UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

CENTRO DE BIOTECNOLOGIA DO ESTADO DO RIO GRANDE DO SUL

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

SALIVA DE CARRAPATOS: ESTUDO PROTEÔMICO E CARACTERIZAÇÃO DE PROTEÍNAS SALIVARES NA RELAÇÃO PARASITO-HOSPEDEIRO

Lucas Tirloni

Porto Alegre, agosto de 2015

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Tese submetida ao Programa de Pós-graduação em Biologia Celular e Molecular (PPGBCM) da UFRGS como parte dos requisitos para a obtenção do grau de Doutor em Ciências.

Orientador: Dr. Itabajara da Silva Vaz Jr.

Coorientador: Dr. Carlos Termignoni

Coorientador: Dr. Albert Mulenga

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“De tudo ficaram três coisas...
A certeza de que estamos começando...
A certeza de que é preciso continuar...
A certeza de que podemos ser interrompidos
antes de terminar...”

Fernando Sabino

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LISTA DE ABREVIATURAS, SÍMBOLOS & UNIDADES

Å	Angstrom
AAS-19	*Amblyomma americanum serpin 19*
ADP	difosfato de adenosina
APC	proteína C ativada
aPTT	tempo de tromboplastina parcialmente ativada
ATP	trifosfato de adenosina
BCA	ácido bicinconínico
BCIP	5-bromo-4-cloro-3-indolilfosfato
BLAST	*basic local alignment search tool*
BMGY	meio de cultura tamponado contendo glicerol
Bm86	*Boophilus microplus 86 kDa protein*
BSA	albumina sérica bovina
cDNA	DNA complementar
CD43	*cluster of differentiation 43*
CEUA	Comissão de Ética no Uso de Animais
C-terminal	carbóxi-terminal
DAB	diaminobenzidina 3,3'
DAPI	*Dermacentor andersoni primary infestation*
DEPC	dicarbonato de dietila
DNA	ácido desoxirribonucleico
DTT	ditiotreitol

ELISA	*enzyme-linked immunosorbent assay*
ECP	proteína catiônica de eosinófilos
EDN	neurotoxina derivada de eosinófilos
EPO	peroxidase de eosinófilos
FEF	fêmea totalmente engurgitada
GeLC-MS/MS	eletroforese unidimensional seguida de cromatografia líquida acoplada à espectrometria de massas em tandem
HAT	*hypoxanthine-aminopterin-thymidine medium*
hpi	*hour post infestation*
HRP	*horseradish peroxidase*
HSP	proteína de choque-térmico
IgG	imunoglobulina G
IgM	imunoglobulina M
GST	glutationa S-transferase
GO	*Gene Ontology*
INCT-EM	Instituto Nacional de Ciência e Tecnologia em Entomologia Molecular
IRIS	*Ixodes ricinus immunosuppressor*
IRS-2	*Ixodes ricinus serpin 2*
LC-MS/MS	cromatografia líquida acoplada à espectrometria de massas em tandem
MAbs	anticorpos monoclonais

MW	massa molecular
N	Norte
NBT	nitroazul de tetrazólio
NCBI	*National Center for Biotechnology Information*
N-terminal	amino-terminal
OD	densidade óptica
ORF	fase aberta de leitura
PA	ativador de plasminogênio
PAR2	receptor ativado por protease 2
PAR4	receptor ativado por protease 4
PBS	solução salina tamponada com fosfato
PBS-T	solução salina tamponada com fosfato contendo Tween
PEF	fêmea parcialmente engurgitada (*partially engorged female*)
PT	tempo de protrombina
pH	potencial hidrogeniônico
PRP	plasma rico em plaquetas
RCL	alça do centro de reação
rIL-5	interleucina 5 recombinante
RmS	serpina de *Rhipicephalus microplus*
rRmS	serpina de *Rhipicephalus microplus* recombinante
RNA	ácido ribonucleico
RNAi	RNA de interferência

RNAm	RNA mensageiro
RPM	rotação por minuto
RT	tempo de recalcificação
RT-PCR	transcrição reversa – reação em cadeia da polimerase
S	Sul
SCCA-2	antígeno celular de carcinoma escamoso 2
SDS	dodecilsulfato de sódio
SDS-PAGE	eletroforese em gel de poliacrilamida na presença de dodecilsulfato de sódio
SEM	erro padrão da média
SG	glândula salivar
SI	estequiometria de inibição
TF	fator tecidual
TIL	*trypsin-like inhibitor*
TSA	*transcriptome shotgun assembly*
t-PA	ativador de plasminogênio tipo tecidual
TT	tempo de trombina
U	unidade enzimática
u-PA	ativador de plasminogênio tipo uroquinase
V_i	velocidade máxima na presença de inibidor
V_{max}	velocidade máxima
V_0	velocidade máxima na ausência de inibidor

W	Oeste
WBBL	sangue bovino total
YPD	extrato de levedura, peptona e dextrose

RESUMO

Carrapatos são ectoparasitos hematófagos que causam grandes impactos na saúde pública e na produção animal devido à sua capacidade vetorial e também pelos distúrbios associados diretamente ao parasitismo. O carrapato *Rhipicephalus microplus* é considerado o parasito mais importante de bovinos na América Latina e Oceania. Os carrapatos permanecem fixados ao hospedeiro por um longo período de tempo, e a saliva representa um fator crucial na relação parasito-hospedeiro. Os carrapatos secretam na saliva um conjunto de moléculas com atividades que modulam as respostas de defesa do hospedeiro frente ao parasitismo, tais como as reações do sistema hemostático e do sistema imune inato e adaptativo. Considerando a importância econômica do carrapato *R. microplus*, e o papel da saliva no parastismo, pouco se sabe sobre a composição proteica da saliva desse parasito. Os objetivos desse trabalho são caracterizar o proteoma da saliva do carrapato *R. microplus* em diferentes momentos da fase de alimentação no hospedeiro bovino e caracterizar três serpinas salivares. Neste sentido, este estudo foi organizado em três partes. Primeiramente, fizemos uma análise proteômica da saliva de fêmeas adultas em dois estágios de ingurgitamento, compreendendo fêmeas parcialmente ingurgitadas e fêmeas totalmente ingurgitadas. Num segundo momento, analisamos mais a fundo a variação dos componentes proteicos salivares das fêmeas, expandindo a análise proteômica para nove fases de ingurgitamento do parasito. Esses dados mostraram que a saliva de *R. microplus* é uma amostra proteica complexa, com variação nos diferentes tempos de alimentação. Entre as classes de proteínas identificadas, podemos citar proteínas relacionadas com metabolismo de heme e ferro; proteínas relacionadas com processos de oxidação e detoxificação; enzimas como as serino-proteases, cisteíno-proteases, metaloproteases; inibidores de proteases da família das serpinas, cistatinas, TIL e Kunitz; peptídeos antimicrobianos; e famílias de proteínas específicas e conservadas em carrapatos. A terceira parte do trabalho objetivou a caracterização de três serpinas que foram identificadas na saliva (RmS-3, RmS-6 e RmS-17). Essas proteínas foram expressas em *Pichia pastoris* e foram utilizadas em ensaios de caracterização funcional contra um painel de 16 proteases relacionadas com os sistemas de defesa do hospedeiro, revelando um perfil de inibição sobre proteases pró-inflamatórias e pró-coagulantes. Os dados apresentados neste trabalho contribuem para o entendimento do papel de proteínas salivares durante o parasitismo pelo carrapato *R. microplus*.

ABSTRACT

Ticks are hematophagous ectoparasites with worldwide distribution impacting on public health and animal production due to their vectorial capacity and also for disorders associated directly to parasitism. The tick *Rhipicephalus microplus* is considered the most important parasite of cattle in Latin America and Oceania. As hematophagous ectoparasites which remain attached to the host for a long period of time, the saliva of ticks represent a crucial factor in host-parasite relationship. The tick saliva comprises an arsenal of molecules with activities modulating the host defense responses trigged against tick feeding, such as the reactions of the hemostatic system, the innate immune system and the adaptive immune system. Considering the economic importance of the tick *R. microplus*, little is known about the protein composition of the saliva of this parasite. The aims of this study were to characterize the *R. microplus* tick saliva proteome at different feeding times, and to characterize three salivary serpin that were identified in the saliva of this parasite. Thus, this study was organized into three parts. Firstly, we conducted a proteomic analysis of female saliva in two developmental stages of adult tick feeding, including partially engorged and fully engorged females. Secondly, we analyzed in depth the variation of salivary protein components, expanding proteomics analysis to nine stages of tick feeding. These data showed that the *R. microplus* saliva is a complex protein sample, with variations in different feeding times. Among the classes of identified proteins, we can mention proteins related to heme and iron metabolism; proteins related to oxidation and detoxification processes; enzymes such as serine proteases, cysteine proteases, metalloproteases; proteases inhibitors belonging to serpin family, cystatins, TIL, and Kunitz; antimicrobial peptides; and families of tick-specific and conserved proteins. The third part of the study aimed to characterize three serpins that have been identified in saliva (RmS-3, RmS-6 and RmS-17). These proteins were expressed in *Pichia pastoris* and were used for functional characterization against a panel of 16 host proteases related to defense systems. Results revealed an inhibition profile for pro-inflammatory and pro-clotting proteases. The data presented here contributes to understanding the role of salivary proteins into tick-host relationship.

APRESENTAÇÃO

O meu treinamento científico iniciou-se ao final da minha gradução em Biomedicina na Universidade de Cruz Alta em 2009, onde durante a realização dos estágios curriculares em Porto Alegre ingressei no grupo de pesquisa do Dr. Carlos Termignoni, Dr. Itabajara da Silva Vaz Jr. e da Dra. Aoi Masuda. Após a conclusão do curso de graduação, ingressei no mestrado onde tive a oportunidade de trabalhar na identificação e caracterização de uma família de inibidores de serino-proteases (serpinas) do carrapato bovino. No doutorado, trabalhei com estudos proteômicos da saliva de carrapatos, e na caracterização de algumas serpinas salivares dos carrapatos *Rhipicephalus microplus* e *Amblyomma americanum*.

A presente tese é o resultado do trabalho desenvolvido no período de três anos no Programa de Pós-Graduação em Biologia Celular e Molecular da Universidade Federal do Rio Grande do Sul. Durante esses três anos, passei o período de um ano em estágio de doutorado sanduíche no labortório do Dr. Albert Mulenga, no Departamento de Entomologia da Texas A&M University. Os trabalhos aqui apresentados foram realizados sob a orientação do Dr. Itabajara da Silva Vaz Jr. e coorientação do Dr. Carlos Termignoni e do Dr. Albert Mulenga. Os resultados desta tese estão dividos em três capítulos que se referem a cada um dos trabalhos apresentados: (i) um artigo publicado no períodico PlosOne; (ii) um trabalho em fase de análise dos dados e redação de um manuscristo; e (iii) um manuscrito que será submetido ao periódico International Journal for Parasitology. Ao final desta tese constam outros quatro anexos: (i) protocolo de um pedido de patente junto ao Instituto Nacional de Propriedade Intelectual; (ii) um artigo publicado no periódico Parasites & Vectors; (iii) um artigo publicado no periódico International Journal for Parasitology; e (iv) um *Curriculum Vitae* resumido.

1 INTRODUÇÃO

Carrapatos são ectoparasitas hematófagos obrigatórios pertencentes ao filo Arthropoda, classe Arachnida, ordem Acarina, subordem Ixodida e são divididos em três famílias: a família *Argasidae* (*soft-ticks* ou carrapatos moles); a família *Ixodidae* (*hard-ticks* ou carrapatos duros); e a família *Nuttalliellidae* que é representada pelo gênero monoespecífico *Nuttalliella* (ANDERSON, 2002). Adicionalmente, de acordo com a posição do sulco anal, carrapatos da família *Ixodidae* são subdivididos em dois grupos: Prostriata, compreendendo unicamente o gênero *Ixodes*; e o grupo Metastriata, representado pelos gêneros restantes. Até o momento, cerca de 900 espécies de carrapatos foram descritas: *Ixodidae* (692 espécies), *Argasidae* (186 espécies) e *Nuttallielidae* (1 espécie) (SONENSHINE & ROE, 2013).

Os carrapatos são encontrados em quase todos os continentes, parasitando mamíferos, pássaros, répteis e anfíbios (ANDERSON & MAGNARELLI, 2008). Esses artrópodes são vetores de grande variedade de microrganismos patogênicos, protozoários, riquétsias, espiroquetas e vírus (SONENSHINE & ROE, 2013). Embora os mosquitos sejam vetores de patógenos que acometem maior número de pessoas, causando doenças com grande impacto em saúde pública (como a malária, dengue, febre amarela, etc.), os carrapatos são vetores de um número bem maior de patógenos que causam doença em seres humanos e outros animais, ocupando o segundo posto dos artrópodes vetores com importância em saúde pública (SONENSHINE & ROE, 2013). Atualmente, 17 doenças que acometem humanos são transmitidas por carrapatos, além de 19 doenças que afetam animais de companhia e animais de produção (DANTAS-TORRES *et al*., 2012). Dentre as principais espécies de carrapatos vetores de doenças humanas e animais, podemos citar o

Ixodes scapularis (vetor da Doença de Lyme), *Amblyomma cajennense* (vetor da febre maculosa brasileira), *Amblyomma americanum* (vetor da erliquiose, tularemia e *Q fever*), *Rhipicephalus microplus* (vetor do complexo tristeza parasitária bovina), *Ornithodoros coriaceus* (vetor do aborto epizoótico bovino), e *Ornithodoros moubata* (vetor da febre recorrente africana em humanos e da peste suína africana).

Além dessa capacidade de transmissão de patógenos que causam doenças, diferentemente da maioria dos outros artrópodes vetores, os carrapatos representam um risco potencial maior aos hospedeiros devido à grande espoliação de sangue. Dependendo da espécie, um único carrapato pode ingerir de 1 a 3 mL de sangue do hospedeiro durante o parasitismo, causando impacto na fisiologia dos hospedeiros (em infestações com múltiplos parasitos se alimentando). Além da espoliação de grande quantidade de sangue, as reações induzidas pelos componentes salivares injetados no hospedeiro durante o parasitismo também constituem um problema, podendo causar paralisia, toxicose e reações alérgicas (EDLOW & MCGILLICUDDY, 2008; RECK *et al*., 2011).

Além da importância dos carrapatos em saúde pública, devido ao seu papel na transmissão de doenças para humanos, o parasitismo por carrapatos e as doenças transmitidas por esses vetores aos animais domésticos são uma restrição importante para a produção animal. Predominantemente em áreas subtropicais do mundo, as infestações por carrapatos afetam o sustento da agricultura em regiões como a América Latina, Ásia, África e Oceania (JONGEJAN & UILENBERG, 2004).

1.1 O carrapato *Rhipicephalus* (*Boophilus*) *microplus*

O carrapato *Rhipicephalus microplus*, ou carrapato bovino, é considerado o parasito mais importante que afeta a produção pecuária em todo o mundo (JONGEJAN &

UILENBERG, 2004; GRISI *et al*., 2014). É um ectoparasito hematófago que possui o bovino como hospedeiro natural, contudo pode aparecer parasitando outros mamíferos (particularmente outros ruminantes) (MCCOY *et al*., 2013). O carrapato bovino é um parasito monóxeno, ou seja, parasita apenas um hospedeiro durante seu ciclo de desenvolvimento. O ciclo de vida do carrapato bovino compreende um período parasitário e um período de vida livre (Figura 1).

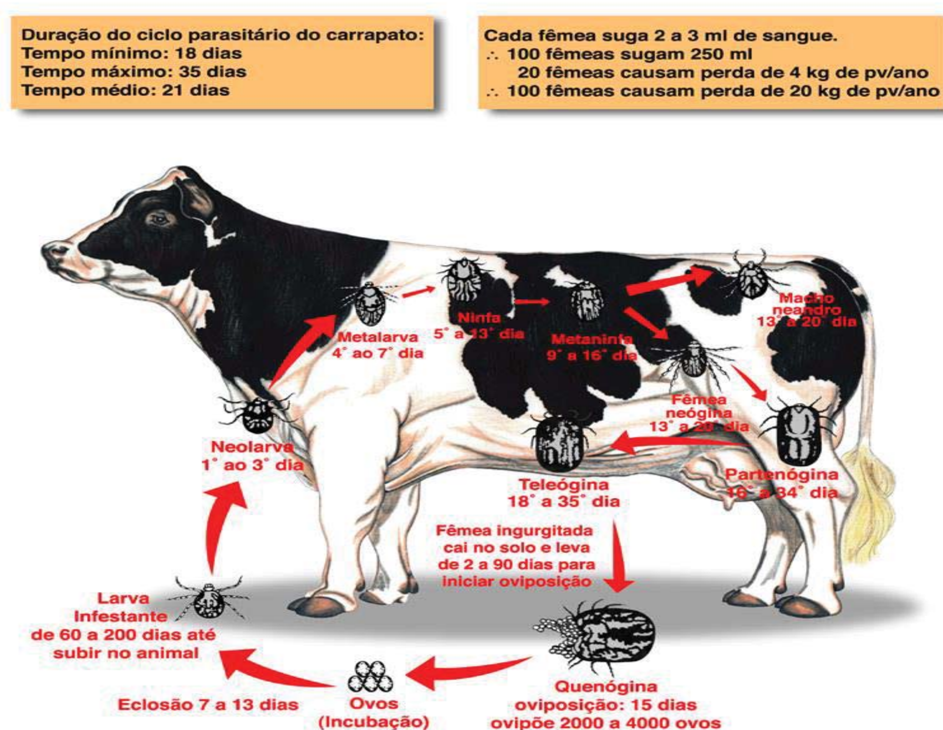

Figura 1. Ciclo de vida do carrapato *Rhipicephalus (Boophilus) microplus*. Figura disponível em https://www2.ufersa.edu.br/portal/view/uploads/setores/98/ENTOMOLOGIA/CARRAPATOS_AHID.pdf

A fase parasitária dura em média três semanas e inicia quando larvas infestantes encontram o hospedeiro. As larvas infestantes encontram-se em aglomerados de centenas ou milhares nas pastagens e, durante o pastoreio, movem-se avidamente ao encontro do corpo do bovino. Após a fixação no hospedeiro, as larvas iniciam sua alimentação, que

primeiramente não é baseada em sangue e sim em exsudato vascular e fluido linfático (SUTHERST *et al.*, 1978). Cerca de seis dias após a fixação, as larvas infestantes realizam muda e tornam-se ninfas. As ninfas alimentam-se de sangue por cerca de seis dias até realizarem nova ecdise, dessa forma dando origem a adultos imaturos (ROBERTS, 1968a; ROBERTS, 1968b). É no estágio de adultos que ocorre o dimorfismo sexual. Os machos também ingerem sangue, porém em quantidades muito menores que as fêmeas. Ao fim do seu repasto sanguíneo, os machos procuram as fêmeas para o acasalamento. A fêmea sexualmente madura é designada partenógina (parcialmente ingurgitada), esta permanece fixada ao hospedeiro ingerindo pouca quantidade de sangue até o momento da fecundação, no que compreende a chamada fase de ingurgitamento lento (ou *slow feeding phase*). Depois de fecundadas, as fêmeas aumentam consideravelmente a ingestão de sangue, na fase de ingurgitamento rápido (ou *rapid engorgement phase*), evento esse que ocorre por volta do 21º dia de parasitismo. Após a rápida ingestão de grandes quantidades de sangue, as fêmeas atingem o tamanho máximo, desprendendo-se do hospedeiro. As fêmeas caem ao solo geralmente no 22º dia pela manhã, sendo assim designadas teleóginas ou fêmeas totalmente ingurgitadas. Esse evento encerra a fase parasitária, dando origem à fase de vida livre do parasito. Já no solo, a fêmea procura local úmido e protegido da luz solar para postura dos ovos. A postura dura em torno de 14 dias, e a eclosão dos ovos em larvas inicia-se cerca de sete dias após a postura, fechando assim o ciclo de vida do parasito (ROBERTS, 1968a; ROBERTS, 1968b).

Supõe-se que o carrapato *R. microplus* possui origem no sudoeste da Ásia e hoje esteja distribuído entre os paralelos 32° N e 35° S, ou seja, nas regiões tropicais e subtropicais que incluem grande parte dos países em desenvolvimento (América Latina, África e Ásia Continental) e importantes regiões produtoras de carne bovina e leite, como

América Latina e Oceania (JONGEJAN & UILENBERG, 2004). O Brasil é o maior produtor de bovinos comerciais do mundo, sendo que 20% da carne consumida internacionalmente é de origem brasileira. Possui um rebanho de 212,8 milhões de cabeças e uma produção anual de 9 milhões de toneladas de carne bovina e 32 bilhões de litros de leite (IBGE, 2012). Esses dois segmentos combinados tem uma produção estimada em 67 bilhões de reais sendo projetado um crescimento de 2% ao ano pelos próximos 10 anos (MINISTÉRIO DA AGRICULTURA, 2014).

O carrapato *R. microplus* é um dos principais problemas econômicos para criadores de bovinos. Estima-se que anualmente as perdas potenciais decorrentes das infestações pelo *R. microplus* cheguem a 2 bilhões de dólares americanos no Brasil (GRISI *et al*., 2014). Diretamente, os principais prejuízos relacionados às infestações por carrapatos são diminuição na produção dos produtos animais, como carne e leite, além da perda da qualidade do couro, uma vez que reações inflamatórias que ocorrem nos locais de fixação do parasita causam cicatrizes que são visíveis mesmo após o curtimento (SUTHERST, 1983). Além dos distúrbios associados diretamente ao parasitismo, o *R. microplus* também é o vetor do complexo causador da tristeza parasitária bovina, que consiste em um complexo de doenças causadas por infecção pelos protozoários *Babesia bovis* e *Babesia bigemia* e pelas bactérias *Anaplasma marginale* e *Anaplasma centrale* (JONGEJAN & UILENBERG, 2004).

Atualmente o controle desse parasito é realizado através do uso de acaricidas químicos compostos por organofosforados, piretróides, formamidinas (amitraz), lactonas macrocíclicas (avermectinas), fluazuron e fipronil (MARTINS & FURLONG, 2001; LABRUNA *et al*., 2009). Os acaricidas são frequentemente criticados pelo seu custo e por necessitarem de período de carência antes do consumo dos produtos de origem animal.

Outra desvantagem dessa forma de controle é a crescente seleção de populações resistentes aos princípios ativos que compõem as formulações desses acaricidas (MARTINS & FURLONG, 2001; POHL *et al*., 2011; RECK *et al*., 2014). Portanto, é crescente a necessidade do estudo e desenvolvimento de métodos alternativos de controle.

O fato de que, após múltiplas infestações, animais naturalmente infestados são capazes de rejeitar entre 80% a 99% das larvas (TATCHELL & MOORHOUSE, 1968; ALLEN, 1994) aponta que a resposta imune pode ser usada como uma aliada no combate ao *R. microplus*. A primeira vacina disponibilizada comercialmente contra *R. microplus*, e a primeira vacina contra um ectoparasito, foi formulada com a proteína recombinante Bm86, um proteína da membrana celular do intestino do carrapato (WILLADSEN *et al*., 1989). A eficácia dessa vacina varia entre 51% e 91%, de acordo com a população de carrapato na região e da condição nutricional dos bovinos vacinados (RODRIGUEZ *et al*., 1995; PATARROYO *et al*., 2002). A variação na eficácia desta vacina deve-se principalmente aos polimorfismos existentes na região codificadora da Bm86 em diferentes populações de *R. microplus*, o que inviabiliza a sua utilização como alternativa de controle em muitos países, incluindo o Brasil e outros países da América do Sul (GARCIA-GARCIA *et al*., 2000).

