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# Protein Components of the Arthrodial Membrane Gland in a Neotropical Harvestman (Arachnida, Opiliones)

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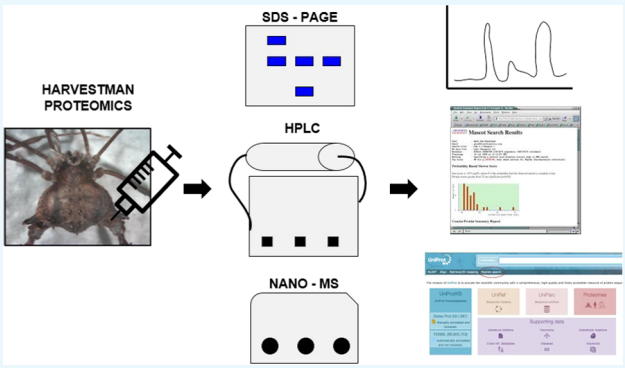
**ABSTRACT:** The content of arthrodial membrane glands in arthropods has seldom been studied. Here, we have analyzed the proteins of the arthrodial membrane gland of the trochanter−coxa articulation of the fourth pair of legs in the harvestman *Mischonyx cuspidatus* via reverse-phase high-performance liquid chromatography (RP-HPLC), polyacrylamide gel electrophoresis (PAGE), and nanoscale liquid chromatography coupled to mass spectrometry (nLC-MS/MS) analysis. Most of the fractions studied are hydrophobic, being proteins with molecular weights of ∼28, 62, and ∼198 kDa. These proteins seem to be homologous to proteins involved in product secretion, cytoskeleton, protein binding, cellular metabolism, and antimicrobial action among others. Lubricant function is also possible based on the literature. We were able to identify 147 proteins in the inner region, 91 proteins from the outer dorsal region, and 36 proteins from the outer ventral region. Some proteins are present only in one of these regions and some are shared by one or more regions. Our work provides, to the best of our knowledge, the first proteome characterization of the content of an arthrodial membrane gland in arachnids. Dataset Identifier: ftp://massive.ucsd.edu/MSV000087195/.




## INTRODUCTION

The body of arthropods is covered by thicker and thinner cuticles depending on the region.[1] The thicker cuticle typically covers most of the body except few areas such as the junctions between the body parts.[2,3] These junctions are known as arthrodial membranes, which are soft and flexible cuticles with elastic properties.[4] Below the arthrodial membrane of some insects, glands thought to have lubricating function have been found.[5,6] These glands have ducts that open at the arthrodial membrane and probably work as the outlet for the secretion for the outer body of the organism.[5,6] It has been shown that the arthrodial membrane of a lobster is composed of water and a small amount of chitin−protein fibers. However, there is no work reporting the chemical composition of arthrodial membrane glands.[7] It is suggested that these glands have a lubricating function that might be related to their location in articulation regions in the body of animals such as the head−thorax, trochanter−femur, coxa−thorax, etc.[5,6]

The arthrodial membrane is also known to be a site of worm colonization and invasion by fungi and bacteria.[8] Spiracles and arthrodial membranes of insects are used by parasites to invade a host body.[9,10] Therefore, the presence of glands per se is not enough information to allow conclusions on their function. A first step toward a functional understanding of the gland is to study its content by identifying the group of molecules in the gland and comparing them with molecules of known function in the literature. The following step would be to identify, isolate, and further run bioassays to confirm its function.

