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HEMÓCITOS DE CARANGUEJEIRAS DA FAMÍLIA THERAPHOSIDAE: ULTRACARACTERIZAÇÃO E PURIFICAÇÃO DE PEPTÍDEOS ANTIMICROBIANOS

TATIANA SOARES

ORIENTADORA: PATRÍCIA MARIA GUEDES PAIVA

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Tese apresentada para o cumprimento das exigências para obtenção do título de Doutor(a) em Bioquímica e Fisiologia pela Universidade Federal de Pernambuco – UFPE.

Orientadora: Patrícia Maria Guedes Paiva

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Tese apresentada para o cumprimento parcial das exigências para obtenção do título de Doutor em Bioquímica e Fisiologia pela Universidade Federal de Pernambuco

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RESUMO

Alguns aracnídeos possuem uma ampla distribuição geográfica e podem viver mais de duas décadas; eles estão presentes desde a Era Paleozóica há cerca de 300 milhões de anos. Esses aracnídeos são as tarântulas, especificamente as aranhas pertencentes ao gênero *Lasiodora*. Infelizmente o gênero ainda encontra-se sob uma revisão sistemática e necessita de informações e compilações científicas de qualquer natureza. Com o intuito de enriquecer as informações sobre esse gênero a presente Tese descreve, pela primeira vez, a caracterização dos hemócitos de *Lasiodora* sp. quanto a ultraestrutura e a purificação de peptídeos antimicrobianos a partir dessas mesmas células. Adicionalmente, apresenta um artigo de revisão que tem como foco os hemócitos da *Lasiodora* sp. sob aspectos de purificação de biomoléculas, caracterização celular e situação taxonômica do gênero. Seis tipos celulares de hemócitos foram identificados e classificados de acordo com a literatura existente, nomeados prohemócitos, granulócitos tipo I e II, esferulócitos, oenocitóide e plasmatócitos. Quanto à purificação de peptídeos antimicrobianos (AMPs) as tarântulas: *Lasiodora* sp., *Acanthoscurria rondoniae* e *Vitalius sorocabae*, migalomorfas da família Theraphosidae, foram escolhidas para testar a hipótese de que moléculas podem ser conservadas em organismos do mesmo táxon. Os componentes antimicrobianos de *Lasiodora* sp. (L), *A. rondoniae* (A) e *V. sorocabae* (V) foram pré-purificados por cromatografia com concentrações crescentes de acetonitrila (5%, 40% e 80%). O material eluído na concentração de 40% de acetonitrila foi submetido a um fracionamento por cromatografia em fase reversa (RP-HPLC), resultando em frações com atividade antimicrobiana. Todas as frações isoladas foram analisadas quanto ao efeito no crescimento da bactéria Gram-positiva *Micrococcus luteus* A270, Gram-negativa *Escherichia coli* SBS363 e levedura *Candida albicans* MDM8. As frações L1, A1 e V1 inibiram o crescimento de *E. coli*, *M. luteus* e *C. albicans*. As frações L2, A2 e V2 apresentaram atividade antimicrobiana somente contra *E. coli* enquanto as frações L3, A3, e V3 apresentaram atividade antimicrobiana contra *E. coli* e *C. albicans*. Todas as frações isoladas foram submetidas à espectrometria de massas (ESI-MS). As frações L1, A1 e V1 correspondem ao peptídeo gomesina, as frações L2, A2 e V2 ao peptídeo migalina, e as frações L3, A3 e V3 ao peptídeo acanthoscurrina. O estudo contribui para a caracterização inédita do gênero e para a elucidação de biomoléculas em hemócitos de aranhas.

Palavras-chave: hemócitos, ultraestrutura, peptídeos antimicrobianos, *Lasiodora* sp, Theraphosidae.

ABSTRACT

Some arachnids have a wide geographical distribution and can live more than two decades; they are present from the Paleozoic Era about 300 million years. These arachnids are tarantulas, specifically spiders belonging to *Lasiodora* genre. Unfortunately the genre still is under a systematic review and needs information and scientific compilations of any kind. In order to provide more details about this genre this thesis describes for the first time, the characterization of hemocytes *Lasiodora* sp. as the ultrastructure and purification of antimicrobial peptides from these same cells. Additionally, features a review article focuses on the hemocytes of *Lasiodora* sp. under aspects of purification of biomolecules, cell characterization and taxonomic situation. Six cell types of hemocytes were identified and classified according to the literature, named prohemocytes, granulocyte type I and II, spherulocytes, oenocytes and plasmatocytes. For the purification of antimicrobial peptides (AMPs) of tarantulas: *Lasiodora* sp., *Acanthoscurria rondoniae* and *Vitalius sorocabae*, mygalomorphs from Theraphosidae family, were chosen to test the hypothesis that molecules can be conserved in the same taxon. The antimicrobial components of *Lasiodora* sp. (L) *A. rondoniae* (A) and *V. sorocabae* (V) was pre-purified by chromatography with increasing concentrations of acetonitrile (5%, 40% and 80%). The material eluted at a concentration of 40% acetonitrile was subjected to fractionation by reversed phase chromatography (RP-HPLC), resulting in fractions with inhibitory activity. All the isolated fractions were analyzed for their effect on the growth of Gram-positive bacterium *Micrococcus luteus* A270, gram negative *Escherichia coli* and yeast *Candida albicans* SBS363 MDM8. The fractions L1, A1 and V1 inhibited the growth of *E. coli*, *C. albicans* and *M. luteus*. The L2 fractions, A2 and V2 showed antimicrobial activity against *E. coli* while only fractions L3, A3, and V3 showed antimicrobial activity against *E. coli* and *C. albicans.* All isolated fractions were subjected to mass spectrometry (ESI-MS). The fractions L1, A1 and V1 correspond to gomesin peptide fractions; the L2, A2 and V2 mygalin and L3, A3 and V3 the acanthoscurrin. The study contributes to the unprecedented characterization of the genus and the elucidation of biomolecules in hemocytes of spiders.

Keywords: hemocytes, ultrastructure, antimicrobial peptides, *Lasiodora* sp, Theraphosidae.

LISTA DE ABREVIATURAS

A - Frações peptídicas isoladas por HPLC a partir de hemócitos de *Acanthoscurria rondoniae*

AIDS- Síndrome da Deficiência da Imunidade Adquirida

AMPs – Antimicrobial Peptides

ESI-MS – Espectrometria de Massa por ionização por Eletrospray

HPLC – High Performance Liquid Chromatography

IL-2 – Interleucina 2

L – Frações peptídicas isoladas por HPLC a partir de hemócitos de *Lasiodora sp.*

LPS - Lipopolissacarídeo

SDS-PAGE – Eletroforese em gel de poliacrilamida com sódio dodecil sulfato

TFA – Ácido trifluoroacético

UFPE – Universidade Federal de Pernambuco

UPE – Universidade de Pernambuco

V- Frações peptídicas isoladas por HPLC a partir de hemócitos de *Vitalius sorocabae*

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1. INTRODUÇÃO

Invertebrados estão constantemente expostos a infecções microbianas, e para se protegerem eles desenvolveram poderosos mecanismos de defesa imunológica semelhante à resposta inata dos vertebrados. Estes mecanismos envolvem respostas celulares e humorais. O primeiro consiste em encapsulamento, nodulação, e, fagocitose de microrganismos por hemócitos; enquanto a resposta humoral compreende fatores relacionados ao reconhecimento dos microrganismos invasores, cascatas de serinoproteases participando da melanização, fatores da coagulação agindo como peptídeos antimicrobianos (AMPs), espécies reativas de oxigênio, e intermediários reativos de nitrogênio (HOEBE *et al.*, 2004; FUKUZAWA *et al.*, 2008).

Mais de 2500 AMPs produzidos por organismos unicelulares (como os protistas) e multicelulares (como os fungos, plantas, invertebrados e vertebrados) têm sido isolados e caracterizados, sendo essas informações estocadas em bancos de dados (BULET *et al.*, 2004; CASTRO & FONTES, 2005; HANCOCK & SAHL., 2006; WANG *et al*., 2009; KHAMIS *et al*., 2015).

Dependendo do organismo e do tecido considerado, os AMPs são armazenados dentro das células secretoras, ou a sua síntese é induzida no momento da infecção (CERENIUS & SÖDERHALL, 2012). Na maioria dos insetos, a síntese de AMP começa algumas horas após uma infecção. Em outros invertebrados, como os caranguejos-ferradura, mexilhões e camarões, os AMPs são constitutivamente produzidos e armazenados em grânulos nos hemócitos (MITTA *et al*., 2000; BACHERE *et al*., 2004; BULET & STOCKLIN, 2005; IWANAGA & LEE, 2005; FUKUZAWA *et al*., 2008; BAUMANN *et al*., 2010).

AMPs armazenados em grânulos nos hemócitos interagem com os organismos invasores por dois processos diferentes: (i) através da fusão dos grânulos onde os AMPs são armazenados dentro de fagossomos e/ou (ii) a liberação destes AMPs no plasma por exocitose (MITTA *et al.*, 1999; DESTOUMIEUX *et al*., 2000; MITTA *et al*., 2000).

A hemolinfa de invertebrados é a principal fonte de peptídeos antimicrobianos (GHOSH *et al*., 2002). Diversos peptídeos antimicrobianos foram isolados a partir do veneno e da hemolinfa de artrópodes, como nos escorpiões (KHAMIS *et al*., 2015).

Gomesina foi o primeiro peptídeo antimicrobiano isolado a partir de hemócitos da aranha *Acanthoscurria gomesiana* (SILVA-JÚNIOR *et al*., 2000). Da mesma espécie foram extraídos ainda outros AMPs com atividade antimicrobiana contra bactérias e/ou fungos sendo eles a theraphosinina (SILVA-JÚNIOR *et al*., 2000), a gomesina (SILVA-JÚNIOR *et al*., 2000), a acanthoscurrina (LORENZINI *et al*., 2003) e a migalina (PEREIRA *et al*., 2007). A partir dos hemócitos da *A. rondoniae* foi purificado o AMP rondonina (RICILUCA *et al*., 2012) com atividade antifúngica, e, até o momento não há relatos de AMPs isolados de *V. sorocabae*.

O gênero *Lasiodora* passa por uma revisão sistemática (coordenada pelo Instituto Butantan) e estudos realizados têm contribuído para o avanço do conhecimento sobre o mesmo. O inibidor de elastase EILaH (do inglês *Elastase Inhibitor from Lasiodora* sp. *Hemocytes*) foi purificado a partir dos hemócitos de *Lasiodora* sp. (SOARES *et al*., 2011) e há relatos da presença de toxinas e AMPs em veneno da mesma espécie (ESCOUBAS *et al*., 1987; KUSHMERICK *et al.*, 2001; KALAPOTHAKIS *et al.*, 2003; VIEIRA *et al.*, 2004; VIZZOTTO, 2009; GUETTE *et al*., 2006; DUTRA *et al*., 2008; HORTA *et al*., 2013; RATES *et al.*, 2013). Estudos sobre a morfofisiologia dos hemócitos são ainda inéditos e até o momento não foram isolados AMPs de hemócitos de *Lasidora* sp..

Fêmeas (n=10) adultas do gênero *Lasiodora* (Figura1), no estágio intermuda, coletadas em Paudalho-PE-Brasil foram utilizadas na presente Tese. Foram escolhidas fêmeas, pois as mesmas possuem comportamento menos agressivo e são mais fáceis de manipular já que são maiores que os machos.

Figura 1. Representante de *Lasiodora* sp..Tamanho da barra: 4cm

Fonte: FERREIRA, 2006.

A detecção dos peptídeos antimicrobianos e avaliação de seu modo de ação podem contribuir para um entendimento mais amplo dos processos envolvidos no sistema imune dos aracnídeos e dos artrópodes em geral. Além disso, a descrição e caracterização de novas moléculas com atividade antimicrobiana podem contribuir também para a produção e utilização de novas drogas na medicina e agricultura.

2. FUNDAMENTAÇÃO TEÓRICA

2.1 Artrópodes

Os artrópodes constituem um grande filo dentro dos animais invertebrados e podem ser subdivididos em quatro subfilos: Myriapoda, Chelicerata [límulos (Fig. 2), escorpiões (Fig. 3), aranhas (Fig. 4) e ácaros (Fig. 5)], Crustacea (copépodos, cracas, camarões, lagostas e caranguejos) e Insecta (insetos) (STOLLEWERK *et al*., 2001).

Figura 2. O caranguejo-ferradura *Limulus polyphemus*.

Fonte: RI BUGS (2007) Foto de Dann Thombs.

Figura 3. O escorpião *Tityus serrulatus*.

Fonte: FASTSERV (2011).

Figura 4. A aranha *Lasiodora parahybana*.

Fonte: SKLIPKANI (2002).

Figura 5. O carrapato *Boophilus microplus.*

Fonte: USP/ICB Foto de Marcelo Palmeira (2011).

Os artrópodes ocupam quase todos os nichos ecológicos e estão constantemente expostos ao ataque de inúmeros inimigos naturais, muitos dos quais são potencialmente patogênicos. Para sobreviver a esses ataques, desenvolveram um eficiente sistema de defesa, sendo a imunidade inata a primeira linha de proteção contra bactérias, fungos e patógenos virais (FUKUZAWA *et al.*, 2008).

Em contraste com insetos e crustáceos, para a maioria dos grupos de quelicerados o sistema imune não é bem investigado. Isso é especialmente verdadeiro para animais pertencentes a um táxon numeroso e ecologicamente importante como as aranhas e escorpiões, que contêm espécies de interesse médico (KUHN-NENTWIG, 2014).

2.2 Aranhas

As aranhas (ordem Araneae) são o mais diverso e bem sucedido grupo de invertebrados terrestres, excluindo os insetos, os quais são suas presas primárias (RASH e HODGSON, 2002). Estão distribuídas em praticamente todo o planeta habitando todos os ecossistemas, com exceção do ar e do mar aberto (FERREIRA, 2006).

As aranhas pertencem ao filo Artropoda, subfilo Chelicerata, classe Aracnida, ordem Aranea (STOLLEWERK *et al.*, 2001). A ordem Aranea pode ainda ser dividida em 2 subordens: Mesothelae, que compreende uma única família de aranhas primitivas (Liphistiidae) com 85 espécies documentadas. As aranhas da subordem Mesothelae são caracterizadas por apresentarem o abdômen segmentado, enquanto que as aranhas da outra subordem, Opisthothelae, não apresentam segmentação externa do abdômen. A subordem Opisthothelae é dividida em duas Infra-ordens: Labdognatha e Orthognatha. A maioria das Labdognathas respira através de um par de pulmões libriformes e um par de traquéias, que permitem uma troca gasosa mais eficiente, permitindo que sejam menos sedentárias que as Orthognathas. As Labdognathas possuem quelíceras diaxiais, que se movimentam de um lado para o outro e sintetizam a seda para a construção de teias (DUTRA, 2006). As Orthognathas possuem quelíceras paraxiais, que se movimentam de cima para baixo, e normalmente sintetizam a seda apenas para construir a ooteca e linhas de reboque. Com algumas poucas exceções, não constroem teias elaboradas (DUTRA, 2006). Este grupo inclui as maiores aranhas conhecidas.

As aranhas migalomorfas (Arthropoda, Chelicerata, Araneae, Mygalomorphae) compreendem as maiores aranhas vivas, comumente chamadas de "tarântulas" (BERTANI *et al*., 2012). Com quase mil espécies descritas (PLATNICK, 2015), é uma infra-ordem de aranhas abundante. Uma das famílias de migalomorfas é a Theraphosidae representada por cerca de 980 espécies distribuídas em 128 gêneros (sendo *Lasiodora* um desses) ocorrendo em diferentes habitats (PLATNICK, 2015).

As migalomorfas são caracterizadas por suas quelíceras paraxiais que estão situadas paralelamente ao corpo, e se movem de cima para baixo. Possuem dois pares de pulmões foliáceos desenvolvidos e ausência de traquéia, o que confere a característica primitiva do grupo (FERREIRA, 2006).

Morfologicamente, o corpo de uma aranha consiste de duas partes principais: uma porção anterior, o prossoma ou cefalotórax, e uma parte posterior, o opistossoma ou abdome, que são conectadas por uma estrutura denominada pedicelo. O cefalotórax suporta quatro pares de pernas, um par de quelíceras em frente à boca (característica que determina o subfilo Chelicerata) e um par de pedipalpos localizados entre a quelícera e o primeiro par de pernas, que nos machos são modificados na sua extremidade em órgãos copuladores. Ainda no prossoma são encontrados os olhos do animal que podem ser em número de dois, seis ou oito, e são de extrema importância na taxonomia do grupo. O abdome por sua vez abriga os sistemas respiratório, circulatório, digestivo e reprodutor (FOELIX, 1996).

As aranhas como todos os artrópodes, apresentam um exoesqueleto e durante o crescimento passam pelo processo de muda. Em geral, na maioria das aranhas, quando o estágio reprodutivo é atingido cessam o crescimento e as trocas de exoesqueleto. Nas grandes caranguejeiras, diferentemente das outras aranhas, as fêmeas adultas continuam realizando a muda uma vez ao ano ou em intervalos irregulares (SILVA-JÚNIOR, 2000).

