Not so Dangerous After All? Venom Composition and Potency of the Pholcid (Daddy Long-Leg) Spider *Physocyclus mexicanus*

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**Abstract**

Pholcid spiders (Araneae: Pholcidae), officially “cellar spiders” but popularly known as “daddy long-legs,” are renowned for the potential of deadly toxic venom, even though venom composition and potency has never formally been studied. Here we detail the venom composition of male *Physocyclus mexicanus* using proteomic analyses and venom-gland transcriptomes (“venomics”). We also analyze the venom’s potency on insects, and assemble available evidence regarding mammalian toxicity. The majority of the venom (51% of tryptic polypeptides and 62% of unique tryptic peptides) consists of proteins homologous to known venom toxins including enzymes (astacin metalloproteases, serine proteases and metalloendopeptidases, particularly neprilysins) and venom peptide neurotoxins. We identify 17 new groups of peptides (U₁–U₁₇-PHTX) most of which are homologs of known venom peptides and are predicted to have an inhibitor cysteine knot fold; of these, 13 are confirmed in the proteome. Neprilysins (M13 peptidases), and astacins (M12 peptidases) are the most abundant venom proteins, respectively representing 15 and 11% of the individual proteins and 32 and 20% of the tryptic peptides detected in crude venom.
evidence suggests that the neprilysin gene family is expressed in venoms across a range of spider taxa, but has undergone an expansion in the venoms of pholcids and may play a central functional role in these spiders. Bioassays of crude venoms on crickets resulted in an effective paralytic dose of 3.9 µg/g, which is comparable to that of crude venoms of *Plectreurys tristis* and other Synspermiata taxa. However, crickets exhibit flaccid paralysis and regions of darkening that are not observed after *P. tristis* envenomation. Documented bites on humans make clear that while these spiders can bite, the typical result is a mild sting with no long-lasting effects. Together, the evidence we present indicates pholcid venoms are a source of interesting new peptides and proteins, and effects of bites on humans and other mammals are inconsequential.

**Keywords**
neprilysin; astacin metalloproteinase; insecticidal neurotoxin; proteome; PD$_{50}$

**INTRODUCTION**

Pholcid spiders (Araneae: Pholcidae) are an example of disconnect between public perception, and scientific understanding. Commonly called “cellar spiders” some pholcids are also referred to as “daddy long-legs” and have been maligned in popular culture as having venoms with the highest mammalian potency of all spiders, that would be lethal if they only had fangs that could penetrate human skin. Pholcids, in reality, are a family of spiders that has been diversifying for over 200 million years (Dimitrov et al., 2012; Eberle et al., 2018) and are among the top six percent of the most diverse spider families (1,714 species in 94 genera, World Spider Catalog, February 2019). Reports of high mammalian potency emerge from rumor and non-scientific anecdote. We are not aware of any scientific work to date that has attempted to characterize venom composition of any pholcid species, and given their evolutionary depth and diversity, they provide remarkable opportunity to discover new toxins and lend insight into the broader patterns of venom diversity within spider venoms, while directly investigating a widespread urban myth.

Spiders of the family Pholcidae are interesting animals in their own right. Found on all continents except Antarctica, they range in size from minute to ~1 cm in adult body length. They tend to have long, thin (hair-like) legs that are readily dropped defensively and twitch to deter predators after being removed from the body (e.g., Johnson and Jakob, 1999). As generalist foragers of arthropods, they build tangly, non-adhesive webs in protected places, and diversification within the family has involved extensive diversification of web microhabitats (Eberle et al., 2018). In prey capture, they either wait for prey to encounter their web, or some species leave their web and enter webs of other spiders and capture them and/or consume their egg sacs or prey (Jackson and Brassington, 1987). When pholcids encounter prey, they immobilize them by first wrapping with silk, and then biting to envenomate and eventually feed (Jackson and Brassington, 1987). Many common pholcids readily feed on other spiders, including black widows (*Latrodectus*) (pers. obs.) that have highly potent venoms on both arthropods and vertebrates (Wang et al., 2007). Given that spiders are dangerous prey, this observation inspires the question of how potent pholcid venoms are on arthropods. While Kirchner and Opderbeck (1990) provide data suggesting
venoms of *Pholcus phalangioides* only weakly affect insect prey that are ultimately killed by digestive enzymes, we have much to learn about the details of pholcid venom composition and how the venoms affect prey. Although small, their fangs can certainly penetrate the cuticle of many insects.

Beyond insects, the fangs of adult male *Physocyclus mexicanus* are able to pierce uncalloused human skin (Kristensen, pers obs). In fact, the only recorded human bites we are aware of that are publically available are from Mythbusters (Episode 13, premiered 15 Feb 2004), in which male *P. mexicanus* bit from aggression resulting in a brief mild sting ([https://www.discovery.com/tv-shows/mythbusters/videos/daddy-longlegs-minimyth](https://www.discovery.com/tv-shows/mythbusters/videos/daddy-longlegs-minimyth)). The same episode also reports the only direct tests of mammalian toxicity of daddy-long leg venom with injections of male *P. mexicanus* venom into mice. In comparative injections of the same volume (one µl, ∼80 µg, Kristensen pers obs), venom from Western black widows *Latrodectus hesperus*, were more potent than *P. mexicanus*. Despite the fact that obtaining one µl required venom extraction from 50–100 adult males (Kristensen pers obs), the Mythbuster assays suggest that much larger amounts of venom would be required to obtain an LD$_{50}$ for *P. mexicanus* venom on mice. Together, these first reports suggest bites from this species are not harmful to mammals. We expand on these observations in our discussion.

Shifting focus from potential risks to humans, venoms of pholcids and all spiders are exciting phenotypes because their rich chemical diversity contains a natural storehouse within which we can discover new molecular activities, and study patterns of diversity and mechanisms of evolution that have generated these phenotypes (e.g., Escoubas, 2006; King and Hardy, 2013). Venoms contain a complex mixture of bioactive components—proteins, peptides, and small molecules—that vary widely across the over 48,000 described species of spiders (Pineda et al., 2018; World Spider Catalog., 2019). A growing body of research characterizing venoms from diverse representatives of spiders is allowing inference of the phylogenetic distribution of venom components. Pholcids are in a higher lineage of spiders called Synspermiata (Michalik and Ramirez, 2014), and more specifically in the “lost trachea clade” (Wheeler et al., 2016). Within the last few years, select venoms in this clade have been characterized allowing inference of the presence and distribution of gene families that are expressed in venom. Venomic sampling to date includes venoms of *Plectreurys tristis* (Zobel-Thropp et al., 2014a), *Scytodes thoracica* (Zobel-Thropp et al., 2014b), and venoms within the family Sicariidae that includes brown recluse spiders (review in Chaves-Moreira et al., 2017). Adding a representative pholcid venom to this comparative set allows inference about the phylogenetic distribution and relative abundance of widespread gene families that contribute to spider venoms.