A pesquisa por novos antígenos que possam aumentar a eficácia de vacinas contra o carrapato tem sido intensificada nos últimos anos (PARIZI *et al*., 2012). Um dos passos limitantes para o desenvolvimento de vacinas é a identificação e validação de proteínas-alvo que quando bloqueadas irão comprometer o desenvolvimento do parasito. Uma estratégia para o estudo de novos antígenos consiste na identificação, clonagem e produção *in vitro* de antígenos recombinantes e caracterização das possíveis funções biológicas dessas proteínas. Uma seleção racional de novos antígenos poderá ser obtida através do estudo de vias fisiológicas cruciais para o desenvolvimento do parasito.

1.2 O processo hematofágico

A hematofagia é o meio pelo qual os animais hematófagos adquirem a sua principal fonte de nutrientes, o sangue. A hematofagia está presente em mais de 15.000 espécies e 400 gêneros de artrópodes, incluindo os carrapatos (RIBEIRO, 1995; MANS, 2011). Os artrópodes hematófagos necessitam ingerir grandes quantidades de sangue para o seu desenvolvimento e sobrevivência, provendo assim os nutrientes necessários para realização da muda para os diferentes estágios de desenvolvimento e produção de ovos. Portanto, o estudo de proteínas relacionadas com a aquisição de sangue pelos carrapatos pode resultar na identificação de novas formas de controle desse parasito, além da identificação de novos compostos que possam ter outras aplicações biotecnológicas (STEEN *et al*., 2006; MARITZ-OLIVIER *et al*., 2007).

Durante o processo hematofágico, o primeiro passo a ser tomado pelos carrapatos para a aquisição de sangue é encontrar um hospedeiro no ambiente. Esse processo se dá por um complexo de reações comportamentais específicas, tendo o órgão de Haller um papel fundamental nesse processo. O órgão de Haller está situado na parte dorsal do primeiro par de patas dos carrapatos, e contem cápsulas com múltiplas sensílas compostas por diferentes quimio- e termo-receptores que contribuem para a identificação e localização dos hospedeiros (WALLADE & RICE, 1982). Após a localização, os carrapatos aderem-se ao hospedeiro e escolhem um lugar propício para dar início ao processo hematofágico. Nesse momento, o carrapato deve dar início ao rompimento da barreira mecânica da pele e acessar o sangue do hospedeiro.

De modo geral, o processo de obtenção de sangue realizado pelos artrópodes hematófagos ocorre por duas vias diferentes: mosquitos e percevejos utilizam seus aparatos bucais para canular as vênulas e arteríolas profundas da pele. Já os carrapatos adquirem

sangue a partir do fluído acumulado após a dilaceração dos tecidos e de pequenos vasos sanguíneos, que se acumula no sítio de alimentação do carrapato, também conhecido como bolsão hemorrágico (LAVOIPIERRE, 1965). O rompimento das barreiras da pele é um processo mecânico que se dá inicialmente pela varredura da pele do hospedeiro, objetivando a identificação de um sítio propício para a fixação do carrapato. Nesse processo, diferentes estruturas presentes no aparato bucal são utilizadas (Figura 2).

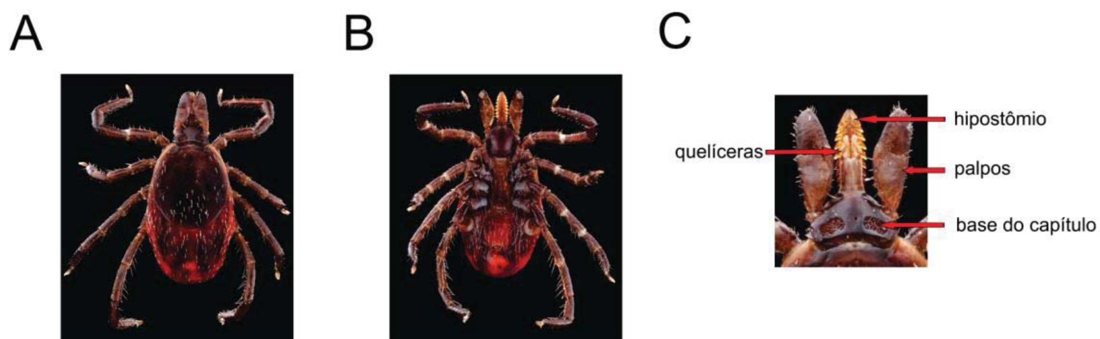

Figura 2. Morfologia do carrapato *Ixodes scapularis* (família *Ixodidae*). (A) Vista dorsal de uma fêmea adulta; (B) vista ventral de uma fêmea adulta; e (C) detalhe do aparato bucal de uma fêmea adulta, representado pela base do capítulo, palpos, quelíceras e hipostômio. Figura adaptada de SONENSHINE & ROE (2013).

Utilizando suas quelíceras, os carrapatos rompem a epiderme do hospedeiro para posterior introdução do hipostômio (RICHTER *et al*., 2013). Este por sua vez, quando introduzino na pele, dilacera os tecidos adjacentes provocando lesão do tecido do hospedeiro, com a formação do chamado bolsão hemorrágico, que contem sangue e outros fluídos tissulares oriundos do rompimento dos vasos sanguíneos e dos tecidos. A Figura 3 ilustra a análise histológica do local de fixação durante o parasitismo por ninfas do carrapato *Dermancentor andersoni*. Enquanto o carrapato se prepara para inserir as quelíceras e o hipostômio na pele do hospedeiro, a contração dos ductos salivares fazem com que a

salivação comece. A saliva é secretada no hospedeiro antes da aquisição de sangue, ocorrendo concomitantemente no momento em que as quelíceras e o hipostômio rompem os tecidos do hospedeiro, e também ao longo do processo hematofágico (BINNINGTON, 1978; BINNINGTON & KEMP, 1980; RICHTER *et al*., 2013).

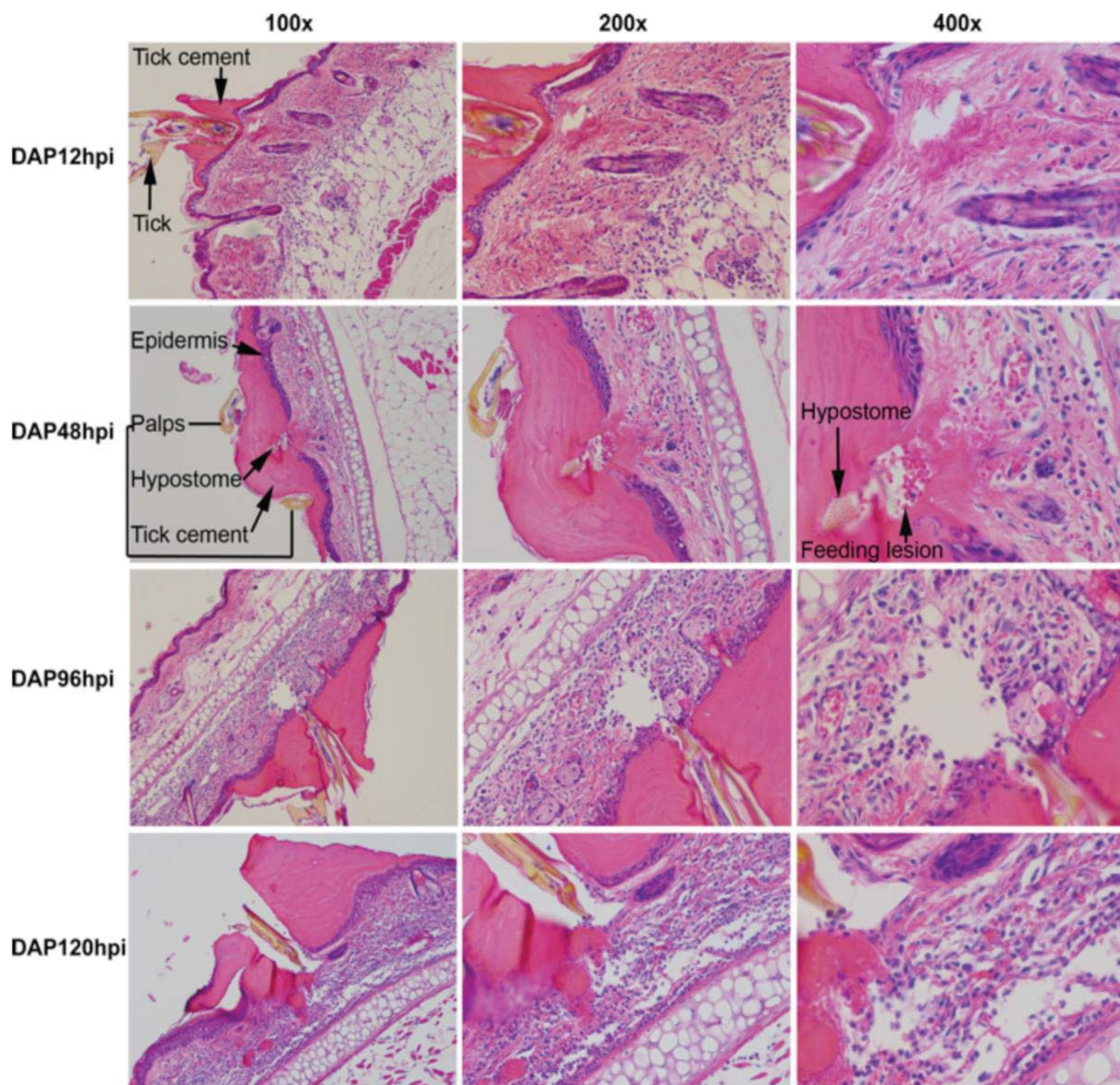

Figura 3. Análise histológica do parasitismo por ninfas de *Dermacentor andersoni*. Esta figura foi extraída do trabalho de HEINZE *et al*. (2014), que mostra com detalhes a histologia do local de fixação durante o parasitismo. Biópsias dos sítios de fixação durante o parasitismo por ninfas de *D. andersoni* foram fixadas em parafina, seccionados e corados com hematoxilina e eosina. Estruturas relevantes estão indicadas com setas: carrapato (*tick*), cemento (*tick cement*), palpos (*palps*), hipostômio (*hypostome*), bolsão hemorrágico (*feeding lesion*). Os cortes representam infestação

primária por ninfas de *D. andersoni* (*D. andersoni primary* – DAP) em diferentes períodos (12 horas após infestação – 12hpi; 48 horas após infestação – 48hpi; 96 horas após infestação – 96hpi; e 120 horas após infestação – 120hpi).

Durante a alimentação, os carrapatos vão alternando ciclos de ingestão de sangue e salivação (BINNINGTON, 1978; BINNINGTON & KEMP, 1980). Diferentemente da maioria dos artrópodes hematófagos, onde o processo hematofágico leva de minutos a horas, os carrapatos necessitam permanecer fixados no hospedeiro por diversos dias. Durante o processo de salivação, os carrapatos secretam juntamente com a saliva o cemento, que consiste em material composto por proteínas, lipídeos e carboidratos. O cemento possui função adesiva e é importante na formação do cone de cemento (Figura 3), uma estrutura que permitirá que os carrapatos permaneçam fixados no hospedeiro durante todo o período de alimentação (KEMP *et al.*, 1982). Quando completamente fixados ao local de alimentação, os carrapatos ixodídeos se alimentam lentamente por diversos dias, na fase de ingurgitamento lento. Durante essa fase, além da aquisição lenta de sangue, dá-se início ao desenvolvimento de tecidos relacionados com reprodução, desenvolvimento das glândulas salivares, e o crescimento da cutila, que irá permitir que o carrapato estenda o seu tamanho durante a fase de ingurgitamento rápido . É nessa última fase, logo após o acasalamento, que os carrapatos ingerem grandes quantidades de sangue, aumentando consideravelmente de tamanho e peso. As fêmeas podem aumentar de 100-200 vezes o seu peso ao final dessa fase, e a quantidade de sangue ingerido é determinante na quantidade de ovos produzidos (FLYNN & KAUFMAN, 2011).

Ao provocar laceração dos tecidos e vasos sanguíneos, o sangue e fluídos tissulares acumulados no local da lesão são ingeridos pelos carrapatos. Esse material servirá de fonte de nutrientes para o desenvolvimento do parasito. O material é ingerido pela bomba

faríngea, passando pelo esôfago e chegando assim ao intestino, onde o sangue e os fluídos ingeridos serão digeridos (FRANTA *et al*., 2010). Além de ser o processo crucial para a obtenção e ingestão de sangue para o desenvolvimento dos parasitos, a hematofagia é o processo pelo qual os carrapatos podem transmitir patógenos para o hospedeiro, sendo a saliva secretada durante esse processo considerada a rota primária pela qual os patógenos são inoculados nos hospedeiros. O processo de transmissão de patógenos se dá principalmente na fase de ingurgitamento lento (BOWMAN *et al*., 1997; NUTTALL & LABUDA, 2004; HOVIUS, 2009).

1.3 As respostas de defesa do hospedeiro e o papel da saliva na hematofagia

Os carrapatos e outros artrópodes hematófagos podem parasitar uma grande variedade de hospedeiros vertebrados e para que possam obter acesso à sua fonte de alimento desenvolveram uma série de mecanismos que permitem a aquisição de sangue. Os hospedeiros vertebrados possuem três eficientes sistemas de defesa que tornam a aquisição de sangue difícil: o sistema hemostático, a imunidade inata e a imunidade adquirida (RIBEIRO *et al*., 1985; RIBEIRO, 1987b; RIBEIRO, 1995). A introdução das quelíceras e do hipostômio na pele do hospedeiro provoca lesões teciduais e endoteliais, que ativam os sistemas de defesa do hospedeiro (RIBEIRO, 1987b; RIBEIRO, 1995). Portanto, os carrapatos, assim como outros artrópodes hematófagos, utilizam uma série de recursos para garantir o aporte sanguíneo e permanecer se alimentando, mesmo diante da ativação das reações de defesa do hospedeiro (RIBEIRO, 1995). Neste contexto, a saliva inoculada no hospedeiro durante a alimentação possui um papel chave no processo hematofágico.

A saliva dos hematófagos é composta por mediadores farmacológicos que exercem funções modulando as respostas do hospedeiro, garantindo assim o repasto sanguíneo

(RIBEIRO *et al*., 1985; RIBEIRO, 1987b; RIBEIRO, 1995). A saliva dos carrapatos e outros artrópodes hematófagos é composta por componentes proteicos e outras moléculas (como lipídeos) que apresentam uma grande variedade de atividade farmacológicas. Para obterem sucesso no processo hematofágico, as principais funções farmacológicas que estes componentes salivares devem exercer são: vasodilatação, inibição da agregação plaquetária, inibição da coagulação sanguínea, atividade anti-inflamatória e imunossupressora, além da modulação endotelial (RIBEIRO *et al*., 1985; RIBEIRO, 1987b).

Inicialmente, para garantir o aumento do fluxo sanguíneo no local de fixação, os carrapatos secretam na saliva compostos lipídicos que são capazes de induzir a dilatação dos vasos sanguíneos, antagonizando a vasoconstrição produzida como resposta à perda de sangue logo após a injúria do tecido (RIBEIRO *et al*., 1985; RIBEIRO *et al*., 1988; VALENZUELA, 2004). A lesão dos vasos sanguíneos, em decorrência da inserção do hipostômio leva à ativação do sistema hemostático, compreendendo agregação plaquetária, coagulação sanguínea e fibrinólise. As moléculas anti-hemostáticas são os componentes mais bem estudados e caracterizados na saliva de hematófagos (VALENZUELA, 2004; CHAMPAGNE, 2005; STEEN *et al*., 2006; MARITZ-OLIVIER *et al*., 2007), sendo esse grupo de moléculas dividido em antiplaquetários e em moléculas anticoagulantes.

A agregação plaquetária é a primeira linha de defesa para evitar a perda de sangue durante a lesão vascular (KROLL & SCHAFER, 1989). As plaquetas são ativadas por diferentes agonistas (incluindo a trombina, o colágeno, o ADP e catepsina G de neutrófilos) e após ativadas agregam-se para formar o tampão de plaquetas. Esse tampão é formado para obstruir a lesão vascular, evitando assim a perda de sangue. A mudança na superfície das plaquetas, com a exposição de alguns fosfolipídeos específicos, serve como substrato para a promoção da coagulação sanguínea. Quando ativadas, as plaquetas degranulam e secretam

diversas substâncias que desempenham diversas atividades, incluindo vasoconstrição (KROLL & SCHAFER, 1989). A capacidade da saliva de carrapatos de inibir a agregação plaquetária decorre da presença de moléculas específicas que bloqueiam a interação entre plaquetas, inibindo assim o processo de agregação, ou da presença de proteínas com atividade enzimática que destroem ou inibem os agonistas que ativam a agregação plaquetária (RIBEIRO *et al*., 1991; MANS *et al*., 1998; VALENZUELA, 2004).

A coagulação sanguínea compreende um sistema enzimático de ativação em cascata, onde os fatores de coagulação que circulam na forma de zimogênios são sequencialmente convertidos enzimaticamente na sua forma ativa. A maioria dos fatores da coagulação são enzimas pertencentes ao grupo das serino-proteases. Essa cascata enzimática culmina na formação de trombina, a enzima chave da coagulação que é responsável pela conversão de fibrinogênio em fibrina, o principal componente do coágulo sanguíneo (MACFARLANE, 1964; HOFFMAN & MONROE, III, 2001; HOFFMAN, 2003). De maneira geral, os componentes salivares de carrapatos que possuem atividade anticoagulante atuam principalmente inibindo moléculas de trombina ou de fator Xa (ZHU *et al*., 1997; HORN *et al*., 2000; IWANAGA *et al*., 2003; CIPRANDI *et al*., 2006). Os inibidores de coagulação encontrados em carrapatos diferem de tamanho e mecanismo de ação, variando desde pequenos peptídeos a moléculas maiores, como proteínas pertencentes à família Kunitz e à família das serpinas (MANS & NEITZ, 2004; CHAMPAGNE, 2005; STEEN *et al*., 2006; MARITZ-OLIVIER *et al*., 2007).

A fibrinólise é o processo fisiológico pelo qual o coágulo de fibrina formado durante a coagulação sanguínea é dissolvido. Esse processo se dá através da ativação do zimogênio plasminogênio em plasmina. Os ativadores de plasminogênio (PA) são do tipo tecidual (t-PA) e do tipo uroquinase (u-PA). Ambos os ativadores convertem o

plasminogênio em plasmina, que hidrolisa a fibrina em fragmentos solúveis, além de hidrolisar o fibrinogênio (COLLEN, 1999). A interferência no sistema fibrinolítico do hospedeiro é outro recurso anti-hemostático que já foi descrito para a saliva de carrapatos. Já foi demonstrado que a saliva de *I. scapularis* possui uma metaloprotease com atividade fibrinolítica (FRANCISCHETTI *et al*., 2003).

Os carrapatos permanecem fixados ao hospedeiro por um longo período de tempo, podendo variar de minutos a horas, como no caso dos argasídeos, ou por diversos dias, como no caso dos carrapatos ixodídeos (ANDERSON & MAGNARELLI, 2008). Portanto, além de inibir as respostas de vasoconstrição e a hemostasia, para o sucesso na obtenção de sangue, os carrapatos necessitam modular as respostas do sistema imune do hospedeiro, sendo a imunidade inata no início da infestação primária (basicamente para os carrapatos que possuem três hospedeiros), e tanto a imunidade inata como a imunidade adaptativa nas infestações secundárias ou subsequentes (HEINZE *et al*., 2012a; HEINZE *et al*., 2012b; HEINZE *et al*., 2014). Logo após que as quelíceras e o hipostômio são inseridos na pele do hospedeiro, as lesões teciduais e vasculares ativam uma série de reações inflamatórias. O ATP liberado pelas células lesadas é responsável pela dor aguda logo após injúria tecidual (COOK & MCCLESKEY, 2002). A lesão vascular expõe os componentes do sangue à superfície negativa da membrana basal, e à exposição de outras moléculas com cargas negativas, levando à ativação do sistema contato. Esse sistema consiste na ativação da pré-calicreína, que age sobre certos precursores gerando os peptídeos conhecidos como cininas, sendo a bradicinina um potente mediador algésico, que além de nociocepção, também induz vasodilatação periférica, aumento do fluxo sanguíneo, extravasamento vascular e quimiotaxia e ativação de mastócitos e macrófagos (BHOOLA *et al*., 1992). Os compostos serotonina e histamina, que são liberados pelas plaquetas ativadas e mastócitos, também são

indutores de dor, além de atuarem como vasodilatadores (SOMMER, 2006). A lesão tecidual também ativa o sistema complemento, que consiste em um sistema em cascata, resultando no opsonização de antígenos e lise de células estranhas ao organismo (WALPORT, 2001a; WALPORT, 2001b).

A inflamação ocorre como resposta em decorrência de uma injúria local, envolvendo neutrófilos, macrófagos, mastócitos, basófilos, eosinófilos e linfócitos, bem como quimiocinas, enzimas do plasma, mediadores inflamatórios lipídicos e citocinas. Células do sistema imune da epiderme e da derme, incluindo mastócitos, eosinófilos, células dendríticas e macrófagos, fazem o primeiro contato com o aparato bucal e a saliva secretada pelos carrapatos (FRANCISCHETTI *et al*., 2009). Essas células liberam fatores quimiotáticos que recrutam outras células inflamatórias, como os neutrófilos, para o sítio de fixação do parasita. Em adição a esses mediadores quimiotáticos, o ATP liberado pelas células lesionadas; a trombina gerada pela ativação da cascata de coagulação; e outras moléculas pró-inflamatórias ativam neutrófilos que se acumulam no local da lesão e liberam o conteúdo dos seus grânulos (KUROKI & MINAKAMI, 1989). A ativação de neutrófilos é acompanhada pela liberação para o ambiente extracelular de diversas serino-proteases como, a catepsina G, a elastase e a proteinase-3 (PHAM, 2006; PHAM, 2008). A atividade proteolítica dessas enzimas é responsável pela degradação de proteínas do tecido conjuntivo, incluindo a elastina, o colágeno e proteoglicanos. Além do mais, essas proteases estão envolvidas na ativação de linfócitos, na clivagem de moléculas apoptóticas e na ativação e degradação de citocinas (PHAM, 2006; PHAM, 2008).