2.2.1 Classificação do gênero *Lasiodora*

A aranha brasileira *Lasiodora* sp. (Mygalomorphae, Theraphosidae), é conhecida com o nome trivial de caranguejeira (HORTA *et al.*, 2013), somente membros da

família Theraphosidae são tarântulas verdadeiras (ESCOUBA e RASH, 2004).

As tarântulas podem ser encontradas em áreas tropicais e semitropicais, savanas, desertos, florestas úmidas ou ambientes semitemperados. Representantes do gênero *Lasiodora* sp. ocorrem principalmente na região Nordeste do Brasil, especialmente na Floresta Atlântica, havendo registros ainda na região Sudeste e Centro-Oeste do país (BERTANI, 2001).

O gênero, pertencente à família Theraphosidae, descrito por C. Koch em 1850 (BERTANI, 2001; BERTANI, 2012), encontra-se atualmente com sua classificação em andamento coordenada pelo Dr. Rogério Bertani do Instituto Butantan, São Paulo, Brasil (Tabela 1). Até agora são 39 espécies pertencentes ao gênero *Lasiodora*, de acordo com Platnick (2015).

Tabela 1. Classificação do gênero *Lasiodora*.

Filo	Arthropoda
Subfilo	Chelicerata
Classe	Arachnida
Ordem	Araneae
Sub-ordem	Ophistothelae
Infra-ordem	Mygalomorphae
Família	Theraphosidae
Gênero	*Lasiodora*

Das 37.972 espécies de aranhas descritas até o momento (totalizando 3526 gêneros), apenas 860 espécies pertencem à família Theraphosidae, composta por 107 gêneros, dentre eles podemos citar o gênero *Acanthoscurria* (Fig. 6) (ESCOUBAS e RASH, 2004).

Figura 6. Representantes do gênero *Acanthoscurria gomesiana* (à esquerda) e *A. rondoniae* (à direita).

Fonte: SILVA-JÚNIOR, 2000; RICILUCA *et al*., 2012, respectivamente.

Lasiodora sp. (Fig. 7) em relação às demais aranhas é de grande porte, podendo atingir 25 cm de comprimento e seus exemplares apresentam cor preta ou marrom e possui no abdome pêlos urticantes tipo I e III e/ou IV, que podem ser lançados quando o animal sente-se ameaçado (FERREIRA, 2006).

Figura 7. *Lasiodora sp.* Barra: 4 cm.

Foto: Tatiana Soares.

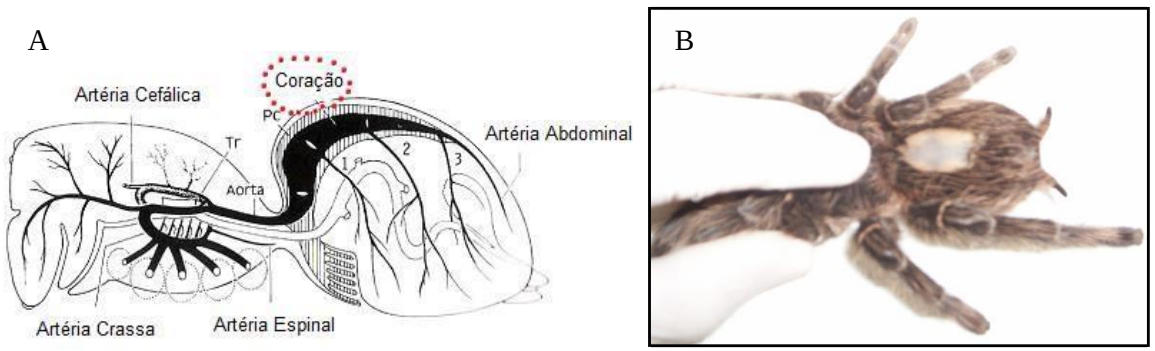
As dificuldades taxonômicas da família Theraphosidae são bem conhecidas (RAVEN, 1985), e mesmo com o pequeno avanço nesses últimos anos ainda há muito que ser feito (BERTANI *et al*., 2012). As diferentes espécies de *Lasiodora* são difíceis de distinguir (BRAZIL e VELLARD, 1926) o que agrava ainda mais esse conflito taxonômico.

Uma identificação morfológica confiável de tarântulas é mais difícil devido às características muito semelhantes de muitas espécies (ESCOUBAS e RASH, 2004). Os métodos clássicos são baseados no exame dos órgãos genitais masculinos, forma de apêndices do corpo e na contagem de pêlos. Além disso, a dificuldade de acesso aos habitats, muitas vezes significa que a classificação é baseada em alguns espécimes preservados (ESCOUBAS e RASH, 2004). Mais trabalho de campo é necessário a fim de recolher espécimes, e aplicar técnicas modernas de identificação biotecnológicas.

2.3 Hemolinfa e Hemócitos

As aranhas possuem um sistema circulatório aberto por onde corre um fluido corporal análogo ao sangue dos vertebrados: a hemolinfa (FERREIRA, 2006). O coração, órgão responsável pela circulação desse líquido, é localizado dorsalmente no interior do opistossoma ou abdome (Fig. 8), formado por um tubo muscular suspenso por ligamentos dorsais, laterais e ventrais (FOELIX, 1996).

Figura 8. Sistema circulatório de aranhas. A) Esquema do sistema circulatório. Destaque para o vaso dorsal (coração). Fonte: adaptado FOELIX (1996). B) Visualização do coração por transparência após raspagem dos pelos dorsais da aranha *Lasiodora* sp..



Fonte: foto Tatiana Soares.

A hemolinfa fresca de uma aranha apresenta cor azulada devido à presença de cobre contido no pigmento respiratório hemocianina, e exibe uma grande variedade de células denominadas hemócitos. Segundo FOELIX (1996), substâncias orgânicas presentes nesse fluido incluem proteínas, como a hemocianina (cerca de 80%), aminoácidos livres (principalmente a prolina), carboidratos (glicose) e ácidos graxos (palmítico, linoléico e esteárico).

Em quelicerados e crustáceos, a hemocianina parece desempenhar um papel imunológico importante e participa no sistema de imunidade inata (CERENIUS & SÖDERHÄLL, 2004; NAGAI *et al.*, 2001). *In vitro*, componentes da cascata de coagulação e diversos fatores antimicrobianos derivados dos hemócitos podem induzir a hemocianina a expressar atividade de fenoloxidase (NAGAI *et al*., 2001; ADACHI *et al*., 2003).

Hemocianinas são proteínas multiméricas envolvidas na ligação e transporte de oxigênio em todas principais linhagens de artrópodes (COATES & NAIRN, 2013; STARRETT *et al*., 2013). Evidências reunidas recentemente revelaram as múltiplas funcionalidades da hemocianina (COATES & NAIRN, 2014). *Lasiodora erythrocythara* foi o primeiro organismo do gênero estudado a determinar a influência de cofatores orgânicos, o efeito na especificidade relacionada com o CO_2 e temperatura na afinidade do oxigênio e hemocianina (BRIDGES, 1988).

Em quelicerados e crustáceos, a hemocianina desempenha uma função imunológica importante, assim como na homeostasia e muda (CERENIUS & SÖDERHÄLL, 2004; NAGAI *et al.*, 2001; ADACHI *et al.*, 2003; GLAZER *et al.*, 2013; KUBALLA & ELIZUR, 2008; KUBALLA *et al*., 2011). Experimentos *in vitro* demonstraram que componentes da cascata de coagulação e fatores antimicrobianos podem induzir a hemocianina a expressar atividade de fenoloxidase (NAGAI *et al.*, 2001; ADACHI *et al*., 2003; BAIRD *et al*., 2007; JEANICKE & DECKER, 2008; KUBALLA *et al*., 2011), atividade antimicrobiana (RICILUCA *et al*., 2012; COATES & NAIRN, 2014; QIU *et al*., 2014) e atividade antiviral (ZHANG *et al*., 2004; PAN *et al*., 2005).

A hemolinfa de artrópodes é amplamente estudada em seu aspecto bioquímico; lectinas, inibidores de protease e peptídeos antimicrobianos tem sido isolado a partir de plasma e hemócitos. Como exemplo, já foram descritos o inibidor de protease no plasma do bicho-da-seda *Antheraea mylitta* (SHRIVASTAVA & GHOSH, 2003), o inibidor de serino proteinase encontrado no plasma da lagarta *Manduca sexta* (WANG e JIANG, 2004), o inibidor tripsina e subtilisina dos hemócitos do camarão *Litopenaeus vannamei* (VEGA e ALBORES, 2005), e o peptídeo antimicrobiano dos hemócitos do carrapato *B. microplus* (FOGAÇA *et al*., 2006). Nosso grupo de trabalho caracterizou um inibidor de elastase de neutrófilos humanos purificado a partir dos hemócitos da *Lasiodora* sp. (EILaH) com 8274 Da com a sequência amino terminal LPC(PF)PYQQELTC (SOARES *et al*., 2011).

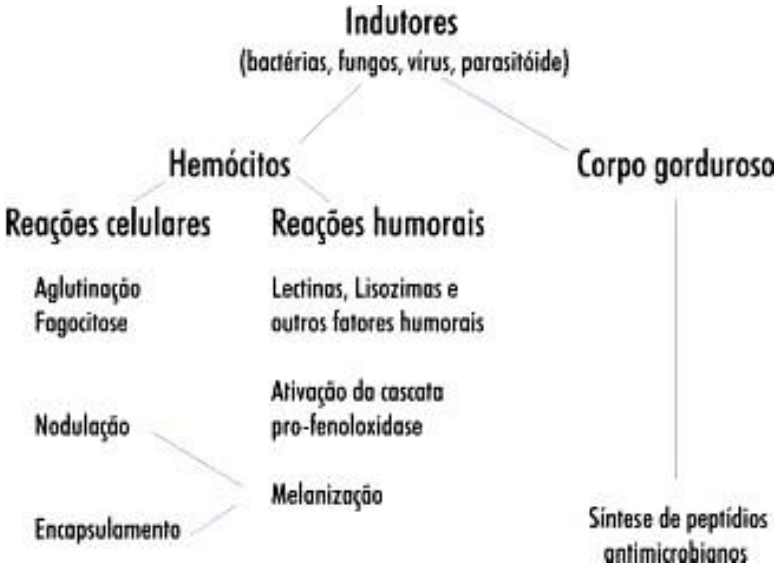
Os hemócitos circulantes parecem estar envolvidos na coagulação da hemolinfa e no combate às infecções, sendo extremamente sensíveis ao lipopolissacarídeo bacteriano respondendo através da liberação de componentes granulares (IWANAGA & LEE, 2005). Estruturalmente são distinguidos quatro tipos de células, sendo os mais comuns os granulares, que apresentam muitos grânulos densos concentrados em seu citoplasma, atribuindo-lhes a função de esclerotização da exocutícula (FOELIX, 1996), outros parecem atuar como fagócitos ou células de armazenagem, ou ainda impedindo o extravasamento da hemolinfa (MUTA & IWANAGA, 1996). Fukuzawa e colaboradores (2008) observaram três tipos de hemócitos na *A. gomesiana*: os prohemócitos, os granulócitos e os cianócitos. Durante a troca de exoesqueleto, ou muda, a porcentagem relativa de diferentes tipos de hemócitos é alterada drasticamente.

Os hemócitos têm a habilidade de defender os invertebrados contra patógenos, parasitas e outros corpos estranhos, que penetrem na hemocele. As reações de defesa são mediadas pela fagocitose, encapsulamento e reparação de danos (LAVINE e STRAND, 2002). Em insetos, esses mecanismos de defesa são bem caracterizados (Fig. 9).

Injúrias mecânicas ou a presença de objetos estranhos como microrganismos resultam na deposição de melanina ao redor do tecido danificado ou do corpo estranho. A melanina servirá fisicamente de escudo a um invasor e, portanto, impede ou retarda o seu crescimento, mas talvez ainda mais importante durante a formação da melanina, é a produção de intermediários altamente reativos e tóxicos como as quinonas (CERENIUS

e SÖDERHÄLL, 2004). Todo o mecanismo de melanização faz parte do sistema de imunidade inata, e diversas proteínas presentes na hemolinfa são envolvidas nesse processo, dentre elas, proteases da cascata de coagulação. A ativação dessas proteases é cuidadosamente regulada pelo sistema da fenoloxidase que consiste numa cascata de proteínas capazes de se ligar a polissacarídeos e outro composto tipicamente associado a microrganismos, tais como peptidoglicanos e lipopolissacarídeos (SILVA, 2002).

Figura 9. Representação esquemática das respostas imunológicas nos insetos.

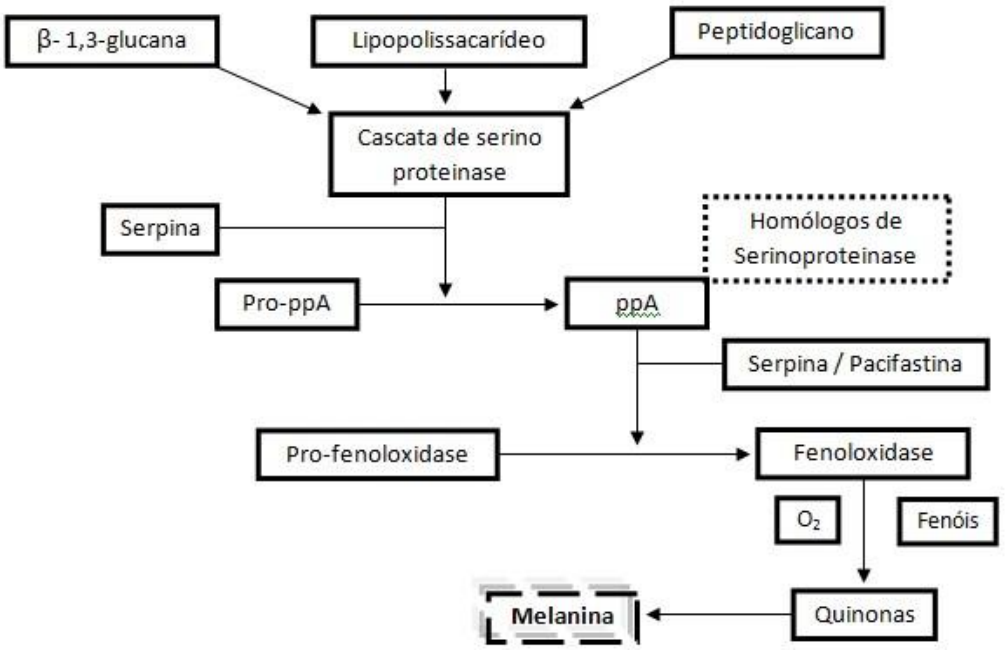


Fonte: SILVA (2002).

Fenoloxidase é uma enzima que cataliza a oxidação de compostos fenólicos presentes na hemolinfa. O produto final dessa oxidação é a melanina, que participa de três importantes processos fisiológicos: esclerotização da cutícula, cicatrização de lesões e defesas imunológicas (AZZOLINI, 2003). A fenoloxidase encontra-se como uma pro-enzima, chamada pro-fenoloxidase que é ativada proteoliticamente por uma ou duas serino proteases em resposta ao lipopolissacarídeo (LPS, componente da parede celular das bactérias Gram-negativas), peptidoglicanos (componente celular das bactérias Gram-positivas), β-1,3 glicanos (componente da parede celular de fungos e algas), parasitóides, enzimas proteolíticas (tripsina e quimotripsina) e injúrias nos tecidos. Oxidações subseqüentes de fenóis pela fenoloxidase levam à produção de quinonas que são polimerizadas para formar melanina (Fig. 10) (NAPPI e OTTAVIANI, 2000;

CERENIUS e SÖDERHÄLL, 2004; AZZOLINI, 2006).

Figura 10. Sistema ativador da pro-fenoloxidase (proPO) de artrópode. O sistema é ativado pelo reconhecimento protéico de β-1,3-glicanos, lipopolissacarídeos, peptidoglicanos, ou por outros componentes como fatores endógenos produzidos sobre a lesão tecidual. A cascata de serinoproteinases, a qual não foi ainda caracterizada, pode resultar na clivagem do zimogênio da enzima ativadora da pro-fenoloxidase (pro-ppA) em fenoloxidase ativa.



Fonte: adaptado CERENIUS e SÖDERHÄLL (2004).

As proteases da cascata da fenoloxidase ainda não estão bem caracterizadas, mas é proposto que essas sejam precisamente reguladas através da presença de inibidores de proteases específicos que previnem uma ativação descontrolada (CERENIUS e SÖDERHÄLL, 2004).