The goal of this study was to use transcriptomic and proteomic methods to characterize venom proteins of the North American pholcid, *Physocyclus mexicanus*. Our sampling includes venom from adult males, and transcriptomes with mRNAs from adult males and females sampled from the same population used in the Mythbusters episode. To our knowledge, this investigation is the first proteomic characterization of pholcid venom. We consider venom composition of this species in the context of what is known about venoms of other Synspermiata spiders, and discuss evidence for abundant and diverse astacins,
comparable to their near relative *Plectreurys tristis*, and an expanded role of nephrilysin homologs and diverse peptides with and without homology to known toxins. To better understand venom effects on natural prey, we use bioassays to evaluate potency on crickets. We also summarize information available on the propensity of these animals to bite humans and the effects of venom on mammals. Together, these results provide an important contribution to comparative venom analyses, and more formally address the urban myth of daddy long-leg spiders having exceptional venom potency on humans.

**MATERIALS AND METHODS**

**Spider and Venom Collection**

All spiders used in this work were adult *Physocyclus mexicanus* Banks from a local population in a single building at Spider Pharm (Yarnell, AZ 34° 13′ 12.34″ N; 112° 44′ 56.43″ W; 4,796 ft elev). After spiders were unfed for at least 1 week, venom was extracted using electrostimulation as in Binford and Wells (2003). This was done under two circumstances. The first at Lewis & Clark College (L&C) was intended to induce upregulation of expression to optimize capture of venom mRNAs. In parallel, at Spider Pharm, spiders were milked for proteomics analyses and insect bioassays, taking care to avoid contamination with regurgitate. Electrostimulation did not yield sufficient, pure samples of venom from females in either lab. Rather, at L&C of 12 adult females, six yielded tiny drops of venom, three of these were contaminated by vomit. However between 500 and 600 adult males were milked at Spider Pharm to obtain sufficient uncontaminated venom for proteomics and bioassays. Therefore, all methods requiring crude venom were done with venom from males only collected at Spider Pharm, but venom-gland transcriptomes were obtained from a combination of glands from males and females as described below. Voucher specimens are retained in the L&C Natural History collections, and deposited in The National Museum of Natural History.

**Proteomic Analyses**

**LC-MS/MS Detection of Tryptic Peptides From Crude Venom**—Crude venom extracted at SpiderPharm (∼80 µg from ∼100 male spiders) was sent to the Arizona Proteomics Consortium (University of Arizona) for proteomic analysis. Proteins were analyzed from an in-gel tryptic digest (Figure 1A) with LC-MS/MS using an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) as in Zobel-Thropp et al. (2014a,b). The sample contained a mixture of two digests using trypsin (Princeton Separations) in the presence of ProteaseMAX (Promega, manufacturer’s protocol). One digest was from the excision of an entire lane containing 10 µg of crude venom (12% Tris-glycine SDS-PAGE) and the other was an excised region of <10 kDa only from a separate gel (25 µg crude venom, 16.5% Tris-tricine SDS), thereby attempting to enrich the sample with small venom polypeptides and peptides which are prone to diffusing out of the protein gel. Samples were desalted using OMIX C18 tips (Agilent Technologies), injected onto a Proxeon Easy nano-HPLC (Proxeon, Odense) precolumn (100 µm, 2 cm, Fisher Scientific) and separated on a C18 column (75 µm, 10 cm, Fisher Scientific) at a flow rate of 300 nL/min over a 145 min gradient of 5–40% solvent B (A = H₂O, B = acetonitrile, both with 0.1% (v/v) formic acid). Eluate was delivered by a nanospray source (Nanomate, Advion) with a voltage of 1.85 kV
to the Orbitrap Velos. Survey scans were acquired at 60,000 resolution and the top 14m/z values were selected for CID (width = 2 amu) at 35% relative energy in the Velos linear trap and then placed on an exclusion list for 45s.

Resulting MS/MS data was searched using SEQUEST on Discoverer (v. 1.3.0.339, Thermo Fisher Scientific) against masses of theoretical fragments from two databases. The first included a combined set of sequences generated by the Binford lab including Illumina HiSeq NGS data from 12 Synspermiata species (described below), and Sanger cDNA library sequences from 10 species as in Zobel-Thropp et al. (2014b) (described below). The second included all chelicerate sequences in NCBI (downloaded 2/17/2016), totaling 964,541 sequences. Matches required <10 ppm precursor error. The reversed database was searched to provide quality scores for FDR filtering. The SEQUEST output was organized in Scaffold (version Scaffold_4.8.6, Proteome Software Inc., Portland, OR; Searle, 2010). Peptides were identified with 95% minimum threshold and 0% false discovery rate (FDR) and proteins were identified with 95% minimum threshold, two peptides minimum per protein, and 0% FDR. Details are in Table S2. While our goal for this analysis is thorough representation of peptides present in male crude venom, there are a number of standard reasons peptides or proteins may be missed. These include (but are not limited to) sample degradation or low concentration for mass spectrometry requirements, incomplete gel extraction, poor or no tryptic cleavage, lack of peptide binding to the C18 column, poor ionization or fragmentation, or posttranslational modification that were not included in the data search. Proteome profiling data will be available through the PRIDE repository, and amino acid and cDNA sequences for all proteins detected in this study are deposited in GenBank (NGS accession numbers MN004877-MN004966) and included in Table S1.

Transcriptome Database

**RNA Isolation**—A single pool of RNA isolated from 12 adult *P. mexicanus* was used for generation of Sanger cDNA libraries, and Illumina 3000 transcriptomes. To up-regulate venom transcripts, venom was extracted from 12 spiders across two consecutive days. Six were milked on 1 day (three females and three males) and six spiders were milked the following day (three females and three males). Then, on the same day—three and 4 days after extraction—all 12 spiders were anesthetized with CO$_2$ and venom glands were extracted and flash-frozen immediately in liquid nitrogen. Given differential success in obtaining venom, only glands from spiders from which venom was extruded were used for mRNA isolation. We pooled glands between sexes to optimize total mRNA, and generate a thorough within-species transcriptome with the goal of annotating the proteome. Transcriptional timing is not known for pholcids, however this window of time has been successful for isolating venom gland mRNAs from other Synspermiata spiders, and combining three and four-day transcripts likely increased the breadth of transcripts captured (Binford et al., 2005; Zobel-Thropp et al., 2014a,b). Total RNA was isolated using the ChargeSwitch Total RNA Cell kit (Invitrogen) following the manufacturer’s protocol.

