production is not completely abol-

ished but is suppressed during nonbreeding periods. Alternatively, these genes might function in other capacities outside of the breeding season. Large numbers of leukocytes infiltrate the stroma, secretory epithelium, and gland lumena during gland regression (1), and this might lead to an increase of immune gene expression in the out-of-season transcriptome that is unrelated to toxin production.

To our knowledge, the platypus is the only venomous animal that seasonally produces venom. Gene expression comparisons between venom glands obtained in and out of the breeding season offer a unique opportunity to inform us of venom gene regulation, providing insights into the putative functions of these genes. Although the large number of genes significantly up-regulated in the in-season venom gland (2,587) was likely due in part to the lack of biological replicates and subsequent underestimation of biological variation, our results suggest that venom gland gene expression changes dramatically during the breeding cycle. This corresponds with increases in gland weight and venom volumes and correlates directly with increases in the weight and activity of the testis and in the size of the androgen-producing Leydig cells in the testis (1). Up-regulated nonvenom genes are enriched in genes involved in cellular processes required for venom synthesis, such as the regulation of gene expression and RNA splicing (Fig. 2*A*). Similarly, a large number of genes (1,821) are up-regulated out of the breeding season. It is interesting to consider the activity of the crural gland at this time; we noted an abundance of genes functioning in cytoskeleton organization and protein secretion regulation (Fig. 2*B*). This might point to differences in the abundance of cell populations during the venom cycles. Indeed, secretory epithelial cells have been reported to be lost in the venom gland after the mating season, suggestive of seasonal changes in cell types (2). Care should be taken when interpreting the result of the gene enrichment analyses because of the lack of biological replicates, and the use of human functional annotations to infer relationships. Biological replicates of venom glands would likely reduce the number of differentially expressed genes, producing more robust results. However, it is difficult to obtain samples, given the lack of a breeding colony for research purposes, and the two venom glands sequenced in this study were obtained opportunistically from animals found dead. It should also be noted that protein synthesis might not always correlate with gene expression.

This is the first time, to our knowledge, that deep sequencing of the venom gland transcriptome has been used in combination to characterize the venom components from any venomous species. This rapid, genome-wide approach can be applied to any venomous species to identify venom peptides, even in species for which a genome assembly is not available. Given full-length transcripts, it will be possible to compare transcriptomic abundance and protein abundance using informatics strategies such as spectral counting (65).

Future functional studies will allow us to understand the role of the identified venom proteins in the context of toxicity, which will shed light on their contribution to the observed symptoms of platypus envenomation. Animal venoms are a valuable source of novel drugs (67). Our findings might pave the way for future therapeutics and biomedical studies.

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Author contributions: E.S.W.W. analyzed the datasets and wrote the manuscript. D.M. performed the proteomics experiments under the supervision of G.K. E.M. performed transcriptomic analysis under the supervision of A.T.P. S.G. and K.M. performed cDNA extractions of full-length genes. M.B.R. and P.T.S. collected the venom samples. C.W. constructed the cDNA libraries for transcriptomic sequencing. W.W. sequenced the transcriptomes. E.S.W.W., D.M., C. W., A.T.P., and K.B. contributed to the design of the experiment. K.B. conceived of the project. All authors revised the final manuscript.

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