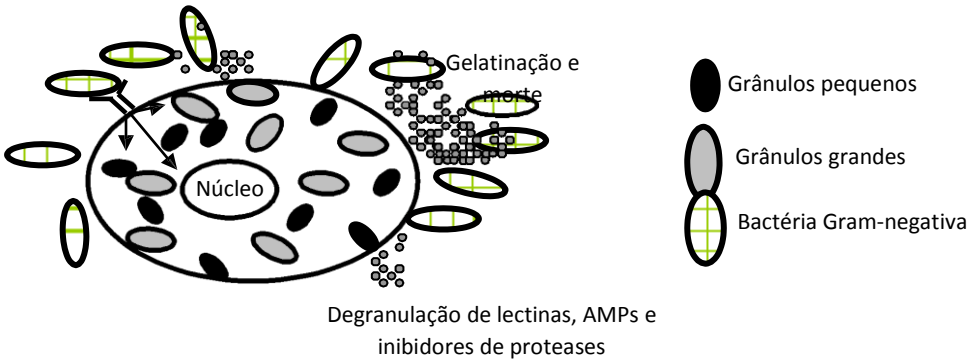
Uma vez atingido o local de infecção os hemócitos podem secretar componentes da cascata de coagulação e peptídeos antimicrobianos na cavidade livre da hemocele (FUKUZAWA *et al.,* 2008). A produção de peptídeos antimicrobianos mediados por receptores semelhantes a Toll, coagulação da hemolinfa, formação da melanina e ativação do complemento mediado por lectinas são as respostas imunes mais proeminentes (IWANAGA e LEE, 2005). O fenômeno da coagulação da hemolinfa foi primeiramente identificado como um sistema de defesa no caranguejo-ferradura *L.*

polyphemus, por BANG (1956).

No caranguejo-ferradura, os hemócitos são conhecidos como granulócitos ou amebócitos, por possuírem grânulos de vários tamanhos, e essas células são bastante sensíveis à endotoxinas bacterianas, em geral o LPS. Quando detecta essa molécula em suas superfícies, os hemócitos liberam seus grânulos através de uma rápida exocitose (Fig. 11). Dois componentes granulares liberados da reação de coagulação são os fatores C e G. Esses zimogênios de serino proteases são autocataliticamente ativados pelo LPS e β-1,3-D-glicano, principais componentes da parede celular de bactérias Gram-negativas e fungos, respectivamente (MUTA e IWANAGA, 1996); a ativação resulta na transformação do coagulogênio em coagulina (Fig. 12). Os invasores da hemolinfa ou hemocele são fagocitados ou imobilizados pelo coágulo e em seguida são mortos por ação de lectinas, substâncias antimicrobianas e inibidores de proteases encontradas nos grânulos.

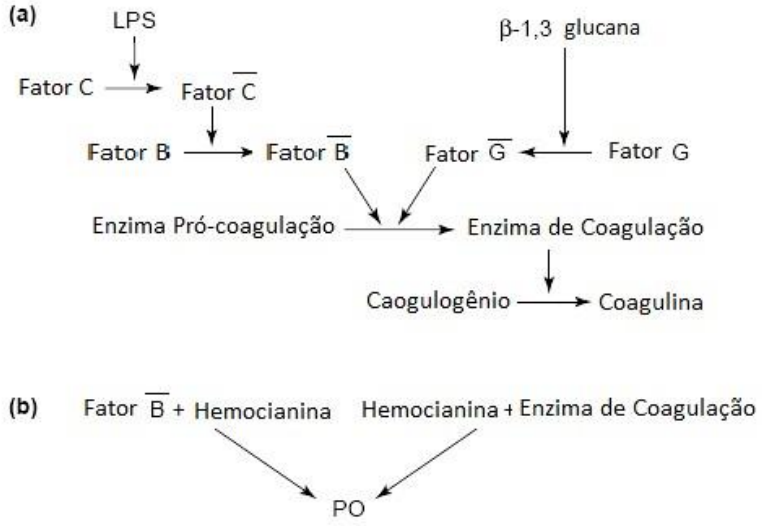
Os hemócitos estão sendo bastante investigados, porque contêm uma diversidade de peptídeos antimicrobianos, lectinas, proteases e inibidores de proteases.

Figura 11. Sistema de defesa nos hemócitos do caranguejo-ferradura. O hemócito detecta LPS nas bactérias Gram-negativas e inicia a exocitose dos grandes e pequenos grânulos. Os fatores de coagulação são ativados por LPS ou β-1,3-D-glicano, resultando na coagulação da hemolinfa. Os grandes grânulos contêm inibidores de proteases.



Fonte: adaptado de Muta e Iwanaga (1996).

Figura 12. Cascata de coagulação em carangueijo-ferradura. (a) A exposição ao LPS leva à proteólise autocatalítica de fator C, ao passo que a exposição a β-1,3-D-glicano resulta na ativação do fator G. Factor C pode atuar como um receptor de reconhecimento padrão. (b) Hemocianina pode ser convertida para fenoloxidase por meio de interações não-catalítica com um fator ativado da enzima B ou de coagulação.



Fonte: Theopold *et al.* (2004).

2.3.2 Peptídeos Antimicrobianos

Doenças infecciosas causadas por fungos e bactérias tem afetado a humanidade desde os primeiros dias de civilização (KHAMIS *et al*., 2015). No entanto, a descoberta da penicilina por Fleming (1929) proporcionou uma potente defesa e sobrevivência dos mamíferos contra patógenos (SILVA *et al*., 2011). Baseadas na penicilina, várias outras moléculas e diferentes classes de antibióticos têm sido desenvolvidas. Contudo, todos esses agentes têm perdido a eficiência e se tornando banais contra cepas bacterianas resistentes.

Esse problema sustenta a busca por novos agentes que possuam atividade antimicrobiana contra espécies resistentes a antibióticos convencionais e incentiva um interesse nos peptídeos de pequeno-médio porte chamados peptídeos antimicrobianos (AMPs) (STEGEMANN e HOFFMANN, 2008). Tais moléculas são produzidas em bactérias, insetos, plantas e vertebrados (GUANÍ-GUERRA *et al*., 2010; PASUPULETI *et al.*, 2012).

AMPs são componentes importantes do sistema imune inato, usados pelo hospedeiro para se proteger de diferentes tipos de patógenos (LATA *et al*., 2007; SUNDARARAJAN *et al*., 2012). Os AMPs possuem um largo espectro de atividade potente contra bactérias Gram-positivas e Gram-negativas, fungos, protozoários, parasitas, células cancerosas e diferentes tipos de vírus (THOMAS *et al*., 2010). Sua ação efetiva de defesa contra um amplo espectro de microrganismos e sua capacidade de matar rapidamente tornaram os AMPs substitutos altamente eficazes para os antibióticos convencionais (PETERS *et al*., 2010; THOMAS *et al*., 2010).

AMPs interagem com microrganismos alvo por permeação e penetração de membrana (BROGDEN, 2005; RADEK e GALLO, 2007), afetando subsequentemente a formação de septos de membrana citoplasmática (mecanismos complementares adicionais envolvem rompimento da parede celular, ácidos nucleicos e proteínas envolvidas na biossíntese), em última análise, matam o organismo alvo (PETERS *et al*, 2010; CHEN *et al*, 2012; FJELL *et al*., 2012).

AMPs constituem um grupo heterogêneo de peptídeos no que diz respeito às suas estruturas primárias e secundárias, seus potenciais antimicrobianos, os seus efeitos sobre as células do hospedeiro, e a regulação da sua expressão (PASUPULETI *et al.*, 2012). A maioria dos AMPs têm uma carga positiva fornecida pelos resíduos de arginina e lisina, e uma estrutura anfipática que lhes permite interagir com membranas bacterianas (EPAND e VOGEL, 1999; AUVYNET e ROSENSTEIN, 2009). Estes peptídeos são moléculas tipicamente curtas com menos de 100 aminoácidos (a maioria tem entre 12 e 50 aminoácidos), existem em todas as classes de vida e são evolutivamente conservados (YEAMAN e YOUNT, 2003; HANCOCK e SAHL, 2006; SANG e BLECHA, 2008; AUVYNET e ROSENSTEIN, 2009; PETERS *et al*, 2010; PASUPULETI *et al.*, 2012). Assim, AMPs foram classificados em famílias e sub-famílias com base em suas sequências primárias e estruturas (KAISER e DIAMOND, 2000; YEAMAN e YOUNT, 2003). No entanto, há evidências experimentais consideráveis que mesmo pequenas variações na estrutura de peptídeos podem conduzir a diferenças significativas na atividade antimicrobiana (THOMAS *et al*., 2010).

Centenas de AMPs naturais foram identificados e caracterizados, com informações em bancos de dados públicos, por exemplo, DAMPD (SUNDARARAJAN

et al., 2012), CAMP (THOMAS *et al.*, 2010), APD2 (WANG *et al*., 2009), APD (WANG e WANG, 2004) e ANTIMIC (BRAHMACHARY *et al*., 2004). O aumento da demanda por AMPs promoveu mais interesse em desenvolver sinteticamente essas moléculas (NUSSLEIN *et al.*, 2006; MARCOS *et al.*, 2008) sendo o projeto de novos AMPs sintéticos um desafio devido à vasta variedade de propriedades que os caracterizam (GURALP *et al*., 2013).

Esforços têm sido feitos para estudar as propriedades físico-químicas de AMPs (PORTO *et al.*, 2010; TORRENT *et al.*, 2011; MACCARI *et al.*, 2013). No entanto, uma abrangente e sistemática análise dessas propriedades, e caracterização de diferentes famílias para ampliação do conhecimento, ainda não estão disponíveis.

Algumas classificações são propostas uma delas leva em consideração a grande variedade estrutural dos peptídeos, e agrupa-os em dois conjuntos característicos de estruturas secundárias: aqueles com estrutura em α-hélice e em folha-β (JENSSEN *et al*., 2006). As estruturas helicoidais ocorrem em grande parte no meio extracelular e são formadas durante a interação do peptídeo com a superfície externa de membranas, pois os peptídeos têm como característica apresentarem conformação randômica em soluções aquosas (YEAMAN e YOUNT, 2003). A estabilidade da forma helicoidal em peptídeos ou proteínas depende de diversos fatores, como, por exemplo, a disposição de resíduos de aminoácidos na sequência peptídica. Resíduos de aminoácidos com cadeias laterais de mesma carga (positiva ou negativa) que estejam muito próximos entre si podem desestabilizar a forma devido a interações repulsivas. A presença de resíduos de prolina, glicina ou de D-aminoácidos na sequência primária destes peptídeos também desestabiliza as estruturas α-helicoidais, podendo ainda afetar a ação destas moléculas contra células bacterianas (PAPO e SHAI, 2003), mostrando que a conformação α-helicoidal é de fundamental importância para a manutenção da atividade biológica.

Estudos recentes mostram ainda que distorções da α-hélice canônica são fundamentais para o modo de ação de peptídeos que penetram membranas fosfolipídicas (peptídeos trans-membrana) (BORDAG e KELLER, 2010). Muitos destes peptídeos podem apresentar adaptações estruturais durante a interação com membranas fosfolipídicas, resultando em uma inclinação ou torção da estrutura helicoidal, e ainda reorientações de cadeias laterais (NYHOLM *et al*., 2007).

Em aranhas, moluscos e camarões, os peptídeos antimicrobianos são sintetizados constitutivamente nos hemócitos e armazenados em seus grânulos (BACHERE *et al.*, 2004). Vizzotto (2009) indica que os peptídeos antimicrobianos encontrados em animais podem ser agrupados de acordo com suas propriedades químicas e estruturais em duas classes: lineares e cíclicos. Os lineares, não apresentam o aminoácido cisteína em sua composição, e podem ser subdivididos nos que formam uma α–hélice anfipática após contato com a membrana celular e os cíclicos em um determinado tipo de aminoácido, tais como prolina, histidina e triptofano. Os cíclicos são peptídeos que apresentam resíduos de cisteína em sua estrutura, podendo ter extremidades amino-terminal abertas ou fechadas (VIZZOTTO, 2009).

Vários peptídeos antimicrobianos foram isolados a partir do veneno e da hemolinfa artrópodes, como escorpiões e aranhas (KUHN-NENTWIG, 2003). Utilizando a aranha caranguejeira *A. gomesiana*, Silva-Júnior (2000) purificou e caracterizou quatro moléculas presentes em sua hemolinfa (Tabela 2).

Tabela 2. Peptídeos antimicrobianos presentes na aranha *Acanthoscurria gomesiana*. Fonte: Silva-Júnior (2000).

Peptídeos	Fonte	Massa Molecular	Atividade Antimicrobiana	Seqüência de aminoácidos	Similaridade
Theraphosinina	Plasma	4.052,5 Da	Batéria Gram-positiva: *M. luteus* A270	**ETDXEAHRXRASRGPLV NDINGXENGXYNPN** (31 aminoácidos – seqüência parcial)	Não apresenta similaridade com proteínas e peptídeos antimicrobianos
Mygalomorphina	Hemócitos	415,9 Da	Bactéria Gram-negativa: *E. coli* SBS363	**.....Cys-Asx.....** (seqüência parcial)	5-S-GAD de dípteros
Gomesina	Hemócitos	2.270 Da	Bactérias Gram-negativas e Gram-positivas, Fungos e *Leishmania (L.) amazonensis*	**Z*CRRLCYKQRCVTYCR GR** (18 aminoácidos – seqüência total) ***Z** – ácido piruglutâmico	**Taquiplesinas e Polifemusinas (limulídeos), Androctonina (escorpião) e** Protegrina (suínos)
Acanthoscurrina (2 isoformas)	Hemócitos	10.132 Da 10.249 Da	Bactéria Gram-negativa: *E. coli* SBS363 e Fungo: C. albicans	DVYKGGGGGRYGGGRY GGGGGYGGGLGGGGLG GGGLGGGKGLGGGGLG GGGLGGGLGGGGLG (62 aminoácidos – seqüência parcial)	Peptídeos ricos em glicina: a)relacionados com defesa em plantas e, b)antifúngicos de insetos

A primeira foi a theraphosina, peptídeo de 4052,5 Da, purificado a partir do plasma, com atividade contra *M. luteus* sem apresentar similaridade com outros peptídeos. As outras foram isoladas a partir dos hemócitos, e foram denominadas: mygalina (um peptídeo de 415,9 \Da com atividade contra *E. coli*.), gomesina (peptídeo de 2270,4 Da com alta similaridade com taquiplesinas e protegrinas, apresentando atividade ampla contra bactérias, fungos, leveduras e *Leshimania*), e, acanthoscurrina (peptídeo rico em glicina apresentando duas isoformas com 10132,4 e 10249,1 Da, atividade contra *E. coli* e *Candida albicans*, e similaridade com proteínas antifúngicas de insetos e proteínas relacionadas com defesa em plantas).

Foram purificadas a partir dos hemócitos de *Cupienius salei* as ctenidinas com mais de 70% de resíduos de glicina assemelhando-se à acanthoscurrina (BAUMANN *et al.*, 2010). As ctenidinas são constitutivamente expressas em hemócitos e tecido nervoso, sendo a sua expressão independente de desafios imunológicos, apresentando atividade contra *E. coli* e bactérias Gram-negativas (BAUMANN *et al.*, 2010).

Além da identificação de peptídeos antimicrobianos isolados de hemócitos da aranha migalomorfa *A. gomesiana* (LORENZINI *et al.*, 2003; SILVA *et al.*, 2000), apenas informações limitadas sobre o sistema imune inato de aranhas está disponível (FUKUZAWA *et al*., 2008). O nosso grupo de trabalho conseguiu identificar no extrato de hemócitos de *Lasiodora* sp. atividade antibacteriana contra *Bacillus subtilis* and *Enterococcus faecalis*, enquanto EILaH foi ativo somente contra *E. faecalis* (SOARES *et al*., 2011). Além disso, foi realizada uma purificação parcial de AMPs na hemolinfa de *Lasiodora* sp. com atividade contra *E. coli* e *Candida tropicalis* (FERREIRA *et al.*, 2006).

Um estudo atual demonstrou que a mygalina não é citotóxica para as células murinas *in vitro* e não afeta a proliferação celular ou produção de IL-2 (MAFRA *et al*., 2012). No entanto, para entender melhor como o processo pró-inflamatório mediado pela mygalina é preciso realizar estudos adicionais para determinar como ela modula essas vias de sinalização (MAFRA *et al*., 2012). Além disso, por ser um fator pró-inflamatório que regula a resposta imune, a mygalina deve ser mais explorada para utilização terapêutica por si só ou em combinação com outras moléculas, como a gomesina com características anti-câncer (SOLETTI *et al*., 2010; MAFRA *et al*., 2012).

Atividades antimicrobianas foram detectadas na hemolinfa da aranha *A. rondoniae* (Fig. 6) devido à presença de um peptídeo antifúngico, rondonina, purificado por cromatografia líquida de fase reversa de alta eficiência (RP-HPLC) (RICILUCA *et al*., 2012). Rondonina tem uma sequência de aminoácidos de IIIQYEGHKH e uma massa molecular de 1236.776 Da, correspondendo ao primeiro relato de um fragmento de hemocianina com atividade antifúngica (RICILUCA *et al*., 2012).

Embora sejam numerosos os relatos sobre peptídeos purificados em aranhas do gênero *Acanthoscurria* e *Cupiennius*, que são da mesma Ordem Araneae, não há estudos semelhantes em *Lasiodora* sp. Assim, a identificação de novos compostos antimicrobianos do sistema imunológico de aranhas e de outros compostos que possam estar relacionados direta ou indiretamente contribuirá para o conhecimento do sistema imune inato em aracnídeos.

3. OBJETIVOS

3.1 Objetivo Geral

Ultracaracterizar os hemócitos da aranha *Lasiodora sp*. e purificar peptídeos antimicrobianos (AMPs) produzidos por hemócitos de *Lasiodora* sp., *Acanthoscurria rondoniae* e *Vitalius sorocabae*.