**Sanger cDNA Library Construction and Screening**—We constructed the venom gland cDNA library using the SMART™ cDNA library construction kit (Clontech). Details of all procedures including library construction, cDNA packaging, screening and sequence
analysis are the same as in Zobel-Thropp et al. (2014b). For this library, we used 94 ng of total RNA for first strand synthesis of cDNA and followed the manufacturer’s protocol for library construction. The titer for this library was $1.4 \times 10^6$ pfu/ml. We screened 1,685 clones using PCR and sequenced 311 cDNAs ≥500 bp on an Applied Biosystems 3730 Analyzer at the Genomic Analysis and Technology Core (University of Arizona) using TripEx2 vector-specified primers. We trimmed vector sequence and assembled sequences using Sequencher 5.1 (Gene Codes Corp.). Due to chromatogram ambiguity, we discarded 32 sequences, resulting in 279 high quality sequences for transcriptomic analysis.

**Illumina 3000 Transcriptomes**—From the same *P. mexicanus* RNA sample used for the Sanger cDNA library, we sequenced a transcriptome using Illumina 3000 at the Center for Genome Research and Biocomputing (CGRB) at Oregon State University. This was done as part of a larger project that included RNA samples from 23 transcriptomes from 11 species. All sequences resulting from these taxa were combined into a single dataset used to annotate the *P. mexicanus* proteome. The taxon representation in the dataset includes venom-gland and whole body transcriptomes from *Plectreurys tristis* and *Scytoedes thoracica* (venom gland RNA is from the same samples used in Zobel-Thropp et al., 2014a,b, respectively), *Austrarchaea mainae*, *Periegops suterii*, *Hexophalma dolichocephalus*, *Drymus sarrana*, *Loxosceles spinulosa*, *L. rufescens*, *L. arizonica*, and *L. reclusa*. For each of these samples, total RNA pools (ChargeSwitch, Invitrogen) were quantified and analyzed for quality using a Nano chip on the Agilent 2100 bioanalyzer at the CGRB. For NGS transcriptome construction, each total RNA pool was enriched for mRNA and prepared for multiplexed sequencing with PrepX reagents for RNA-Seq library construction (Wafergen). The final libraries were checked for quality and size distribution followed by qPCR analysis for quality control. Transcriptomes were multiplexed into two Illumina HiSeq 3000 150 bp paired end lanes.

**Transcriptome Assembly**—Each transcriptome sample was individually *de novo* assembled using Trinity (Grabherr et al., 2011). Coding regions were identified from the *de novo* transcriptome assemblies with TransDecoder (Haas et al., 2013). The three-step TransDecoder process was used: first, TransDecoder.LongOrfs was used to identify the long open reading frames; second, BLASTP (Altschul et al., 1990) was used against a set of known proteins [spider proteins from GenBank (Benson et al., 2005), ArachnoServer (Pineda et al., 2018), previously isolated Sanger cDNAs from the Binford lab, *Latrodectus hesperus* (https://www.hgsc.bcm.edu/arthropods/western-black-widow-spider-genome-project), *Loxosceles reclusa* (https://www.hgsc.bcm.edu/arthropods/brown-recluse-spider-genome-project), *Centruroides exilicauda* (https://www.hgsc.bcm.edu/arthropods/bark-scorpion-genome-project)]; third, TransDecoder.Predict was used in conjunction with the TransDecoder.LongOrfs output and the protein output to predict the likely coding regions. Output contigs from TransDecoder were combined with the known proteins described above and the final two steps of TransDecoder were run a second time.

To make sure the TransDecoder steps did not remove any essential contigs a second comparison was done against the original Trinity assemblies. Contigs in the Trinity assembly not identified from the TransDecoder process were aligned to previously isolated...
Sanger venom-gland cDNAs (Binford lab, unpublished) to identify important but missing genes. Sequences identified here that had hits in the above alignment were then aligned back to the TransDecoder peptides, if no hit was found they were added to the final TransDecoder set.

Proteome Annotation

Our analysis pipeline for sequence annotation is illustrated in Figure 1. The final set of proteins was annotated into functional categories based on results from combined searches in the Conserved Domain Database (CDD v3.16, https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), BLAST searches in NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and ArachnoServer (Pineda et al., 2018), http://www.arachnoserver.org public databases, and our database containing sequences from several Synspermiata venom gland cDNA libraries constructed in the Binford lab (unpublished). Hits were recognized as significant if e-values were ≤10^{-5}. All of the sequences that were detected by peptide spectra in mass spec analyses (116 unique polypeptides) were annotated manually using the following steps that largely follow those in Zobel-Thropp et al. (2014a).

We identified 38 unique polypeptides with close homology to proteins known to be involved in conserved metabolic, housekeeping, or maintenance functions. Hits had strong e-values (e10^{-20} to 0.0), and all matches agreed between hits in CDD, InterProScan, and NCBI (Table S1). Polypeptides that hit domains previously described as venom proteins were categorized as “proteins with venom function.” Sequences that had no hits in CDD, InterProScan, or NCBI (60) included peptides with characteristics consistent with venom peptides (secreted with characteristic disulfide patterns) and were categorized as “venom peptides.” Finally, 18 polypeptides were categorized as “proteins of unknown function,” because they either had strong matches to proteins that were not considered housekeeping/metabolic proteins (14, e10^{-29} to 0.0) or they did not have any hits in any databases (4), so potential function in venom could not be determined.

With the exception of sequences that hit housekeeping/metabolic proteins in our initial homology searches, we identified homologous sequences within venom proteomes across our Synspermiata dataset, and aligned those with homologs in NCBI using MAFFT followed with manual editing in Mesquite [Version 3.51 (build 898), http://mesquiteproject.org]. We identified conserved characteristics consistent with known venom toxins (e.g., cysteine-rich), including signal peptide cleavage sites with SpiderP (http://www.arachnoserver.org/spiderP.html) and knottin or ICK predicted folding patterns using Knotter1d (http://www.dsimb.inserm.fr/KNOTTIN/knoter1d.php) tools. All full-length sequences were analyzed for signal peptide cleavage sites using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/). Protein coding sequences were considered full-length if they contained a methionine and a stop codon.