Harvestmen belong to the class Arachnida and have ∼6700 described species.[11] They typically inhabit humid areas where they shelter in caves, rocks, trees, and fallen logs.[12] As in other arthropods, leg joints are separated by an arthrodial membrane. Scanning electron microscopy images of these areas have shown that the external region of the arthrodial membrane in harvestmen may bear dorsal pores of ∼1 μm diameter.[13] Such pores suggest that harvestmen release glandular secretions,[13] but to the best of our knowledge, there are no studies on the chemical composition of these secretions. In this work, we carried out proteomic characterization of the secretion content found in the arthrodial membrane of a Neotropical species, *Mischonyx cuspidatus* Rower 1913 (Arachnida, Opiliones, Gonyleptidae), considering both the outer (dorsal and ventral) and inner contents of the arthrodial membrane gland. We

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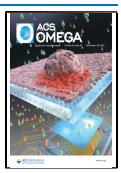

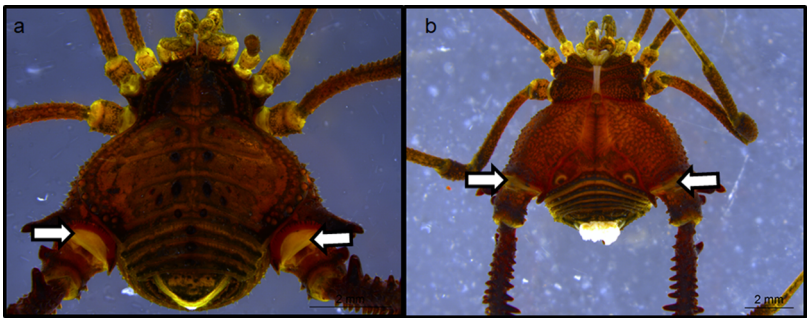


**Figure 1.** Male harvestman *M. cuspidatus* in (a) dorsal and (b) ventral views. Arrows indicate the arthrodial membrane of the region between the coxa−trochanter.

expect that such description may help in the understanding of the function of this gland (Figure 1).

## RESULTS

**Protein Profile of Inner Secretion of the Arthrodial Membrane Gland by SDS-PAGE.** The nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (15%) of raw extract of the inner secretion of the arthrodial membrane gland resulted in several bands (Figures 2

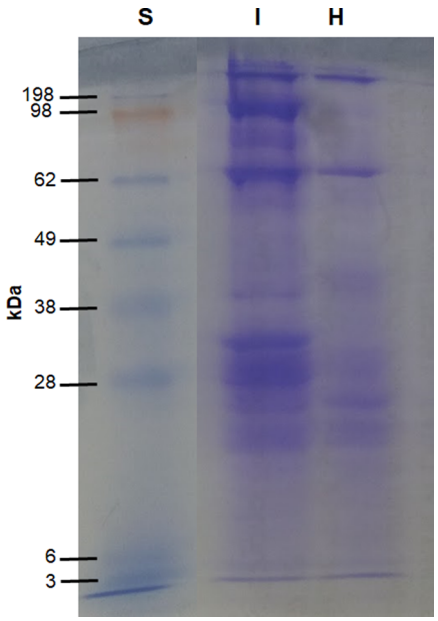


**Figure 2.** Nonreducing SDS-PAGE (15%) gel showing the protein profile from the standard stain (S) (left lane), the inner secretion of the arthrodial membrane gland (I) (middle lane), and hemolymph from the patella region (H) (right lane) of the harvestman *M. cuspidatus* male (SeeBlue Plus2 Prestained Protein Standard, Invitrogen). Numbers at the left indicate the mobility of molecular mass markers.

and S1): one group with molecular weight between ∼62 and ∼198 kDa and another group with molecular weight at ∼28 kDa. The hemolymph fraction shows bands with low molecular mass at ∼62 and ∼28 kDa.

**Protein Fractionation by Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC).** The RP-HPLC analysis profile of the extracts of the inner and outer (both dorsal and ventral) secretions revealed some fractions (three−seven), hydrophilic and several in the hydrophobic fractions of each region (Figure 3). The profile of dorsal and ventral chromatograms (Figure 3) showed the same profile as the inner region.

**Mass Spectrometry and Bioinformatics.** Mass spectrometry analysis of the inner and outer gland secretions allowed us to identify several peptide and protein fragments that are homologous to peptides and proteins described in the literature in other arthropod species (Table S1). We found 147 protein sequences in the inner region, 91 protein sequences on the dorsal region, and 36 protein sequences on the ventral region (Tables S1 and S2). Many of the identified sequences are unique (Supporting Information: Dataset Identifier: ftp://massive.ucsd.edu/MSV000087195/; Figures S2−S5).