3.2 Objetivos Específicos

3.2.1 Ultracaracterizar os hemócitos de *Lasiodora sp*. por microscopia óptica (luz e confocal) e de transmissão;

3.2.2 Classificar morfologicamente os hemócitos de *Lasiodora sp.* utilizando a literatura existente;

3.2.3 Purificar AMPs a partir de extratos de hemócitos de *Lasiodora* sp., *Acanthoscurria rondoniae* e *Vitalius sorocabae*;

3.2.4 Caracterizar os AMPs isolados quanto à atividade antimicrobiana;

3.2.5 Determinar a massa molecular dos AMPs isolados;

3.2.6. Identificar os AMPs por espectrometria de massa;

3.2.7. Coletar dados e escrever artigo de revisão sobre o gênero *Lasiodora*.

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5. CAPÍTULO 1

ULTRASTRUCTURAL CHARACTERIZATION OF THE HEMOCYTES OF *Lasiodora* sp. (KOCH, 1850) (ARANEAE: THERAPHOSIDAE)

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Ultrastructural characterization of the hemocytes of *Lasiodora* sp. (Koch, 1850) (Araneae: Theraphosidae)

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ABSTRACT

This paper is the first descriptive review of hemolymph cell types in the circulation of the tarantula spider *Lasiodora* sp. These animals are more long-lived than other arthropods, and may live for approximately twenty years. Such remarkable longevity may result from a highly successful immune system, which in turn is directly correlated with hemocyte function. Since the literature on the genus *Lasiodora* sp. is limited, the main goal of the present study was to identify the different cell types by optical and transmission microscope. Six hemocyte types were characterized and called prohemocyte, granulocyte type I, granulocyte type II, spherulocyte, oenocytoid and plasmatocyte. Prohemocytes presented a large nucleus, elongated granulocytes type I showed the nucleus with the same cell format, elliptical granulocytes type II showed the central nucleus of identical shape, spherulocytes exhibited the nucleus filling almost the whole cell, oval oenocytoids showed eccentric nucleus and less dense cytoplasm, and irregular plasmatocytes showed a nucleus and no granules in cytoplasm. These polymorphic granulocytes presented a round, elongated, elliptical, oval or irregular profile with large and varied numbers of granules, except for plasmatocytes, that were agranular. Different densities and different concentrations of these granules were found at the periphery of the cell. The possible reasons and implications of differences and similarities between arthropods hemocytes are discussed. It can be concluded that there are six cell types in *Lasiodora* sp. This study is of the first step in the elucidation of the role these cells play in the circulatory and immune system in spiders.

1. Introduction

Arthropods protect themselves against infection based on innate immunity, in a set of reactions that can be divided into humoral and cellular (Ribeiro and Brehélin, 2006). The ability of hemolymph cells to recognize and rapidly respond to tissue damage or infections is an integral part of the tissue repair response and immunity. One of the humoral defenses comprises mechanisms of melanization and degranulation that recognize and destroy invading microorganisms (Smith and Söderhäll, 1983; Okino et al., 1995; Lavine and Strand, 2002; Fukuzawa et al., 2008). The identification of types of hemocytes is important as far as understanding the immune cellular response in invertebrates is concerned (Kadota et al., 2003).

Arthropod hemocytes, according to morphological characteristics, are classified as prohemocytes, plasmatocytes, granulocytes, spherulocytes, coagulocytes, discoid hemocytes, adipohemocytes, cystocytes, oenocytoids and cyanocytoids (Xylander and Nevermann, 2006; Xylander, 2009).

Studies addressing arthropod hemocytes have mainly been motivated by the significance of these cells in host–pathogen interactions and, due to this fact, the mainstream of hemocyte research focuses on insects, an animal group containing many important vectors of diseases that affect humans. The majority of data available on hemocytes, including their classification schemes and terminology, concerns this group of arthropods (Nevermann et al., 1991; Borovičková and Hypša, 2005; Araújo et al., 2008; Cunha et al., 2009; Laughton et al., 2011). A review conducted by Gupta (1985) grouped the hemocytes of insects into seven main types:

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prohemocytes, plasmatocytes, granulocytes, spherulocytes, adipohemocytes, oenocytoids, and coagulocytes.

Observing heart sections of the spider *Eurypelma marxi* by transmission microscope, Sherman (1973) classified the hemocytes according to insect nomenclature. The author found three types of hemocytes: plasmatocytoids (quite abundant), oenocytoids (5% of hemocyte population), and granular hemocytes. Furthermore, Foelix (1996) classified the spider cell types in four types: granulocytes, leberidiocytes, cyanocytes and prohemocytes. Leberidiocytes were first described in the horseshoe crab (Fahrenbach, 1970), cystocytes were a preparation artifact (Nevermann et al., 1991) and cyanocytes were reduced in number after the evolution of terrestrial arthropods with trachea and respiratory pigments (Xylander, 2009).

After insects, spiders represent the most diverse and successful terrestrial invertebrates (Rash and Hodgson, 2002). The Brazilian spider *Lasiodora* sp. is known by trivial name of tarantula and is widely distributed in Northeastern Brazil (Bertani, 2001). The identification of the species of the genus *Lasiodora* sp. (C. L. Koch, 1950) requires the development of a suitable key, an effort currently in progress (Bertani et al., 2012). The aim of the present study was to characterize, for the first time, morphological types of *Lasiodora* sp hemocytes by optical and transmission electron microscopy.

2. Materials and methods

2.1. Animal and sample collection

Adult female *Lasiodora* sp. spiders were kept in plastic boxes with water and food given weekly. The spiders were maintained in the vivarium of the Biochemistry Department, Federal University of Pernambuco, Recife, Pernambuco, Brazil. To avoid hemocyte degranulation and coagulation, 3% NaCl supplemented with 2 mM propranolol was initially used (Soares et al., 2011). Nevertheless, microscopic analysis revealed that propranolol was not satisfactorily effective in preventing degranulation. Then the haemolymph was collected in the presence of sodium citrate buffer (0.14 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid, 10 mM EDTA, pH 4.6 (2:1, v/v)) (Soderhall and Smith, 1983) which was efficient to avoid hemocyte degranulation and coagulation with no change in cell morphology. Hemocytes were removed from plasma by centrifugation at 800 × *g* for 10 min at 4 °C.

2.2. Hemocyte characterization

2.2.1. Optical microscopy (OM)

Hemolymph of one animal was obtained by cardiac puncture directly on to a glass slide where it was allowed to dry at room temperature for 20–30 min. Cells were fixed in methanol for 10 min. After the fixative had dried, the hemocytes were stained with Giemsa (diluted 1:9 in buffered distilled water) (Sigma®) for 10–15 min, and the slides were rapidly washed with buffered distilled water (Brayner et al., 2005). Following this, the slides were dehydrated in ethanol, mounted in Entellan (Sigma®) and observed under an optical microscope.

2.2.2. Optical microscopy on live cells

To view live hemocytes, hemolymph samples (5 μL) were collected and diluted in GRACE medium, 1:500 (Sigma®). They were then directly deposited on cell culture plates (MatTEK) and allowed to settle for 30 min at room temperature. After this, the hemocytes were examined using differential interference contrast (DIC) microscopy (Leica SP2 confocal microscope).

2.2.3. Transmission electron microscopy (TEM)

Hemolymph (2 mL) was pooled and centrifuged at 800 × g for 10 min at 4 °C. The pellet was washed in sodium citrate buffer, pH 4.6. The cells were ressuspended and fixed in 4% glutaraldehyde (Sigma®) in 0.2 M cacodylate buffer (Sigma®), pH 7.2, overnight (Brayner et al., 2005). The samples were washed in 5% sucrose solution in 0.2 M cacodylate buffer, pH 7.2 and post-fixed with osmium tetroxide (1%) (Sigma®) in cacodylate buffer for 1 h. After dehydration in graded acetone (Merck) series, the samples were embedded in EMBED 812/Araldite (Electron Microscopy Sciences, Fort Washington, PA). Ultrathin sections were picked up on uncoated 200-mesh copper grids, double-stained with uranyl acetate (Sigma®) and lead citrate (Sigma®), and observed using a Jeol JEM 100Cx electron microscope.

2.2.4. Total and differential hemocyte counts

Fresh hemolymph (100 μL) in diluted GRACE medium (1:1) was collected from fifteen spiders. Then, the number of hemocytes in the *Lasiodora* sp. samples (10 μL of hemolymph/slide, for each spider) was determined using a Neubauer chamber (iNCYTO C-Chip DHC-N01) according to the manufacturer's instructions. Four slides were evaluated for each specimen.

The same fresh hemolymph described above was used in the determination of percentages of the total hemocyte population comprised by different cell subpopulations. This was carried out by counting the different hemocyte types and calculating their relative percentages in Giemsa-stained smears (four slides were prepared from fresh hemolymph with 10 μL of hemolymph/slide, for each spider). At least 400 cells were counted from each spider and examined using bright-field microscopy at 100× magnification. Both the total and the differential count methods were carried out in triplicate.

3. Results

3.1. Hemocytes cell types

Six morphological types of the circulating cells were identified in the hemolymph of the adult female spider *Lasiodora* sp. maintained in the vivarium of the Biochemistry Department: prohemocyte, granulocyte type I, granulocyte type II, spherulocyte, oenocytoid and plasmatocyte.

3.2. Prohemocytes

Prohemocytes display a spherical shape of approximately 10–15 μm in diameter. The large and centrally located nucleus almost fills the whole cell (Fig. 1A). Chromatin is condensed, cytoplasm was homogeneous with some vesicles and only a few structures can be seen, with conspicuous granules (Figs. 2A and 3A) and mitochondria (Fig. 3A). The photomicrographs showed a range of granules, from empty to partially filled and almost completely filled granules (Fig. 3A). This cell type represented 8.1% of the total hemocyte population.

3.3. Granulocytes type I

Granulocytes I, the largest cells found in hemolymph, presented an elongated shape of approximately 25–30 μm in diameter (Fig. 1B and Fig. 2B) with a centrally located nucleus and similar to the cells' shape (Fig. 3B). The cytoplasm was rich in round or elongated polymorphic mitochondria (Fig. 3B). The electrondense granules observed in granulocytes I were the most prominent granules found in all hemocyte cell types assessed. Lobed nucleus with

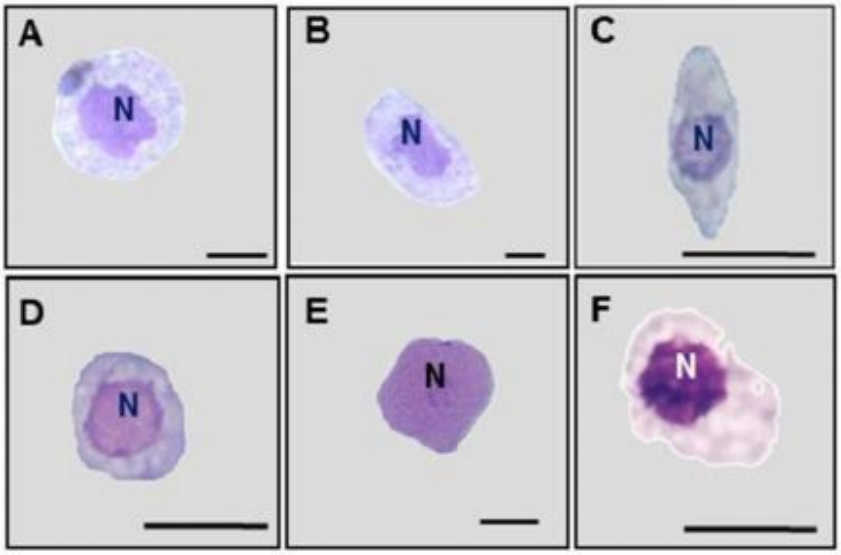

Fig. 1. (A–F) Optical microscopy of *Lasiodora* sp. hemocytes. (A) Prohemocyte with a large nucleus (N). Bar: 5 μm. (B) An elongated granulocyte type I showing the nucleus (N) with the same cell format. Bar: 10 μm. (C) Elliptical granulocyte type II with the central nucleus of identical shape (N). Bar: 25 μm. (D) Spherulocyte exhibiting the nucleus filling almost the whole cell (N). Bar: 25 μm. (E) Oval oenocytoid with an eccentric nucleus (N) and less dense cytoplasm. Bar: 10 μm. (F) Irregular plasmatocyte showing a nucleus (N) with no granules. Bar: 20 μm.

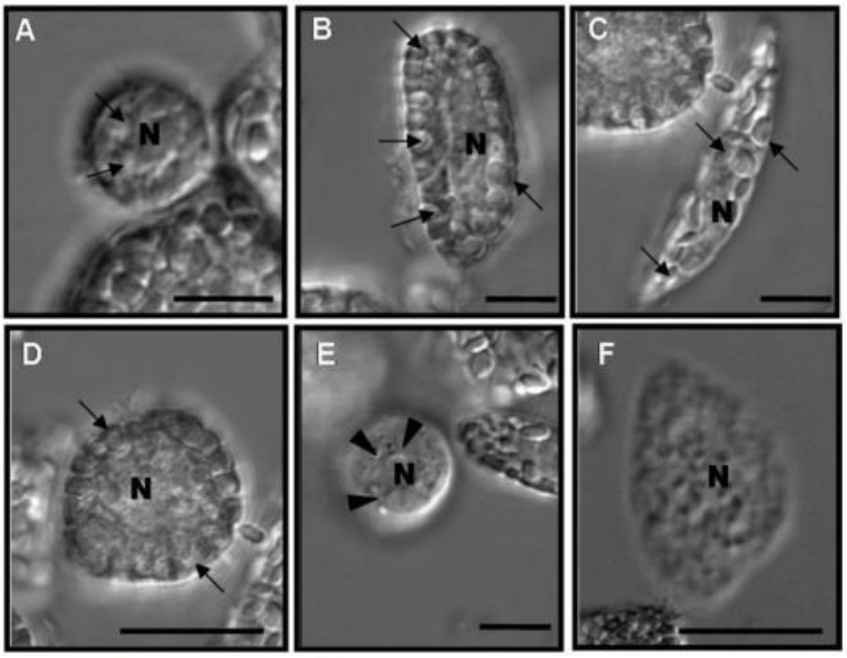

Fig. 2. (A–F) Microscopy for differential interference contrast (DIC) of *Lasiodora* sp. hemocytes. (A) Prohemocyte and a large nucleus (N). Bar: 13 μm. (B) Granulocyte type I with elongated nucleus (N). Bar: 13 μm. (C) Elliptical granulocyte type II with the central nucleus of identical shape (N). Bar: 13 μm. (D) Spherulocyte exhibiting the nucleus filling almost the whole cell (N) and the granules located at the cell's periphery deforming the membrane (arrows). Bar: 15 μm. (E) Oval oenocytoid with an eccentric nucleus (N) and less dense cytoplasm. Bar: 13 μm. (F) Irregular plasmatocyte showing a nucleus (N) with no granules. Bar: 13 μm. The polymorphic granulocytes were round (prohemocytes/oenocytois), elongated/irregular (granulocytes type I), elliptical (granulocytes type I) or oval (spherulocytes). The arrows or head-arrows indicate granules.

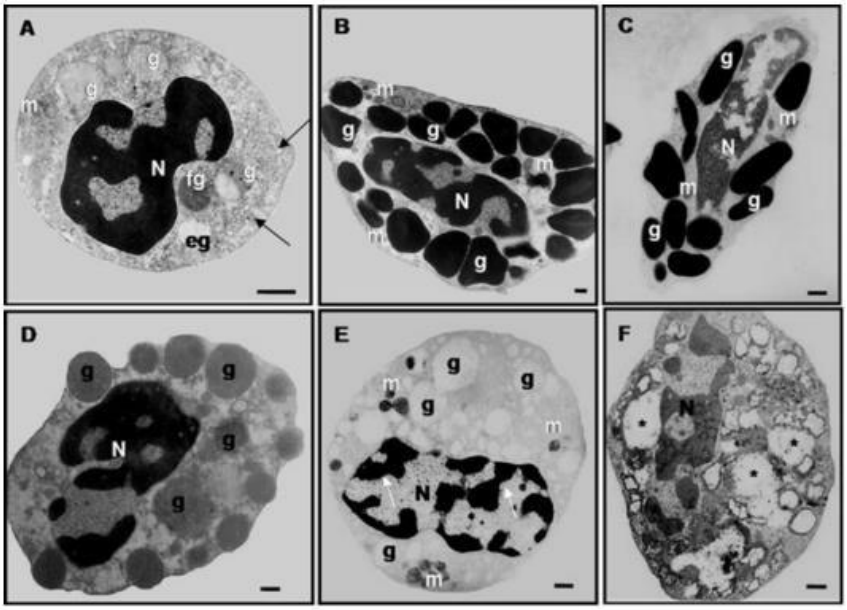

Fig. 3. (A–F) Transmission electron microscopy of *Lasiodora* sp. hemocytes. (A) Prohemocytes, large nucleus (N), mitochondria (m), and granules (g) in different formation stages: empty granule (e.g.) could be found at the same time as another full granule (fg). The arrows indicate some vesicles in the cytoplasm. (B) Elongated granulocyte type I with several large electron-dense granules (g) and mitochondria (m) in the cytoplasm. The nucleus (N) was also present. (C) A granulocyte type II filled with the typical granules (g) that were mostly spherical and bulge the plasma membrane at different sites and mitochondria (m) in the cytoplasm. The nucleus (N) fills the elliptical cell format. (D) Spherulocytes exhibiting the nucleus (N) and specific granules (g), the spherules that deforming the plasmatic membrane. (E) Round oenocytoids with eccentric nucleus (N) and heterochromatin attached at the nuclear membrane, less dense granules (g) and mitochondria (m). (F) Irregular plasmatocytes showing nucleus (N), no granules and some vesicles (asterisks) could be seen. Bars 1 μm.

heterochromatin was observable by electron micrographs (Fig. 3B). This cell type represented 35.2% of the total hemocyte population.