Sanger cDNA Library Annotation—The cDNA library consists of 251 sequences that were aligned, analyzed, and separated into 24 clusters and 60 singletons (individual sequences) (Figure S1, Table S3). Of those in the original dataset (279), sequences from two clusters and 11 singletons were deemed “no open reading frame,” and 28 sequences were
omitted from the dataset because they hit a sequence in NCBI, but when translated they were repetitive and did not contain an ORF. Our annotation of the Sanger cDNA sequences followed the same steps as those detailed above.

Given evidence of high levels of index swapping in our Illumina sequences, we only use our Illumina sequences as a database source for mass spec analyses. We limit our analysis of the transcriptome to sequences isolated from our Sanger cDNA library of *P. mexicanus* for which we are confident in the taxonomic and tissue source. The cDNA library includes combined transcripts of both male and female spiders and highly expressed homologs that we did not detect in the male only proteome may provide insight into female venom composition.

All non-redundant sequences >200 bp were submitted to GenBank and granted accession numbers (KP138796-KP138905).

**Phylogenetic Analyses**—We estimated phylogenetic relationships of the most abundant toxins detected in our proteomes to help interpret diversity within the group, and to infer how these toxins are related to homologs in NCBI and proteomes of other Synspermiata. To do this we identified sets of related sequences using BLAST with a threshold of \( e < 10^{-5} \), aligned them initially with MAFFT online server (http://MAFFT.cbrc.jp/alignment/server/) by manual alignment in Mesquite v3.2. Bayesian phylogenies were inferred using MrBayes XSEDE through the CIPRES portal. Analyses were run for 50 million generations, or until the average standard deviation of split frequencies decreased below 0.01.

**Venom Potency Analysis**

**Insect Bioassays**—To assess the potency of pholcid venom, we quantified the dose at which 50% of crickets (*Acheta domesticus*) injected were paralyzed after 60 min (PD\(_{50}\)). We diluted venom in physiologically buffered lepidopteran saline solution (5 mM KH\(_2\)PO\(_4\)/100 mM KCl/4 mM NaCl/15 mM MgCl\(_2\)/2 mM CaCl\(_2\), pH 6.5) and established a dosage range distributed around the PD\(_{50}\) by performing preliminary injections. Experimental doses were 0.25, 0.50, 1.0, 1.5, and 2.0 µg of crude venom protein with two replicate assays each on 15 crickets per dose by injection into the dorsal mesothorax using a PB-600 dispenser with a gastight syringe (Hamilton Co.). With each replicate, we injected an additional 15 control crickets with 0.4 µl of saline, a volume equal to the largest dose volume. Paralysis was scored every 10 min for 1 h based on the ability of a cricket to right itself. PD\(_{50}\) values were generated for both absolute amounts of venom injected (µg) and for weight standardized dose (µg/g cricket) using the EPA Probit Analysis Program for calculation of LC/EC values (version 1.5 SAS).

**RESULTS AND DISCUSSION**

We detail below the chemical composition of *P. mexicanus* venoms with evidence that they include an expanded presence of neprilysin and astacin-like metalloproteases, and a diverse set of peptide toxins. The venom causes rapid, flaccid paralysis on crickets with potency that is within the norm of those estimated for other Synspermiata spiders. A collection of
observations of bites on humans and subsequent effects indicate that *P. mexicanus* can bite through human skin, however the effect is minimal.

**Venom Composition**

A total of 2,021 tryptic peptides were detected via Orbitrap MS of our crude venom sample. These match 116 unique polypeptide sequences from our combined databases with >95% identity (Figure 1A). Of these, 33% have close homology with proteins that are likely involved in basic cellular function (Table S1), however these are represented by only 20% of the unique peptide spectra (Figure 1B). Within the remaining 78 polypeptides, the majority are homologs of known venom proteins (39) or venom peptides (21), and the remaining 18 are homologs of proteins of unknown function (Table S1; Figure 1B).

**Toxic (and Potentially Toxic) Venom Proteins**—Metallopeptidases are the most abundant components we detected in the venom, as quantified either by number of spectra, or unique polypeptides. These are primarily neprilysins (M13 peptidases) (17 polypeptides, 53% of the unique spectra) and astacin metalloproteases (M12 peptidases) (13 polypeptides, 18% of unique spectra) (Figure 1B). Other peptidases were present in lower abundance (1–2 polypeptides each) including neuroendocrine convertase (S8 peptidase), and chymotrypsin-like (S1 peptidase). Beyond peptidases, we also detected in low abundance (1–3 polypeptides each) chitinase, two cysteine rich secretory proteins (CRISP), and phospholipase A2.

**Neprilysins (M13 peptidases):** The apparent dominance of neprilysins in *P. mexicanus* venom is unprecedented in spider venom proteomes, and likely indicates an important functional role. Neprilysins are thermolysin-like zinc metalloendopeptidases (Turner et al., 2001). They are typically membrane-bound proteins that can turn off peptide signaling events at the cell surface, and metabolize regulatory peptides (Turner et al., 2001). As venom components, they are predicted to degrade extracellular matrices around synapses, and possibly facilitate toxin access to targets (Undheim et al., 2013). Of the 17 unique sequences we detected in the venom proteome, only one was full length (MN004884) and was specifically hit uniquely by 110 spectra. This transcript has a signal peptide and codes for a mature protein of 697 residues with an estimated molecular weight of 79.7 kDa (Table S1). MN004884 aligns well with homologs from a broad set of taxonomic sources (Figure 2) and retains the conserved zinc binding motif HExxH… ExxA/GD (Turner et al., 2001) present in Gluzincins (Sterchi et al., 2008). The other sequence fragments range in length from 90 – 504 amino acids, and were all detected by anywhere from 4–24 unique spectra (Table S1).

Within spiders, neprilysins have been found in venoms of the barychelid mygalomorph *Tritamine loki* (Undheim et al., 2013), however in lower abundance than we see in *P. mexicanus*. We have detected single proteins in each of *Loxosceles arizonica* and *L. reclusa* venom proteomes, but in no other Synspermiata species. Interestingly, even with updated annotations (unpublished) based on our expanded NGS database, we have not detected neprilysin homologs in *P. tristis* venom (Zobel-Thropp et al., 2014a), the closest relatives of *P. mexicanus* with characterized venoms.
Outside of spiders, homologs of neprilysins have been detected in snake venoms including timber rattlesnakes (annotated as SVMPs) *Crotalus horridus* (Rokyta et al., 2013, 2015), and cobras *Naja kaouthia* (Tan et al., 2017), however, they do not dominate any of the other venoms. Phylogenetic analysis with taxon inclusion of all venom expressed spider neprilysins, representative arthropod homologs detected in NCBI, the *Crotalus* venom toxin and a human homolog, indicate that the diverse set of homologs detected in *P. mexicanus* venom are monophyletic and sister to neprilysins in *Loxosceles* venoms (Figure 2A). The *T. loki* sequences are highly divergent from the Synspermiata clade. The latter shares a closer relationship with diverse chelicerate proteins detected in genomes than they do with the venom-expressed *T. loki*, making convergent recruitment into venoms from a diverse neprilysin lineage a reasonable explanation of this pattern. The detection of single proteins in *Loxosceles* suggests that neprilysins may be phylogenetically widespread in venoms at low levels with expansion of the diversity and perhaps functional role within a lineage including *P. mexicanus*.