Essas reações desencadeadas podem interromper o fluxo sanguíneo ou iniciar um comportamento de defesa por parte do hospedeiro, pela sensação de dor e prurido, que poderiam culminar na rejeição ao parasito por parte do hospedeiro (FRANCISCHETTI *et*

al., 2009). Nas infestações subsequentes ocorre um maior envolvimento da resposta imune adaptativa com produção de anticorpos capazes de sensibilizar mastócitos e basófilos, que em conjunto com os eosinófilos e neutrófilos, são as células predominantes do infiltrado inflamatório no sítio de fixação (CARVALHO *et al*., 2010; HEINZE *et al*., 2012a; HEINZE *et al*., 2014). Nesse contexto, o sucesso do processo hematofágico depende de um complexo sistema de proteínas e outras moléculas secretadas pelo carrapato através da saliva, que além de modularem o sistema hemostático, necessitam modular o sistema imunológico do hospedeiro, permitindo a fixação do carrapato e sua alimentação durante vários dias (RIBEIRO *et al*., 1985; RIBEIRO, 1987b; RIBEIRO, 1995). A capacidade de inibição da resposta inflamatória decorrente do parasitismo, é um exemplo de adaptação dos hematófagos ao parasitismo. Diversos mecanismos anti-inflamatórios presentes em carrapatos são descritos, incluindo: hidrólise da bradicinina por enzimas (RIBEIRO & MATHER, 1998; BASTIANI *et al*., 2002), ligação de proteínas salivares à serotonina, histamina e leucotrieno (PAESEN *et al*., 2000; MANS, 2005; BEAUFAYS *et al*., 2008; MANS *et al*., 2008; MANS & RIBEIRO, 2008), inibição do sistema complemento (RIBEIRO, 1987a; VALENZUELA *et al*., 2000; MEJRI *et al*., 2002; TYSON *et al*., 2007), inibição de proteases envolvidas na resposta inflamatória (KOTSYFAKIS *et al*., 2006; PREVOT *et al*., 2009a; CHMELAR *et al*., 2011a), além modulação da atividade de neutrófilos (RIBEIRO *et al*., 1990; MONTGOMERY *et al*., 2004) e macrófagos (BRAKE *et al*., 2010; BRAKE & PEREZ DE LEON, 2012). Adicionalmente, na saliva de carrapatos são encontradas moléculas capazes de modular a resposta imune adaptativa do hospedeiro (KOTAL *et al*., 2015).

Ainda que a modulação das respostas de defesa do hospedeiro pela saliva permita o sucesso durante a hematofagia, ao longo de repetidas infestações é demonstrado que os

hospedeiros são capazes de desenvolver resistência ao parasitismo. Essa resistência é evidenciada pela diminuição do número de fêmeas que completam o ciclo e pela diminuição no peso e fecundidade das fêmeas. Nesse sentido, o estudo de moléculas bioativas da saliva do carrapato pode servir para a identificação de novos alvos para o controle do carrapato, além de servir como meio para compreensão dos mecanismos envolvidos na interação parasito-hospedeiro.

No que concerne ao controle imunológico, mas especificamente vacinas, as proteínas secretadas na saliva do carrapato podem constituir antígenos mais eficazes do que antígenos ocultos (como no caso da proteína Bm86). Como antígenos secretados e expostos ao hospedeiro durante o parasitismo, as proteínas salivares podem permitir a manutenção da memória imunológica quando ocorrer a re-exposição do hospedeiro vacinado aos carrapatos.

1.4 Identificação de proteínas salivares: sialotranscritomas e sialomas

As glândulas salivares são responsáveis por diversas funções na fisiologia dos carrapatos, sendo estruturas vitais tanto para o desenvolvimento dos parasitos, assim como para o desenvolvimento e transmissão de patógenos (BOWMAN & SAUER, 2004). Além da secreção de compostos proteicos e lipídicos com atividades farmacológicas que modulam as respostas de defesa do hospedeiro, a salivação desempenha outras importantes funções para a fisiologia dos carrapatos, incluindo: a absorção de vapor de água por carrapatos de vida livre e a excreção do excesso de água ingerida durante a alimentação, objetivando a concentração do sangue ingerido (SAUER *et al.*, 1995; SAUER *et al.*, 2000; SIMO *et al.*, 2012).

Em fêmeas, três tipos de ácinos podem ser descritos compondo a estrutura da glândula salivar dos carrapatos: os ácinos tipo I estão associados com osmoregulação; os ácinos do tipo II e III estão envolvidos na síntese e secreção de proteínas e no transporte de água (BINNINGTON, 1978; SAUER *et al*., 1995). Estudos morfológicos e estruturais têm demonstrado que a glândula salivar dos carrapatos sofre mudanças durante os processos de estímulo ao hospedeiro, fixação e alimentação (BINNINGTON, 1978; BINNINGTON & KEMP, 1980; BOWMAN & SAUER, 2004). Durante a alimentação, as glândulas salivares sofrem notáveis mudanças morfológicas, principalmente os ácinos de tipo II e tipo III. Essas mudanças morfológicas são acompanhadas pelo aumento na síntese de proteínas (BINNINGTON, 1978; MCSWAIN *et al*., 1982).

Nos últimos anos, com o desenvolvimento e aplicação de técnicas de análise molecular em grande escala, como a transcritômica e a proteômica, novas informações acerca da composição molecular de diferentes tecidos/estágios dos carrapatos estão sendo geradas, enriquencendo assim a literatura e gerando informações que podem ser utilizadas para o estudo sobre a fisiologia desses parasitos. De modo especial, os sialotranscritomas (do grego, *sialo* = saliva) estão gerando informações importantes sobre a transcrição na glândula salivar desses parasitos (RIBEIRO & FRANCISCHETTI, 2003; FRANCISCHETTI *et al*., 2009; MANS, 2011). Os sialotranscritomas de diversas espécies de carrapatos ixodídeos têm sido descritos, incluindo *A. americanum* (MULENGA *et al*., 2007a; ALJAMALI *et al*., 2009; KARIM & RIBEIRO, 2015), *A. maculatum* (KARIM *et al*., 2011a), *A. triste*, *A. parvum*, *A. cajennense* (GARCIA *et al*., 2014), *A. variegatum* (NENE *et al*., 2002; RIBEIRO *et al*., 2011), *Hyalomma marginatum* (FRANCISCHETTI *et al*., 2011), *I. scapularis* (VALENZUELA *et al*., 2002; RIBEIRO *et al*., 2006), *I. ricinus* (LEBOULLE *et al*., 2002; SCHWARZ *et al*., 2014; KOTSYFAKIS *et al*., 2015), *I. pacificus*

(FRANCISCHETTI *et al*., 2005), *R. pulchellus* (TAN *et al*., 2015), *Haemaphysalis flava* (XU *et al*., 2015), dentre outros.

Os sialotranscritomas fornecem uma visão global dos perfis de expressão gênica em glândulas salivares do carrapato. Contudo, a inferência direta de que a presença de certos transcritos em glândula salivar remete diretamente ao que é secretado no hospedeiro através da saliva deve ser um tópico abordado com cautela. Recentemente, um trabalho da análise de transcritos e proteínas realizado com glândulas salivares de *I. ricinus* revelou que a dinâmica entre transcritos e proteínas não é totalmente concordante (SCHWARZ *et al*., 2014; KOTSYFAKIS *et al*., 2015). Além disso, os sialotranscritomas por si só não fornecem informações únicas sobre proteínas que de fato são secretadas na saliva. Nesse contexto, a análise proteômica dos componentes salivares representa um tópico interessante e complementar de ser abordado e estudado.

Em condições experimentais os carrapatos produzem pequeno volume de saliva, o que faz com que seja difícil de trabalhar com esse material biológico, fazendo com que muitos pesquisadores optem por trabalhar com glândula salivar (FRANCISCHETTI *et al*., 2008; FRANCISCHETTI *et al*., 2011; SCHWARZ *et al*., 2014; TAN *et al*., 2015). Experimentalmente, em carrapatos a salivação pode ser induzida pela administração de dopamina, um neurotransmissor que estimula a secreção da glândula salivar, ou pelo uso da pilocarpina, um agente colinomimético que induz a liberação de dopamina nos nervos salivares resultando em salivação (MCSWAIN *et al*., 1992; SAUER *et al*., 2000).

Atualmente, graças aos avanços em diferentes áreas da proteômica é possível identificar um número expressivo de proteínas utilizando diminutas quantidades de amostra (na ordem de microgramas). Os avanços em técnicas de preparação de amostra, na separação de proteínas/peptídeos por cromatografia líquida (LC), na sensibilidade e acurácia dos

espectromêtros de massa (MS), e nos avanços em análise computacional são fatores de grande importância para o avanço das análises proteômicas (YATES, III, 2013). Análises proteômicas baseadas em espectrometria de massas de alta resolução tem permitido a identificação e quantificação de milhares de proteínas, bem como suas modificações e localização (YATES, III, 2013; MEISSNER & MANN, 2014). Em proteômica, o termo *bottom-up* refere-se à identificação de proteínas por análises de peptídeos gerados através de proteólise. Quando as análises *bottom-up* são realizadas a partir de uma mistura de proteínas, o termo *shotgun proteomics* é usado (em analogia ao *shotgun genomic sequencing*) (YATES, III, 1998; YATES, III, 2013; ZHANG *et al*., 2013). Análises proteômicas por *shotgun proteomics* fornecem uma medida indireta de proteínas por meio dos peptídeos derivados a partir da proteólise de proteínas intactas. Essa mistura de peptídeos gerada é fracionada e analisada por LC-MS/MS. A identificação dos peptídeos é realizada por comparação dos espectros de massa derivados da fragmentação dos peptídeos com os espectros de massa teóricos gerados a partir de digestão *in silico* de um banco de dados de proteínas do organismo em estudo. A inferência da identidade da proteína é realizada através da identificação dos peptídeos nas sequências de aminoácidos presentes no banco de dados utilizados nas buscas. Devido ao fato de que os peptídeos identificados podem ser unicamente atribuídos a uma única proteína, ou, partilhados por mais de uma proteína, métodos estatísticos podem ser utilizados para aprimorar a identificação e/ou confirmação de uma dada proteína (TABB *et al*., 2002; MCDONALD *et al*., 2004; CARVALHO *et al*., 2012). Dessa maneira, essas novas abordagens de análises proteômicas têm substituído ferramentas e métodos utilizados anteriormente, tais como electroforese em gel bidimensional seguida por LC-MS/MS (NAGELE *et al*., 2004).

Até pouco tempo, somente o proteoma da saliva dos carrapatos *A. americanum* e *I. scapularis* tinham sido alvo de estudos proteômicos, resultando na identificação de um número não muito expressivo de proteínas (na ordem de algumas dezenas de proteínas identificadas) (MADDEN *et al*., 2002; VALENZUELA *et al*., 2002). Nos últimos dois anos, utilizando técnicas mais sofisticadas de LC-MS/MS, foi possível obter maiores informações sobre o proteoma da saliva de algumas espécies de carrapatos. Em 2013 foi publicado o proteoma da saliva do carrapato argasídeo *Ornithodoros moubata*, compreendendo uma comparação da saliva entre fêmeas e adultos. Este estudo permitiu a identificação de 193 proteínas salivares (DIAZ-MARTIN *et al*., 2013). No mesmo ano, o proteoma da saliva de *R. sanguineus* foi publicado. Nesse estudo, os autores utilizaram a técnica de GeLC-MS/MS, onde a amostra é processada por SDS-PAGE em uma dimensão (1D), as bandas são excisadas e digeridas com uma protease específica, os petídeos são extraídos e analizados por LC-MS/MS (LUNDBY & OLSEN, 2011). Nesse trabalho, utilizando saliva de fêmeas parcialmente ingurgitadas (5-7 dias se alimentando em coelhos) foi possível identificar 19 proteínas de carrapato. De maneira interessante, foi demonstrado que o carrapato é capaz de reciclar e secretar algumas proteínas do próprio hospedeiro, revelando a presença de 56 proteínas de origem do hospedeiro na saliva de *R. sanguineus* (OLIVEIRA *et al*., 2013). A identificação de proteínas do carrapato (19 proteínas) foi menor que as proteínas identificadas para o hospedeiro (56 proteínas). Essa observação pode ser atribuída à qualidade/complexidade do banco de dados utilizado nas busca. Os autores utilizaram um banco contento aproximadamente 2.000 sequências de *R. sanguineus*, um número bem menor em relação ao banco de dados utilizado para as buscas do hospedeiro (ANATRIELLO *et al*., 2010; OLIVEIRA *et al*., 2013). Recentemente, combinando um estudo transcritômico de glândula salivar gerado a partir de sequenciamento de última

geração (*Next Generation Sequencing* - NGS) e um estudo proteômico envolvendo a análise da saliva do carrapato *D. andersoni* em diferentes estágios de alimentação (dois e cinco dias de alimentação) foi possível identificar aproximadamente 700 proteínas presentes na saliva desse parasito (MUDENDA *et al.*, 2014). Até o momento, o proteoma da saliva de *D. andersoni* representa o mais abrangente estudo da composição proteica da saliva de um carrapato ixodídeo.

A identificação dos componentes salivares dos carrapatos representa um avanço no estudo da fisiologia desses parasitos. A identificação e caracterização desses componentes salivares auxiliam na compreensão de como os carrapatos modulam as respostas de defesa do hospedeiro, gerando não só dados para a melhor compreensão da relação parasito-hospedeiro, mas também gerando bases para novos estudos sobre a caracterização molecular e bioquímica das proteínas salivares. Estes estudos podem auxiliar no desenvolvimento de novos métodos de controle e/ou novos compostos com aplicações biotecnológicas.

1.5 Inibidores de serino-proteases e a saliva

A manutenção do equilíbrio homeostático dos diversos sistemas de defesa do hospedeiro é um processo que envolve a interação de diversas proteínas. Na sua grande maioria, esses processos são coordenados por reações de cascatas enzimáticas, através da ativação sequencial de serino-proteases, incluindo proteases pró-coagulantes (trombina, fator Xa, fator XIa, e outros fatores de coagulação), peptidases pró-inflamatórias (elastase de neutrófilos, proteinase-3, catepsina G, calicreína, quimase, triptase, e outras enzimas do tipo tripsina e quimotripsina) e peptidases do sistema complemento (fator B, fator C, fator D e o componente 2) (DAVIE *et al.*, 1979; MATSUNAGA *et al.*, 1994; KORKMAZ *et al.*, 2008; CATTARUZZA *et al.*, 2014). O controle dessas cascatas enzimáticas ocorre pela

interação das proteases com seus inibidores específicos. Levando em conta o papel dessas proteases e seus respectivos inibidores na manutenção da homeostase dos sistemas de defesa dos hospedeiros, os carrapatos codificam proteases e inibidores de proteases para quebrar esse equilíbrio (MULENGA *et al*., 2001).

Os sialotranscritomas e sialomas de carrapatos descrevem a presença de uma variedade de proteínas pertencendo a diferentes famílias de inibidores de proteases, incluindo proteínas pertecentes à família das serpinas, cistatinas, tiropinas, TIL (*trypsin-like inhibitors*), inibidores do tipo Kunitz e Kazal (MANS, 2011). A presença desses inibidores na saliva de carrapatos pode ser relacionada com as funções anti-hemostáticas, anti-inflamatórias e imunomodulatórias descritas na saliva (MANS & NEITZ, 2004; CHAMPAGNE, 2005; STEEN *et al*., 2006; MARITZ-OLIVIER *et al*., 2007).

Serpinas (*serine protease inhibitors*) são inibidores proteicos que participam de uma variedade de funções fisiológicas em diferentes organismos (SILVERMAN *et al*., 2001; GETTINS, 2002). Tipicamente, serpinas possuem na sua estrutura terciária três feixes de folhas-β (A-C), entre 7-9 α-hélices (A-I), além da alça do centro da reação (*reactive center loop* – RCL). Além dessas estruturas, outras regiões são importantes para a atividade inibitória das serpinas (Figura 4). O RCL é a região da serpina que liga ao sítio ativo da protease alvo sendo crucial para a atividade inibitória. Em α_1-antitripsina, os resíduos de aminoácidos do RCL são nomeados e numerados sequencialmente, compreendendo os resíduos P_{17}-$P_{4'}$. (SCHECHTER & BERGER, 1967). Conforme este sistema de notação, a ligação suscetível de hidrólise é a ligação peptídica ente os resíduos P_1-$P_{1'}$. Esta notação é usada também para se referir à ligação de inibidores na enzima, mesmo no caso de inibidores cuja ligação não é hidrolisada pela protease. Inicialmente a serpina liga-se à protease através da formação de um complexo não-covalente do tipo Michaelis, através da interação dos

resíduos P_1 e $P_{1'}$ que estão acessíveis (Figura 5A) para a interação com o sítio ativo da protease (SILVERMAN *et al*., 2001; GETTINS, 2002). O ataque da serina do sítio ativo da peptidase leva à formação de uma ligação éster entre a serina-195 da serino-peptidase e a carbonila do resíduo de aminoácido P_1 da serpina, resultando na hidrolise da ligação peptídica, formando o que se conhece por acil-enzima. A especificidade da serpina é dirigida aos resíduos P_1-$P_{1'}$ que devem estar posicionados e acessíveis para a interação com a protease, atuando como um substrato.

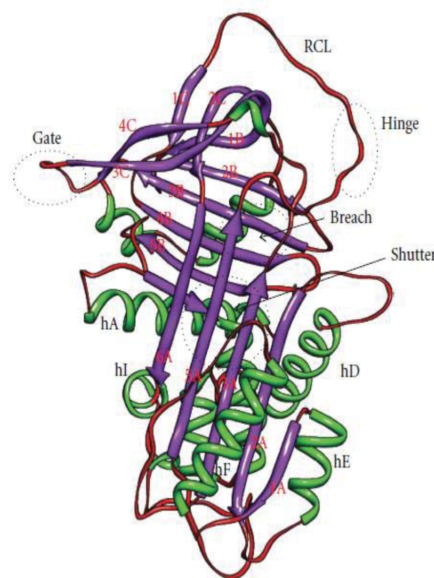

Figura 4. Estrutura terciária da α_1-antitripsina humana. Os feixes de folhas-β (A, B e C) estão representados em púrpura. As α-hélices (A-I) estão representadas em verde. O RCL está representado em vermelho. A região $P_{15} – P_9$ do RCL é chamada região da dobradiça *"hinge region"*. O local onde o RCL inicialmete se insere após clivagem pela protease é denominado *"breach region"* e está localizado ao topo da folha-β A. De acordo com KHAN *et al*. (2011).

O RCL hidrolisado é inserido dentro do feixe de folhas-β A, movendo a protease, ligada covalentemente, juntamente com ele até o polo oposto da serpina. O resultado é a translocação da protease em aproximadamente 70 Å e a distorção do seu sítio ativo (HUNTINGTON *et al*., 2000) (Figura 5B). Essa distorção do sítio ativo previne a etapa final

da catálise, a hidrólise da ligação éster acil-enzima, e resulta na formação do complexo serpina-protease ligado covalentemente de modo irreversível (HUNTINGTON *et al.*, 2000; KHAN *et al.*, 2011).

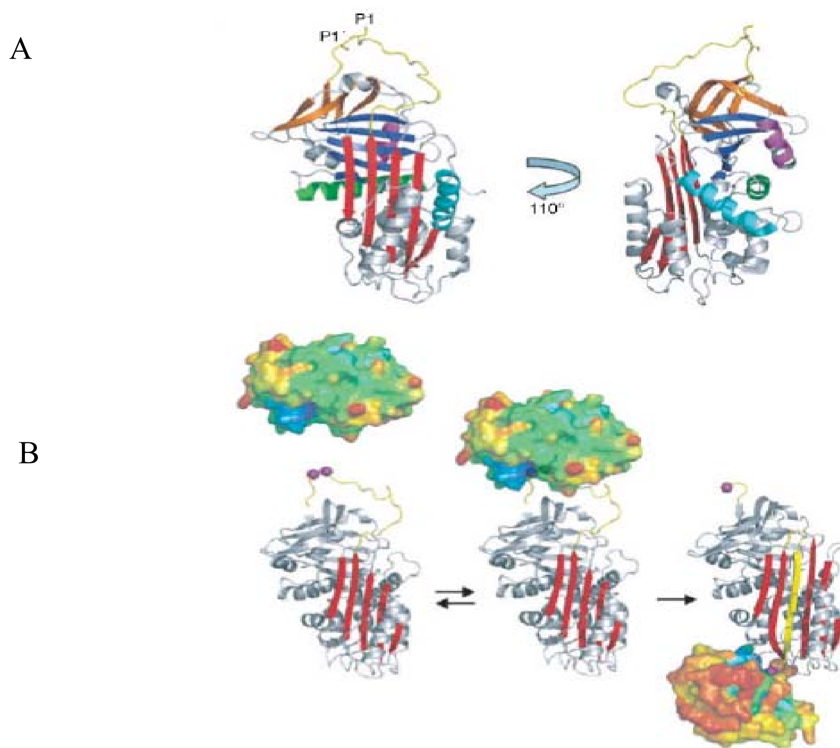

Figura 5. Mecanismo de inibição das serpinas. (A) ilustração da estrutura da α_1-antitripsina no seu estado nativo. Os resíduos $P_1 - P_{1'}$ estão expostos e disponíveis para o ataque proteolítico. (B) O mecanismo de inibição da protease está exemplificado em dois passos. No primeiro passo, a serpina interage de modo reversível com a protease para formar o complexo do tipo Michaelis. Após a formação do intermediário acil-enzima, o RCL é hidrolisado e rapidamente inserido dentro do feixe de folhas-β A. Nesse processo de inserção do RCL, a protease é arremessada para o polo oposto da serpina, e sua arquitetura catalítica é destruída. De acordo com RAU *et al.* (2007).

Em mamíferos, as serpinas regulam a cascata de coagulação sanguínea, fibrinólise, reparo tecidual, angiogênese, resposta inflamatória e resposta imune (SILVERMAN *et al.*, 2001; RAU *et al.*, 2007). Portanto, presume-se que os carrapatos usam desses inibidores para desequilibrar os sistemas de defesa do hospedeiro durante o parasitismo (MULENGA

et al., 2001). O número de trabalhos descrevendo serpinas em carrapatos é crescente, podendo ser citada a descrição desses inibidores em *A. americanum* (MULENGA *et al*., 2007b; PORTER *et al*., 2015; KIM *et al*., 2015), *A. maculatum* (KARIM *et al*., 2011b), *I. scapularis* (RIBEIRO *et al*., 2006; MULENGA *et al*., 2009), *I. ricinus* (LEBOULLE *et al*., 2002; PREVOT *et al*., 2006; CHMELAR *et al*., 2011b), *R. microplus* (JITTAPALAPONG *et al*., 2010; RODRIGUEZ-VALLE *et al*., 2012; TIRLONI *et al*., 2014; RODRIGUEZ *et al*., 2015), *R. appendiculatus* (MULENGA *et al*., 2003), *R. haemaphysaloides* (YU *et al*., 2013) e *H. longicornis* (SUGINO *et al*., 2003; IMAMURA *et al*., 2005; IMAMURA *et al*., 2006). Similarmente, os estudos proteômicos da saliva de *A. americanum* (RADULOVIC *et al*., 2014) e *D. andersoni* (MUDENDA *et al*., 2014) descrevem a presença desses inibidores como proteínas secretadas no hospedeiro com papel na relação parasito-hospedeiro.