3.4. Granulocytes type II

Granulocytes II presented an elliptical shape of approximately 35–40 μm in diameter (Figs. 1 and 2C), with a centrally located nucleus and shaped like the cell (Fig. 3C). The cytoplasm presented few mitochondria (Fig. 3C); the electron-dense granules were also prominent, differing only in shape, which was more elliptical than in granulocytes I. Lobed nucleus with heterochromatin attached to the nuclear membrane were observable by electron micrographs (Fig. 3C). This cell type represented 29.6% of the total hemocyte population.

3.5. Spherulocytes

The spherulocytes showed a round cell shape, with average diameter of 15–25 μm, displaying a round nucleus which almost fills the whole cell (Figs. 1 and 2D) and condensed chromatin. These cells showed a considerable number of large inclusions (spherules) responsible for the cells' irregular shape and for deformed membrane (Fig. 3D). Clot formation indicates that spherulocytes may be involved in the process of coagulation of hemolymph (Fig. 4). This cell type represented 11.1% of the total hemocyte population.

3.6. Oenocytoids

Oenocytoids were observed to be round cells with homogeneous cytoplasm (Figs. 1 and 2E) measuring approximately 15–20 μm in diameter. The ultrastructure revealed many electrodense mitochondria, some granulocytes with different electrodensity (when compared with other cell types) and heterochromatin

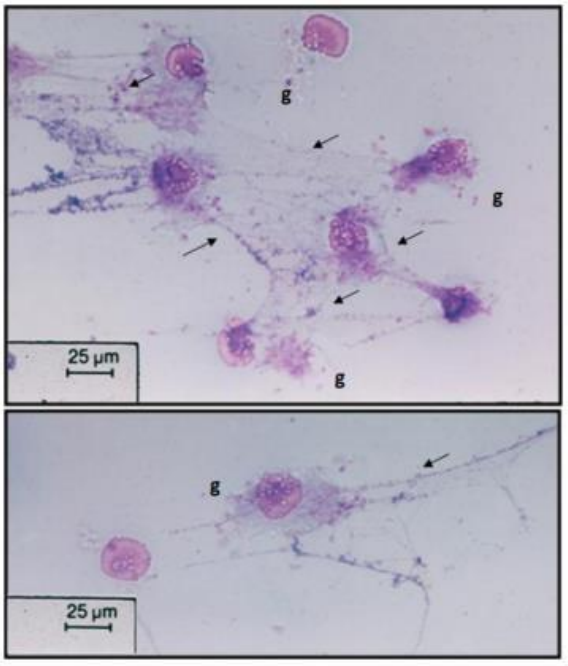

Fig. 4. Spherulocyte degranulation and cloting process of *Lasiodora*sp. by optical microscopy. The coagulation cascade was clearly activated because of the presence of mesh clotting (arrows) and the exocytosis of contents of hemocytic granules (g) by spherulocytes. This event may suggest the possible relationship of these cells in the coagulation and tissue renewal of *Lasiodora* sp. hemolymph. Bars 25 μm.

Table 1
Ultrastructural characteristics of *Lasiodora* sp. hemocytes shared by cells of Arthropoda and Mollusca species.

Lasiodora sp. hemocyte	Ultrastructural characteristics of *Lasiodora* sp.	Arthropoda and mollusca species
Prohemocytes	Granules in different formation stages.	See reference on *Biomphalaria straminea* (Preston, 1910) (Mollusca).
	Large nucleus and few granules and mitochondria in the cytoplasm.	See reference on *Scutigera coleoptrata* (Lamarck, 1801) (Arthropoda).
Granulocytes type I	Numerous electron dense granules in the cytoplasm.	See reference on *Acanthoscurria gomesiana* (Ausserer, 1871) (Arthropoda).
	The largest and dense granules.	See reference on *Tachypleus tridentatus* (Leach, 1819) (Arthropoda).
Granulocytes type II	Large, electron dense, granules showing a round to oval granule profile.	See reference on *Astacus leptodactylus* (Borlase, 1758) (Arthropoda).
	Granules mostly elliptical that subtly deform plasma membrane.	See reference on *Rhapidostreptus virgator* (Silvestri, 1910) (Arthropoda).
Spherulocytes	Spherical cell which granules deform the plasma membrane.	See reference on *Bombyx mori* (Linnaeus, 1758) (Arthropoda).
		See reference on *Scutigera coleoptrata* (Lamarck, 1801) (Arthropoda).
Oenocytoids	Few Golgi complexes and rough endoplasmic reticula were observed in the cytoplasm.	See reference on *Mythimna unipuncta* (Ochsenheimer, 1816) (Arthropoda).
	Round shape with eccentric nucleus.	See reference on *Culex quinquefasciatus* (Linnaeus, 1758) (Arthropoda).
Plasmatocytes	Agranular plasmatocytes which have none or just a few grana.	See reference on *Rhapidostreptus virgator* (Silvestri, 1910) (Arthropoda).
	Vesicles in the cytoplasm.	See reference on *Ornithodoros moubata* (Koch, 1844) (Arthropoda).

Shigenaga et al. (1993), Inoue et al. (2001), Hilken et al. (2003); Ling et al. (2003), Brayner et al. (2005), Ribeiro and Brehélin (2006), Giulianini et al. (2003), Fukuzawa et al. (2008), Xylander (2009), Cavalcanti et al. (2012).

attached to the nuclear membrane (Fig. 3E). This cell type represented 3% of the total hemocyte population.

3.7. Plasmatocytes

The plasmatocytes were very polymorphic, varied from spindle-shaped to round cells (Figs. 1 and 2F) measuring approximately 15–20 μm in diameter and presenting agranular cytoplasm. Heterochromatin clumps were present (Fig. 3F). In TEM, vesicles were observed (Fig. 3F). This cell type represented 13% of the total hemocyte population.

4. Discussion

Hemocytes are identified based on morphology and ultrastructure traits, apart from physiological function (Kadota et al., 2003). The correspondence of characters up to the ultrastructural level suggests that usage of a common nomenclature for hemocytes is reasonable due to comparative-morphological and functional, although homology of hemocytes within the arthropods has not yet been proven (Xylander and Nevermann, 2006). As there is no specific cell classification for spiders, in this study the literature about other phyla was used to name the cells found in *Lasiodora* sp. circulation, as well as to compare these cells (Table 1). The cell description was based on the evaluation of several cells with the same characteristics, and the percentage of each cell type was determined considering as 100% the total hemocytes sampling, similarly to previous studies (Brayner et al., 2005; Araújo et al., 2008; Cavalcanti et al., 2012).

Spider hemocytes originate from the heart cell wall (Foelix, 1996). The prohemocytes found in this study presented granules in different formation stages, similar to those found in granulocytes from *Biomphalaria straminea* (Cavalcanti et al., 2012), even though the snail belongs to another phylum.

Granulocytes type I and II, named because of the numerous dense granules in cytoplasm, were the most abundant cell type observed in *Lasiodora* sp, similarly to what was found for the spider *Acanthoscurria gomesiana* by Fukuzawa et al. (2008). The horseshoe crab *Tachypleus tridentatus*, an animal that belongs to the same phylum of spider, has just one type of cell present in hemolymph, called amebocyte or granulocyte, whose cytoplasm is filled with two types of granules: (i) larger but less dense, and (ii) smaller but dense (Shigenaga et al., 1993).

The polymorphic granulocytes of *Lasiodora* sp. presented round (prohemocytes and oenocytoids), elongated/irregular (granulocytes I), elliptical (granulocytes II) and oval (spherulocytes) shapes with a large and varied amount in number. These granules had differences in electron density, when compared with the other granulocytes of *Lasiodora* sp. and they also could be found at the periphery of the cell. The granules can be differentiated based on number, distribution and shape. The exception was agranular plasmatocytes, like the ones found in Myriapoda (Arthropoda), which have none or just a few grana (Xylander, 2009). The plasmatocytes found in this present study were also similar to those found by Inoue et al. (2001) in *Ornithodoros moubata* (Acari), with vesicles in the cytoplasm.

The granulocytes type I were similar to those found by Shigenaga et al. (1993) in horseshoe crab, especially regarding the format of the L-granules, which were less dense. The granulocytes type II were similar to those found by Xylander (2009) in diplopods, *Chicobolus* spp. and *Rhapidostreptus virgator*, with granules mostly elliptical and that subtly deform plasma membrane at different sites.

Arthropods have open circulatory systems and must seal wounds and use efficient clotting systems to keep bacteria from entering the hemocoel (Theopold et al., 2004). Collection of *Lasiodora* sp. hemolymph promoted the clotting process, and we suggest that tissue injury caused by the needle led to exocytosis of contents of hemocytic granules by spherulocytes. This event may suggest the possible relationship of these cells in the coagulation of *Lasiodora* sp. hemolymph. The spherules were clearly visible by differential interference contrast in spherulocytes (Sh) of *Lasiodora* sp. similar to what was described for silkworm (Ling et al., 2003). *Lasiodora* sp. spherules were spherical and deformed the plasma membrane, resulting in deformation of the spherulocytes' membrane. Fukuzawa et al. (2008) reported that injection of fluorescent particles into the spider leg activated the coagulation cascade, and release of granules could also be observed. Giulianini et al. (2003) reported that discharge of spherules, even after weak activation of spherulocytes, was probably involved in nodule formation. Tissue injury in *Lasiodora* sp. might trigger the coagulation cascade and in this sense, the spherulocytes are related to tissue renewal with agreement of Gupta (1985), Ratcliffe et al. (1985) and Perez and Fontanetti (2011). The clot presumably serves to immobilize bacteria, reducing the risk of their systemic dispersal (Armstrong and Levin, 1979).

Wago (1991) affirmed that the oenocytoids are easily classified even under the light microscope because of their relatively large morphology, fragile nature, and opaque appearance when compared to other hemocytes. These characteristics were also found in *Lasiodora* sp. oenocytoids. These cells have a strong mitochondrial enzyme activity because of the presence of a huge number of mitochondria in MET. Leberidiocytes (Fahrenbach, 1970), cystocytes (Nevermann et al., 1991) and cyanocytes (Xylander, 2009) were not detected in *Lasiodora* sp. spiders.

Xylander (2009) reported that similarity of characters, even at electron microscopic level, allows using the generalized nomenclature for arthropod hemocytes. In this scenario, our examination reveals differences and similarities between arthropods hemocytes, thus highlighting the difficulties in establishing a unique classification of arthropods hemocytes. Furthermore, physiological investigations are necessary to clarify the homology of the different hemocyte types.

5. Conclusions

Six morphological types of the circulating cells, collected by cardiac puncture, were identified in the hemolymph of the tarantula spider *Lasiodora* sp. The ultrastructure revealed different cell type, the hemocytes were named by prohemocyte, granulocyte type I and II, spherulocyte, oenocytoid and plasmatocyte. In comparison with the studies published so far, our examination corroborates ultrastructural data about hemocytes classification of *Lasiodora* sp.

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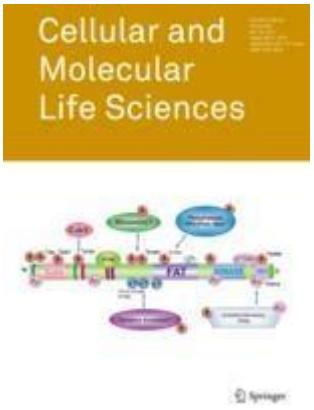
6. CAPÍTULO 2

HEMOCYTES OF SPIDER: ARE THE ANTIMICROBIAL PEPTIDES CONSERVED?

MANUSCRITO A SER SUBMETIDO AO PERIÓDICO:

"Cellular and Molecular Life Sciences"



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Hemocytes of spider: are the Antimicrobial Peptides conserved?

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ABSTRACT

As part of innate immune system, antimicrobial peptides provide protection against a wide variety of microorganisms in both vertebrates and invertebrates. These peptides are very diverse with respect to amino acid sequence and secondary structure but share certain properties, and hypothetically may be conserved in the organisms at the same taxon. To test this hypothesis we searched in three spiders (*Acanthoscurria rondoniae*, *Lasiodora* sp. and *Vitalius sorocabae*) which belong to Theraphosidae family the conserved antimicrobial peptides. According to our results, the peptide similar to gomesin has antimicrobial activity against *Micrococcus luteus*, *Escherichia coli* and *Candida albicans*. Additionally, the peptide similar to acanthoscurrin and the peptide

similar to mygalin are active against *M. luteus* and *E. coli*. All these peptides were found in *A. rondoniae*, *Lasiodora* sp. and *V.* sorocabae. These results suggest that maybe the immune system of arachnids is more complex and has maintained not only these but other molecules as important to your success. The study contributes to the characterization of the immune system of aracnids and elucidation of the biochemical role of biomolecules in hemocytes of spiders.

Key-words: hemocytes, antimicrobial peptides, spider, Theraphosidae.

1. Introduction

The emergence of new diseases as well as the increasing resistance of bacteria against antibiotics over the past decades has become a growing threat to humans. This has driven a sustained search for new agents that possess antibacterial activities against bacteria being resistant against conventional antibiotics and prompted an interest in short to medium-sized peptides called antimicrobial peptides (AMPs) (STEGEMANN & HOFFMANN, 2008; HAYASHI *et al*., 2013). Such molecules are produced by plants, bacteria, invertebrates, and vertebrates (GUANÍ-GUERRA, 2010).

AMPs are important components of the innate immune system, used by the host to protect itself from different types of pathogens (LATA *et al*., 2007). These molecules constitute a heterogeneous group of peptides with respect to their primary and secondary structures, antimicrobial potentials, effects on host cells, and regulation of their expression. Most antimicrobial peptides are small (12– 50 amino acids), have a positive charge provided by Arg and Lys residues, and an amphipathic structure that enables them to interact with bacterial membranes (AUVYNET & ROSENSTEIN, 2009).

In invertebrates, AMPs can be produced differently depending on the species, and are found constitutively present in granular hemocytes (LAMBERTY *et al*., 2000). In arachnids, several AMPs were isolated and characterized with antimicrobial properties.

Three AMPs (gomesin, acanthoscurrin and mygalin) were isolated from hemocytes of mygalomorph spider *Acanthoscurria gomesiana*. Another example is the three isoforms of AMP named ctenidins identified in the hemocytes of *Cupiennius salei* (BAUMANN *et al*, 2010).

Representing one of the three main spider lineages, the suborder Mygalomorph, include the tarantulas, trapdoor spiders and others less well-known groups (STARRETT *et al*., 2013). Mygalomorphs are essentially worldwide in distribution, with centers of generic diversity in all tropical regions as well as temperate austral areas of South America, southern Africa and Australia (RATES *et al*., 2013; RAVEN, 1985). Currently the spiders are distributed in 3.935 genera and 44.906 species (PLATNICK, 2015).

With almost one thousand species described (PLATNICK, 2015), it is one of the richest spider families. One of these families is the Theraphosidae family which is represented by around 976 species divided in 128 genera, which *Lasiodora* is included, distributed across the globe, occurring in various habitats (PLATNICK, 2015).

The tarantulas *Acanthoscurria rondoniae*, *Lasiodora* sp. and *Vitalius sorocabae*, mygalomorphs of the Theraphosidae family were chosen to test the hypothesis that some molecules may be conserved in organisms of the same taxon. To corroborate this idea, still talking about conservation molecules in spiders, we can mention the work of Starrett *et al* (2013) which deals with the conservation across distantly related species of hemocyanin that play a crucial role in oxygen storage and transport in spiders.

The three spiders are commonly known in Brazil as caranguejeiras (HORTA *et al*., 2013). The different species of *Lasiodora* spiders are difficult to distinguish (BRAZIL & VELLARD, 1926) is one of the reasons that still named as sp.

2. Material and Methods

2.1 Animals and sample collection

The spiders *(Acanthoscurria rondoniae*, *Lasiodora* sp. and *Vitalius sorocabae*) were kept alive in the biotherium of the Laboratório Especial de Toxinologia Aplicada - LETA of the Institute Butantan (São Paulo, Brazil) and Laboratório de Glicoproteínas

(Pernambuco, Brazil) respectivelly. These animals were collected under License Permanent Zoological Material n^0. 11024-3-IBAMA and Special Authorisation for Access to Genetic Patrimony n^0. 001/2008. The hemolymph (approximately 1 ml/spider) from animals of either sex at intermolt stages was collected by cardiac puncture with an apyrogenic syringe. To avoid hemocyte degranulation and coagulation, the hemolymph was collected in the presence of sodium citrate buffer, pH 4.6 (2:1, v/v) (Soderhall and Smith, 1983). The hemocytes were removed from plasma by centrifugation at 800 xg for 10 min at 4°C. Entire hemocytes were washed once with sodium citrate buffer and lysed in acetic acid 2M.