By comparing the peptide and proteins (obtained via database) present in the inner and outer (in both dorsal and ventral) regions, we observe that some molecules are present in more than one region (Figure 4), a pattern that is also clear looking at the Venn diagram. Nine molecules are present in the inner and dorsal region, one molecule is present in the inner and ventral region, and two molecules are present in the dorsal and ventral region. The diagram shows that several molecules are exclusive to one of the regions; however, many molecules appeared more than once in the three regions and in different fractions obtained from the liquid chromatography fractionation (Table S3). The inner region of the gland presented 121 unique molecules, the dorsal region presented 57 molecules, and the ventral region presented 27 molecules (Figure 4).

The analysis of peptide and protein functions of the identified molecules in the three regions of the gland (i = inner, d = dorsal, and v = ventral) via UNIPROT (see Table S2) showed that they may be the product of glandular secretions, be structural ribosomal, and have a role in metabolism, defense against pathogens, and signaling.

## DISCUSSION

In this work, we were able to identify a total of 274 molecules (proteins and/or peptide homologues) that we attribute to be accumulated in or produced by the arthrodial membrane gland of the coxa−trochanter region of *M. cuspidatus*. We found hydrophilic and hydrophobic molecules putatively homologous to peptides and/or proteins with functions such as cellular metabolism, signaling and binding, defense, and microbial activity. In addition, we observed high similarities between the inner and outer regions.

SDS-PAGE of the extract of the inner secretion of the arthrodial membrane gland resulted in two major groups of molecules with high (>100 kDa) and low (62 and ∼28 kDa) molecular weights. The hemolymph that was used as a control


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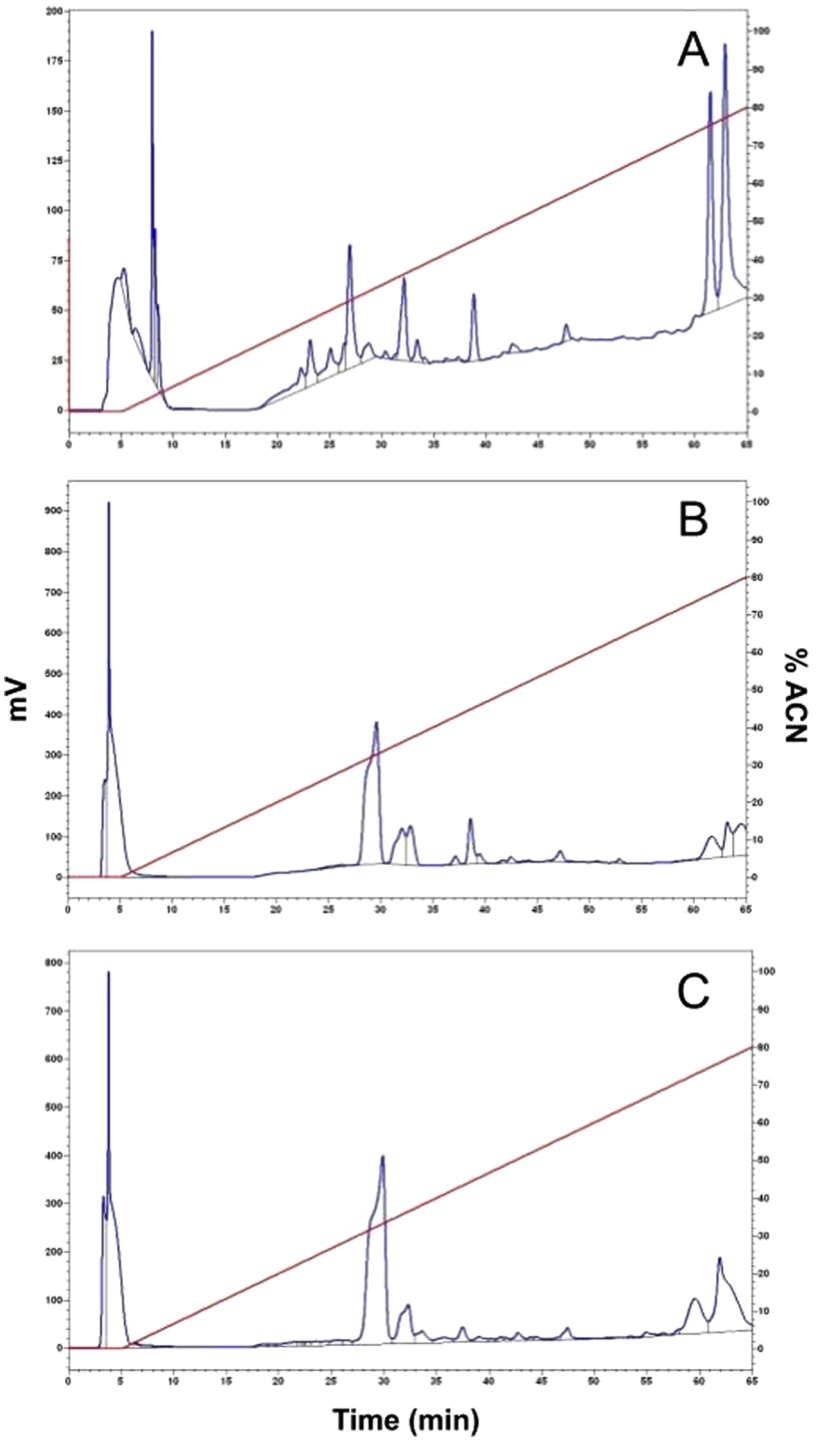