**Astacin metalloproteinases (M12 peptidases):** Homologs of astacin metalloproteinases (M12 peptidases) are the second most abundant venom proteins detected in *P. mexicanus* proteomes. Among the 13 distinct polypeptides, seven are full length and all have signal peptides (Table S1). The diversity of homologs includes seven polypeptide subgroups with <95% aa identity and separate into four distinct clades (Figure 3). Similar to neprilysins, M12 peptidases represent a gene family that is widespread in spider venoms, and homologs have been well described as contributors to venom function in *Loxosceles* (annotated as *Loxosceles* astacin-like proteases – LALPs; da Silveira et al., 2007; Trevisan-Silva et al., 2013), with up to three distinct proteins detected in proteomes of South American species (reviewed in Chaves-Moreira et al., 2017). The diversity of astacin-like metalloproteinases in the *P. mexicanus* venom proteome is comparable to that detected in *P. tristis* (Zobel-Thropp et al., 2014a). There are scattered M12 peptidase homologs in other proteomes of Synspermiata (Figure 3), however, only *P. tristis* and *P. mexicanus* have more than three distinct homologs. Phylogenetic analyses including all venom proteome-confirmed M12 peptidase homologs across Synspermiata resolve into a monophyletic *P. mexicana* group that is derived from a paraphyletic *P. tristis* set of proteins (Figure 3). This pattern is consistent with an evolutionary expansion of the role of astacins in venom before the common ancestor of these two species, with further diversification in the pholcid lineage after the divergence from the shared ancestor with *Plectreurys*.

All of the sequences we detect have the conserved catalytic domain HEXXHXXGXXHEXXRXDR- and a MXY region that is involved with a sequence turn and Zn-dependent activity (da Silveira et al., 2007). LALPs have proteolytic effects on gelatin, fibronectin, fibrinogen and extracellular matrices, and thus, as venom toxins, they are hypothesized to be spreading factors that act in synergy with other toxins (Trevisan-Silva et al., 2013). The hydrolytic effects of LALPs are suspected to contribute to hemostatic disturbance during mammalian envenomation, thereby influencing the dermonecrotic or systemic effects of *Loxosceles* bites (Trevisan-Silva et al., 2013).

Astacin-like metalloproteinases have also been detected as major components in digestive fluids secreted from the mouths of the mygalomorph *Acanthoscurria geniculata* (Walter et
al., 2017) and araneomorphs *Stegodyphus mimosarum* (Walter et al., 2017), *Nephilengys cruenta*ta (Fuzita et al., 2016), and *Argiope aurantia* (Foradori et al., 2006) indicating a phylogenetically widespread presence in spider digestate. We are confident that the homologs we detect in venom proteomes represent presence in venom rather than contamination by regurgitate, because our venom collection techniques carefully exclude digestate. Expanded phylogenetic analyses including a broad taxonomic set of proteins obtained in oral digestate and venom resolve a monophyletic clade of venom-expressed homologs in *P. mexicanus* that is sister to all but one divergent venom-expressed homolog in *P. tristis* (data not shown), reflecting potential diversification in this gene family in the context of venom expression.

**Other venom proteins:** The remaining two peptidases in *P. mexicanus* venom are serine proteases and homologs of known venom toxins. Chymotrypsin-like homologs (S1 peptidases) were present with low diversity and abundance. The two homologs are fragments that each hit proteins with CUB domains. The other is a homolog of neuroendocrine convertase 1 (S8 peptidase) which are furin-like proteases, homologs of which cleave N- and C-termini in processing mature latrotoxins (Ushkaryov et al., 2004) and may be common in venoms across multiple lineages.

We detect three other non-peptidase toxic proteins that are homologs of well-established and widespread venom toxins. These include two CRiSP homologs, one that is full length (425 aa, 48.8 kDa); three chitinase homologs, one full length and corresponds to a 493 aa mature protein (est 54.7 kDa), and a single fragment of a bee venom-like phospholipase A2 (PLA2) with weak support (only 4 tryptic peptides). Of these, the chymotrypsins, chitinase, and PLA2 have not been detected in *P. tristis*, perhaps because of their low abundance, or, they may have a dynamic presence in venom extracts. However, the presence of CRiSP homologs adds to evidence that these are predictable widespread venom components in spider venoms (Fry et al., 2009; Undheim et al., 2013).

**Proteins of Unknown Function**—Beyond proteins with homology to established venom components, there are proteins in the proteome, some well-represented with respect to peptide spectra, that are homologs of uncharacterized and unnamed genes. Given high rates of discovery of new venom components, we carefully assessed these as potential candidates for venom activity. An intriguing protein is abundant (combined 239 peptide spectra, 62 of which are unique peptides) but not diverse; it is represented by two identical polypeptides, one full length (599 aa), with an estimated size 67.7 kDa. This protein has clear homology to a domain of unknown function (DUF885), however Fold Function and Alignment Searches (FFAS, http://ffas.godziklab.org/ffas-cgi/cgi/ffas.pl) detect sequence elements of lipoproteins, and folds similar to M32 carboxypeptidases, which are metalloproteases. High sequence similarity across arachnid genomes, suggests they perform a conserved, but undescribed function, which could be involved in venom polypeptide processing.

Of the remaining polypeptides with limited homology to known proteins, four are full length. One is a homolog of a protein we have previously detected in *Scytodes* venom (RP23-like) that is annotated as “mite allergen.” This basic protein (est pI 9.97) has a signal peptide, is estimated 20.7 kDa (188 aa) and has homologs across arachnid genomes.
Curiously, proteins referred to as allergens and “mite allergens” in Loxosceles venoms are not homologs of this toxin (MN004921). The two remaining proteins are both secreted and do not have any cysteines. The first (MN004924) is acidic (pI 4.14) estimated to be 22.5 kDa, and is leucine and glutamic acid rich. The other two (MN004934 and MN004937) are from a group of five listed to have leucine-rich repeat domains, based on results from the CDD and InterProScan searches. Of the five, three are homologs (MN004924 is estimated to be 34.9 kDa with a pI of 4.95), and the other two are not homologous. One is estimated to be 34.4 kDa (pI 9.05). Four distinct polypeptides had no hits in any databases (KP138800, KP138796, MN004925, and MN004926); they are distinct from each other, and we have named them “hypothetical proteins.” None of these are found in other venom proteomes within Synspermiata, so we conclude they are not abundant or widespread contributors to venoms in this higher lineage.