2.2 Peptide extraction and purification

Lysed hemocytes collected from the hemolymph of unchallenged spiders were homogenized with a pistile in 5 ml of 2M acetic acid. The supernatant were submitted to solid phase extraction onto Sep-Pak C18 cartridges (Waters Associates) equilibrated with acidified water (0.05% trifluoroacetic acid (TFA)). Three stepwise elutions were performed with 5, 40, and 80% acetonitrile in acidified water. The 40% Sep-Pak fraction was concentrated in a vacuum centrifuge and reconstituted in Milli-Q water (Millipore™). The reconstituted 40% Sep-Pak fraction was subjected to reverse phase chromatography on a semi-preparative Jupiter C18 column equilibrated in acidified water. The sample was eluted with a linear 2-60% gradient of acetonitrile in acidified water over 120 min at a flow rate of 1.5 mL/min. Eluent absorbance was monitored at 225nm. Fractions corresponding to absorbance peaks were collected by hand, concentrated under vacuum (Speed-Vac Savant) and reconstituted in Milli-Q water. The presence of antimicrobial activity was determined by a liquid growth inhibition assay.

2.3 Structural characterization

Briefly, 0.35 μL of sample in Milli-Q water was mixed with 0.35 μL of saturated matrix α-cyano-4-hydroxycinnamic acid solution deposited onto the sample slide and

dried on the bench. The analysis was performed with the spectrometer operating in positive mode, which detects positively charged ions.

To determine the ions features, the sample were subjected Q-TOF Ultima API (Micromass) spectrometer operating in positive ionisation mode. The spectrum was analysed and subject to mascot database to compare our results.

2.4 Bioassays

During the purification procedure, the antimicrobial activities of samples were monitored by a liquid growth inhibition assay against the Gram-negative bacteria *Escherichia coli* SBS363, Gram-positive bacteria *Micrococcus luteus* A270 that were cultured in poor (NaCl, Bacto Peptone, pH 7.4) broth (PB), whereas yeast strain *Candida albicans* MDM8 was cultured in yeast extract/peptone/ dextrose (PDB) medium. Determination of antimicrobial peptide was performing using 5-fold microtiter broth dilution assay in 96-well sterile plates at a final volume of 100µL. Mid-log phase culture were diluted to a final concentration of 1 $x10^5$ colony forming units/mL. Dried HPLC were dissolved in 200µL of water ultrapure and 20µL applied into each well and added to 80µL of the bacterium/yeast dilution. The fractions were tested in triplicate. The microtiter plates were incubated for 18h at 30ºC; growth inhibition was determined by measuring absorbance at 595 nm.

3. Results

Purification of antimicrobial molecules from hemocytes

The antimicrobial peptides from hemocytes of the spiders *A. rondoniae* (A), *V. sorocabae* (V) and *Lasiodora* sp. (L) from the Theraphosidae family was extracted under acidic conditions after lysis and dissolved in acidified Milli-Q water as previously described. The supernatant obtained by centrifugation was applied to a Sep-PakC18 column and subjected to three successive extractions of increasing concentrations of

acetonitrile (5%, 40% and 80% ACN) to pre-purify antimicrobial peptides. The material eluted at 40% ACN was subjected to fractionation by RP-HPLC, which resulted in fractions with antimicrobial activity (Fig. 1, 2 and 3).

Antimicrobial activity of antimicrobial molecules measured by liquid growth inhibition

All fractions were analysed in the liquid growth inhibition assay using Gram positive bacteria *M. luteus* A270, Gram-negative bacteria *E. coli* SBS363, and the yeast *C. albicans* MDM8. We found nine fractions (L, A and V 1-3) which showed different antimicrobial activity (Table 1). L1, A1 and V1 were active on *E. coli*, *M. luteus* and *C. albicans* while L3, A3, and V3 showed antimicrobial activity against *E. coli* and *C. albicans*. L2, A2 and V2 did not show antifungal activity on *C. albicans* but were antibacterial agent on *E. coli*.

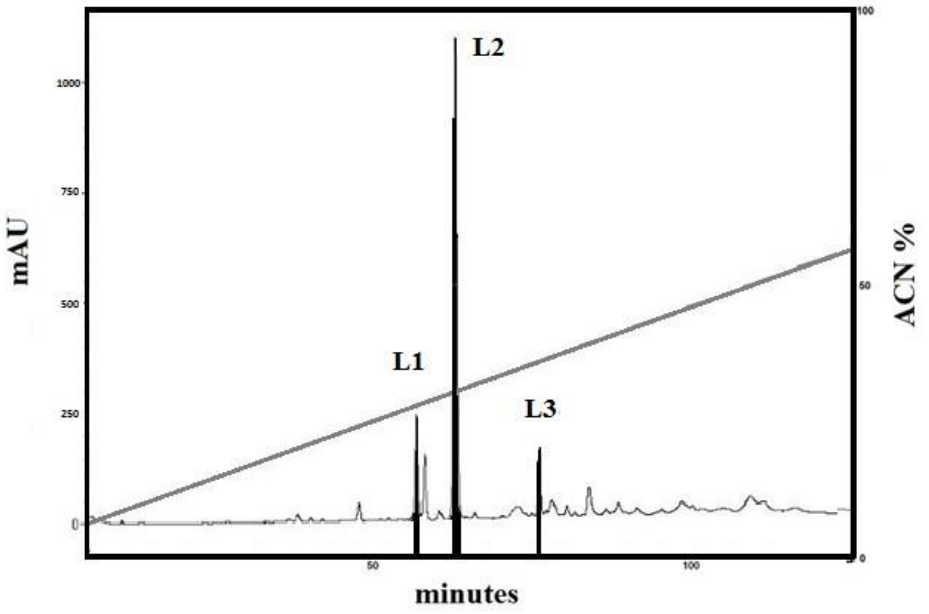

Figure 1 - Purification from *Lasiodora* sp hemocytes by reversed phase HPLC. An acidic extract obtained from hemocytes was submitted to solid phase extraction on Sep-Pak C18 cartridges. The fraction eluted with 40% was analysed on a semi preparative Jupiter C_{18} column with a linear 2 to 60% acetonitrile gradient in acidified water over 60 min (dotted line), at a flow rate of 1,5 mL/min.

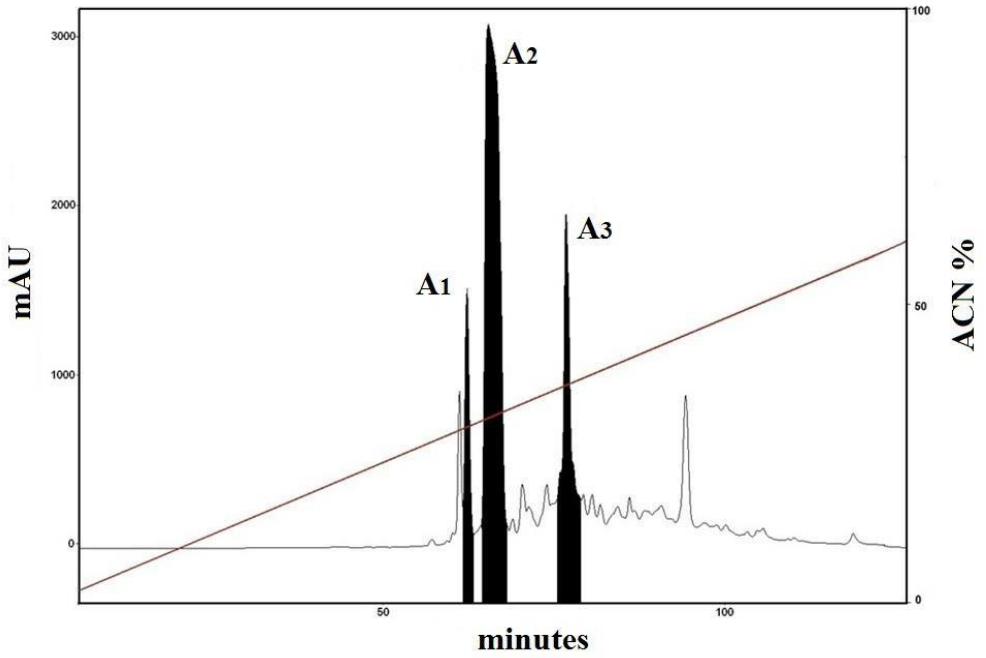

Figure 2 – Purification from *Acanthoscurria rondoniae* hemocytes by reversed phase HPLC. An acidic extract obtained from hemocytes was submitted to solid phase extraction on Sep-Pak C18 cartridges. The fraction eluted with 40% was analysed on a semi preparative Jupiter C_{18} column with a linear 2 to 60% acetonitrile gradient in acidified water over 120 min, at a flow rate of 1,5 mL/min.

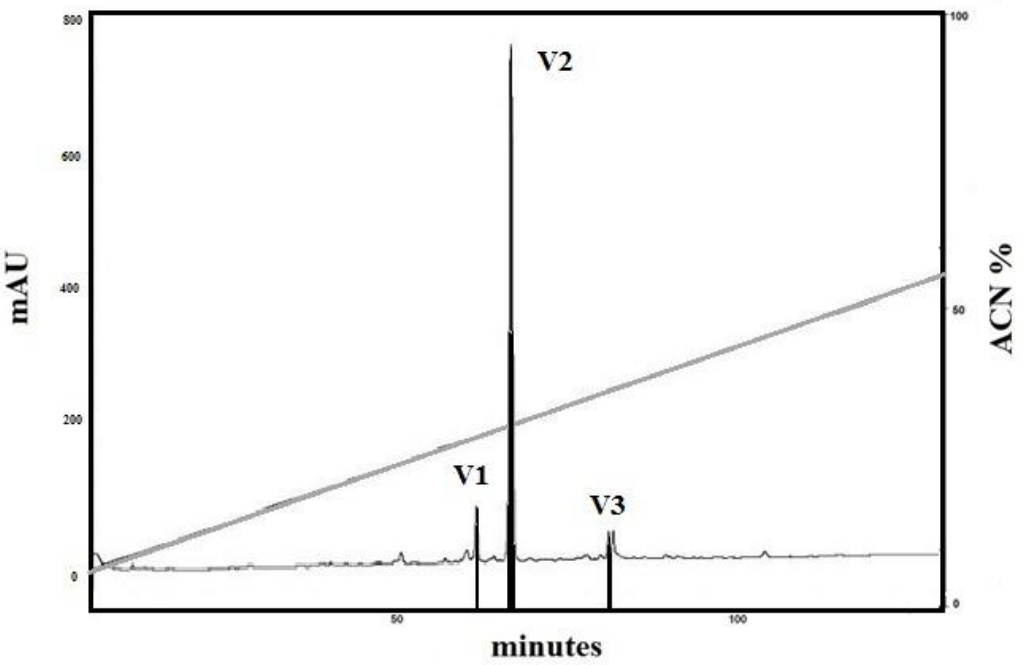

Figure 3 – Purification from *Vitalius sorocabae* hemocytes by reversed phase HPLC. An acidic extract obtained from hemocytes was submitted to solid phase extraction on Sep-Pak C18 cartridges. The fraction eluted with 40% was analysed on a semi preparative Jupiter C_{18} column with a linear 2 to 60% acetonitrile gradient in acidified water over 60 min, at a flow rate of 1,5 mL/min.

ESIMS/MS and LC/MS analysis

The fragmentation pattern of the AMPs were investigated by ESIMS/ MS (tandem electrospray ionization mass spectrometry). All spectra were acquired in the positive-ion mode, and collision induced dissociation (CID) experiments were performed using a relative collision energy 35% (1–1.5 eV).

The antimicrobial peptides L1-3, A1-3 and V1-3 were analyzed in a Database swissprot and was revealed that the fractions were similiar to antimicrobial peptides previously described in *A. gomesiana*. (Table 1). L1, A1 and V1 were identified as gomesin, L2, A2 and V2 were identified as mygalin and L3, A3 and V3 as acanthoscurrin (Fig. 4-6).

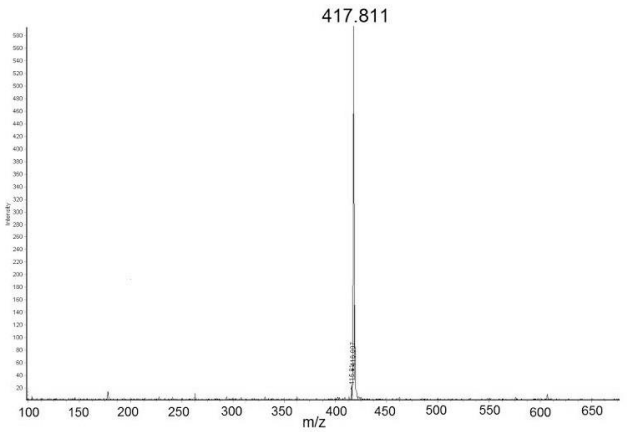

Figure 4 – ESIMS/MS spectrum of *Lasiodora* sp. Gomesin. Analysis of fraction L1 spectrometry showed a single molecule with an m/z of 417.811.

Table 1. Antimicrobial peptides isolated from hemocytes of Mygalomroph spiders.

Organism	Peptide	Molecular mass	Antimicrobial activity	Fractions
Lasiodora sp.	Gomesin	Database swissprot	*E. coli* SBS363, *M. luteus* A270 and *C. albicans* MDM8	L1
A. rondoniae	Gomesin	2270.3 Da	*E. coli* SBS363, *M. luteus* A270 and *C. albicans* MDM8	A1
V. sorocabae	Gomesin	Database swissprot	*E. coli* SBS363, *M. luteus* A270 and *C. albicans* MDM8	V1
Lasiodora sp.	Mygalin	417.x Da	*E. coli* SBS363	L2
A. rondoniae	Mygalin	418.65 Da	*E. coli* SBS363	A2
V. sorocabae	Mygalin	418.2 Da	*E. coli* SBS363	V2
Lasiodora sp.	Acanthsocurrin	Database swissprot	*E. coli* SBS363 and *C. albicans* MDM8	L3
A. rondoniae	Acanthsocurrin	10111.8 Da	*E. coli* SBS363 and *C. albicans* MDM8	A3
V. sorocabae	Acanthsocurrin	Database swissprot	*E. coli* SBS363 and *C. albicans* MDM8	V3

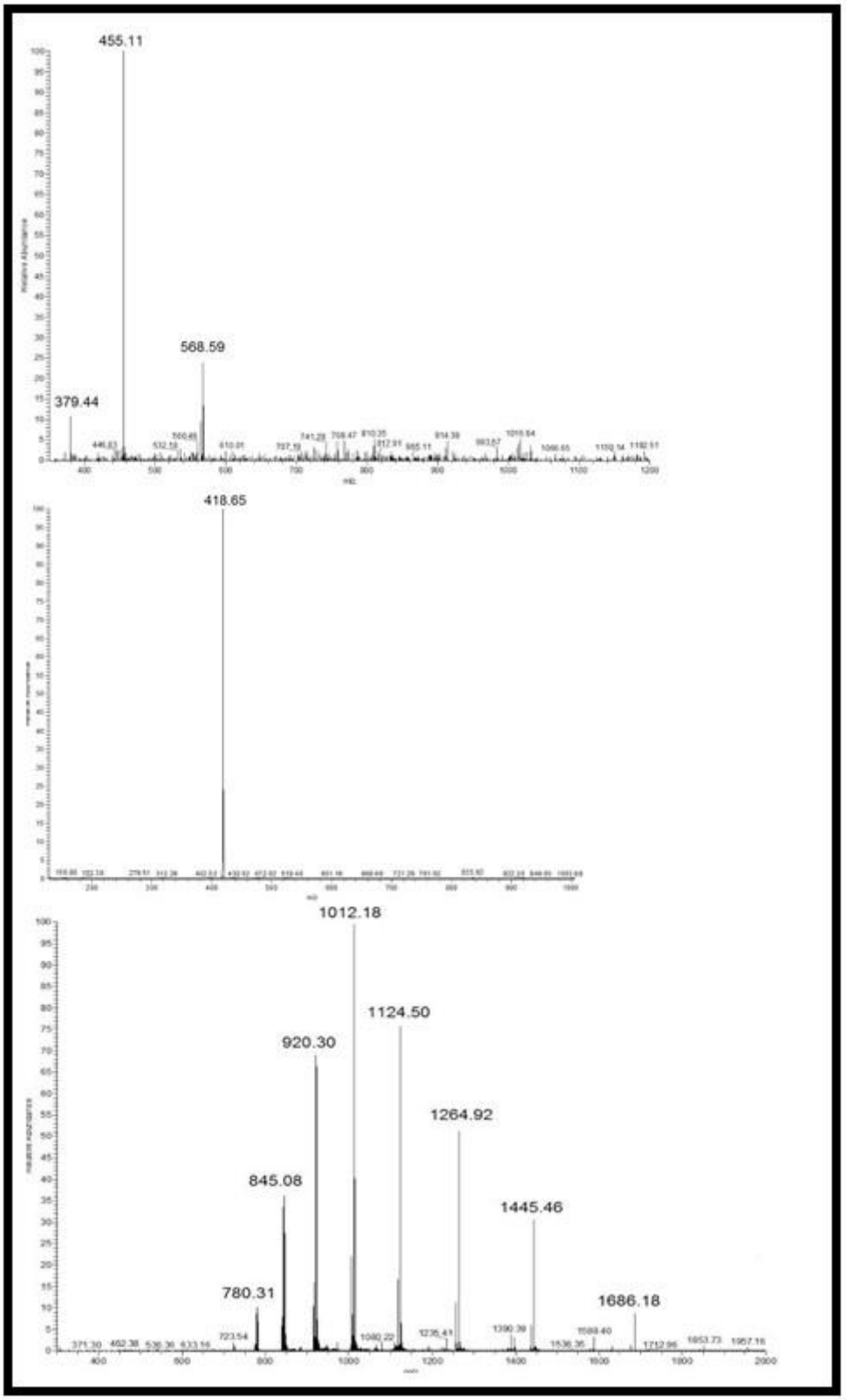

Figure 5 – ESIMS/MS spectrum of *A. rondoniae* gomesin, mygalin and acanthoscurrin. Analysis of fraction A1-3 spectrometry showed molecules with an m/z of 455.11, 418.65 and 1012.18 Da and 1124.50 Da, respectively.