**Figure 3.** Chromatographic profile of the purification step of secretion of the arthrodial membrane of the region between the coxa−trochanter in *M. cuspidatus*: (A) inner secretion, (B) dorsal (outer), and (C) ventral (outer) regions.

of the extraction of glandular content had a low (~28 kDa) molecular weight, suggesting that the inner secretion and hemolymph are two different compounds. However, it is likely that there are similar compounds as glands are nourished by hemolymph.[16]

Through RP-HPLC, we found several hydrophobic fractions. Many fractions have similar retention times mainly between the outer regions (both dorsal and ventral). For example, fractions four and seven (Figure 3b,c) presented similar molecules with similar sequences (Table S3). These fractions probably correspond to the same peptides or proteins, as evidenced by mass spectrometry.

In our search, all molecules identified by the mass spectrometry analysis matched peptides or proteins from the Arthropoda database to some extent. Many molecules were found in a unique region (inner or outer—both dorsal and ventral), but some were found in more than one region. We were able to identify a higher number of proteins in the inner region (147 proteins of which 121 are unique to this region). In addition, we identified nine shared molecules in the inner



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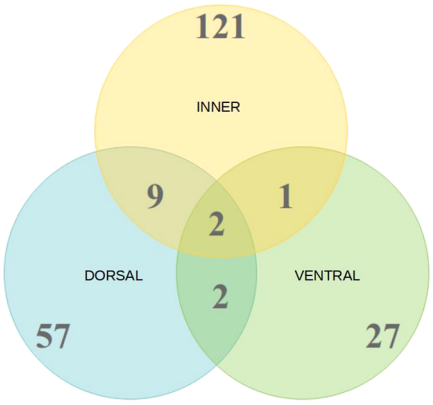


**Figure 4.** Venn diagram of the putative proteins of the arthrodial membrane of the harvestman *M. cuspidatus*. The numbers indicate the quantity of proteins found in three regions of the arthrodial membrane. Inner: proteins found inside the gland; dorsal (outer): proteins found on the dorsal region of the arthrodial membrane; and ventral (outer): proteins found on the ventral region of the arthrodial membrane.

region and one molecule in the dorsal region. The exact biological role of the arthrodial membrane gland is not yet known but we have evidence that the dorsal region has several pores that are likely to serve as outputs for these molecules (NFSS unpublished data).[13] Cytochrome c oxidase subunit 1 protein is the only protein shared by the inner and ventral regions, but we do not know why this molecule is found outside the animal.