**Venom Peptide Toxins**—While we limited our discussion of venom proteins to those confirmed in the proteome, we discuss the venom peptides in the proteome in the context of homologous sequences in our transcriptomes. We do this because patterns emerge in comparisons between these data sets that potentially reflect sexual dimorphism given that our proteome is male specific and our transcriptome combines male and female gland extracts.

**Overview:** In our combined transcriptome we detected a total of 103 sequences with homology to venom peptides, or cysteine motifs that are characteristic of venom peptides (91 in our Sanger cDNA library (taxon source confident, Table S3) and 12 NGS sequences detected as matches to our proteome). Among the 103 sequences, 49 are non-redundant and full length. These consist of 17 distinct groups, 13 of which are predicted knottins, and the other four with characteristics of spider venom peptides as described below. The peptides range in cysteine number from 7 to 10 and in predicted size from 3.6 kDa-11.9 kDa (mature, processed sequences) (Table S1). These sequences constitute over one-third (36%) of the venom gland transcriptome (Figure S1) and 18% of the proteome that is annotated as proteins with venom function (Figure 1B). We assigned the 17 groups of “pholcitoxins” with “unknown” functional notations (U₁-U₁₇) and a “PHTX-Pmx” name according to established nomenclatural convention (King et al., 2008). Suffixes of ascending letters distinguish distinct sequences within a group (e.g. 1a, 1b, 1c, etc.) and those with an underscore followed by a number differ only in the signal sequence region (accession numbers KP138863-KP1388905 and MN004909-MN004920).

**Peptides detected in proteome:** Of the 17 peptide groups identified in the transcriptome, 13 (with 36 distinct sequences) were detected in the venom proteome. These vary in abundance (as quantified by numbers of unique tryptic peptides) and diversity (as quantified by the numbers of non-redundant sequences) (Figure 4). Over 25% (61/220) of the tryptic peptides that hit peptide toxin matches were to U₁₇-PHTX-Pmx. The three 7.8 kDa peptides in this group are homologs of U₁-HXTX-Iw1 that is abundant in the Australian hexathelid funnel-web spider Illawarra wisharti. While homologs appear to be widespread in spider venoms, the target of U₁-HXTX-Iw1 remains unknown despite much experimental effort (reviewed in Wilson, 2015).
The most diverse peptide group in *P. mexicanus* venom is the U₅-PHTX set of 13 non-redundant sequences that correspond to ~5 kDa peptides and are homologs of ω-PLTX-Pt1a (Figure 4; Table S1). ω-PLTX-Pt1a blocks presynaptic voltage-gated Ca²⁺ channels in invertebrates [(King, 2007), PLTX-II in Branton et al. (1987)], specifically Dmca1A channels (Kuromi et al., 2004) and are the most abundant peptide toxins in venoms of *P. tristis* (Zobel-Thropp et al., 2014a). While ω-PLTX-Pt1a is the top hit in BLAST searches of U₅-PHTX, ω-PLTX-Pt1a is homologous to two other well-characterized toxins from *P. tristis*, U₁₂-PLTX-Pt1c and δ/ω-PLTX-Pt1a. U₁-PLTX-Pt1c (Plt-XI) is a highly potent insecticidal toxin, however, the molecular mechanism of activity remains unknown (Quistad and Skinner, 1994). The activity of δ/ω-PLTX-Pt1a is better understood and is an excitatory toxin with activity on both Ca²⁺ and Na⁺ channels (Zhou et al., 2013). All of these toxins share a cysteine pattern of -C₆C-II-C₂C-I-C-I-C- and some require O-palmitoylation for activity.

The *P. mexicanus* venom proteome is well represented in diverse homologs with the same cysteine pattern as ω-PLTX-Pt1a. These include U₃-, U₄-, U₅-, U₆-, U₈-, U₉- and U₁₁-PHTX. While the top BLAST hits for these toxin families varied, they are alignable and share the same motif, and thus represent a superfamily (Figure 5). Collectively, the motif spacing is summarized with (1–4)C₆C(4–10)CC(1–2)C₂C₁C(4–9)C₁C(3–8)C(0–5). All of the sequences in this superfamily have residues that may be palmitoylated (threonine or serine, in addition to cysteine) near the C-terminus. Together, members of this superfamily make up a large proportion of the venom peptides in *P. mexicanus* venom (~45% of tryptic peptides, ~70% of the unique polypeptides). Moreover, given the similarities and homology with toxins of known activity, members of this superfamily in *P. mexicanus* are strong candidates for contributing to toxicity of these venoms and may include novel target specificities.

Of the remaining four peptide families in the proteome, U₁₄-PHTX is the most abundant and diverse with 28 tryptic peptides and four unique polypeptide sequences. These peptides have 8 cysteines with the pattern (2–4)C(6–7)C(5–7)CC(7–8)CRC(8–9)CHC(8–14). While they have no matches in databases, the cysteine pattern is similar to, but larger than, µ-agatoxins and they may share a similar fold with cysteine bonding of I-IV, II-V, III-VIII, VI-VII. The predicted molecular weight is ~6.65 kDa. U₁₀, U₁₅, and U₁₆ are each represented by single polypeptides and U₁₀ is well represented in the proteome with 17 tryptic peptide hits. Like U₁₄-PHTX, U₁₀ is unique to pholcids with no hits in databases, however it has seven cysteines and codes for a peptide with estimated molecular mass 5.7 kDa. U₁₅-PHTX is a clear homolog of U₃- PLTX-Pt1a, originally described as Plt-X (Quistad and Skinner, 1994) in *P. tristis* venom, which is insecticidal, however they are not instantly paralytic but lead to slow lethality in some lepidopteran larvae. U₁₆-PHTX has significant homology with U₁- PLTX, however, it has an extended N terminus that is lacking a cysteine that is present in the rest of the superfamily.