4. Discussion

Several antimicrobial peptides have been isolated from the venom and hemolymph arthropods as scorpions and spiders (KUHN-NENTWIG, 2003). Using the spider tarantula *Acanthoscurria gomesiana* Silva-jr (2000) purified and characterized four molecules present in their hemolymph. The first was the theraphosina, 4052.5 Da peptide, purified from plasma, with activity against *M. luteus* without showing similarity to other peptides. The others three were isolated from hemocytes, as following: the first AMP was the gomesin, with a molecular mass of 22270.4 Da with activity on Gram-neagtive bacteria *E. coli* SBS363; Gram positive bacteria *M. luteus* A270; yeast *C. albicans* MDM8 and *Leishmania amazonenses* (SILVA-JUNIOR *et al*., 2000); the second AMP was the acanthoscurrin, present in two isoforms with molecular mass of 10111 Da and 10225 Da and activity on *E. coli* SBS363 and *C. albicans* MDM8 (LORENZINI *et al*., 2003); and the third one was the mygalin, with 417 Da and activity on *E. coli* SBS363 (PEREIRA *et al*., 2007).

About the species choosen in this study, until now there are any work about AMPs purified from hemocytes of *Lasiodora* sp., *A. rondoniae* and *V. sorocabae*. Recently, from *A. rondoniae* was purified from plasm an AMP with 1236.776 Da named rondonin with a strong activity (can kill yeast in ten minutes) on *C. albicans* MDM8 (RICILUCA *et al*., 2012).

In this work, we purified three molecules with antimicrobial activities that have never been before identified from the hemocytes of the tarantula spiders *Lasiodora* sp., *A. rondoniae*, and *V. sorocabae*. We suggest according these findings that some molecules may be conserved because they are important to immunological system, especially in spiders of the same family.

In addition to defences against predators, survival depends on the presence of an efficient immune system that can quickly remove or inactivate pathogenic organisms. The immune system of the tarantula spiders is particularly interesting because, in addition to having a life expectancy of more than 20 years (FOELIX, 1996), tarantula spiders are also phylogenetically very old with fossil records dating from the Devonian period (400 million years ago). A clear trend in recent literature indicates that some

fragments of hemocyanins may be conserved or have similar activities in different spiders (COATES and NAIRN, 2014), corroborating the hypothesis of conserved molecules.

The increased prevalence of multi-drug resistant (MDR) pathogens heightens the need to design new antimicrobial agents. Antimicrobial peptides (AMPs) exhibit broad-spectrum potent activity against MDR pathogens and kills rapidly, thus giving rise to AMPs being recognized as a potential substitute for conventional antibiotics (KHAMIS *et al.*, 2015).

5. Acknowledgements

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7. CAPÍTULO 3

HEMOLYMPH AND HEMOCYTES OF TARANTULA SPIDERS: PHYSIOLOGICAL ROLES AND POTENTIAL AS SOURCES OF BIOACTIVE MOLECULES

CAPÍTULO DE LIVRO PUBLICADO

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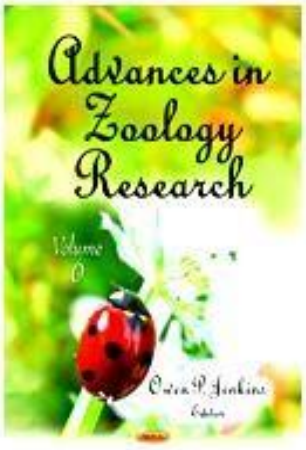

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Chapter 8

HEMOLYMPH AND HEMOCYTES OF TARANTULA SPIDERS: PHYSIOLOGICAL ROLES AND POTENTIAL AS SOURCES OF BIOACTIVE MOLECULES

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ABSTRACT

Arachnids compose the most important and numerous group of chelicerates and include spiders, scorpions, mites and ticks. Some arachnids have a worldwide distribution and can live for more than two decades. This is in part due to their efficient defense system, with an innate immunity that acts as a first line of protection against bacterial, fungal and viral pathogens. The adaptive success of the spiders stimulates the study of their defense mechanisms at cellular and molecular levels with both biological and biotechnological purposes. The hemocytes (plasmatocytes, cyanocytes, granulocytes, prohemocytes, and leberidocytes) of spiders are responsible for phagocytosis, nodulation, and encapsulation of pathogens as well as produce substances that mediate humoral mechanisms such as antimicrobial peptides and factors involved in the coagulation of hemolymph and melanization of microorganisms. This chapter discusses on the morphophysiology of tarantula spider hemocytes and bioactive molecules isolated from hemocytes and hemolymph. In addition, there is a special focus on the Brazilian tarantula *Lasiodora* sp., which is currently under systematic review. Although there are many gaps to be filled, significant progress has been achieved on the identification of *Lasiodora* sp.hemocytes and study of bioactive molecules present in hemocytes of these spiders.

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1. Introduction

The arthropods have developed an innate immunity that is the first line of protection against bacteria, fungi and viral pathogens. The high diversity of invertebrates evidences the efficiency of their defense system and indicates that the absence of acquired immunity did not hinder their successes (Coates and Nairn, 2014).

The Chelicerata is a sub-phylum of arthropods and includes the classes Arachnida, Xiphosura and Picnogonida. The arachnids compose the most important and numerous groups of chelicerates and are represented by the forms most known by humans, such as spiders, scorpions, mites and ticks. Spiders are the most diverse and successful group of terrestrial invertebrates (Rash and Hodgson, 2002) and the tarantula spiders are among the largest living spiders and the richest spider groups (Bertani et al. 2012; Platnick, 2015). The adaptive success of spiders encourages studies based on their immunity.

Cellular and humoral pathways accomplish the innate immunity of arthropods (Hoebe et al. 2004; Fukuzawa et al. 2008). The hemocytes of spiders, which can be identified based on their morphology, ultrastructure, and physiological roles, are responsible for phagocytosis, nodulation and encapsulation. The humoral mechanisms involve the action of antimicrobial peptides (AMPs), coagulation of the hemolymph and melanization of microorganisms (Jiravanichpaisal et al. 2006, 2010). In spiders, in addition to AMPs, the defensive mechanisms involve other molecules such as phenoloxidase, coagulation factors, complement factors, lectins, proteases, and protease inhibitors, which can be found in the hemolymph and hemocytes of these animals.

Detection, isolation and evaluation of mode of action of these molecules can contribute to a broader understanding of the processes involved in the immune system of arachnids. Moreover, the isolation and characterization of new antimicrobial molecules may also contribute with strategies for control of human and phytopathogens.

2. Spiders

Spiders belong to the order Araneae of the class Arachnida. The Araneae can still be divided into two groups: Mesothelae and Opisthothelae (Ruppert, 1994). The suborder Mesothelae comprises a single family (Liphistiidae) of primitive spiders that are characterized by the segmented abdomen, while the Opisthothelae spiders do not show external segmentation of the abdomen (Dutra, 2006). The infraorder Mygalomorphae, which is the most basal of the Opisthothelae group, include spiders that have a pair of chelicera parallel to the direction of the body and a pair of leaf lungs. The Mygalomorphae comprises the families Theraphosidae, Dipluridae and Hexatelidae (Vizzotto, 2009).

Morphologically, the body of a spider consists of two main parts (Figure 1): an anterior portion or carapace called prosome and a posterior portion (the abdomen) called opisthosome. The structure that connects the prosome and the opisthosome is called pedicel. The prosome supports four pairs of legs, a pair of chelicerae and a pair of pedipalps (modified in males on copulatory organs) (Platnick, 1971; Foelix, 1996). In addition, there are the eyes (Figure 1) in the prosome, which can be found in number of two, six or eight, and are extremely important

for taxonomy. The opisthosome contains the respiratory, circulatory, digestive and reproductive systems (Foelix, 1996).

The Mygalomorphae spiders are commonly called as tarantulas. Thousands of mygalomorph species are described, distributed in the families Actinopodidae, Antrodiaetidae, Atypidae, Barychelidae, Ctenizidae, Cyrtaucheniidae, Dipluridae, Hexathelidae, Idiopidae, Mecicobothriidae, Migidae, Microstigmatidae, Nemesiidae, Paratropididae, and Theraphosidae. The Theraphosidae family is represented by around 976 species divided in 128 genera, distributed across the globe and occurring in various habitats (Platnick, 2015). This family possesses a genus (*Lasiodora*) that is under systematic review and includes a group of spiders found in Brazilian northeast (*Lasiodora* sp.), which will be discussed forward.

Figure 1.A tarantula spider from *Lasiodora* genus, evidencing the major anatomical divisions in mygalomorphs. (A) A top view of the body, emphasizing the division in opisthosome and prosome. (B) A frontal view of the body, highlighting the position of the eyes.

Similar to all arthropods, the spiders have an exoskeleton that surrounds the body of the animal and is constituted by chitin secreted by the monolayered epidermis. Numerous trunk and appendicular muscles are attached to this structure. The external layer of the exoskeleton is sclerotized (Bitsch and Bitsch, 2002).

During the growth process, the arthropods pass by the molt and, in general, most of the spiders cease growth and the exchange of exoskeleton when achieve the reproductive stage (Silva Jr., 2000).

However, in large tarantulas, the adult females continue to carry out the molt once a year or at irregular intervals (Silva Jr., 2000). Accidents caused by spiders are classified in distinct groups according to the effects of venom in humans: those that produce necrotic ulceration and those that not produce necrosis (Schwatrz et al. 2012). Although many people consider tarantula spiders as the most dangerous, their bites do not induce local necrosis or systemic effects, being only painful. Nevertheless, tarantulas have been a relatively popular pet for several years. Indeed, these arachnids are popular as household pets and several websites in social networks can be found containing informations about the maintenance of spiders as pets, making it a fascinating hobby.

3. Hemolymph and Hemocytes

The fresh hemolymph of a spider shows a blue color due to the presence of copper contained in the respiratory pigment hemocyanin. The hemolymph is analogue to the blood of vertebrates and shows a variety of circulating cells called hemocytes. This organic fluid include substances such as hemocyanin (accounting for about 80%), amino acids (glutamine is the most abundant), carbohydrates (mainly glucose), and fatty acids, including palmitic, linoleic and stearic acids (Foelix, 1996; Tillinghast and Townley, 2008; Coates and Nairn, 2013). One of the main ways of collecting the hemolymph is by cardiac puncture in the presence of anticoagulant (Soares et al. 2011). The heart of tarantula spiders can be clearly viewed after scraping of the dorsal hairs (Figure 2).

Figure 2. The heart (indicated by the arrow) of a *Lasiodora* spider viewed by transparency after scraping of the dorsal spider hairs.

The hemocyanins are multimeric copper-containing proteins that transport oxygen in the hemolymph, but also have multiple other functions (Coates and Nairn, 2013, 2014; Starrett et al. 2013). In chelicerates, hemocyanin appears to play an important role in innate immunity system, homeostasis and molt (Nagai et al. 2001; Adachi et al. 2003; Cerenius and Söderhäll, 2004; Kuballa and Elizur, 2008; Kuballa et al. 2011; Glazer et al. 2013). *In vitro* coagulation cascade components and antimicrobial factors can induce hemocyanin to exert phenoloxidase activity (Nagai et al. 2001; Adachi et al. 2003; Baird et al. 2007; Kuballa et al. 2011; Jeanicke and Decker, 2008) as well as antimicrobial and antiviral activities (Zhang et al. 2004; Pan et al. 2005; Riciluca et al. 2012; Coates and Nairn, 2014; Qiu et al. 2014).

Mechanical injuries and the presence of invader objects or microorganisms in hemolymph may also result in the deposition of melanin around the damaged tissue or the foreign body. The melanin physically holds an attacker, preventing or slowing its growth. Also, reactive and toxic intermediates, such as quinines, are produced during the formation of melanin and act causing the microorganism death (Cerenius and Söderhäll, 2004). The melanization mechanism is part of the innate immune system and is present in the hemolymph as a protein complex mixture of several molecules including proteases. The activation of these proteases is carefully regulated by phenoloxidase system, which consists

of a cascade of proteins capable of binding to polysaccharides and other compounds typically associated with microorganisms such as peptidoglycans and lipopolysaccharides (Silva, 2002).

The goal of the immune response is to remove or perceive the invader, through non antigen-specific mechanisms that involve specific cells and molecules such as enzyme inhibitors, antimicrobial peptides and lectins. The hemocytes are the cells that have the ability to defend invertebrates against pathogens, parasites and other foreign bodies that had penetrated the hemocele. Defense reactions directly exerted by hemocytes are phagocytosis, encapsulation and damage repair (Lavine and Strand, 2002). The circulating hemocytes are also involved in the release of antimicrobial peptides, the coagulation of the hemolymph, the melanin formation and the complement activation. The hemocytes are extremely sensitive to bacterial lipopolysaccharide (LPS), responding through the release of granular components, including antimicrobial peptides. The production of these peptides is also mediated by Toll-like receptors (Iwanaga and Lee, 2005). Upon reaching the site of infection, the hemocytes can secrete the components of the coagulation cascade in the hemocele (Fukuzawa et al. 2008). The hemolymph coagulation phenomenon was firstly identified as a defense system in the horseshoe crab *Limulus polyphemus* (Bang, 1956).

In the horseshoe crab, the hemocytes are known as granulocytes because they have granules of various sizes. When a hemocyte detects bacterial endotoxins (such as LPS groups), it releases their granules through a rapid exocytosis. Among the released granular content, there are two serineprotease zymogens (called factors C and G), which are catalytically activated in response to LPS and β-1,3-D-glucan, the main components of cell wall of Gram-negative bacteria and fungi, respectively (Muta and Iwanaga, 1996). This activation results in the conversion of the protein coagulogen to coagulin, which forms a gel that hinders the spread of the microorganism. The invaders detained by the gel (clot) are phagocytosed by hemocytes and then killed by action of lectins, antimicrobial compounds and protease inhibitors found in these cells.

According to Xylander and Nevermann (2006) and Xylander (2009), the arthropod hemocytes can be classified as prohemocytes, plasmatocytes, granulocytes, spherulocytes, coagulocytes, discoid hemocytes, adipohemocytes, cystocytes, oenocytoids and cyanocytoids. In contrast to insects and crustaceans, the immune system of the most groups of chelicerates is not well-investigated. Studies addressing arthropod hemocytes have mainly focused on vectors of diseases that affect humans directly (Araújo et al. 2008, Castilho et al. 2006, Hillyer and Strand, 2014) in detriment to ecologically important and species-rich taxa, such as scorpions and spiders, which also contain many species of medical importance (Kuhn-Nentwig et al. 2014).

Sherman (1981) classified the hemocytes of the tarantula spider *Eurypelma marxi* according to insect nomenclature in plasmatocytoids, oenocytoids and granular hemocytes. Foelix (1996) classified the spider cells in four types: granulocytes, leberidocytes, cyanocytes and prohemocytes. The more actual classification for hemocytes of spiders comprises five cell types, named based on the nomenclature used for insects: plasmatocytes or hyaline cells (immune response), cyanocytes or oenocytes (involved in respiration and immune response), granulocytes (mainly acting in the immune response), prohemocytes (stem cells) and leberidocytes (present in moulting individuals) (Kuhn-Nentwig et al. 2014).

The cells differ in shape and cytochemical/electron microscopic staining of their cytoplasma and granules. Frequently, the researchers use more than one type of microscopy

to insure the different types of cells. Other aspects that may influence the results are the method of punction and spider health state. These are one of the reasons for that the authors differ so much on the spider hemocytes classification.

Lectins, protease inhibitors, and antimicrobial peptides have been isolated from both hemolymph plasma and hemocytes of arthropods. As some examples, it has been reported the protease inhibitor from plasma of silkworm *Antheraea mylitta* (Shrivastava and Ghosh, 2003), the serine proteinase inhibitor from plasma of *Manduca sexta* (Wang & Jiang, 2004), the trypsin and subtilisin inhibitor from hemocytes of shrimp *Litopenaeus vannamei* (Vega and Albores, 2005), and an AMP from hemocytes of the tick *Boophilus microplus* (Fogaça et al. 2006). Foradori et al. (2006) investigated the digestive fluid of spider *Argiope aurantia* and isolated two peptidases of lower molecular mass, called p16 and p18. The authors also showed evidences of the presence of serine peptidase inhibitor. Wan et al. (2013) reported that the chymotrypsin inhibitor of the spider *Araneus ventricosus* also acts as elastase inhibitor and microbial serine protease inhibitor.