Em *A. americanum*, serpinas salivares têm sido descritas e caracterizadas, incluindo a serpina AamS6 (MULENGA *et al*., 2007b; CHALAIRE *et al*., 2011), um inibidor de papaína e enzimas do tipo tripsina que é capaz de inibir a coagulação sanguínea e a ativação do sistema complemento (MULENGA *et al*., 2013). A serpina 19 (AAS19) é descrita como uma serpina possuindo a estrutura primária do RCL 100% conservada entre diferentes espécies de carrapatos ixodídeos. Esta serpina é descrita como uma proteína com atividade anticoagulante, inibindo a atividade enzimática de fatores de coagulação e a agregação plaquetária induzida por trombina (KIM *et al*., 2015). Em *I. scapularis* uma serpina foi descrita possuindo atividade sobre trombina e agregação plaquetária (IBELLI *et al*., 2014). Já em *I. ricinus*, serpinas foram descritas inibindo as proteases pró-inflamatórias catepsina G, elastase e quimase. A serpina IRIS possui atividade anti-elastase e apresenta atividade imunomodulatória, além de interferir na hemostasia. A serpina IRS-2 inibe catepsina G e

quimase de mastócitos, apresentando atividades anti-inflamatória e anti-coagulante (PREVOT *et al*., 2006; PREVOT *et al*., 2009b; CHMELAR *et al*., 2011b). Além disso, IRS-2 é uma serpina com atividade inibitória sobre a diferenciação da resposta Th17 em linfócitos (CHMELAR *et al*., 2011b; PALENIKOVA *et al*., 2015). Em *R. haemaphysaloides* duas serpinas foram descritas com potencial para inibir peptidases do tipo quimotripsina (YU *et al*., 2013). Em *R. microplus,* análises em bancos de transcritos revelam a presença de 26 sequências que codificam para serpinas (TIRLONI *et al*., 2014; RODRIGUEZ *et al*., 2015). A serpina RmS-3 é descrita como sendo uma proteína secretada durante o parasitismo. Anticorpos geradas contra epítopos dessa proteína, quando administrados a carraptos por alimentação artificial impactam na fertilidade dos carrapatos (RODRIGUEZ-VALLE *et al*., 2012).

Esses trabalhos acima descritos demonstram que serpinas de carrapatos possuem atividade anticoagulante e imunossupressora. Além do mais, estudos demonstram que em hospedeiros imunizados com serpinas recombinantes e infestados com *I. ricinus* (PREVOT *et al*., 2007), *H. longicornis* (SUGINO *et al*., 2003) ou *R. appendiculatus* (IMAMURA *et al*., 2006; IMAMURA *et al*., 2008), os parasitos apresentam mortalidade e deficiência na aquisição de sangue durante o parasitismo. Essas observações demonstram a importância das serpinas na fisiologia do carrapato.

2 OBJETIVOS

No desenvolvimento deste trabalho objetivou-se aplicar análises proteômicas para proporcionar uma visão abrangente dos componentes salivares do carrapato *R. microplus* em diferentes estágios de ingurgitamento, além da caracterização funcional de três serpinas salivares. Nesse sentido, os objetivos específicos focam em:

- Identificar o proteoma da saliva de fêmeas de *R. microplus*, comparando dois estágios de desenvolvimento (partenógenas e teleógenas);

- Identificar o proteoma da saliva de fêmeas de *R. microplus*, comparando a saliva de fêmeas em diferentes estágios de ingurgitamento;

- Caracterizar funcionalmente três serpinas salivares recombinantes.

3 PARTE EXPERIMENTAL & RESULTADOS

Esta seção está organizada em três capítulos que compreendem a descrição do trabalho experimental da tese desenvolvida durante o período de execução do doutorado. Num primeiro momento analisamos a composição proteica da saliva de fêmeas em dois estágios de desenvolvimento: fêmeas parcialmente ingurgitadas e fêmeas totalmente ingurgitadas. Esses resultados são apresentados na forma de artigo científico publicado no periódico *PLoS One*. Os dados obtidos nesse primeiro estudo nos levaram a dar prosseguimento em um estudo mais amplo, focando na identificação da composição proteica da saliva ao longo do parasitismo, analisando a saliva de fêmeas em vários estágios de ingurgitamento. Esses dados são apresentados na forma de métodos e resultados. Nessas análises proteômicas identificou-se a presença de proteínas pertencentes à família das serpinas. A caracterização funcional dessas proteínas salivares é apresentada na forma de manuscrito que será submetido para publicação.

Além desses três capítulos que compõem o corpo desta tese, ao final da mesma encontram-se dois artigos publicados durante o doutorado, que são apresentados como material anexo: (i) um trabalho publicado no periódico *Parasites & Vectors* focando na análise proteômica da saliva de ninfas e fêmeas totalmente ingurgitadas do carrapato *H. longicornis*; e (ii) um trabalho publicado no periódico *International Journal for Parasitology* focando na caracterização da serpina AAS19 do carrapato *A. americanum.* Esses trabalhos foram desenvolvidos em colaboração com outras instituições, e por motivos de políticas institucionais não foram inseridos no corpo principal desta tese.

3.1 Capítulo I

Proteomic analysis of cattle tick *Rhipicephalus* (*Boophilus*) *microplus* saliva: a comparison between partially and fully engorged females

Artigo científico publicado no periódico **PLoS ONE**

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Proteomic Analysis of Cattle Tick *Rhipicephalus* (*Boophilus*) *microplus* Saliva: A Comparison between Partially and Fully Engorged Females

Lucas Tirloni[1,2], José Reck[3], Renata Maria Soares Terra[1,4,5], João Ricardo Martins[3], Albert Mulenga[2], Nicholas E. Sherman[4], Jay W. Fox[4], John R. Yates, III[8], Carlos Termignoni[1,6], Antônio F. M. Pinto[1,4,5], Itabajara da Silva Vaz, Jr.[1,7]*

1 Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil, **2** Department of Entomology, Texas A&M University, College Station, Texas, United States of America, **3** Instituto de Pesquisas Veterinárias Desidério Finamor, Fundação Estadual de Pesquisa Agropecuária, Eldorado do Sul, RS, Brazil, **4** Department of Microbiology, University of Virginia, Charlottesville, Virginia, United States of America, **5** CAPES, Ministério da Educação do Brasil, Brasília, DF, Brasil, **6** Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil, **7** Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil, **8** Department of Chemical Physiology, The Scripps Research Institute, La Jolla, California, United States of America

Abstract

The cattle tick *Rhipicephalus* (*Boophilus*) *microplus* is one of the most harmful parasites affecting bovines. Similarly to other hematophagous ectoparasites, *R. microplus* saliva contains a collection of bioactive compounds that inhibit host defenses against tick feeding activity. Thus, the study of tick salivary components offers opportunities for the development of immunological based tick control methods and medicinal applications. So far, only a few proteins have been identified in cattle tick saliva. The aim of this work was to identify proteins present in *R. microplus* female tick saliva at different feeding stages. Proteomic analysis of *R. microplus* saliva allowed identifying peptides corresponding to 187 and 68 tick and bovine proteins, respectively. Our data confirm that (i) *R. microplus* saliva is complex, and (ii) that there are remarkable differences in saliva composition between partially engorged and fully engorged female ticks. *R. microplus* saliva is rich mainly in (i) hemelipoproteins and other transporter proteins, (ii) secreted cross-tick species conserved proteins, (iii) lipocalins, (iv) peptidase inhibitors, (v) antimicrobial peptides, (vii) glycine-rich proteins, (viii) housekeeping proteins and (ix) host proteins. This investigation represents the first proteomic study about *R. microplus* saliva, and reports the most comprehensive Ixodidae tick saliva proteome published to date. Our results improve the understanding of tick salivary modulators of host defense to tick feeding, and provide novel information on the tick-host relationship.

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* E-mail: itabajara.vaz@ufrgs.br

These authors contributed equally to this work.

Introduction

The cattle tick *Rhipicephalus* (*Boophilus*) *microplus* is a one-host tick that feeds on bovines. It is considered one of the most harmful cattle parasites in sub-tropical areas of the world due to its economic importance [1]. The economic losses associated with *R. microplus* parasitism are (i) direct, i.e., blood loss and lesions that predispose animals to myiasis and anaemia, reducing weight gain and milk production, and (ii) indirect, via the transmission of tick-borne pathogens such as *Babesia* spp. and *Anaplasma marginale* [2,3].

Like all hematophagous parasites, *R. microplus* salivary secretion is a complex mixture, rich in bioactive compounds that modulate host defenses to tick feeding activity [4–7]. In recent decades, transcriptomic and proteomic analyses of salivary glands (sialomes) of several ticks have provided a better insight into the immunobiology at the tick–host interface [4,5,7–16]. However, in comparison with other hematophagous arthropods, much has yet to be established about the components of *R. microplus* saliva, particularly taking into account the considerable economic losses this parasite causes. *Amblyomma americanum*, *Ixodes scapularis*, *Ornithodoros moubata* and *Rhipicephalus sanguineus* are the only tick species whose saliva has been the object of proteomic analysis [17–20]. To date, no comprehensive analysis of *R. microplus* tick salivary proteins has been performed.

There is evidence that tick salivary protein profiles change during tick feeding [21–23]. However, it is unclear whether the compounds secreted through *R. microplus* saliva vary throughout tick lifecycle. The identification of tick bioactive salivary components may be a potentially useful tool to more fully understand tick modulation of host physiological system. Moreover, this information may become valuable in the potential identification of novel target antigens for the development of anti-*R. microplus* vaccines

and of potential lead compounds for pharmacological applications [24,25]. The aim of this work was to identify proteins secreted in saliva of *R. microplus* female ticks at two different feeding stages, and to gain insight into the putative role(s) these proteins play in regulating the tick-host relationship. For this purpose, we performed a proteomic characterization of saliva from partially engorged and fully engorged *R. microplus* tick females.

Materials and Methods

Ethics statement

All animals used in these experiments were housed in Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul (UFRGS). This study was conducted considering ethic and methodological aspects in agreement with the International and National Directives and Norms by the Animal Experimentation Ethics Committee of the Universidade Federal do Rio Grande do Sul (UFRGS). The protocol was approved by the Comissão de Ética no Uso de Animais (CEUA) - UFRGS.

Ticks

R. microplus ticks, Porto Alegre strain, free of pathogens such as *Babesia* spp. and *Anaplasma* spp. were obtained from a laboratory colony maintained as previously described [26]. Ticks used in this study were exclusively fed on Hereford calves (*Bos taurus taurus*) acquired from a tick-free area. The calves were infested with 10-day-old *R. microplus* larvae.

Saliva collection

Fully engorged female (FEF) ticks were obtained after the spontaneous detachment from the calves. Partially engorged female (PEF) ticks were carefully detached from the calves' skin by hand, between the 17th and 20th days post-infestation. Mean length of PEF and FEF ticks was 4.5 mm (ranging from 4 to 5 mm) and 11 mm (ranging from 9 to 12.5 mm), respectively. Before saliva collection, any host contaminating tissue in tick mouthparts was removed using a scalpel blade and surgical forceps. PEF and FEF ticks were rinsed with sterile distilled water and induced to salivate by dorsal injection of 2 or 5 µL pilocarpine (2% in PBS), respectively [27,28]. The saliva accumulated in the mouthparts was periodically collected using a pipette tip from ticks maintained at 37°C in a humid chamber for approximately 3 h. The saliva was stored at −80°C upon use. Saliva protein concentration was determined according to the bicinchoninic acid method (BCA Protein Assay, Pierce, Rockford, USA), as previously described [29].

In solution digestion, liquid chromatography and tandem mass spectrometry (LC-MS/MS) analysis

Three micrograms of protein from PEF and FEF tick saliva were reduced (10 mM DTT), alkylated (50 mM iodoacetamide) and digested with 1 µg modified trypsin (Promega Co., Madison, WI, USA) overnight at room temperature. LC-MS/MS was performed using a Thermo Electron LTQFT hybrid linear ion trap-FTICR mass spectrometer. Samples were loaded into a capillary C18 column (75 µm×7.5 cm) and injected into the mass spectrometer at approximately 500 nL/min. The gradient elution was 0–90% acetonitrile/0.1 M acetic acid over 2 h. Data was collected in a top 10 mode, meaning that one FT scan (100 K resolution) taken was followed by 10 MS/MS fragmentation spectra of the top intensity ions collected in the linear ion trap. After MS/MS fragmentation was performed on a particular parent ion, m/z was placed on an exclusion list to enable greater dynamic range and prevent repeated analysis of the same peptide. Electrospray voltage was set to 2.5 kV, and capillary temperature was 210°C.

Protein and peptide identification and protein quantitation were carried out in an Integrated Proteomics Pipeline - IP2 (Integrated Proteomics Applications, Inc., San Diego, CA, http://www.integratedproteomics.com/). Mass spectra were extracted from raw files using RawExtract 1.9.9.2 [30] and searched against a local *R. microplus* protein database (Rm-INCT-EM) containing 22,009 sequences produced by our research group using Illumina Sequencing technology (BioProject ID PRJNA232001 at Transcriptome Shotgun Assembly (TSA) database – GenBank) with reversed sequences using ProLuCID [31,32]. Additionally, a bovine protein database (IPI *Bos taurus* -ftp://ftp.ebi.ac.uk/pub/databases/IPI/last_release/current/ipi.BOVIN.fasta.gz) was used to identify host proteins. The search space included all fully-tryptic and half-tryptic peptide candidates. Carbamidomethylation of cysteine was considered as differential modification. Peptide candidates were filtered using DTASelect, with the parameters -p 2 -y 1 -trypstat -pfp .01 –dm [30,33].

1D gel electrophoresis and LC-MS/MS (1D-LC-MS/MS)

Saliva samples (25 µg) of both PEF and FEF were electrophoresed in 12% SDS-PAGE and stained with Coomassie brilliant blue. Subsequently, stained gel band slices (42 to PEF and 15 to FEF) were excised and individually subjected to trypsin digestion, as previously described [34]. The resulting peptides were analyzed using an electrospray ionization (ESI) quadrupole time-of-flight (Q-TOF) MicroTM mass spectrometer (Waters, Milford, MA, USA) coupled to a capillary liquid chromatography system nanoACQUITY UPLC (Waters, Milford, MA, USA). The peptides were eluted from a reverse-phase C18 column toward the mass spectrometer. Charged peptide ions (+2 and +3) were automatically mass selected and dissociated in MS/MS experiments. MS/MS spectra were searched against the database described above (item 2.3) using the MASCOT software version 2.2 (Matrix Science, London, UK) with the following parameters: tryptic specificity, one missed cleavage and a mass measurement tolerance of 0.2 Da in the MS mode and 0.2 Da for MS/MS ions. The carbamidomethylation of cysteine was set as a fixed modification, and methionine oxidation was set as variable modifications. The Scaffold software version 4.0.5 (Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they exceeded specific database search engine thresholds. Mascot identifications required ion scores higher than the associated identity scores of 20 and 35 for doubly and triply charged peptides, respectively. Protein identifications were accepted if they contained at least 2 identified peptides. To be included in this analysis, all peptide sequences had to have 100% identity with assigned proteins.

Functional annotation and classification of proteins

For functional annotation of the proteins, BLAST tools were used to compare the protein sequences to the NCBI (http://www.ncbi.nlm.nih.gov/) and GeneOntology protein database [35]. The ScanProsite and Pfam servers were used to search for conserved protein domains [36,37]. Functional annotation of identified tick proteins was based on previously published tick sialomes with some modifications (immunoglobulin-binding proteins were added to this classification) [4].

Results and Discussion

Blood is the only form of nutrition taken by ticks, and large blood meals are required for their development and survival. Ticks are pool feeders that accomplish feeding by lacerating small blood vessels and sucking up the blood that flows to the wound, the so-called feeding site [4–7]. Within minutes of inserting the hypostome into host skin, ticks secrete an amorphous adhesive substance (cement) that anchors them onto host skin and secures attachment throughout the feeding period [38]. When completely attached to the wound site, most ticks slowly feed off the pooled blood at the feeding site for several days [39]. The tick feeding cycle includes (i) the preparatory feeding phase, when the tick attaches onto host skin and creates the feeding lesion; (ii) the slow feeding phase, when the tick swallows moderate amounts of blood, begins to transmit pathogens, and grows new tissue to prepare itself for (iii) the rapid feeding phase, when it feeds to repletion [38,39]. The tick feeding style triggers tissue repair and other defense responses, like hemostasis, inflammatory reactions, pain or itching, and immune rejection [4–7]. Like other blood-sucking parasites, *R. microplus* ticks have developed a complex and sophisticated collection of pharmacological bioactive proteins and lipids produced by salivary glands that counteract host defenses and allow successful parasitism [4,5]. During blood meal acquisition, salivary glands undergo remarkable growth and differentiation accompanied by significant increase in protein synthesis [21–23]. Ticks concentrate the blood meal by secreting excess water and ions back into the host through salivary secretion [40]. After detachment from the host, a signal triggers tick salivary gland degeneration [41,42]. *R. microplus* ticks attach to its host as unfed larvae, and then proceed to feed and molt through nymphal and immature adult stages in a period that stretches to 12 days. After mating, adult pre-engorged females (PEF) increase blood meal ingestion rapidly, and by the 21st or 22nd day these fully engorged females (FEF) complete feeding and detach [43,44]. Adult ticks used in this study were collected between days 17 and 22 after experimental infestation. Thus, data presented here represent part of the slow feeding phase and of the final rapid feeding phase. Consistent with reports that other tick species change salivary expression profiles during feeding [21–23], data in this study reveals remarkable, quantitative and qualitative differences in saliva content of *R. microplus* at different feeding stages, suggesting modulation of protein expression during these stages. The saliva collection procedure yielded approximately 0.1 µL *per* PEF tick, and on average 0.8 µL of saliva *per* FEF tick. Despite the low amount of saliva secreted by PEF ticks using the pilocarpine-induced method, their salivary secretion had a higher protein concentration (3.22 µg/µL), compared with those obtained from FEF ticks (1.75 µg/µL). This is in accordance with an increased expression of saliva proteins that are important in hematophagy, during slow feeding phase (PEF). Most of these proteins may have been turned off in FEF. This could also be explained by fast degeneration of salivary glands in FEF ticks immediately after detaching from the host [41,42]. In the same way, as the salivary gland is responsible for hydrodynamic equilibrium in ticks [45] it is supposed that it excretes more water in the rapid feeding phase (FEF) than in the slow feeding phase (PEF), so the volume of saliva is higher in FEF, however protein concentration is lower. The proteomic analysis of *R. microplus* saliva allowed identifying 187 and 68 proteins from tick and cattle, respectively. Sequences from tick identified proteins were deposited as Transcriptome Shotgun Assembly project at DDBJ/EMBL/GenBank under the accessions GBBO00000000 and GBBR00000000. The versions described in this paper are the first version, GBBO01000000 and GBBR01000000, respectively

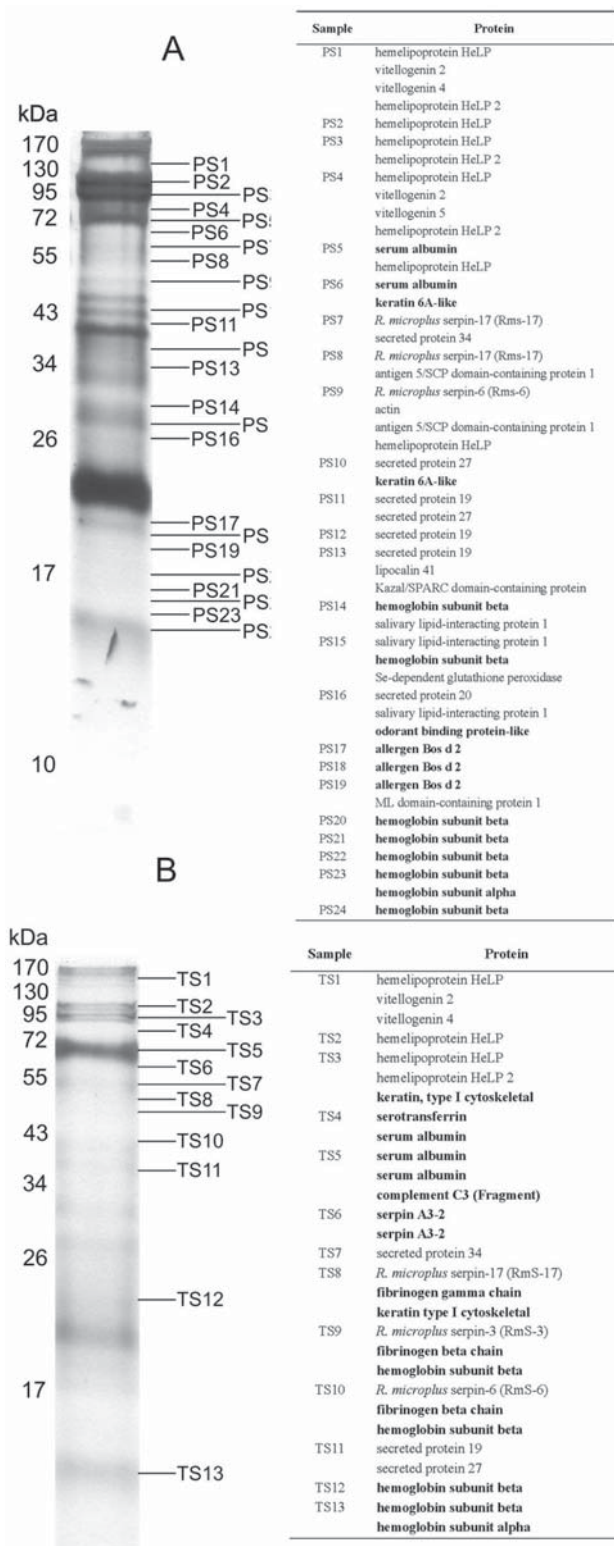
Based on SDS-PAGE analysis summarized in Figure 1, PEF saliva has a wider variety of proteins than FEF, as revealed by the number of identified proteins (147 to PEF and 112 to FEF) as well as in number of spectral counts, which can represent a semi-quantitative approach (Table 1, 2 and 3). These data represent an apparent difference between PEF and FEF saliva. Interestingly, we observed high amounts of host proteins, which are presented predominantly in FEF saliva (Table 4). The tick proteins identified in this study were classified as (i) putative secreted proteins and (ii) putative housekeeping proteins, and were then divided into groups according to their molecular function (Tables 1, 2, 3 and Figure 2) consistent with previous published tick sialomes [4].

Hemelipoprotein and other transporter proteins

Hemelipoproteins are the most abundant proteins in PEF and FEF saliva, based on protein band intensity (Figure 1) and spectral count (Table 3). In SDS-PAGE, these proteins appeared as two predominant bands between 95 and 130 kDa (Figure 1) consistent with a previous study that reported that the major hemelipoprotein present in *R. microplus* hemolymph (HeLp) consists of two subunits (92 and 103 kDa) [46,47]. Although HeLp has no full-sequence deposited in any protein database, peptides corresponding to N-terminal sequence of HeLp subunits match the sequences for hemelipoproteins identified in tick saliva here, corresponding to HeLp-A and HeLp-B subunits [46]. HeLp has the ability to bind eight heme molecules, the prosthetic group released from hemoglobin digestion, and deliver them to tick tissues [46]. As a predominant protein in hemolymph, the presence of HeLp in *R. microplus* saliva could be explained by the phenomenon of hemolymph components incorporation by salivary glands, leading to secretion in saliva [48]. However, in other tick species, the transcriptional profile and protein localization of these hemelipoproteins in salivary glands of adult and unfed ticks suggest that they could act in different pathways during blood-feeding [18,49,50]. Previous studies have described these proteins in saliva from other ticks, which indicates that they are a conserved feature among different tick species [17,18,20], suggesting that HeLp may play vital role(s) in tick feeding and survival.

Since this protein could transport other compounds such as cholesterol, phospholipids and free fatty acids, in addition to heme [47], it is possible that they are secreted in the feeding site carrying small pharmacologic active molecules. It may also be postulated that hemelipoproteins perform non-classical yet unknown functions at the tick-feeding site. Recently, the main hemelipoprotein form in *Dermacentor marginatum* was shown to be a carbohydrate-binding protein with galactose- and mannose-biding specificity able to agglutinate red blood cells [51]. In addition, as ticks use the pool-feeding strategy to feed [39], hemolysis at the feeding site is plausible due to the presence of digestive peptidases in saliva (Table 1 and 2). It is known that both heme and the heme-binding protein hemopexin have pro-inflammatory and anti-inflammatory properties, respectively [52–54]. Thus, the presence of hemelipoproteins could lower free heme concentration at the feeding site, preventing inflammation.