**Not detected in proteome:** Four peptide families in the transcriptome with characteristics of venom toxins were not detected in the proteome. One group U₃-PHTX is among the most abundant and diverse peptides in the transcriptome (second only to U₅-PHTX) with 16 total sequences, 7 non-redundant. These are homologs of a component in *Lycosa singoriensis*.
with unknown molecular target and function \((U_{18}\text{-LCTX-Ls1a}, \text{Zhang et al., 2010})\). Nine transcripts (three non-redundant) in the \(U_7\text{-PHTX}\) family are members of the superfamily described above, which are diverse and abundant in the male proteome (Figure 5). \(U_{12}\text{- and } U_{13}\text{- PHTX}\) are related to one another, are cysteine rich, and homologous to peptides isolated from a range of spider venom transcriptomes. They are distinctively large peptides (11.5–12 kDa), are not predicted knottins, and are remote homologs of Dickkopf-related proteins that are involved in development in a wide range of non-arthropod metazoans. Beyond standard analytical reasons why a protein may not be detected in a proteome (see Methods), lack of detection of these peptide families in the proteome could reflect them serving a non-venomous function in the cell, or potentially expression in female but not male \(P.\text{mexicanus}\) venoms.

**Insecticidal Potency**

The potency of male \(P.\text{mexicanus}\) venom, measured by PD\(_{50}\) on crickets, is comparable to that of venom from female \(P.\text{tristis}\) and other Synspermiata taxa (Figure 6, Zobel-Thropp et al., 2014a). Interestingly crude venom from all Synspermiata taxa tested to date on \(Acheta\text{ domestica}\) are more potent than comparable estimates of effective doses on multiple taxa using a different cricket model, \(Grillus\text{ assimilis}\) (Figure 6, Manzoli-Palma et al., 2003). However, there are distinct physiological symptoms of envenomation whereby crickets injected by \(P.\text{mexicanus}\) venom undergo rapid, irreversible flaccid paralysis in which the legs lay loosely extended and do not twitch. In contrast, crickets injected with venom of \(P.\text{tristis}\) (Zobel-Thropp et al., 2014a), and sicariid venoms (Zobel-Thropp et al., 2010, 2012) undergo rapid and irreversible excitatory paralysis with legs flexed and twitching. \(P.\text{mexicanus}\) envenomated crickets also develop darkened areas around the mouth and sternum, and on the ventral abdomen (Figure 6), symptoms we have not observed after injection of other Synspermiata venoms (Zobel-Thropp et al., 2010, 2012, 2014a).

The components responsible for flaccid paralysis and areas of discoloration on crickets following injection with \(P.\text{mexicanus}\) venom are unknown. Neprilysins are candidates for contributing to these effects given their abundance in the venoms and uniqueness within Synspermiata. While melanism in crickets has been described as a symptom of intoxication by insecticides (Fisher and Brady, 1980) to our knowledge the mechanisms underlying this are unknown. Flaccid paralysis in insects can be induced by some polyamine toxins (Quistad et al., 1991), by \(Na_v\) channel toxins (Bende et al., 2013, 2015) and a variety of peptide toxins that affect \(Ca^{2+}\) channels (e.g., Knaus et al., 1987; Troncone et al., 1995; Lipkin et al., 2002; King and Hardy, 2013). Characterization of the non-polypeptide and peptide components in \(P.\text{mexicanus}\) may identify homologous or convergent factors that are causing these symptoms.

**Notes on \(P.\text{mexicanus}\) Behavior and Effects of Bites on Humans**

While we have not performed controlled behavioral experiments a dense natural, local colony of \(P.\text{mexicanus}\) at Spider Pharm in Yarnell, Arizona, has allowed opportunity for anecdotal observations of their behaviors including their propensity to bite humans. We include an overview of observations, emphasizing those that inform the role of venom and may inspire future experimental work.
Propensity to Bite and Effects on Humans—Contrary to popular opinion about pholcid spiders, *P. mexicanus* readily bite humans when they are disturbed by moving or cleaning activities, but bites are rarely confirmed by direct observation (Kristensen, pers obs.). While working in the lab with an open colony of these animals, members of Spider Pharm staff are frequently bitten when their habitats are disturbed. Most bites are brief nips as the spiders are moving rapidly over exposed skin, possibly with a slight pinch or tug resulting in a mild and brief sting. All confirmed bites by this species have been by adult or subadult males.

In one case, an adult male *P. mexicanus* bite on the back of the neck produced a mild sting that became a small welt which blackened and eventually opened into a 1 mm diameter wound. Images of the wound were diagnosed by a dermatologist with expertise in spider envenomation (Van Stoeker, pers comm) as a typical reaction to foreign protein rather than necrosis resembling a triggered immune response comparable to loxoscelism. In a second case, three subadult males that were disturbed by moving trays of larvae walked down the fingers of the left hand and simultaneously bit the knuckle at the base of the little finger. This resulted in an immediate mild, temporary sting. The knuckle was sore and slightly inflamed for several days. Interestingly, the case of human bites recorded on Mythbusters (Episode 13, premiered 15 Feb 2004) involved adult males that were aggregated and engaged antagonistically with one another. In that case, when human arms were extended deeply into the enclosure spiders did not initially bite, though several spiders pressed their chelicerae against the skin without biting. A bite occurred on the back of the hand after the hand was pulled back closer to males fighting near the entrance (Kristensen pers obs).

Potential Venom Dimorphism, Male Aggression, and Feeding Biology—The difference between sexes in our ability to obtain venom by electrostimulation inspires consideration of potential sexual dimorphism in venom composition and/or functional role [repeated independently in the Binford Lab and the Kristensen Lab (Spider Pharm)]. The easier to obtain, higher yields of males could reflect larger volumes of venom in males, or different morphological/physiological circumstances of extrusion. There could also be seasonal differences in venom volume. Adult males and females are similar in size, but males have more ornate and armored or reinforced chelicerae. Cheliceral armor is a known dimorphic trait in pholcids that is a proposed adaptation for mating (Huber, 1999) but may also be an adaptation for fighting and defense. Males are aggressive to one another when at high densities. For example, after placement in enclosures for the Mythbuster staged encounters, males were concentrated on the cloth and interacted aggressively including biting that resulted in fatalities (Kristensen pers obs). There appears to be less aggression in natural stable colonies, and interactions are typically settled quickly and non-lethally. While most of the aggressive interactions observed were between males, females also exhibit aggression, especially when guarding clusters of hatchlings.