In arthropods, protease inhibitors play important roles controlling endogenous activity of proteases involved in digestion and activation of phenoloxidase cascade as well as of microbial proteases that act as virulence factors (Kanost, 1999). Protease inhibitors are classified into five groups (serine, threonine, cysteine, aspartyl and metalloprotease inhibitors) according to the mechanism employed at the active site of proteases that they inhibit (Fear et al. 2007).

Protease inhibitors are essential for organisms for controlling protein damage caused by self and non-self proteases (Simonet et al. 2002). Most of the invertebrate protease inhibitors were isolated initially from insect hemolymph and could be classified in two groups: Kunitz-type family, corresponding to low molecular mass proteins; and serpin superfamily, corresponding to proteins of approximately 45 kDa (Polanowski and Wilusz, 1996). Another two main families of arthropods proteases inhibitors are pacifastin and cystatin. The pacifastin family constitutes a family of peptidic inhibitors of serine proteases that are considered to be important regulators of several physiological processes in arthropods (Breugelmans et al. 2008). Cystatins are involved in various physiological and cellular processes, including immune responses, protein homeostasis, signaling pathways, and apoptosis (Wan et al. 2013).

Serine proteases involved in hemolymph coagulation and activation of prophenoloxidase processes, which are restricted to arthropods, are controlled by serine protease inhibitors of Kunitz, serpin and pacifastin families present in the hemolymph of arthropods. These inhibitors can act on specific proteases or on more than one type of proteases (Theopold et al. 2004; Fear et al. 2007). Inhibitors of serine proteases could also mediate the production of antimicrobial peptides (Fogaça et al. 2006).

Present in all living organisms, the AMPs consist in small molecules with about one hundred amino acids in length (Pasupuleti et al. 2012) and are evolutionarily conserved (Hancock and Sahl, 2006). The AMPs are used by the host for protection against different types of pathogens and are a heterogeneous group with respect to their primary and secondary structures, antimicrobial action and effects on host cells. The AMPs usually have a positive charge provided by arginine and lysine residues and an amphipathic structure that allows them interact with the bacterial membrane (Auvynet and Rosenstein, 2009).

The AMPs have been classified into families and sub-families based on their primary sequences and structures (Yeaman and Yount, 2003). The AMP families described differ

among each other in physicochemical and chemical structure (Khamis et al. 2015). The AMPs isolated until now have been organized in public databases like APD (Wang and Wang, 2004), ANTIMIC (Brahmachary et al. 2004), APD2 (Wang et al. 2009), DAMPD (Sundararajan et al. 2012) and CAMP (Thomas et al. 2010; Waghu et al. 2014). The database APD contains 2529 antimicrobial peptides described with the following activities: antibacterial, antiviral, antifungal, antiparasitic, anticancer, antiprotozoal, insecticidal, spermicidal, chemotactic, and antioxidant (Wang and Wang, 2009).

Several AMPs have been isolated from the venom and hemolymph of scorpions and spiders (Kuhn-Nentwig, 2003). Silva Jr. (2000) purified and characterized four molecules present in the hemolymph of the tarantula spider *Acanthoscurria gomesiana*. The first was the theraphosin, purified from plasma, and the other three peptides, called mygalin, gomesin and acanthoscurrin, were isolated from hemocytes (Silva Jr., 2000; Lorenzini et al. 2003; Pereira et al. 2007). The theraphosin is a 4052.5 Da peptide with activity against *Micrococcus luteus*. The mygalin is a peptide of 415.9 Da with activity against *Escherichia coli* and able to induce the production of hydrogen peroxide. Gomesin is a 2270.4 Da peptide, with high similarity to tachyplesins and protegrins (peptides from the horseshoe crab), with broad activity against bacteria, fungi, yeasts and *Leishmania*. The gomesin structure includes a pyroglutamic acid as the N-terminal, one α-*C*-terminal amide arginine and four cysteine residues that form two disulfide bonds. The acanthoscurrin is a glycine-rich peptide presenting two isoforms with 10132.4 and 10249.1 Da, which differ by the presence of two additional glycine residues; the acanthoscurrin showed activity against *E. coli* and *Candida albicans* and similarity to insect antifungal proteins and proteins related to defense in plants (Silva Jr., 2000; Lorenzini et al. 2003; Pereira et al. 2007).

A study demonstrated that mygalin is not cytotoxic to murine cells *in vitro* and does not affect cell proliferation or IL-2 production. This peptide activates the innate immune response through induction of Th1 cytokines and proinflammatory mediators, such as TNF-α and nitric oxide, that are essential for defense against infectious pathogens (Mafra et al. 2012). Moreover, as a proinflammatory factor that regulates the immune response, mygalin can be exploited with therapeutic purposes alone or in combination with other molecules such as gomesin, which has anti-cancer activity (Soletti et al. 2010; Mafra et al. 2012).

Besides the identification of AMPs isolated from the hemocytes of *A. gomesiana* (Silva Jr., 2000; Lorenzini et al. 2003), only limited information on the innate immune system of spiders is available. Antimicrobial activity was detected in the hemolymph of spider *Acanthoscurria rondoniae* due to the presence of an antifungal peptide called rondonin (Riciluca et al. 2012). Rondonin has the amino acid sequence IIIQYEGHKH and a molecular mass of 1236.776 Da, corresponding to the first report of a fragment of hemocyanin with antifungal activity (Riciluca et al. 2012). Peptides called ctenidins showing activity against Gram-negative bacteria were purified from the hemocytes of the spider *Cupiennius salei* (Baumann et al. 2010).These peptides, with over 70% glycine residues resembling acanthoscurrin, are constitutively expressed in hemocyte and nerve tissues, and their expressions are independent of immune challenges (Baumann et al. 2010).

The appearance of new diseases and increased resistance of bacteria to antibiotics in recent decades have become an increasing threat to human health. This has driven a constant search for new agents that have antibacterial activity, especially against resistant strains, leading to an increasing interest in AMPs (Kamysz et al. 2003; Godoy et al. 2013).

4. Hemocytes from *Lasiodora* sp.: A Brazilian Tarantula Species under Systematic Review

The tarantulas from *Lasiodora* genus belongs to the Theraphosidae family, can reach the average age of 25 years, and are 15–25 cm long with the legs extended (Bertani, 2001). Members of the genus *Lasiodora* are widely distributed in Brazil, where they are called "caranguejeiras" (Horta et al. 2013). Until now there are 39 species described for the *Lasiodora* genus, according Platnick (2015).

Lasiodora spiders show a black or brown color and has stinging hairs on the abdomen of the types I, III and/or IV, which can be launched when the animal feels threatened (Foelix, 1996). Many American theraphosid spiders possess these hairs covering their opisthosome, which are brushed with the hind legs into the direction of the perceived attack, so defensive bites are rarely necessary (Fuchs et al. 2014).

The different species of *Lasiodora* spiders are difficult to distinguish. Reliable morphological identification of tarantulas is most difficult due to the very similar features of many species. Classical methods are based on the examination of male genitalia, shape of body appendages or hair counts. In addition, the difficulty of accessing habitats often means that putative classification is based on few preserved specimens (Escoubas and Rash, 2004).

Lasiodora spiders can be found at the Northeast, Southeast and Midwest regions of Brazil, especially in the Atlantic Forest (Bertani, 2001). In Brazil, this genus is currently under a process of systematic review, coordinated by Butantan Institute at São Paulo. Despite some significant progress has been achieved, there are still many gaps to be filled and this completion of this systematic review remains a long journey.

Tarantulas can be found in diverse types of environment and this diversity of ecological niches, associated with the diversity of prey capture behavior, contributes to the diversity of their poisons (Vieira et al. 2004). There are many studies on the purification of toxins from the venom of these spiders, including *Lasiodora* species.

Kusmerick et al. (2001) screened the *Lasiodora* sp. venom for activity against ion channels and suggested that venom of this spider evokes vesicular release of acetylcholine from parasympathetic nerve terminals in the heart by activating TTX-resistant Na^+ channels. Kalapothakis et al. (2003) reported that the *Lasiodora parahybana* and *Lasiodora* sp. venom interfere in the heart rate. Vieira et al. (2004) reported the identification of toxins LTx1, LTx2 and LTx3, which are expressed in the venom gland of *Lasiodora* sp. These toxins showed significant similarity at the amino acid level with spider toxins from *Lasiodora parahybana*, *Eurypelma californicum*, *Brachypelma smithii*, *Selenocosmia huwena*. Dutra et al. (2008) showed that the recombinant LTx2 toxin acts on calcium channels of BC3H1 cells, blocking L-type calcium channels.

However, there are few studies on the morphophysiology of hemocytes and bioactive molecules from hemolymph of tarantulas spiders, which could contribute to the systematic classification of *Lasiodora* at species level. In order to contribute in this sense, studies have been conducted *Lasiodora* spiders found in the rain forests at Pernambuco, a state at Northeastern Brazil, which are referred as *Lasiodora* sp.

Soares et al. (2013) classified the hemocytes of *Lasiodora* sp. in six morphological types: prohemocytes, granulocytes type I, granulocytes type II, spherulocytes, oenocytoids and

plasmatocytes. The most abundant cells were the granulocytes and Table 1 summarizes the ultrastructural characteristics of *Lasiodora* sp. hemocytes.

Fukuzawa et al. (2008) suggested that phagocytosis is not the major defense mechanism activated towards a microbial challenge and probably plays a secondary role, being responsible for clearing cellular debris and remodeling damaged tissues. These authors showed that the injection of particles into the legs of the tarantula *Acanthoscurria gomesiana* clearly activated a coagulation cascade. Soares et al. (2013) reported that the same occurred after cardiac punction of *Lasiodora* sp. and the Figure 3 shows this process observed by optical microscopy. Based on this observation the authors suggested that *Lasiodora* sp. spherulocytes participated in the hemolymph coagulation.

Table 1. Ultrastructural characteristics of hemocyte found in *Lasiodora* sp.

Hemocyte type	Relative population* (%)	Cell characteristics
Prohemocytes	8.1	10–15 μm in diameter Spherical shape Central nucleus Few granules and mitochondria
Granulocytes type I	35.2	25–30 μm in diameter Elongated shape Lobed and central nucleus Numerous dense granules and mitochondria
Granulocytes type II	29.6	35–40 μm in diameter Elliptical shape Central nucleus Elliptical granules and few mitochondria
Spherulocytes	11.1	15–25 μm in diameter Uniform (round) cell and nucleus shape Numerous granules near to the plasma membrane
Oenocytoids	3	15–20 μm in diameter Round cell shape Variable nucleus shape Many mitochondria and some granules
Plasmatocytes	13	15–20 μm in diameter Variable cell and nucleus shape Cytoplasm without granules

*The determination of relative hemocyte populations was performed using slides prepared with fresh hemolymph (10μl) in triplicate analysed by bright-field microscopy (magnification: 100x). Reference: Soares et al. (2013).

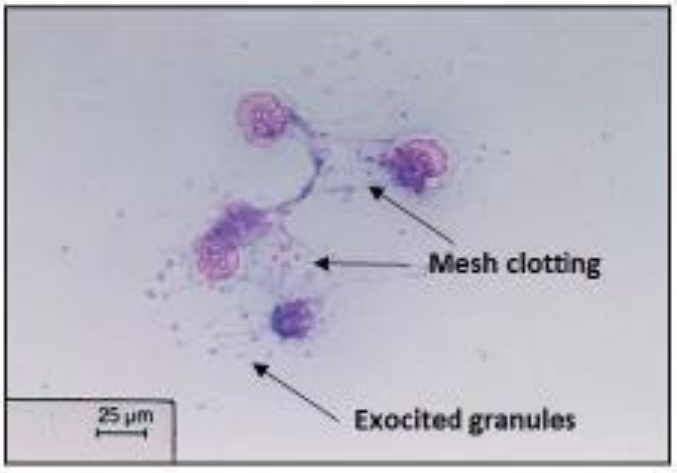

Figure 3. Spherulocyte degranulation and clotting process in *Lasiodora* sp. observed by optical microscopy. The presence of mesh clotting and the exocytosis of contents of granules by spherulocytes evidence that the coagulation cascade was activated.

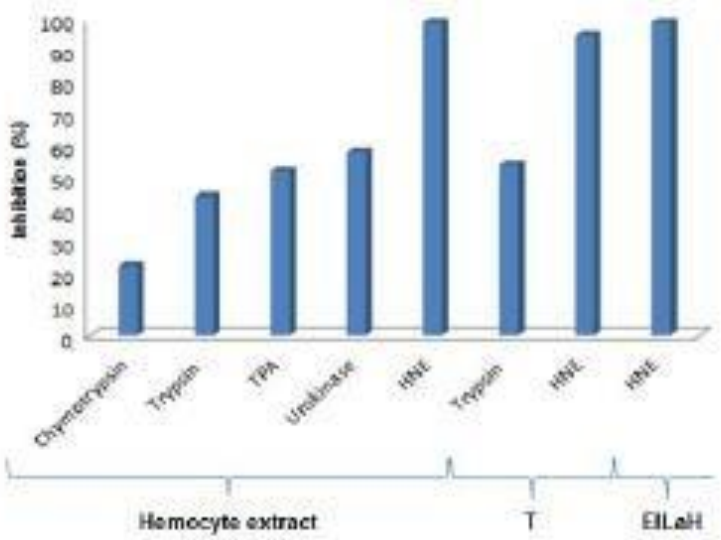

Figure 4. Inhibitory effect on proteases of *Lasiodora* sp. hemocyte extract, a partially purified pool from Trypsin-Sepharose chromatography (T) and isolated elastase inhibitor (EILaH). TPA: tissue plasminogen activator. HNE: human neutrophil elastase.

Hemocytes of *Lasiodora* sp. were also investigated as source of protease inhibitors by Soares et al. (2011). The authors showed that the hemocyte extract was able to inhibit chymotrypsin, trypsin, tissue plasminogen activator, urokinase and mainly human neutrophil elastase (Figure 4). Then, they isolated an antibacterial elastase inhibitor (called EILaH) using affinity chromatography on Trypsin-Sepharose and reversed-phase chromatography. The Figure 5 shows the protein profile of hemocyte extract, Trypsin-Sepharose fraction and isolated inhibitor and Table 2 shows that the inhibitor was active only on *Enterococcus faecalis*.

Table 2. Bacteriostatic activity of preparations from *Lasiodora* sp. hemocytes

Preparation	Bacteria	Minimal Inhibitory Concentration (µg/ml)
Hemocyte extract	*Bacillus subtilis* ATCC-6633	3400
	Enterococcus faecalis ATCC-6057	3400
	Escherichia coli ATCC-25922	NI
	Klebsiella pneumoniae ATCC-29665	NI
	Staphylococcus aureus ATCC-6538	NI
EILaH	*Bacillus subtilis* ATCC-6633	NI
	Enterococcus faecalis ATCC-6057	227.5
	Escherichia coli ATCC-25922	NI
	Klebsiella pneumoniae ATCC-29665	NI
	Staphylococcus aureus ATCC-6538	NI

NI: no inhibition. Reference: Soares et al. (2011).

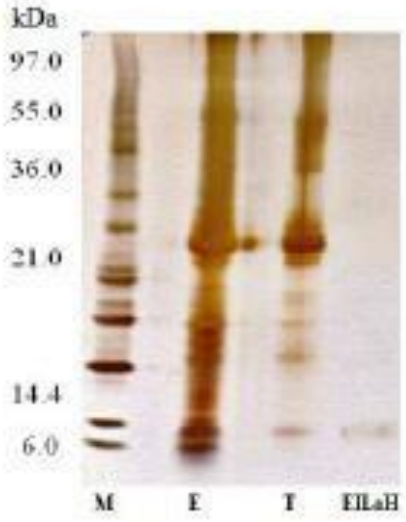

Figure 5. Polyacrylamide gel (12%, w/v) electrophoresis in presence of sodium dodecyl sulfate (SDS-PAGE). Molecular mass standard (M). Extract of *Lasiodora* sp. hemocytes (E), Trypsin-Sepharose column fraction (T) and isolated human elastase inhibitor (EILaH). The isolation of EILaH is described by Soares et al. (2011).

CONCLUSION

Hemocytes and molecules from hemolymph are important components of immunity of spiders, being responsible for phagocytosis, nodulation, encapsulation, coagulation, melanization, and cytotoxicity mechanisms that act against microorganisms. Several types of hemocytes can be found in tarantula spiders, including the *Lasiodora* sp. The identification ofhemocyte types and the isolation of a protease inhibitor from hemocytes of *Lasiodora* sp. were recent contributions for the systematic review of this genus.

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8. CONCLUSÃO

O estudo identificou prohemócito, granulócito tipo I, granulócito tipo II, esferulócito, oenocitóide e plasmatócito na hemolinfa de *Lasiodora* sp. A investigação da presença de peptídeos antimicrobianos em hemócitos de *A. rondoniae*, *Lasiodora* sp. e *V. sorocabae*, revelou que os peptídeos antimicrobianos acanthoscurrina, gomesina e mygalina encontram-se conservados em espécies da família Theraphosidae.