The existence of two molecules found exclusively on the ventral and dorsal regions may be the result of proteins cleaved from the inner region. Since the inner region has some enzymes (Table S1), it is possible that they act on protein cleavage, making them active and performing their functions in the outer region of the gland.[17,18]

We have found proteins that have been related to transport, lysis, and storage of lipids (Tables S1 and S2) and smooth endoplasmic reticulum (NSF, unpublished data). These observations have also been reported previously in studies of the presence of secretory glands between joints of insect appendages.[19,20] Previous authors have suggested a lubricating function for the secretions.[5,6] If this is true, it would probably also apply to harvestmen in our study. In addition, we found similarities to four bioactive molecules (Protein diedel, U-poneritoxin, Ceratoxin-B, Tachystatin-A2) in the inner region and only one in the dorsal region (Peptide ctri9293). These molecules may have antimicrobial activity against Gram-positive and Gram-negative bacteria, viruses, and fungi[21−24] (Tables S1 and S2). Since the arthrodial membrane region is possibly a site of colonization and possible invasion by pathogens,[10] the similarity of molecules with others with antimicrobial activity indicates that the arthrodial membrane gland may have components necessary for the defense of the organism against infection. Finally, antimicrobial molecules are of great medical importance, and since we have evidence that these molecules can be found in the structures of harvestman, this work contributes to a possible new source of bioactive molecules for new drug discovery.[25]

## CONCLUSIONS

We were able to identify and characterize possible peptides and proteins from the secretion of the *M. cuspidatus* arthrodial membrane gland and this study represents an important new data for this type of structure. To our knowledge, this is the first study to describe the composition of an arthrodial membrane gland in arachnids. This set of molecules may be used as a basis for future studies of the arthrodial membrane gland in Opiliones and other arthropods with a similar structure.

## MATERIALS AND METHODS

**Study Species and Laboratory Conditions.** Individuals of *M. cuspidatus* were collected manually (in August 2018 and March 2019) under tree trunks at Parque Ecológico do Tietê (−23.494587, −46.521383), São Paulo City, São Paulo State, Brazil. The animals were collected under SISBIO/ICMBio licence number 61431-1-2018. We used only males because they have a larger arthrodial membrane gland that facilitates the collection of the secretion (Figure 1a,b). Harvestmen were fed with dog food and provided water *ad libitum*.

**Extraction of Glandular Secretion.** We first aimed to extract the inner secretion of the arthrodial membrane gland. After anesthetizing the animals ($n$ = 5) in a freezer at −20 °C (15 min), we made a perforation in the arthrodial membrane using a microneedle and collected the secretion (∼15 μL) with an ultrafine syringe with 1 mL of ultrapure water (Seringa Insulina BD Ultra-Fine U-100, needle 6 mm). Water was used to dilute the secretion and the solution was placed in an eppendorf tube with another 1 mL of ultrapure water and stored in a freezer.

To compare the secretions of the inner region with the outer dorsal and ventral regions (Figure 1a,b), we rubbed clean cotton swabs on the dorsal and ventral outer regions of the gland. Cotton swabs from each region were placed in separate vials containing 50 mL of 50% acetonitrile (ACN, Sigma-Aldrich). We used one cotton swab per individual for the dorsal and one for the ventral region ($n$ = 136 males).