It may be speculated that HeLp is also essential to heme storage and/or detoxification in ticks. An important adaptation that co-evolved with blood feeding is heme sequestration by heme-binding proteins and heme excretion, both of which prevent oxidative stress and tissue damage [55]. Interestingly, *R. microplus* ticks are unable to synthesize heme *de novo* [56], so hemelipoproteins could be critical components of a mechanism for sequestration, storage and utilization of host heme [46,49]. Due to their high



Sample	Protein
PS1	hemelipoprotein HeLP
	vitellogenin 2
	vitellogenin 4
	hemelipoprotein HeLP 2
PS2	hemelipoprotein HeLP
PS3	hemelipoprotein HeLP
	hemelipoprotein HeLP 2
PS4	hemelipoprotein HeLP
	vitellogenin 2
	vitellogenin 5
	hemelipoprotein HeLP 2
PS5	**serum albumin**
	hemelipoprotein HeLP
PS6	**serum albumin**
	keratin 6A-like
PS7	*R. microplus* serpin-17 (Rms-17)
	secreted protein 34
PS8	*R. microplus* serpin-17 (Rms-17)
	antigen 5/SCP domain-containing protein 1
PS9	*R. microplus* serpin-6 (Rms-6)
	actin
	antigen 5/SCP domain-containing protein 1
	hemelipoprotein HeLP
PS10	secreted protein 27
	keratin 6A-like
PS11	secreted protein 19
	secreted protein 27
PS12	secreted protein 19
PS13	secreted protein 19
	lipocalin 41
	Kazal/SPARC domain-containing protein
PS14	**hemoglobin subunit beta**
	salivary lipid-interacting protein 1
PS15	salivary lipid-interacting protein 1
	hemoglobin subunit beta
	Se-dependent glutathione peroxidase
PS16	secreted protein 20
	salivary lipid-interacting protein 1
	odorant binding protein-like
PS17	**allergen Bos d 2**
PS18	**allergen Bos d 2**
PS19	**allergen Bos d 2**
	ML domain-containing protein 1
PS20	**hemoglobin subunit beta**
PS21	**hemoglobin subunit beta**
PS22	**hemoglobin subunit beta**
PS23	**hemoglobin subunit beta**
	hemoglobin subunit alpha
PS24	**hemoglobin subunit beta**

Sample	Protein
TS1	hemelipoprotein HeLP
	vitellogenin 2
	vitellogenin 4
TS2	hemelipoprotein HeLP
TS3	hemelipoprotein HeLP
	hemelipoprotein HeLP 2
	keratin, type I cytoskeletal
TS4	**serotransferrin**
	serum albumin
TS5	**serum albumin**
	serum albumin
	complement C3 (Fragment)
TS6	**serpin A3-2**
	serpin A3-2
TS7	secreted protein 34
TS8	*R. microplus* serpin-17 (RmS-17)
	fibrinogen gamma chain
	keratin type I cytoskeletal
TS9	*R. microplus* serpin-3 (RmS-3)
	fibrinogen beta chain
	hemoglobin subunit beta
TS10	*R. microplus* serpin-6 (RmS-6)
	fibrinogen beta chain
	hemoglobin subunit beta
TS11	secreted protein 19
	secreted protein 27
TS12	**hemoglobin subunit beta**
TS13	**hemoglobin subunit beta**
	hemoglobin subunit alpha

Figure 1. Proteome of *R. microplus* saliva. Saliva (25 µg) from partially engorged females (PEF) (A) and fully engorged females ticks (FEF) (B) was electrophoresed in 12% SDS-PAGE. The bands were excised, submitted for tryptic digestion and identified by LC–MS/MS. Numbers at the left indicate the MW in kDa of the protein standards. Host proteins identified are presented in bold. For further description of protein identification see Table S1 and Table S2.
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concentration in tick saliva, it is possible that relatively high concentrations of hemelipoproteins are present at the feeding site. This may allow re-ingestion of these proteins along with blood. In this scenario, hemelipoproteins may act as heme transporter when hemoglobin digestion begins in the midgut, since the high content of heme in the cytosol of midgut cells suggests a heme transport pathway from the digestive vesicles through the cytosol to reach the midgut basal surface, where heme is transferred to hemolymph to be delivered to the ovary [57,58]. These molecules may be internalized in midgut cells by endocytosis, mediated by specific receptors, as described in mammal cells (e.g. heme-carrier protein hemopexin) [59]. This hypothesis is supported by the results of midgut proteome analysis of *Dermacentor variabilis*, where a hemelipoprotein was identified by LC-MS/MS, but not in the midgut cDNA library [60], suggesting that this protein is delivered from other tissue/secretion. Furthermore, *D. marginatus* major hemolymphatic hemelipoprotein was immuno-localized inside the midgut cells [51]. In the same way, hemelipoproteins may act in an excretory system to remove heme excess, obtained from blood ingestion, binding heme and re-injecting it into the host. This hypothesis of heme-binding agrees with the fact we detected a high amount of hemelipoproteins in PEF than in FEF saliva, and this reduction of hemelipoproteins in FEF saliva was accompanied by an increase in the host heme-binding proteins (Figure 1, Figure 2, Table 3 and Table 4). These findings are compatible with a mechanism in which, towards the end of feeding, the tick replaces hemelipoprotein as heme-carrier by host derived heme-carrier proteins, including serum albumin, hemopexin, apolipoprotein and peroxiredoxin (Figure 2 and Table 4). This may be possible at this stage because, after completing feeding, hemelipoproteins are necessary for vitellogenesis [61]. However, the presence of heme in tick saliva is yet to be demonstrated and needs further investigation. Similarly, ferritin is present only in PEF saliva (Table 1). Ferritin is an important iron reservoir, working as a protective mechanism against free iron overload. It is considered to be crucial for *Ixodes ricinus* development and reproduction [62,63]. Apparently, the absence of ferritin in FEF saliva is functionally compensated by serotransferrin, an iron-carrier protein from the host (Table 4). These observations strongly suggest the existence of a cooperative system between tick and host carrier-proteins, especially those involved in heme and/or iron regulation during blood-feeding. The role of these proteins in tick-host needs further investigation

Lipocalins

Lipocalins are single modular proteins of around 200 amino acids that fold tightly in a β-barrel with potential for binding small hydrophobic molecules in a central pocket. The tertiary structures of lipocalin are greatly conserved, even when amino acid sequence similarities are low [64,65]. In most organisms lipocalins are characterized by the consensus structural conserved regions (SCRs) that are characteristic of kernel lipocalins [66], while tick proteins assigned to the lipocalin family lack the typical SCR [67]. Annotation of the most recently identified tick lipocalins is based on homology with annotated histamine-binding proteins from other tick species, based on the presence of the characteristic tick histamine-binding domain (PF02098) as described in the Pfam database [37,67–69]. PEF and FEF *R. microplus* secrete 50 different lipocalins in saliva (Table 1, 2 and 3). From these identified lipocalins, except for lipocalin 5, which matches the lipocalin domain (PF00061), all other identified *R. microplus* lipocalins possess the tick histamine-binding domain (PF02098), when scanned against the Pfam database or when visually inspected (data not shown) [37,69,70]. MS/MS data show that saliva

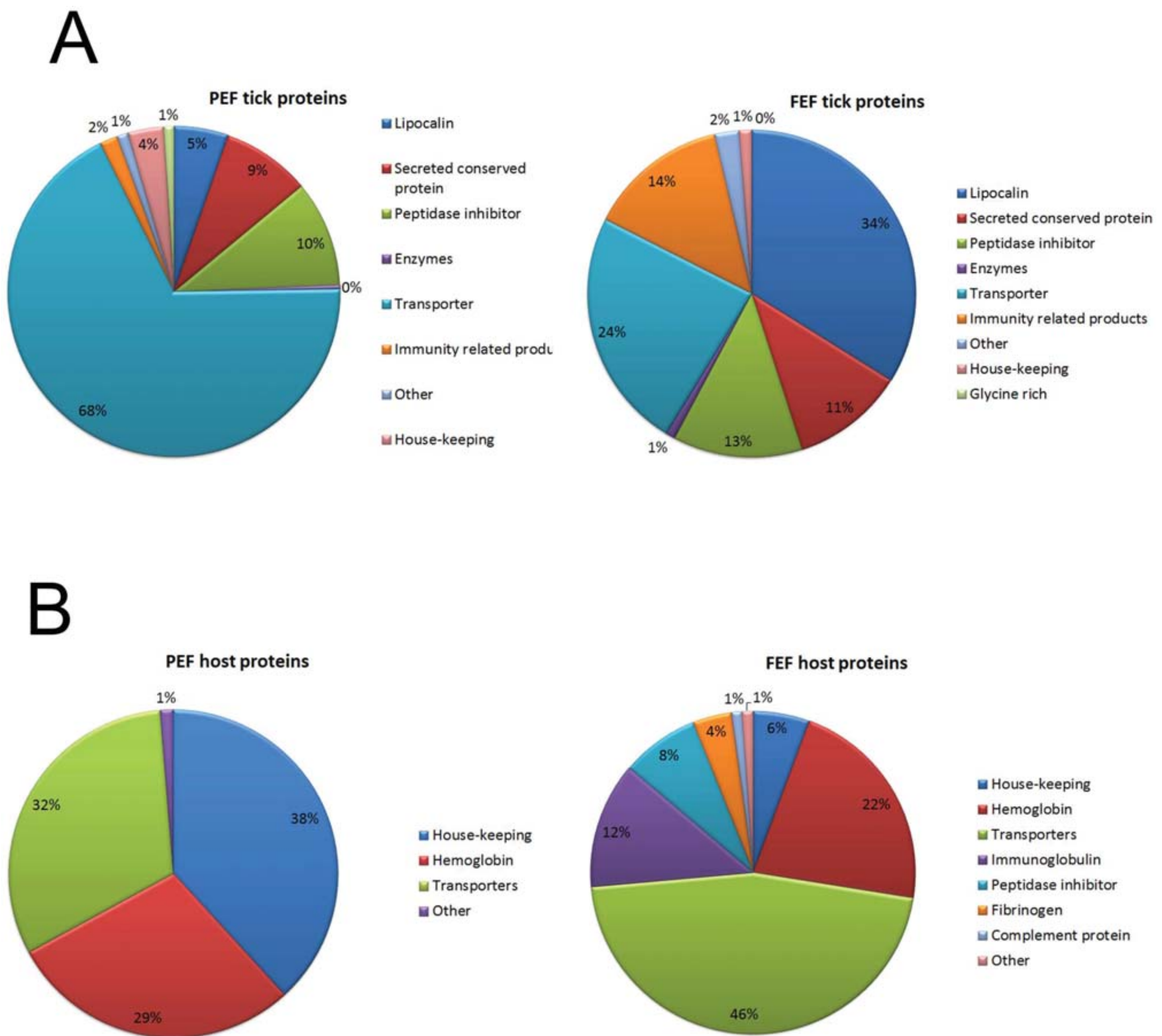

Figure 2. Functional classification of proteins in *R. microplus* saliva. Tick proteins (A) and host proteins (B) identified in *R. microplus* saliva were classified as putative secreted proteins or putative housekeeping proteins, and further in groups according to their function and/or protein family. Pie charts represent the percentage of proteins found in each group with respect to normalized spectral count (in brackets).
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lipocalins spectral counts are higher in FEF than in PEF (Table 1, 2 and 3). The presence of high amounts of lipocalins in cattle tick saliva is comparable with data from the *O. moubata* saliva proteome, showing that lipocalins are the most abundant salivary protein in this species [17]. Some of these *R. microplus* identified lipocalins have similarities with some described tick lipocalins, which have antihemostatic and immunomodulatory activities [68,69,71–80], such as amine-binding molecules. The high content of lipocalins in tick saliva is compatible with their antihemostatic and immunomodulatory roles during tick parasitism [4–7]. Since histamine and serotonin secreted by the host at the feeding site induce cutaneous inflammation, ticks have to overcome their activities in order to complete feeding [4–7]. Sequestering these host molecules may be a mechanism used by *R. microplus* against these defensive reactions that affect thick attachment to hosts [81,82]. The high content of lipocalins in *R. microplus* saliva also could be related to level necessary to block the near micromolar concentration of biogenic amines and prostaglandins that accumulate at the feeding site [4]. The importance of this mechanism for tick feeding is underlined by the fact that *R. microplus*-resistant cattle have its status reverted to susceptible when treated with anti-histamines (H1 antagonists) [83]. Besides, a recent study that demonstrated that tick-resistant cattle sera have a higher IgG titer against lipocalins, compared to susceptible animals, stresses the importance of this class of proteins for blood-feeders [70]. The presence of a high concentration of lipocalins in FEF (Table 1, 2, 3 and Figure 2) is intriguing, because at this stage blood sucking is completed, and the tick does not need to modulate host defense mechanisms. It is possible that lipocalins found in FEF saliva signal the role(s) of these molecules during the

Table 1. Tick proteins identified in PEF saliva by *in solution* digestion.

Protein[a] (75)	MW (kDa)	Spectral count	Coverage (%)	Best match BLAST[b]
PUTATIVE SECRETED PROTEINS				
LIPOCALINS (9)				
lipocalin 1	20.9	25	24	XP_002412631
lipocalin 2	21.0	19	20	ACX53907
lipocalin 3	20.4	14	13	XP_002412631
lipocalin 4	20.6	9	16	XP_002414294
lipocalin 5	14.8	9	21	DAA34565
lipocalin 35	24.7	7	11	ACX53907
lipocalin 36	22.8	5	10	ACX53955
lipocalin 46	26.4	4	10	ACX53986
lipocalin 6	20.3	3	9	ACX53907
SECRETED CONSERVED PROTEINS (16)				
secreted protein 27	37.5	51	29	XP_002403474
secreted protein 1	51.9	40	12	XP_002414081
secreted protein 39	15.5	39	32	ACX54027
secreted protein 28	30.6	29	35	AEE89467
secreted protein 2	13.5	27	22	XP_002424773
secreted protein 3	16.4	19	42	XP_002403368
secreted protein 4	23.2	17	14	XP_002435424
secreted protein 5	25.1	13	20	AAY66581
secreted protein 6	25.2	10	17	DAA34253
secreted protein 7	15.4	8	14	AEH03609
secreted protein 8	25.0	6	15	DAA34045
secreted protein 9	23.6	6	13	DAA34730
secreted protein 10	19.7	5	17	ACX53982
secreted protein 11	14.3	4	14	XP_002399909
secreted protein 12	26.2	4	8	XP_002414536
secreted protein 29	38.5	4	15	AEE89467
PEPTIDASE INHIBITORS (3)				
Serpin				
R. microplus serpin-6 (RmS-6)	44.4	52	31	XP_002402368
Cystatin				
cystatin 1	15.5	9	49	ACX53862
Thyropin				
thyropin 1	29.5	19	7	ACX54001
ENZYMES (6)				
Peptidases				
trypsin-like 1	39.5	9	6	XP_002435936
metallopeptidase 2	58.4	8	7	BAF43575
metallopeptidase 1	44.2	4	6	ADN23566
cathepsin B-like	38.5	2	8	BAF43801
Phospholipases				
phospholipase A2 1	44.8	17	10	XP_002399895
phospholipase A2 2	70.2	2	5	EFX77541
GLYCINE-RICH SUPERFAMILY (12)				
secreted cement protein 1	28.1	51	30	DAA34058
glycine-rich protein 1	13.9	16	36	AAV80791
cuticle protein 1	13.0	14	43	XP_002407787
glycine-rich protein 3	45.5	11	11	DAA34614
large GYY protein 3	15.0	7	27	XP_002411980
glycine-rich protein 4	9.2	7	25	XP_002411974

Table 1. Cont.

Protein[a] (75)	MW (kDa)	Spectral count	Coverage (%)	Best match BLAST[b]
large GYY protein 1	14.0	5	22	XP_002411975
large GYY protein 2	15.8	3	20	XP_002411980
glycine-rich protein 5	9.1	3	35	XP_002411978
glycine-rich protein 2	32.0	3	8	DAA34246
proline-rich protein 1	66.7	2	3	XP_001942898
secreted cement protein 2	30.2	2	8	ACX54028
ANTIGEN 5 PROTEIN FAMILY (1)				
antigen 5/SCP domain-containing protein 1	45.9	33	21	XP_002403125
TRANSPORTERS (1)				
ferritin 1	21.4	10	21	ACJ70653
CALRETICULIN (1)				
calreticulin 1	47.8	5	11	AAR29940
OTHER (1)				
Kazal/SPARC domain-containing protein	32.7	13	21	XP_002413686
PUTATIVE HOUSEKEEPING PROTEINS				
SIGNAL TRANSDUCTION (3)				
metabotropic glutamate receptor 1	59.8	21	19	CAA67993
beta thymosin 1	6.9	5	30	ACX53929
inositol polyphosphate phosphatase	23.5	5	9	XP_002401241
NUCLEAR REGULATION (2)				
histone 2A 1	13.4	21	55	XP_002402622
RNA-binding protein	32.6	2	8	XP_002412054
DETOXIFICATION (4)				
Se-dependent glutathione peroxidase	17.7	63	67	AAY66814
peroxinectin 1	71.3	30	19	XP_002406316
glutathione S-transferase 1	25.6	7	12	AAD15991
glutathione S-transferase	16.8	2	17	AAQ74442
CITOSKELETAL PROTEINS (4)				
microtubule-associated protein 1	13.9	13	47	XP_002399901
tropomyosin 1	25.3	8	19	O97162
alpha tubulin 1	45.8	3	7	XP_002402152
actin-depolymerizing factor 1	17.0	3	17	AA34587
PROTEIN SYNTHESIS. MODIFICATION AND EXPORT MACHINERY (5)				
heat shock protein 70 1	54.3	4	11	DAA34064
heat shock protein 90 1	55.7	3	8	XP_002414808
40S ribosomal protein S28	11.6	3	21	ABR23349
14-3-3 protein zeta 1	28.1	3	10	Q2F637
heat shock protein 70 cognate	51.6	2	9	XP_002407132
METABOLISM. NUCLEOTIDE AND CARBOHYDRATE (3)				
alpha-L-fucosidase	50.5	10	14	XP_002412933
deoxyribonuclease II 1	45.1	4	14	XP_002399332
peptidyl-prolyl cis-trans isomerase 1	21.2	2	13	XP_002410624
TRANSCRIPTION MACHINERY (1)				
elongation fator-1 alpha 1	50.8	29	17	XP_002411147
EXTRACELLULAR MATRIX AND ADHESION (3)				
neural cell adhesion molecule 2	83.1	5	4	XP_002409358
fascilin-like protein	39.2	2	8	XP_002409988
beat protein-like 1	45.3	2	10	XP_002406531

[a]Accession numbers for tick identified proteins were deposited as Transcriptome Shotgun Assembly project at DDBJ/EMBL/GenBank under the accessions GBBO00000000 and GBBR00000000. The versions described in this paper are the first version, GBBO01000000 and GBBR01000000, respectively.
[b]Accession numbers of best matches identities obtained using BLASTP against the non-redundant protein database in GenBank.
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Table 2. Tick proteins identified in FEF saliva by *in solution* digestion.

Protein[a] (41)	MW (kDa)	Spectral count	Coverage (%)	Best match BLAST[b]
PUTATIVE SECRETED PROTEINS				
LIPOCALINS (18)				
lipocalin 49	16.0	56	59	ACX53907
lipocalin 37	16.7	35	41	XP_002414294
lipocalin 7	19.9	27	61	ACX53907
lipocalin 8	20.4	21	33	ACX53907
lipocalin 9	20.3	20	35	ACX53907
lipocalin 38	23.9	19	25	ACX53907
lipocalin 10	20.5	18	31	ACX53907
lipocalin 39	8.3	12	17	XP_002414617
lipocalin 40	24.9	11	18	ACX53986
lipocalin 47	16.3	10	28	ACX53907
lipocalin 41	15.5	8	29	ACX53986
lipocalin 11	20.3	8	23	ACX53907
lipocalin 12	19.9	6	13	ACX53907
lipocalin 13	19.9	5	12	XP_002406507
lipocalin 14	19.4	4	13	ACX53907
lipocalin 42	20.6	3	11	ACX53986
lipocalin 43	19.0	2	15	ACX53907
lipocalin 15	21.1	2	15	ACX53907
SECRETED CONSERVED PROTEINS (8)				
secreted protein 13	27.5	21	23	XP_002414536
secreted protein 14	13.2	20	33	XP_002413811
secreted protein 30	49.9	13	10	XP_002414081
secreted protein 40	6.6	10	11	BAG58161
secreted protein 16	6.9	8	23	YP_001186599
secreted protein 31	15.7	5	11	ACX54027
secreted protein 17	8.5	4	30	ZP_06826700
secreted protein 18	9.5	3	36	XP_001197477
PEPTIDASE INHIBITORS (7)				
TIL domain-containing protein				
TIL domain-containing protein 1	9.3	39	67	ACV83329
TIL domain-containing protein 2	9.2	17	53	ACV83329
TIL domain-containing protein 3	17.5	9	31	XP_002409984
TIL domain-containing protein 4	17.6	9	32	XP_002409984
Thyropin				
thyropin 2	28.2	4	10	ACX54001
thyropin 3	20.9	3	13	ACX54001
Kunitz-type				
Kunitz domain-containing protein 1	78.2	5	4	AAN10061
IMMUNOGLOBULIN-BINDING PROTEIN (2)				
immunoglobulin G-binding protein 2	19.9	18	26	XP_002414615
immunoglobulin G-binding protein 1	17.4	6	12	XP_002411824
ENZYMES (2)				
acetylcholinesterase 1	61.8	9	8	ADO65743
heme-binding aspartic peptidase (THAP)	40.5	5	6	AAG00993
IXODEGRIN FAMILY (1)				
cysteine-rich KGD motif-containing protein 1	19.0	5	5	XP_002411345
CAP SUPERFAMILY (1)				
cysteine-rich protein 2	17.5	35	31	XP_002411345

Table 2. Cont.

Protein[a] (41)	MW (kDa)	Spectral count	Coverage (%)	Best match BLAST[b]
IMMUNITY-RELATED PRODUCTS (1)				
Antimicrobial peptides				
histidine-rich secreted protein 1	17.5	13	17	CAX82541
TRANSPORTERS (1)				
vitellogenin 1	201.8	13	6	AAA92143.1

[a]Accession numbers for tick identified proteins were deposited as Transcriptome Shotgun Assembly project at DDBJ/EMBL/GenBank under the accessions GBBO00000000 and GBBR00000000. The versions described in this paper are the first version, GBBO01000000 and GBBR01000000, respectively.
[b]Accession numbers of best matches identities obtained using BLASTP against the non-redundant protein database in GenBank.
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last stages of the rapid feeding phase, when the tick takes huge amounts of blood or prepares to detach from host skin.