*P. mexicanus* readily capture and eat a diversity of arthropod prey, including spiders. The role of venom is not obvious and prey that are apparently envenomated often appear to be little affected. Like other pholcids, most prey are slowed or restrained by silk first and bitten several times during wrapping, frequently with no obvious immediate effect. In other instances, there is rapid paralysis or death after single bites. More rarely, spiders directly
attack small, weak prey with an initial bite (frontal attack) without the use of silk. Single, short bites made during frontal attacks may result in paralysis or death. Given evidence that venom has a potent effect on some prey, the spiders may meter the amounts of venom injected. The ability to regulate the quantity of venom injected into prey as a function of size, activity levels, and differences in sensitivity to toxins, has been experimentally determined in Cupiennius salei (Wigger et al., 2002; Wullschleger and Nentwig, 2002). The observed differential effects of pholcid bites are consistent with a similar ability to regulate venom injection, and may indicate it is a general attribute of spider envenomation.

In many spider species males reduce or stop feeding when they reach adulthood. This is not the case for P. mexicanus (pers obs; Wilson et al., 2016). Not only do males hunt and capture prey but we have observed them presenting captured prey to females, with and without attempts to mate. We have also observed males with egg sacs in their chelicerae, which could reflect adoption of the egg sacs or infanticide by males. Together, these observations suggest there are interesting sexual dynamics in this species that could influence some degree of sexual dimorphism in venoms.

**CONCLUSIONS**

We conclude from the data and observations presented in this work that venoms of male P. mexicanus consist of a rich set of components, predominantly metalloproteinases and peptides. While activity of none of these components has been directly characterized, they include homologs of proteins and peptides that are demonstrated contributors to toxicity. Our evidence of potent toxicity in arthropods, in combination with homology to known toxins suggests that detailed activity characterizations have strong potential for discovering new or refined activities. The unprecedented extensive presence of neprilysins is intriguing with respect to contributions to immobilization or consumption of prey. While the information available on the effect of P. mexicanus venom on mammals is limited by lack of careful experimentation, we consider the documented accounts evidence that mouthparts of P. mexicanus are capable of piercing human skin, however envenomation results in a mild sting, and venom from a single P. mexicanus is not lethal to humans or other mammals. Given the tremendous diversity of pholcid spiders, we do not assume that venom composition in males of this species is representative of the entire family, and broader sampling within this group may uncover exciting differences that correlate with known ecological diversification within the group. Moreover, proteomic characterization of female venoms of P. mexicanus are needed to infer the full set of proteins identified in venom gland cDNA libraries that are present in adult members of this species, given the likelihood of at least some degree of sexual dimorphism. With expansive diversity to explore in pholcid venoms, we present these results as reason to be excited about potential discovery of new chemical activities and, by default, to change the public narrative and assume pholcid spiders are not harmful to humans.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.
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FIGURE 1.
Overview of the project. (A) A flow-chart illustrating the sequence of steps of the procedures for transcriptomic and proteomic analyses. The photo is an adult male *Physocyclus mexicanus* carrying an egg sac. The gel image is an electrophoretic separation of male *P. mexicanus* crude venom used for the in-gel tryptic digest. (B) Pie charts of major categories of components identified in the proteome by overall transcript abundance, and proportion of unique tryptic peptides detected by Orbitrap. Asterisks (*) indicate hypothetical proteins with unknown function.
FIGURE 2.
Neprilysins (M13 peptidases) identified in *Physocryptus mexicanus* venom are homologs of neprilysins from diverse organisms. (A) An alignment of the region of the conserved active sites of neprilysins with conserved binding sites labeled based on Sterchi et al. (2008). Sequence names are color coded by taxonomic source groups. (B) A Bayesian phylogeny of neprilysins with branches color coded by source group. Branch width correlates with posterior probability support for that branch. Names of taxa are in color when they have been confirmed to be in venom proteomes. The label ∨ indicates that sequence was identified in a venom gland EST.
FIGURE 3.
A Bayesian phylogeny of astacin metalloproteinases (M12 peptidases) supports evolutionary expansion in the venom of male *Physocyclyx mexicanus*. Branch width correlates with posterior probability support for that branch. Branches are color coded by source taxon. Sicariids are different shades of pink for different species in the genus *Loxosceles*. Taxon names are in color when that polypeptide sequence has been confirmed to be present in a venom proteome and are otherwise labeled by symbols according to the legend.
FIGURE 4

Peptides identified in the venom proteome of *Physocyclus mexicanus* summarized by homologous groups (families). The pie chart on the left indicates the proportions of non-redundant polypeptide sequences detected in the proteome for each group, and represents the sequence diversity within and among the peptide families. The pie chart on the right indicates the proportion of peptide families as measured by abundance of unique tryptic peptides in the proteome. The table details the number of non-redundant sequences (#nr seqs), the number of unique tryptic peptides, the number of cysteines in the mature peptide, whether or not the peptides are predicted to have an ICK fold, and the estimated molecular weight of the mature peptide.
A Bayesian phylogeny of a superfamily of homologous groups of ICK peptides from *Plectreurys tristis* and *Physocyclus mexicanus*. Branch width correlates with posterior probability support for that branch. All polypeptides included in this phylogeny have been confirmed to be present in the venom proteomes. Branches colored red are all from *P. tristis*, and all other colored branches represent groups of peptides identified in this work from *P. mexicanus*. Annotations of activity reflect the discussion in the text. The alignment includes a subset of the sequences included in this phylogeny, reduced to fit by eliminating highly similar sequences. Predicted disulfide bonding pattern is based on Quistad and Skinner (1994).
FIGURE 6.

Physocyclus mexicanus bioassays on crickets (Acheta domestica) result in rapid, flaccid paralysis along with ventral darkened areas. (A) Table presenting the estimated dose at which 50% of crickets are paralyzed at 1 h post injection (PD_{50}) for *P. mexicanus* and other Synspermiata spiders. (B) A ventral view of representative crickets at 1 h post injection by venom from *P. mexicanus* and *P. tristis* for comparison. Notable differences are the relaxed legs and darkened regions on the cricket injected by *Physocyclus* in contrast to the folded legs and lack of darkened regions on *Plectreury*.

| species                  | PD_{50} (ug/g cricket) | Source                          |
|--------------------------|------------------------|--------------------------------|
| *Plectreury* *tristis*   | 3.3                    | Zobel-Thropp et al. (2014a)    |
| *Loxosceles arizonica*   | 2.6                    |                                 |
| *Sicarius cf. damarensis*| 0.4                    | Zobel-Thropp et al. (2010)      |
| *S. cf. hahni*           | 1.5                    |                                 |
| *S. cf. rugosa*          | 3.2                    |                                 |
| *Physocyclus mexicanus*  | 3.9                    | this study                      |
| *Phoneutria nigriventer* | 43.5                   | Manzoli-Palma et al. (2003)    |
| *Nephilengys cruentata*  | 24.6                   |                                 |