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).** To verify the protein profile of secretions of the inner region of the arthrodial membrane gland, we ran the samples in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE[14]). The extracts were stained with Coomassie-R blue. The pure extract (20 μg) of the inner secretion solubilized in 20 μL of ultrapure water with a sample buffer was injected in each lane. It was then subjected to electrophoresis under no reducing conditions on 15% SDS-polyacrylamide gel, using Invitrogen SeeBlue (Life Technologies, São Paulo, Brazil) as a molecular weight marker. Since the extraction of the gland secretion involves the perforation of animal tissue, we used the hemolymph as a means of extraction control. After the animals ($n$ = 5) were anesthetized at 4 °C, we cut the patellar region of leg IV and collected the hemolymph (∼15 μL) with a micropipette. Hemolymph was analyzed on polyacrylamide gel under the same conditions as described for the inner secretion.

**Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC).** Here, our aim was to compare the chromatogram profile of three different regions of the gland, "inner", outer "dorsal", and "ventral". RP-HPLC analysis was performed in a 60 min gradient at a 1 mL/min flow rate. We ran RP-HPLC separation with a C18 column (Jupiter, 4.6 mm × 250 mm) equilibrated with 0.05% trifluoroacetic acid (TFA). The elution gradient for the sample was 0−80% of solution B (0.05% (v/v) TFA in ACN) in solution A (0.05% (v/v) TFA in water). Effluent absorbance was monitored at


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225 nm, and the fractions were hand collected, concentrated under vacuum, and reconstituted in ultrapure water. For both the inner and outer regions (dorsal and ventral), we used a 10 $\mu$g/$\mu$L of secretion for the chromatograms.

**Mass Spectrometry (LC/MS).** The fractions obtained on the RP-HPLC analysis were run into a nLC Easy (Thermo Fisher Scientific, Bremen, Germany) coupled to an LTQ XL Mass Spectrometer (Thermo Fisher Scientific). The samples were diluted in 10 $\mu$L of 0.1% formic acid (FA) prior to injection. Liquid chromatography was performed using a house-prepared analytical and precolumn C18 column as previously described.[15] A linear gradient from 5 to 80% of acetonitrile in 0.1% FA during 15 min at a 1 $\mu$L/min flow was used. The spectrometer was set to a positive parameter.

**Bioinformatics.** To identify the molecules, the raw files generated by the LTQ XL Mass Spectrometer were loaded in MASCOT Deamon R (Matrix Science, Inc., Boston, MA) version 5.4.2 software. Since there is no harvestman database in the Uniprot/Swissprot databank, we ran the search against the Arthropoda databank downloaded from Swiss-Prot in July 2019. We use the following parameters for the search: enzyme = none; variable modifications = "oxidation (M)"; fixed modifications = none; mass values = monoisotopic; protein mass = unrestricted; protein and peptide mass tolerance = "1 Da"; significance threshold = $p < 0.05$; and ion score or expected cutoff = −1. We use only predicted proteins with a score greater than or equal to 40. This score was chosen as the cutoff between real hits and possible false positive results.

The peptides and proteins identified in the secretion of the arthrodial membrane gland of *M. cuspidatus* were further searched in the site UNIPROT for their biological function.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c02525.

List of proteins identified in *M. cuspidatus*; functional classification of proteins found in *M. cuspidatus*; sequences of some proteins identified in *M. cuspidatus*; nonreducing SDS-PAGE of crude extract of the inner content of the arthrodal membrane gland; mass spectrum of five studied proteins; and deconvolution plot, mass error, and peptide sequence obtained from the main proteins (inner, dorsal, and ventral) via MASCOT software (PDF)

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### Author Contributions

Conceptualization and methodology: N.F.d.S.S., R.H.W., P.I.d.S.J., and J.R.M.C.d.S.; validation, formal analysis, investigation, resources, and data curation: N.F.d.S.S.; writing—original draft preparation, writing—review and editing, and visualization: N.F.d.S.S. and R.H.W.; supervision: R.H.W., P.I.d.S.J., and J.R.M.C.d.S.; project administration and funding acquisition: N.F.d.S.S., P.I.d.S.J., and J.R.M.C.d.S. All authors have read and agreed to the published version of the manuscript.


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### Notes

The authors declare no competing financial interest.

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