Secreted conserved proteins

Transcriptomical analyses of salivary gland of hard and soft ticks have provided reliable data on blood-feeding behavior [4,5,7–16]. The repertoire of tick salivary gland transcripts found is much broader and complex than anticipated, with many proteins without similarities to proteins in the NCBI database. Most of these new proteins were identified just as hypothetical secreted conserved proteins [4]. Proteins included in this group are the most abundant proteins in *R. microplus* saliva, and PEF saliva is richer in these proteins than FEF saliva (Table 1, 2, 3). The presence of these proteins in *R. microplus* saliva, as observed in the present study, confirms that some previously described hypothetical secreted conserved proteins are actually secreted proteins. Members of this type of proteins in *R. microplus* are 70–460 amino acid proteins (predicted molecular weight varying from 6.6 to 51.9 kDa) and some of them migrate as 34–60 kDa proteins when separated in SDS-PAGE (Figure 1 and Table S1), suggesting that they have post-translational modifications. Given the higher number of these proteins present in tick saliva, it is reasonable to conclude that they have a role in tick feeding. The *A. americanum* AV422 protein (*Aam*AV422) is a member of the secreted conserved protein group that is differentially up-regulated in response to contact with host and/or exposure to feeding stimuli [84,85]. This protein is secreted and injected in the host within the first 24 h of tick attachment onto the host. Apparently, *Aam*AV422 is involved in the mediation of tick anti-hemostasis and anti-complement functions, since r*Aam*AV422 delays plasma clotting time in a dose responsive manner, prevents platelet aggregation and reduces the formation of terminal complement complexes [84,85]. *R. microplus* secreted protein 20 is 99% identical to *Aam*AV422, and is secreted in PEF and FEF saliva (Table 3). Like *Aam*AV422, it may act as an anti-hemostatic and anti-complement protein [85]. Further studies are necessary to better characterize this group of salivary proteins, and may represent an opportunity to discover new targets for parasite control.

Peptidase inhibitors

The tick feeding style of lacerating host tissue and sucking host blood from the pool formed at the bite site is expected to strongly trigger host defense responses as hemostasis, inflammation, and complement systems [4,5,86]. These responses are dependent on the action of several peptidases, such as procoagulant (thrombin, factor Xa and other coagulation factors), pro-inflammatory (neutrophil elastase, proteinase-3, chymase, tryptase, kallikrein, cathepsin L, cathepsin B, cathespin S, cathepsin C and cathepsin G) and complement enzymes (factors B, C, D and component 2) [4,5,86,87]. These host defenses are highly regulated by specific endogenous inhibitors, maintaining homeostasis. From this perspective, it has been suggested that ticks secrete peptidase inhibitors to disrupt host defenses, facilitating feeding [88].

Serpins. proteins that belong to the serpin (serine protease inhibitor) superfamily are expressed in all branches of life [89]. They have a role in the control of several endopeptidase cascades in many organisms [90]. In mammalians, most serpins play crucial roles, controlling endopeptidases involved in blood coagulation, fibrinolysis, inflammation, and complement activation [89,91]. It is assumed that tick secreted serpins disrupt host homeostatic balance in order to facilitate parasitism [88]. Recently, 18 full-length serpin encoding sequences were described in *R. microplus* [92], three of which (RmS-3, RmS-6 and RmS-17) were identified in PEF and FEF saliva (Table 3). Notably, PEF saliva has a high number of spectral counts of this protein family (Table 3), suggesting that inhibition of serine endopeptidases involved in host defense system is important earlier in blood tick feeding. It was shown that tick-resistant cattle sera have high titers of antibodies against RmS-3, compared to tick-susceptible animals, suggesting its importance in the tick-host relationship [93]. Furthermore, the administration of an antibody against RmS-3 linear epitope by artificial feeding decreases the reproductive capacity of *R. microplus* females by 81% [93]. However, the precise role of these inhibitors in *R. microplus* saliva remains unclear. The presence of these serpins in *R. microplus* saliva could be responsible, at least partially, for the anti-thrombin [94] and anti-thrombotic [95] properties of its saliva, including their local and systemic alterations [26]. Moreover, some other pharmacological activities of *R. microplus* saliva may be associated to serpins, such as immunomodulatory activity [96–99]. The potential effect of these proteins on host systems are supported by several studies showing serpins from hematophagous parasites act as anti-coagulant and anti-inflammatory agents, being essential for a successful blood meal [96–102]. Clearly, data showing that the use of serpins as vaccinal antigens impairs tick development reinforces the importance of these proteins in regulating tick physiology [103–107].

α2-macroglobulin (α2M). these are large glycoproteins and are present in the body fluids of both invertebrates and vertebrates, being secreted as glycosylated polypeptides with a molecular mass of about 180 kDa [108]. Three α2M were identified in PEF and FEF saliva (Table 3), and based on spectral counts all three seem to be most abundant in PEF, relatively to FEF. In vertebrates, α2M proteins have been found to regulate host cell apoptosis [109], inhibit several serum peptidases like thrombin [110], factor Xa [111] and kallikreins [112], mediate T-cell proliferation [113] and induce proliferation and activation of

Table 3. Tick proteins identified both in PEF and FEF saliva by *in solution* digestion.

		PEF		FEF		
Protein[a] (70)	**MW (kDa)**	**Spectral count**	**Coverage (%)**	**Spectral count**	**Coverage (%)**	**Best match NCBI[b]**
PUTATIVE SECRETED PROTEINS						
LIPOCALINS (23)						
lipocalin 16	20.7	130	37	97	65	ACX53907
lipocalin 17	20.9	62	49	64	61	ACX53907
lipocalin 18	21.7	43	21	25	34	ACX53907
lipocalin 45	17.3	38	10	7	10	ACX53907
lipocalin 19	20.5	28	15	47	46	ACX53907
lipocalin 20	21.1	24	24	43	57	ACX53907
lipocalin 21	21.1	23	22	12	38	XP_002412631
lipocalin 22	20.5	23	17	33	27	XP_002415124
lipocalin 50	18.7	22	33	9	19	ACX53907
lipocalin 23	21.1	22	36	11	13	ACX53907
lipocalin 24	21.0	22	18	42	56	ACX53907
lipocalin 25	20.7	19	21	49	59	XP_002412631
lipocalin 48	15.4	18	30	5	18	ACX53907
lipocalin 26	20.6	17	27	43	45	ACX53907
lipocalin 27	19.8	16	18	33	28	ACX53907
lipocalin 44	23.9	14	5	25	25	ACX53907
lipocalin 28	20.7	11	16	8	10	XP_002414294
lipocalin 29	20.7	10	16	14	26	ACX53907
lipocalin 30	20.4	9	18	50	32	ACX53907
lipocalin 31	20.5	5	10	9	27	ACX53907
lipocalin 32	22.6	2	14	11	19	ACX53986
lipocalin 33	20.8	2	11	52	51	ACX53907
lipocalin 34	20.9	2	23	9	15	ACX53907
SECRETED CONSERVED PROTEINS (15)						
secreted protein 19	36.3	314	64	27	35	XP_002402717
secreted protein 20	25.1	82	44	48	42	DAA34225
Bm05	19.1	66	27	23	23	ABV53333
secreted protein 32	21.7	64	47	5	14	DAA34730
secreted protein 33	37.8	46	20	5	7	XP_002403474
secreted protein 21	72.2	45	59	19	47	XP_728368
secreted protein 22	15.8	44	18	16	13	ADN23561
secreted protein 34	37.5	37	21	15	16	XP_002402718
secreted protein 23	25.1	31	34	8	8	DAA34045
secreted protein 35	9.7	21	64	9	36	XP_002408964
secreted protein 36	21.6	16	12	14	16	XP_002414083
secreted protein 24	11.3	11	27	16	43	XP_002413811
secreted protein 25	16.7	7	16	10	21	XP_002408703
secreted protein 26	15.2	5	28	16	36	XP_002410662
secreted protein 37	42.4	2	7	9	13	XP_002411420
PEPTIDASE INHIBITORS (10)						
Serpins						
R. microplus serpin-17 (RmS-17)	43.2	206	77	37	32	ABS87360
R. microplus serpin-3 (RmS-3)	43.4	185	60	71	43	AAP75707
R. microplus serpin-3 (RmS-3)	43.4	175	65	66	39	AAK61377
R. microplus serpin-17 (RmS-17)	43.2	146	78	14	31	ABS87360
R. microplus serpin-6 (RmS-6)	44.3	68	41	28	18	ABI94056

Table 3. Cont.

		PEF		FEF		
Protein[a] (70)	**MW (kDa)**	**Spectral count**	**Coverage (%)**	**Spectral count**	**Coverage (%)**	**Best match NCBI[b]**
R. microplus serpin-6 (RmS-6)	44.3	64	41	27	18	ABI94056
Cystatin						
RmCys2b	15.4	24	58	2	21	AGB35873
Alpha2-macroglobulin						
alpha2 macroglobulin 2	164.0	313	38	26	12	ACJ26770
alpha2 macroglobulin 1	85.1	20	11	8	4	XP_002405338
alpha2 macroglobulin 3	87.1	5	7	4	2	AAN10129
ENZYMES (2)						
chitinase 1	48.4	3	5	7	10	ACX33152
serine carboxypeptidase 1	35.6	2	6	7	7	XP_002404034
8.9 kDa FAMILY (2)						
8.9 kDa protein 1	11.7	24	12	12	19	ACG76246.1
8.9 kDa protein 2	11.7	13	8	7	36	ACX53877
MUCIN (1)						
mucin 1	25.5	47	26	5	7	AAA97877
IMMUNITY RELATED PRODUCTS (4)						
Antimicrobial peptides						
microplusin-like 2	10.7	125	40	34	45	AAY66495
BmSEI-like 1	11.5	51	51	167	63	ABH10604
BmSEI-like 2	11.0	29	21	185	43	ABH10604
microplusin-like 1	16.0	8	9	4	19	ABB79785
TRANSPORTERS (8)						
hemelipoprotein HeLP	146.8	3945	77	353	47	ABK40086
hemelipoprotein HeLP 3	94.0	2810	72	207	39	ABK40086
vitellogenin 2	19.0	601	44	6	4	XP_002401768
hemelipoprotein HeLP 2	30.6	512	61	42	47	ABK40086
vitellogenin 4	10.8	381	48	32	16	BAJ21514
vitellogenin 5	64.4	77	36	2	4	XP_002401765
salivary lipid-interacting protein 1	20.4	61	27	7	21	XP_002414779
vitellogenin 3	21.7	9	4	28	10	BAH02666
PUTATIVE HOUSEKEEPING PROTEINS						
CYTOSKELETAL PROTEINS (1)						
actin 1	41.8	99	39	15	22	AAP79880
IMMUNITY RELATED PRODUCTS (1)						
Toll-like receptor 5	38.3	22	12	2	6	DAA34254
EXTRACELLULAR MATRIX AND ADHESION (3)						
ML domain-containing protein 1	13.5	28	35	10	18	XP_002434499
neural cell adhesion molecule 3	40.5	13	26	5	16	XP_002414299
neural cell adhesion molecule 1	63.1	16	8	3	4	XP_002409358

[a]Accession numbers for tick identified proteins were deposited as Transcriptome Shotgun Assembly project at DDBJ/EMBL/GenBank under the accessions GBBO00000000 and GBBR00000000. The versions described in this paper are the first version, GBBO01000000 and GBBR01000000, respectively.
[b]Accession numbers of best matches identities obtained using BLASTP against the non-redundant protein database in GenBank.
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macrophages [114]. Tick saliva α2M may be linked to interference in inflammation and immunomodulation, and it may be an additional salivary anti-coagulant. It is still unclear whether these α2M act as immunomodulators or as anticoagulants, this role needs to be elucidated. However, the fact that such inhibitors (as α2M proteins and serpins) are secreted mostly in PEF saliva (Table 3) reinforces the idea that inhibition of host-defenses endopeptidases is important as early as in the beginning of the blood meal.

TIL domain-containing proteins. proteins belonging to the TIL (trypsin inhibitor-like) domain-containing group have been reported in blood-feeding mosquitoes and tick sialomes [5].

Table 4. Host proteins identified in PEF and FEF saliva by *in solution* digestion.

		PEF		FEF	
Protein (68)	**Accession number**	**Spectral count**	**Coverage (%)**	**Spectral count**	**Coverage (%)**
PEF (17)					
actin, alpha skeletal muscle	IPI00697648.1	64	24	-	-
allergen Bos d 2	IPI00708946.1	38	36	-	-
keratin, type I cytoskeletal 14	IPI00721270.4	36	14	-	-
beta actin	IPI00905257.2	34	17	-	-
keratin, type II cytoskeletal 75	IPI00700471.2	26	9	-	-
keratin, type II cytoskeletal 7	IPI00694214.1	20	7	-	-
odorant binding protein-like	IPI00722909.1	20	41	-	-
histone H2A	IPI00698058.5	16	35	-	-
keratin, type II cytoskeletal 79	IPI00707469.2	16	4	-	-
keratin 15	IPI00692588.3	15	6	-	-
KRT4 protein	IPI00709590.5	13	7	-	-
secretoglobin	IPI00838546.1	10	26	-	-
keratin, type I cytoskeletal 24	IPI00698285.3	8	6	-	-
histone H4 replacement-like	IPI00716205.3	8	17	-	-
heat shock protein HSP 90-alpha	IPI00699622.3	5	5	-	-
lipocalin 2 (oncogene 24p3)-like	IPI00685784.3	2	14	-	-
annexin A1	IPI00703345.2	2	8	-	-
FEF (38)					
serotransferrin	IPI00690534.1	-	-	87	38
alpha-2-macroglobulin	IPI00871133.1	-	-	54	19
immunoglobulin kappa light chain	IPI00699011.3	-	-	46	42
immunoglobulin light chain	IPI01028259.1	-	-	41	42
immunoglobulin light chain	IPI00838162.2	-	-	40	44
immunoglobulin light chain	IPI00855695.1	-	-	40	39
immunoglobulin light chain	IPI00867205.1	-	-	40	44
fibrinogen gamma chain	IPI00843209.1	-	-	33	33
immunoglobulin M heavy chain	IPI00714264.4	-	-	29	27
fibrinogen beta chain	IPI00709763.5	-	-	28	22
fibrinogen alpha chain	IPI00691819.1	-	-	25	15
complement C3 (Fragment)	IPI00713505.2	-	-	24	10
SERPINA1 Alpha-1-antiproteinase	IPI00695489.1	-	-	20	17
SERPINA3-2 Serpin A3-2	IPI00930024.1	-	-	20	19
apolipoprotein A-I	IPI00715548.1	-	-	17	35
immunoglobulin iota chain-like, partial	IPI00907960.2	-	-	17	24
carbonic anhydrase 2	IPI00716246.2	-	-	15	22
SERPINA3-1 Uncharacterized protein	IPI00968658.1	-	-	15	23
SERPINA3-3 Serpin A3-4	IPI00971592.1	-	-	15	17
serpin A3-7 isoform X1	IPI00971595.1	-	-	15	19
SERPINA3 Serpin A3–5	IPI00707034.6	-	-	14	16
hemopexin	IPI00690198.4	-	-	12	14
SERPINA3-6 Serpin A3-6	IPI00829575.1	-	-	12	15
immunoglobulin lambda-like polypeptide 1-like	IPI01002118.1	-	-	11	15
immunoglobulin light chain	IPI00718725.5	-	-	10	19
SERPINA3-7 Endopin 2C	IPI00705594.1	-	-	9	11
peroxiredoxin-2	IPI00713112.1	-	-	9	23
transthyretin	IPI00689362.1	-	-	8	35
cathelicidin-2	IPI00691669.1	-	-	6	16

Table 4. Cont.

		PEF		FEF	
Protein (68)	**Accession number**	**Spectral count**	**Coverage (%)**	**Spectral count**	**Coverage (%)**
alpha-2-HS-glycoprotein	IPI00707101.1	-	-	6	13
protein unc-45 homolog A	IPI00716476.2	-	-	6	3
vitamin D-binding protein	IPI00823795.1	-	-	5	11
cathelicidin-4	IPI00686754.1	-	-	4	19
immunoglobulin kappa light chain	IPI00889485.1	-	-	4	19
zinc finger CCCH domain-containing protein 7B	IPI00693044.4	-	-	3	1
flavin reductase	IPI00718510.2	-	-	3	13
immunoglobulin kappa light chain	IPI00906505.1	-	-	3	19
AF4/FMR2 family member 3	IPI01017768.1	-	-	2	3
PEF and FEF (13)					
hemoglobin subunit beta	IPI00716455.1	210	66	258	74
serum albumin	IPI01028455.1	174	31	452	64
serum albumin	IPI00708398.2	164	30	431	64
hemoglobin subunit alpha	IPI00710783.2	152	77	230	90
keratin 6A-like	IPI01002591.1	44	8	20	10
KRT6A protein	IPI00845184.1	41	12	7	4
keratin 13-like isoform 2	IPI00912554.1	36	14	32	10
keratin 6A-like	IPI01001566.1	29	5	9	3
keratin 2-like	IPI01003176.2	28	5	10	5
keratin, type II cytoskeletal 5	IPI00697851.1	18	4	7	4
polyubiquitin-C	IPI00726431.1	11	4	12	4
cathelicidin-1	IPI00718108.1	9	23	8	30
peptidoglycan recognition protein 1	IPI00701640.1	7	13	6	30

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Ixodidin, an example of this group of inhibitors, was isolated from *R. microplus* hemolymph. In addition to antimicrobial activity, ixodidin has anti-trypsin and anti-elastase activities [115]. Only FEF saliva has peptides matching this group of proteins, including ixodidin (Table 2). These proteins may act similarly to host endopeptidases inhibitors, increasing the inhibition of the target endopeptidases. Additionally, presence of these proteins at the final phase of blood meal acquisition suggests that they have a possible role as an antimicrobial protein to prevent (or control) infection in ticks after blood-meal acquisition. Their interfering role in tick-vectoring ability, regulating the quantity or even the specificity of pathogens ticks transmit remains to be addressed.

Thyropin. thyropin (thyroglobulin type-1 domain protease inhibitors) is a family of proteins characterized by the presence of thyroglobulin type-1 domain repeats [116,117]. The well characterized type-1 domain-containing protein was described in the sea anemone *Actinia equina* and has been shown to inhibit either cysteine or cation-dependent peptidases [118], including cathepsin L, cathepsin S, papain and cruzipain [117,119]. PEF and FEF saliva contains three thyropins (Table 1 and 2). It is possible that these proteins inhibit some host cysteine endopeptidases, contributing to the immunomodulatory effects of tick saliva. This hypothesis has yet to be proved, since thyropins have not been functionally characterized in ticks to date. Proteins containing these domains are present in several tick sialomes [4], and their presence was previously also detected in *O. moubata* and *R. sanguineus* saliva [17,19].

Cystatin. cystatins comprise a large family of reversible and tight-binding inhibitors of papain-like enzymes and legumains [120], which are involved in biological processes like antigen processing and presentation, phagocytosis, neutrophil chemotaxis during inflammation and apoptosis [121–124]. Two proteins of the cystatin family were identified in PEF and FEF, with higher spectral counts in PEF saliva (Table 1 and 3). The most abundant (RmCys2b – AGW80658.1) is a member of type 2 cystatin [125] and is present predominantly in PEF saliva (Table 3). It is able to inhibit cathepsin B, cathepsin L and cathepsin C (L. F. Parizi, personal communication). As these enzymes are important in some immunologic processes, these cystatins in *R. microplus* saliva could act as immunomodulators during the slow feeding phase of cattle tick parasitism, as previously shown for other tick cystatins, facilitating blood feeding and pathogen transmission [126–130]. The importance of these inhibitors in blood feeding was underscored in studies that showed that neutralization of cystatins (through gene silencing in ticks or vaccines) significantly reduces tick feeding ability [128,131,132].

Kunitz-type inhibitors. members of the Kunitz-type family are particularly well characterized as inhibitors of a large number of serine endopeptidases [133]. One protein containing Kunitz domains was found only in FEF saliva (Table 2). Interestingly, this protein contains nine *in tandem* Kunitz domains, a remarkable difference among well characterized inhibitors of this class in other ticks, which range between one and five domains [25,134]. These inhibitors have been characterized as acting upon thrombin, factor Xa, factor XIIa, trypsin and elastase [25]. This raises the

suggestion they contribute to *R. microplus* saliva anticoagulant activity [26,94,95].

Glycine-rich proteins

This group of proteins is described in several tick sialomes and has distinct subdivisions [4]. In ticks, proteins containing glycine-rich (Gly-rich) and proline-rich (Pro-rich) repeat motifs are associated with tick-cement functions [135,136]. Ten proteins of this superfamily were found exclusively secreted in PEF saliva (Table 1). These proteins have been identified also in *O. moubata* and *R. sanguineus* saliva [17,19]. The presence of these proteins at this stage lends strength to the hypothesis that they are important in the formation of a cement cone that affords tick attachment to the host during initial feeding phase. Three of these proteins contain the motif [LPAE]-P-G, that are known as targets of proline hydroxylase (data not shown) [137,138], a post-translational modification which allows cross-linking between proteins, a characteristic present in cement proteins [139]. The identification of these proteins at this developmental stage is in accordance with a previous study on *A. americanum*, where genes codifying for this superfamily of proteins are up regulated at the early stages of parasitism [84].

Enzymes

Peptidases. parasite secreted enzymes may play a wide array of roles in host tissues. Analysis of PEF tick saliva allowed the identification of two metallopeptidases (Table 1). In this sense, metallopeptidases, frequently associated with vascular damage, tissue remodeling and degradation of serum compounds [140] may have a role modulating host responses against ticks. As shown in other ticks, this salivary metallopeptidases may be linked to fibrin(ogen)lysis [141], bradykinin degradation [142], and angiogenesis inhibition [143]. In PEF saliva, a trypsin-like enzyme similar to factor-D from *D. variabilis* was identified (Table 1). This enzyme may interfere with host inflammation and blood clotting, acting as plasminogen activator or protein C activator, similarly to what has been reported for *I. scapularis* saliva [144]. The secretion of metallopeptidases and trypsin-like enzymes in tick saliva is stage-dependent, since the analysis performed here indicates that FEF saliva does not have significant amounts of these enzymes. The presence of these proteins in PEF saliva could also be explained by the fact that host defense modulation is crucial for blood feeding at this time.

In FEF tick saliva, only one endopeptidase was identified, the tick heme-binding aspartic peptidase (THAP) (Table 2). Here, we report, for the first time, the presence of THAP in cattle tick saliva. THAP is able to hydrolyze hemoglobin and vittelin, and thus is supposed to have a role in *R. microplus* digestion and embryogenesis [145,146]. It may be hypothesized that THAP acts as a digestive enzyme secreted in the host during the fast engorgement phase. During blood meal acquisition, THAP may start the digestion process of blood components in the hemorrhagic pool at the tick attachment site. Similarly, this activity could explain the presence of a cathepsin-B in PEF saliva (Table 1), as this type of enzymes has been described to hydrolyze hemoglobin in other tick species [147,148]. In the same way, saliva of both PEF and FEF secretes a serine-carboxipeptidase (Table 3). Since a serine-carboxipeptidase from midgut was able to hydrolyze bovine hemoglobin in *Haemaphysalis longicornis*, it suggests that it also may be involved in digestion of the blood meal at feeding site [149]. In this way, the presence of these digestive enzymes in saliva may be associated with the presence of heme-binding proteins, since the free-heme delivered by hemoglobin digestion at the feeding site has to be sequestered, because heme has pro-inflammatory properties [52] and impairs blood meal acquisition.

Phospholipase A2. phospholipases A2 (PLA2) are secreted enzymes that have been implicated in several biological processes, such as modification of eicosanoid generation, inflammation and host defense [150,151]. Two PLA2 proteins were found in PEF saliva (Table 1). Secretory PLA2 are common and important components of bee and snake venoms, and have hemolytic, antiplatelet aggregation, and anticoagulant effects through their ability to interact with cells or by the degradation of phospholipid, thus generating free arachidonic acid [152]. Likewise, in *A. americanum* these proteins are suggested to act in the hemolytic activity of saliva [153,154]. The presence of PLA2 in PEF is in accordance with those digestive enzymes described above, which also may play a role in host blood cells lyses, facilitating the tick digestive process at feeding site. Additionally, these enzymes may act as antiplatelet and anticoagulant agents [152], facilitating blood feeding and reinforcing the notion that defense modulation in PEF is crucial for blood feeding.

Immunity-related proteins

Antimicrobial peptides. antimicrobial peptides (AMPs) are widely distributed in nature and are essential components of the first defense line against infections [155]. In invertebrates, which have only innate immunity, AMPs are extremely effective and work as powerful weapons against bacteria and fungi [156]. Microplusin is an AMP from *R. microplus* that belongs to the group of cysteine-rich AMPs with histidine-rich regions at N- and C-termini, which have been implicated in sequestration of zinc, a microbial growth factor [157,158]. Proteins of the microplusin-like and histidine-rich families are present in the saliva of both PEF and FEF (Table 2 and Table 3). The role(s) of these proteins in tick saliva may be associated with the prevention of microbial proliferation at the tick-feeding site. Moreover, since a lot of saliva is ingested together with the diet, especially in pool feeders, it could be assumed that the AMP may also act in the midgut of ticks.

Putative housekeeping proteins

In *R. microplus*, we identified putative housekeeping proteins, predominantly in PEF saliva (Table 1 and 3). Putative housekeeping proteins in tick saliva have been identified in *O. moubata* and *R. sanguineus* [17,19]. The presence of this kind of protein in tick saliva is supported by observations showing apocrine and merocrine secretion in tick salivary glands [159]. Moreover, these housekeeping proteins can be secreted in non-classical pathways to the extracellular environment [160,161]. Presence of these proteins in tick saliva is underlined by the fact that hosts infested with *A. americanum* develop antibodies against housekeeping proteins during different tick feeding stages (A. Mulenga, personal communication).

The presence of housekeeping proteins in tick saliva may have further biological importance, since these proteins may play different roles in the tick-host interface. For example, since HSP70 is present in PEF saliva, it may be involved in tick-host relationship (Table 1). In an experimental model of disease, HSP70 administration prevents inflammatory damage and promotes the production of anti-inflammatory cytokines [162]. Similarly, a study showed that HSP70 from *Mycobacterium turbeculosis* has anti-inflammatory properties, inhibiting pro-inflammatory cytokine production by IL-10 driven down-regulation of transcriptional factor in dendritic cells [163]. Other examples of housekeeping protein involve enzymes linked to detoxification (Table 1). Glutathione S-transferase (GST) is a protein that catalyzes the

conjugation of glutathione with several xenobiotic and endogenous substances [164]. In this sense, GST seems to be closely associated with detoxification and acaricide resistance [165]. Additionally, it has been proposed that GST secreted by parasite salivary glands has immunomodulatory activity due to the alteration of cytokine gene expression profile, modulation of immune cell proliferation and decrease in oxidative ability of phagocytes [166]. Further studies are necessary to elucidate the role of this class of proteins in tick saliva, since this appears to be a conserved feature among different tick species [17,19].

Host proteins

A large number of bovine proteins were identified in the saliva of both PEF and FEF, being present predominantly in FEF saliva, relatively to PEF saliva (Table 4). The presence of host proteins in tick saliva has been reported in other ticks species [17–20]. These proteins are the majority secreted proteins in *R. sanguineus* saliva [19]. It was demonstrated that ticks transport intact proteins across the digestive system to the hemolymph [167]. Furthermore, some of the host proteins described in *R. microplus* proteome have been found in salivary glands of other tick species [12,18,20,48], suggesting that the presence of host proteins in tick saliva may be a real and common recycling system present in ticks, not a result of contamination during saliva collection. Furthermore, the presence of different classes of host proteins in the saliva of the two tick developmental stages suggested the existence of this selective uptake process (Table 4 and Figure 2). For example, in PEF saliva we observed a predominance of housekeeping proteins (actin, nuclear proteins like histone and HSP90) and hemoglobin subunits peptides (Table 4 and Figure 2). In FEF saliva this pattern switches dramatically due to: (i) transporter and/or proteins associated with metabolism of heme and iron, like serum albumin, peroxiredoxin, serotransferrin, apolipoprotein and hemopexin; (ii) immunity, like immunoglobulins chains and C3 complement protein; (iii) peptidase inhibitors of the serpin superfamily; and (iv) other proteins (Table 4 and Figure 2). Similarly, rabbit proteins involved in heme and iron metabolism (as serum albumin, serotransferrin and hemopexin); immunity (C3 complement protein); and serpins were identified in *R. sanguineus* saliva [19]. However, as in *R. sanguineus* saliva was collected from 5–7 days partially fed adults ticks [19], it is not possible to compare these differences among different developmental stages, as found in *R. microplus*.

We are mindful of the possibility that tick saliva proteins in FEF may not represent exactly what occurs at the end of the blood feeding. However, it is remarkable that the majority of host proteins in FEF saliva have heme-binding and endopeptidase inhibitory functions similar to some of the tick proteins in PEF saliva (Figure 2). A quite interesting question is: if these proteins are returned intact, can they exert their biological function in the host? For instance, mammalian serpins were detected in FEF saliva (Table 4), so the question is: do these host serpins inhibit host serine endopeptidases of defense pathways as the tick prepares to detach? Whether these proteins are returned to the host as intact proteins or products of partial hydrolysis remains to be clarified. However, as in *R. sanguineus* saliva [19], it seems that host serum albumin is secreted intact into the host, since SDS-PAGE analysis reveals a ~60 kDa protein (Figure 1), which is intact [168]. Taken together with previous results that show the existence of a separate pathway for uptake and digestion of albumin in relation to hemoglobin incorporation into midgut cells [57], these results may be evidence of the existence of a system to recycle serum albumin. However, if serum albumin secreted into host is carrying some molecule along needs to be further clarified. In addition, it is important to note that several of these mammalian proteins, when undergoing limited proteolysis, generate peptides, some of which are bioactive, presenting antimicrobial action [169,170], as well as vasoactive peptides [171] which may enhance parasitism.

The presence of immunoglobulin chains in tick saliva could be explained as a part of the tick self-defense system, since immunoglobulin remains as an active protein in tick hemolymph [172]. In addition, the existence of immunoglobulin-binding proteins in both the tick salivary gland and hemolymph indicates that hemolymph and salivary gland cooperate to remove foreign proteins that could be deleterious for tick development during feeding [48]. An observation that support this hypothesis is that, in *R. microplus*, immunoglobulin-binding proteins from tick were found in the same developmental stage at which host immunoglobulin was found, in FEF saliva (Table 2 and 4). Differently from *R. microplus*, saliva immunoglobulin was not identified in *R. sanguineus* [19]. In spite of that, as these proteins were identified only in FEF in *R. microplus*, the presence in FEF saliva of *R. sanguineus* cannot be ruled out.

Despite reports of the presence of host proteins in tick saliva, this remains a neglected issue in the study of tick biology. It is interesting to note that while long-term blood feeders like *R. microplus* and *R. sanguineus* saliva contains considerable amounts of host proteins, the saliva of the short-term blood feeder, such as *O. moubata*, contains only a few host proteins [17,19]. The demonstration of these proteins in tick saliva raises several questions to be further explored, and may reveal novel insights into tick-host relationship.

Conclusion

The advancements in transcriptomic and proteomic analyses in recent years have opened unprecedented opportunities to identify putative targets for tick control into the variety of tick salivary transcripts and proteins. Saliva of ticks are far more complex than anticipated, having hundreds of different tick proteins as well as a high content of host proteins, which could have a role in several pathways associated with tick survival. A complete identification of tick salivary compounds and their identification and characterization remains a major research challenge that will help understand how host modulation by ticks occurs. The proteomic approach allows a comprehensive analysis of saliva composition and provides novel information to guide further studies about molecular, biochemical, immune biological, pharmacological as well as physiological characterization of these proteins. In *R. microplus* it is technically challenging to study defined feeding time points, and this is the reason why all previous studies have utilized saliva of fully engorged ticks. It is conceivable that after detaching from the host (or most probably just before detaching) ticks stop secreting proteins, indeed, salivary gland degeneration starts at this point. So, all studies conducted with saliva or salivary glands from FEF ticks must be carefully interpreted. This study, comparing saliva from PEF and FEF ticks, helps identify tick proteins that are important in the tick feeding process. These data could contribute to the understanding of tick salivary gland physiology and the tick-host relationship as well clues to approach new immunologically based tick control.

To date, only a few reports have explored *R. microplus* saliva. Compared to other hematophagous parasites, there is relatively little information on the molecular composition of *R. microplus* saliva. This is the first comprehensive proteomic study on *R. microplus* saliva. It is important to note that ticks produce minute amounts of saliva, which makes it difficult to work with as biological material, and as such it is less well characterized than

salivary glands. Although some proteins reported here have already been cloned from cDNA libraries of tick tissues, they were never purified from or identified in *R. microplus* saliva.

Despite the success of tick transcriptomic studies, which provide a global view of gene expression profiles in tick salivary glands, proteomic analysis of saliva provides unique information regarding proteins that are actually secreted. In conclusion, considering the great importance of this parasite, this study improves knowledge on the tick salivary arsenal composition and gives novel insights to clarify the mechanisms associated with the tick-host relationship.

Supporting Information

Table S1 Tick and host proteins identified in partially engorged female saliva by 1D-LC-MS/MS.
(DOCX)

Table S2 Tick and host proteins identified in fully engorged female saliva by 1D-LC-MS/MS.
(DOCX)

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Author Contributions

Conceived and designed the experiments: LT JR RMST JRM AM NES JWF JRY CT AFMP ISV. Performed the experiments: LT JR RMST NES AFMP. Analyzed the data: LT JR RMST JRM AM NES JWF JRY CT AFMP ISV. Contributed reagents/materials/analysis tools: LT JR RMST JRM AM NES JWF JRY CT AFMP ISV. Wrote the paper: LT JR RMST JRM AM NES JWF JRY CT AFMP ISV.

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Table S1. Tick and host proteins identified in PEF saliva by 1D-LC-MS/MS.

Sample	Protein[a]	Accession number[b]	MW (kDa)	Spectral count	Coverage (%)
PS1	hemelipoprotein HeLP	-	147	23	18
	vitellogenin 2	-	190	8	7
	vitellogenin 4	-	108	3	4
	hemelipoprotein HeLP 2	-	31	2	11
PS2	hemelipoprotein HeLP	-	147	14	11
PS3	hemelipoprotein HeLP	-	147	23	18
	hemelipoprotein HeLP 2	-	31	4	22
PS4	hemelipoprotein HeLP	-	147	13	13
	vitellogenin 2	-	190	5	4
	vitellogenin 5	-	68	3	5
	hemelipoprotein HeLP 2	-	31	2	11
PS5	**serum albumin**	IPI01028455.1	69	5	7
	hemelipoprotein HeLP	-	147	2	2
PS6	**serum albumin**	IPI01028455.1	69	12	19
	keratin 6A-like	IPI01002591.1	63	2	4
PS7	*R. microplus* serpin-17 (Rms-17)	KC990116	43	6	13
	secreted protein 34	-	37	2	10
PS8	*R. microplus* serpin-17 (Rms-17)	KC990116	43	9	21
	antigen 5/SCP domain-containing protein 1	-	46	3	9
PS9	*R. microplus* serpin-6 (Rms-6)	KC990105	44	6	21
	Actin	AAP79880	42	4	15
	antigen 5/SCP domain-containing protein 1	-	46	3	9
	hemelipoprotein HeLP	-	147	3	3
PS10	secreted protein 27	-	37	3	9
	keratin 6A-like	IPI01002591.1	63	2	4
PS11	secreted protein 19	-	36	6	22
	secreted protein 27	-	37	3	9
PS12	secreted protein 19	-	36	2	10
PS13	secreted protein 19	-	36	7	22
	lipocalin 41	-	16	2	22
	Kazal/SPARC domain-containing protein	-	33	2	10
PS14	**hemoglobin subunit beta**	IPI00716455.1	16	2	22
	salivary lipid-interacting protein 1	-	20	2	16
PS15	salivary lipid-interacting protein 1	-	20	4	24
	hemoglobin subunit beta	IPI00716455.1	16	3	30
	Se-dependent glutathione peroxidase	-	18	2	18
PS16	secreted protein 20	-	25	7	30
	salivary lipid-interacting protein 1	-	20	2	16
	odorant binding protein-like	IPI00722909.1	20	2	19
PS17	**allergen Bos d 2**	IPI00708946.1	20	2	14
PS18	**allergen Bos d 2**	IPI00708946.1	20	4	26
PS19	**allergen Bos d 2**	IPI00708946.1	20	3	21
	ML domain-containing protein 1	-	13	3	25

PS20	**hemoglobin subunit beta**	IPI00716455.1	16	4	30
PS21	**hemoglobin subunit beta**	IPI00716455.1	16	4	30
PS22	**hemoglobin subunit beta**	IPI00716455.1	16	9	54
PS23	**hemoglobin subunit beta**	IPI00716455.1	16	6	46
	hemoglobin subunit alpha	IPI00710783.2	15	3	25
PS24	**hemoglobin subunit beta**	IPI00716455.1	16	4	30

[a]Identified bovine proteins are presented in bold.
[b]Acession numbers for tick identified proteins were deposited as Transcriptome Shotgun Assembly project at DDBJ/EMBL/GenBank under the accessions GBBO00000000 and GBBR00000000. The versions described in this paper are the first version, GBBO01000000 and GBBR01000000, respectively

Table S2. Tick and host proteins identified in FEF saliva by 1D-LC-MS/MS.

Sample	Protein[a]	Accession number	MW (kDa)	Spectral count	Coverage (%)
TS1	hemelipoprotein HeLP	-	147	5	4
	vitellogenin 2	-	190	4	4
	vitellogenin 4	-	108	2	3
TS2	hemelipoprotein HeLP	-	147	19	16
TS3	hemelipoprotein HeLP	-	147	19	19
	hemelipoprotein HeLP 2	-	31	4	27
	keratin, type I cytoskeletal	IPI00698285.3	55	2	4
TS4	**Serotransferrin**	IPI00690534.1	78	11	19
	serum albumin	IPI00708398.2	70	10	17
TS5	**serum albumin**	IPI01028455.1	69	28	38
	serum albumin	IPI00708398.2	70	28	38
	complement C3 (Fragment)	IPI00713505.2	187	2	2
TS6	**serpin A3-2**	IPI00700622	46	4	12
	serpin A3-2	IPI00930024.1	46	4	12
TS7	secreted protein 34	-	37	2	10
TS8	*R. microplus* serpin-17 (RmS-17)	KC990116	43	4	10
	fibrinogen gamma chain	IPI00843209.1	50	3	9
	keratin type I cytoskeletal	IPI00721270.4	52	2	4
TS9	*R. microplus* serpin-3 (RmS-3)	KC990102	43	7	23
	fibrinogen beta chain	IPI00709763.5	56	6	16
	hemoglobin subunit beta	IPI00716455.1	16	3	22
TS10	*R. microplus* serpin-6 (RmS-6)	KC990105	44	5	14
	fibrinogen beta chain	IPI00709763.5	56	4	11
	hemoglobin subunit beta	IPI00716455.1	16	3	22
TS11	secreted protein 19	-	36	3	14
	secreted protein 27	-	37	2	9
TS12	**hemoglobin subunit beta**	IPI00716455.1	16	5	39
TS13	**hemoglobin subunit beta**	IPI00716455.1	16	29	56
	hemoglobin subunit alpha	IPI00710783.2	15	22	60

[a] Identified bovine proteins are presented in bold.
[b]Acession numbers for tick identified proteins were deposited as Transcriptome Shotgun Assembly project at DDBJ/EMBL/GenBank under the accessions GBBO00000000 and GBBR00000000. The versions described in this paper are the first version, GBBO01000000 and GBBR01000000, respectively.

3.2 Capítulo II

A proteomic study of *Rhipicephalus microplus* saliva through feeding time

Lucas Tirloni; Adriana Seixas; Antônio F. M. Pinto; Jolene K. Diedrich; John R.Yates III; Albert Mulenga; Carlos Termignoni and Itabajara da Silva Vaz Jr

Trabalho em fase de análise dos dados e redação do manuscrito.

Neste capítulo, descreve-se o estudo proteômico da saliva do carrapato *R. microplus* em diferentes fases de ingurgitamento. De acordo com o peso, os carrapatos foram agrupados em nove diferentes grupos, representando diferentes fases de ingurgitamento. Desta maneira, analisando a saliva dos parasitos desses diferentes grupos, obtemos uma cobertura mais ampla do processo hematofágico, com a amostragem de saliva nas diferentes fases de alimentação. A saliva coletada foi analisada por espectrometria de massas resultando na identificação de 284 proteínas do carrapato e 47 proteínas de origem do hospedeiro bovino. Esse trabalho está em fase de análise dos dados e preparação para a redação de um manuscrito. Parte das análises e resultados estão contidas em material digital.

Contribuição dos autores

L.T, A.S, A.F.M.P, J.K.D, J.R.Y, A.M, C.T, I.S.V: delineamento experimental; L.T, A.F.M.P, J.K.D: execução dos experimentos; L.T, A.S, A.F.M.P, J.K.D, J.R.Y, A.M, C.T, I.S.V: análise e interpretação dos dados.

Material and Methods

Ethics statement

Animals used in these experiments were housed at Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul (UFRGS). This study was conducted according to the ethic and methodological aspects preconized by the International and National Directives and Norms by the Animal Experimentation Ethics Committee of the UFRGS. The protocols were approved by the Comissão de Ética no Uso de Animais - CEUA – UFRGS.

Ticks and saliva collection

R. microplus ticks (Porto Alegre strain), free of pathogens such as *Babesia spp* and *Anaplasma spp* were reared on Hereford calves (*Bos taurus taurus*), which were brought from a naturally tick-free area (Santa Vitória do Palmar, RS, Brazil; 33°32'2'' S, 53°20'59'' W) and maintained in individual boxes. Calves were infested with approximately 20,000 10-day-old larvae (from 1 g of *R. microplus* eggs) and after 21 days, adult females attached on the host were manually collected. Adult females were collected, weighted and divided in nine groups: (group Rm-01; n=33 ticks) 6.8 ± 1.1 mg; (group Rm-02; n=22 ticks) 15.8 ± 1.3 mg; (group Rm-03; n=16 ticks) 25.1 ± 1.3 mg; (group Rm-04; n=40 ticks) 35.0 ± 2.5 mg; (group Rm-05; n=25) 52.5 ± 2.8 mg; (group Rm-06; n=21 ticks) 88.0 ± 12.6 mg; (group Rm-07; n=16 ticks) 172.5 ± 16.1 mg; (group Rm-08; n=21 ticks); 270.2 ± 14.9 mg; and a group representing fully engorged females (group FEF; n=12 ticks) 347.5 ± 9.0 mg.

Ticks were rinsed in Milli-Q water, dried on a paper towel and placed dorsal-side down on a glass slide containing tape. Salivation was induced by injecting 1 – 5 uL of 2% pilocarpine hydrochloride (in 1X PBS pH 7.4) on the ventral side of the lower right coxa

using Hamilton syringe (Hamilton Company, Reno, NV, USA). Subsequently after injections, saliva was periodically collected using a Hamilton syringe (every 15 min over approximately 4 h). Protein concentration was determined using the bicinchoninic acid method (BCA Protein Assay, Pierce, Rockford, USA) and samples stored at -70 °C upon use.

Protein digestion and sample preparation

Saliva of *R. microplus* ticks for each specific feeding group was digested in solution with trypsin. Saliva were diluted in 8 M urea/0.1 M Tris, pH 8.5, reduced with 5 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP, Sigma-Aldrich, St Louis, MO, USA) and alkylated with 25 mM iodoaceamide (Sigma-Aldrich). Proteins were digested overnight at 37°C in 2 M urea/0.1M Tris pH 8.5, 1 mM $CaCl_2$ with trypsin (Promega) with a final ratio of 1:20 (enzyme: substrate). Digestion reactions, in a final concentration of 0.15 μg/mL, were quenched with formic acid (5% final concentration) and centrifuged for debris removal.

Precolumns and analytical columns

Reversed phase pre-columns were prepared by first creating a Kasil frit at one end of a deactivated 250 μm ID/360 μm OD capillary (Agilent Technologies, Santa Clara, CA, USA). Kasil frits were prepared by dipping 20 cm capillary in 300 μL Kasil 1624 (PQ Corporation, Malvern, PA, USA) and 100 μL formamide solution, curing at 100°C for 3 h and adjusting the length. Pre-columns were packed in-house (John Yates III's lab, The Scripps Research Institute) with 2 cm of 5 μm ODS-AQ C18 (YMC America, INC., Allentown, PA, USA) particles from particle slurries in methanol. Analytical reversed phase

columns were fabricated by pulling a 100 µm ID/360 µm OD silica capillary (Molex Polymicro Technologies™, Austin, TX, USA) to a 5 µm ID tip. The same packing material was packed until 20 cm directly behind the pulled tip. Reversed phase precolumns and analytical columns were connected using a zero-dead volume union (IDEX Corp., Upchurch Scientific, Oak Harbor, WA, USA).

LC-MS/MS

Peptide mixtures were analyzed by nanoflow liquid chromatography mass spectrometry using an Easy NanoLC II and a Q Exactive mass spectrometer (Thermo Scientific, Waltham, MA, USA). Peptides eluted from the analytical column were electrosprayed directly into the mass spectrometer. Buffer A and B consisted of 5 % acetonitrile/0.1% formic acid and 80% acetonitrile/0.1 % formic acid, respectively. The flow rate was set to 400 nL/min. Saliva samples (1.5 µg per injection in three technical replicates) were separated in 155-min chromatographic runs, as follows: 1-10% gradient of buffer B in 10 minutes, 10-40% of buffer B in 100 min, 40-50% of buffer B in 10 min and 50-90% of buffer B in 10 min. Column was held at 90% of buffer B for 10 min, reduced to 1% of buffer B and re-equilibrated prior to next injection.

The mass spectrometer was operated in a data dependent mode, collecting a full MS scan from 400 to 1,200 m/z at 70,000 resolution and an AGC target of 1×10^6. The 10 most abundant ions per scan were selected for MS/MS at 17,500 resolution and AGC target of 2×10^5 and an underfill ratio of 0.1%. Maximum fill times were 20 and 120 ms for MS and MS/MS scans, respectively, with dynamic exclusion of 15 s. Normalized collision energy was set to 25.

Data Analysis

Tandem mass spectra were extracted from Thermo RAW files using RawExtract 1.9.9.2 (MCDONALD *et al*., 2004) and searched with ProLuCID (XU *et al*., 2006) against a non-redundant database containing an *R. microplus* database (22,009 entries) concatenated with an *Bos taurus* Uniprot reference database (23,868 entries) and reverse sequences of all entries. Searches were done using Integrated Proteomics Pipeline – IP2 (Integrated Proteomics Applications, Inc.). The search space included all fully-tryptic and half-tryptic peptide candidates. Carbamidomethylation of cysteine was used as static modification. Data was searched with 50 ppm precursor ion tolerance and 20 ppm fragment ion tolerance.

The validity of the peptide spectrum matches (PSMs) generated by ProLuCID was assessed using Search Engine Processor (SEPro) module from PatternLab for Proteomics platform (CARVALHO *et al*., 2012). Identifications were grouped by charge state and tryptic status, resulting in four distinct subgroups. For each group, ProLuCID XCorr, DeltaCN, DeltaMass, ZScore, number of peaks matched and secondary rank values were used to generate a Bayesian discriminating function. A cutoff score was established to accept a false discovery rate (FDR) of 1% based on the number of decoys. This procedure was independently performed on each data subset, resulting in a false-positive rate that was independent of tryptic status or charge state. Additionally, a minimum sequence length of six residues per peptide was required. Results were post processed to only accept PSMs with <10ppm precursor mass error.

Protein functional annotation and classification

BLASTP searches against several databases were performed. To check tick proteins identity, following databases were used: non-redundant (NR), Acari and refseq-invertebrate

from NCBI; Acari from Swissprot; the GeneOntology (GO) FASTA subset (LEWIS *et al*., 2000); MEROPS database; and the conserved domains database of NCBI (MARCHLER-BAUER *et al*., 2011) containing the KOG (TATUSOV *et al*., 2003), PFAM (BATEMAN *et al*., 2002), and SMART motifs (SCHULTZ *et al*., 2000). To check host proteins, were used: *Bos taurus* and refseq-vertebrates databases were from NCBI; the conserved domains database of NCBI containing the KOG, PFAM, and SMART motifs; and the GeneOntology (GO) FASTA subset. To functionally classify the protein sequences, a program written and provided by Dr. José M. C Ribeiro in Visual Basic 6.0 (Microsoft, Redmond, Washington, USA) was used (KARIM *et al*., 2011). The functionally annotated catalog for each dataset was manually curated and plotted in a hyperlinked Excel spreadsheet (Table S1 and Table S2).

Graphical visualization

Pie charts were constructed using normalized number of spectral counts for each time point. Normalization was done dividing total spectral counts for each class of protein by the total number of proteins for each time point (considering all replicates). To visualize differential secretion between protein classes and time points, or even between specific contigs into a specific class of tick protein, heat maps were created. For this purpose, z-scores were calculated for each class and in different feeding time points. A z-score indicates how many standard deviations an element is from the mean. A z-score can be calculated from the following formula: $z = (X - \mu) / \sigma$, where z is the z-score, X is the value of the element, μ is the population mean, and σ is the standard deviation. The z-scores were used as input into a matrix and heat maps were created using the function *heatmap.2* in the *gplots* package as part of the stats package in R (WARNES *et al*., 2009).

Results

An overview on R. microplus saliva proteome through time feeding

We successfully harvested pilocarpine-induced saliva of *R. microplus* ticks that were partially fed on bovines. Adult females were collected, weighed and divided in nine groups as showed in Figure 1 (from left to right, group Rm-08 to Rm-01; FEF group is absent). Ticks in these engorgement stages represent feeding phases which adult ticks pass through during blood feeding process.

Figure 1. Adult females were collected, weighted and divided in nine groups: (group Rm-01) 6.8 ± 1.1 mg; (group Rm-02) 15.8 ± 1.3 mg; (group Rm-03) 25.1 ± 1.3 mg; (group Rm-04) 35.0 ± 2.5 mg; (group Rm-05) 52.5 ± 2.8 mg; (group Rm-06) 88.0 ± 12.6 mg; (group Rm-07) 172.5 ± 16.1 mg; (group Rm-08) 270.2 ± 14.9 mg; and a group representing fully engorged females (group FEF) 347.5 ± 9.0 mg. From left to right, group Rm-08 to Rm-01; FEF group is absent.

Proteins in tick saliva were identified using LC-MS/MS. The search of extracted tandem mass spectra against the tick and host protein database using ProLucid in Integrated Protein Pipeline (Integrated Proteomics Applications, San Diego, CA) produced hits to 346 (Table 1) tick and 74 host proteins (Table 2). When subjected to filtering and validation analysis in PatternLab for Proteomics platform, 284 of the 346 tick proteins were determined to be authentic hits as they were detected in a minimum of two of the three runs (Table 1A), while the remaining 62 detected in one the three runs were considered low confidence and not further analyzed (Table 1B). From the 74 host proteins that were detected in *R.*

microplus saliva, 47 met the cut-off criteria (Table 2A), and the remaining 27 did not and were not further analyzed (Table 2B). The 284 tick and 47 host validated proteins were auto-annotated using Visual Basics-based programs developed by Dr. J. M. C. Ribeiro at NIH and classified into 23 tick (Table S1) and 15 host protein classes (Table S2).

Table 01. Identification and distribution of identified tick saliva proteins. This table is presented as an Excel file containing information about the LC-MS / MS data.

Table 02. Identification and distribution of identified host proteins in tick saliva. This table is presented as an Excel file containing information about the LC-MS / MS data

Table S1. Annotation of tick coding sequences identified in the saliva proteome. A hiperlinked Excel file containing information about the annotation of the sequences is provided.

Table S2. Annotation of host coding sequences identified in the saliva proteome. A hiperlinked Excel file containing information about the annotation of the sequences is provided.

Pie charts represent relative abundance of tick protein classes in accordance with normalized number of spectral counts for each tick feeding group (Figure 2). A heat map of normalized spectral count data are represented in Figure 3.

Rm-01

0.50%
0.03%
2.30%
0.58%
0.56%
17.87%
1.27%
8.78%
0.12%
0.31%
0.03%
0.02%
1.63%
0.35%
0.12%
0.17%
3.13%
59.57%
0.02%
1.40%
0.21%
1.02%

Rm-02

0.64%
0.34%
0.70%
24.79%
51.55%
11.34%
0.28%
0.30%
1.26%
0.20%
0.20%
5.09%
0.42%
2.89%

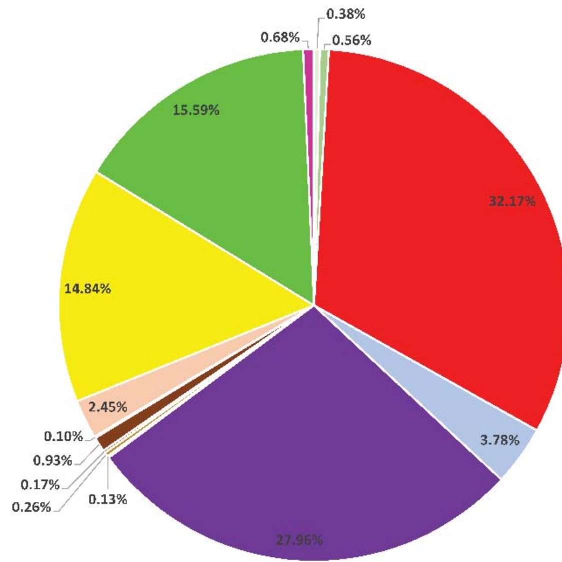
Rm-03

0.27%
0.33%
0.02%
0.43%
19.25%
13.02%
54.23%
0.66%
0.26%
0.88%
0.30%
6.67%
0.08%
0.48%
3.11%

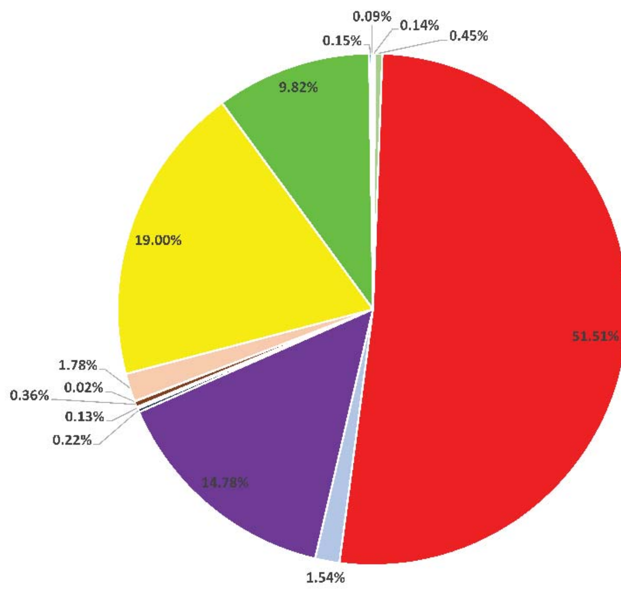
Rm-04

0.43%
0.13%
0.27%
16.53%
12.61%
53.63%
0.69%
0.17%
0.63%
0.07%
13.21%
1.62%

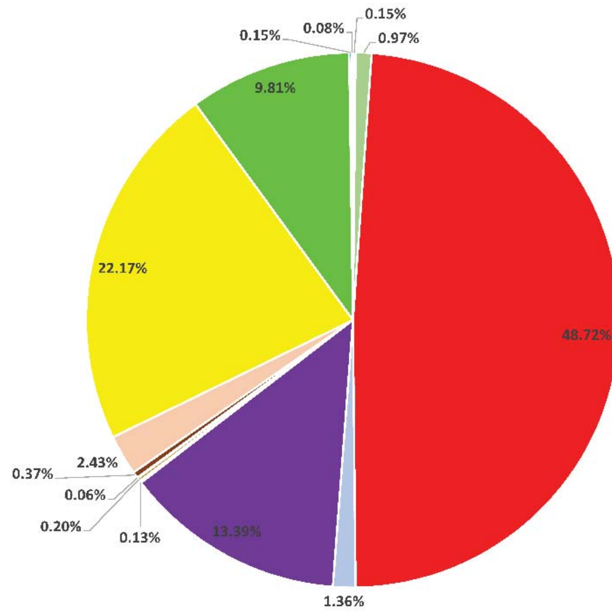
Rm-05



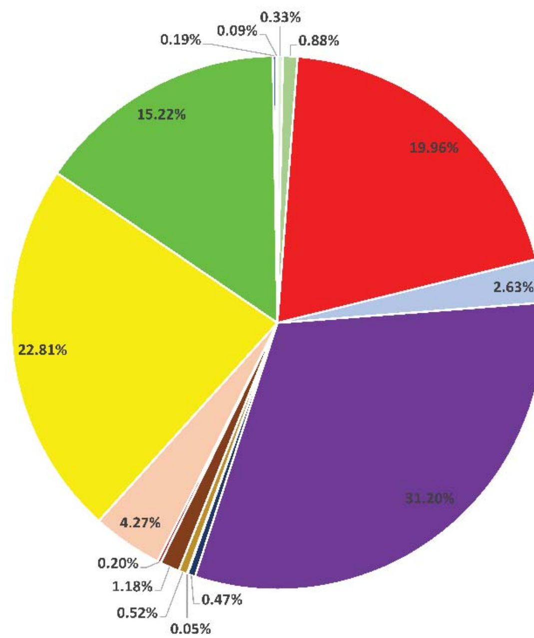
Rm-06



Rm-07



Rm-08



FEF

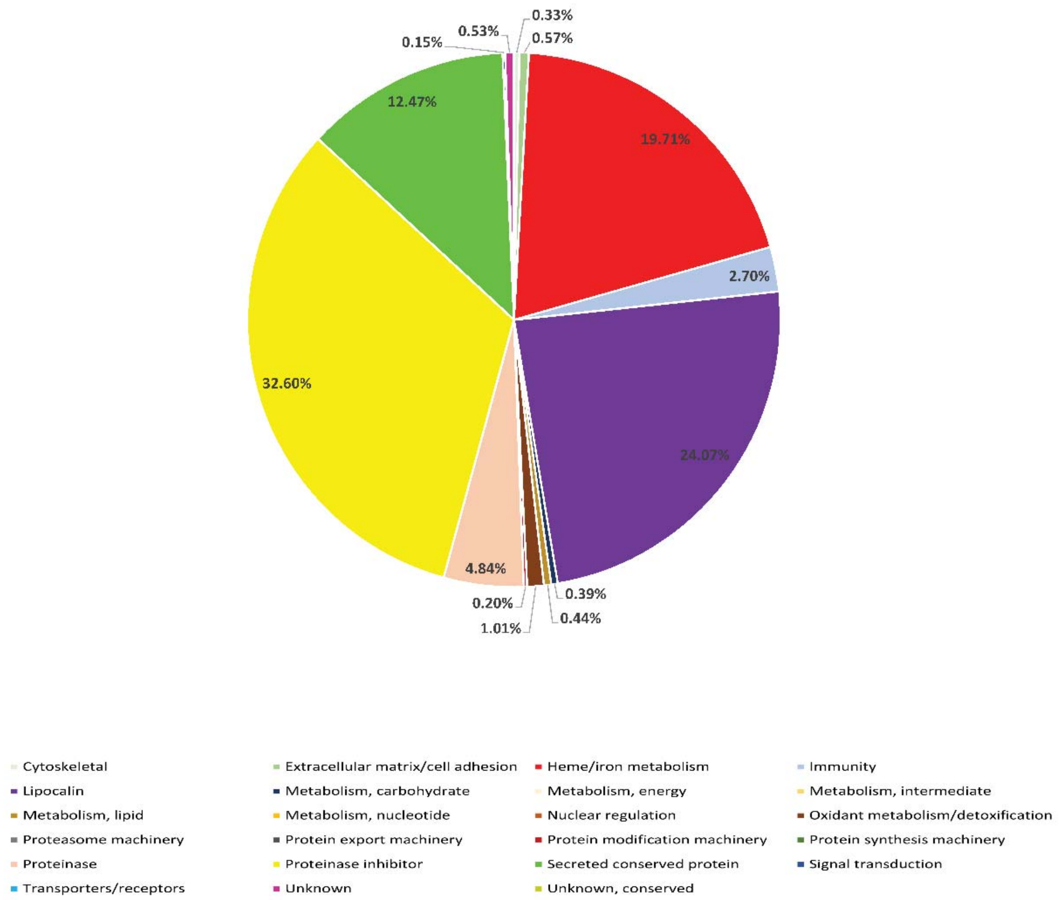


Figure 02. Relative abundance of tick saliva proteins classes. Total spectral counts for each protein class is expressed as a percent of total spectral counts per time point. A key is provided listing the classes of proteins identified in tick saliva.

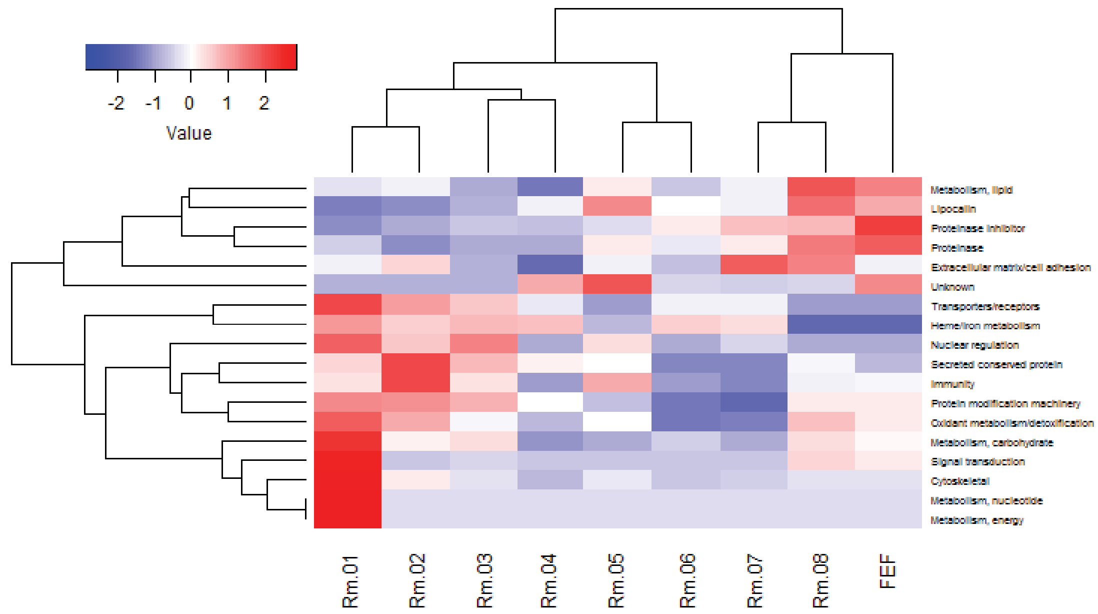


Figure 03. Heat map of normalized data from LC-MS/MS analysis of tick saliva proteins at different feeding stages. The Z score represents the deviation from the mean by standard deviation units. For other details, please see the Graphical Visualisation topic at Material and Methods.

3.3 Capítulo III

The putative role of Rhipicephalus microplus salivary serpins in tick-host relationship

Manuscrito a ser submetido para publicação no periódico **Insect Biochemistry and Molecular Biology**

Contribuição dos autores

L.T, T.K.K, M.L.C, A.A, A.S, C.T, A.M, I.S.V: delineamento experimental; L.T, T.K.K, M.L.C, A.A, A.S, I.S.V: execução dos experimentos; L.T, T.K.K, M.L.C, A.A, A.S, C.T, A.M, I.S.V: Análise e interpretação dos dados; L.T, T.K.K, M.L.C, A.A, A.S, C.T, A.M, I.S.V: redação e revisão do manuscrito.

The putative role of *Rhipicephalus microplus* salivary serpins in the tick-host relationship

Lucas Tirloni[a,b]; Tae Kwon Kim[b]; Mariana Loner Coutinho[a,c]; Abid Ali[d]; Adriana Seixas[a,e]; Carlos Termignoni[a,f]; Albert Mulenga[b] and Itabajara da Silva Vaz Jr[a,c#]

[a]Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

[b]Department of Veterinary Pathobiology, College of Veterinary Medicine, Texas A&M University, College Station, TX, USA.

[c]Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

[d]Institute of Biotechnology Genetic Engineering, The University of Agriculture, Peshawar, Khyber Pakhtunkhwa, Pakistan.

[e]Departamento de Farmacociências, Universidade Federal de Ciências da Saúde de Porto Alegre, Porto Alegre, RS, Brazil.

[f]Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

#Address correspondence to Itabajara da Silva Vaz Jr. itabajara.vaz@ufrgs.br

Itabajara da Silva Vaz Jr. and Albert Mulenga are co-senior authors.

Abstract

The first lines of host defense to tick feeding are serine protease-mediated pathways such as inflammation and haemostasis that are tightly controlled by serine protease inhibitors (serpins). From this perspective ticks are thought to use serpins to evade host defenses during feeding. The cattle tick *Rhipicephalus microplus* encodes at least 24 serpins. RmS-3, RmS-6, and RmS-17 were previously identified in saliva of partially and fully fed ticks. In this study, we screened inhibitor functions of these three salivary serpins against a panel of 16 proteases across the mammalian defense pathway and confirm that *Pichia pastoris*-expressed rRmS-3, rRmS-6, and rRmS-17 are likely inhibitors of pro-inflammatory and pro-coagulant proteases. We show that rRmS-3 inhibited chymotrypsin and cathepsin G with stoichiometry of inhibition (SI) indices of 1.8 and 2.0, and pancreatic elastase with SI higher than 10. Likewise, rRmS-6 inhibited trypsin with SI of 2.6, chymotrypsin, factor Xa, factor Xia, and plasmin with SI higher than 10, while rRmS-17 inhibited trypsin, cathepsin G, chymotrypsin, plasmin, and factor XIa with SI of 1.6, 2.6, 2.7, 3.4, and 9.0, respectively. Additionally, we observed the formation of irreversible complexes between rRmS-3 and chymotrypsin, rRmS-6/rRmS-17 and trypsin, and rRmS-3/rRmS-17 and cathepsin G, which is consistent with typical inhibitory mechanism of serpins. In blood clotting assays, rRmS-17 delayed plasma clotting by 60 s in recalcification time assay, while rRmS-3 and rRmS-6 did not have any effect. Consistent with inhibitor function profiling data, 2.0 μM rRmS-3 and rRmS-17 inhibited cathepsin G-activated platelet aggregation in a dose-responsive manner by up to 96% and 95% respectively. Of significant interest, polyclonal antibodies blocked inhibitory functions of the three serpins. Also notable, antibodies to *Amblyomma americanum, Ixodes scapularis,* and *R. sanguineus* tick saliva proteins cross-reacted with

the three serpins, suggesting the potential of these proteins as candidates for universal anti-tick vaccines.

Keywords: immune response, tick saliva, platelet aggregation inhibitor, cathepsin G

1. Introduction

The cattle tick *R. microplus* is one the most harmful hematophagous ectoparasites of bovines, with significant impacts on the cattle industry worldwide due to its spoliation action and its role as a vector of tick-borne pathogens such as *Babesia* spp and *Anaplasma marginale*, the agents of babesiosis and anaplasmosis, respectively (Jongejan and Uilenberg ,2004;Grisi et al., 2014). Current tick control strategies rely mostly on the use of chemical acaricides, even though selection of resistant tick populations to most used acaricides has been confirmed (Guerrero et al., 2002;Pohl et al., 2011;Guerrero et al., 2012). This is recognized as a worldwide drawback to successful tick control, not to mention environment and food chain contamination hazards. Immunization of cattle against *R. microplus* and other ticks has been recognized as an alternative against tick control strategy (Willadsen et al., 1989;de la Fuente et al., 2007). Thus, in the effort to find effective targets for tick vaccine development, our research group has endeavored to understand how ticks acquire blood meal.

Tick blood feeding occurs as two steps, namely the disruption of host tissue and the suction of blood that flows into the feeding lesion, triggering a host response that includes pain, itching, blood coagulation, inflammation, complement activation, tissue repair response, and adaptive immune response (Francischetti et al., 2009;Heinze et al., 2014). These host defense responses to tick feeding are predominantly mediated by serine proteases such as pro-coagulant (thrombin, factor Xa, factor XIa, and other blood coagulation factors),

pro-inflammatory (neutrophil elastase, proteinase-3, chymase, tryptase, kallikrein, cathepsin G, trypsin-like, and chymotrypsin-like), and complement serine proteases (factors B, factor C, factor D, and component 2) (Davie et al., 1979;Matsunaga et al., 1994;Korkmaz et al., 2008;Cattaruzza et al., 2014). Ticks successfully acquire blood meals by inoculation of saliva proteins in order to counteract host defenses to tick feeding (Ribeiro ,1987;Ribeiro and Francischetti ,2003;Francischetti, Sa-Nunes, Mans, Santos, and Ribeiro ,2009). Proteomic analysis of tick saliva revealed that it contains a great variety of proteins with anti-hemostatic, anti-inflammatory, and immunomodulatory roles, among which proteinase inhibitors that belong to different families such as serpin, Kunitz-type, Kazal-type, cystatin, alpha-2-macroglobulin, thyropin, and trypsin inhibitor-like (TIL) inhibitors (Diaz-Martin et al., 2013;Oliveira et al., 2013;Mudenda et al., 2014;Radulovic et al., 2014;Tirloni et al., 2014a;Carvalho-Costa et al., 2015;Lewis et al., 2015).

Members of the serpin (serine proteinase inhibitors) superfamily are irreversible inhibitors of serine protease mediators of host defense pathways to tick feeding (Gettins ,2002). In mammals serpins are known to regulate blood coagulation cascade, fibrinolysis, wound healing, angiogenesis, as well as inflammatory and immune responses (Silverman et al., 2001;Rau et al., 2007). This knowledge has led to the assumption that ticks inject serpins during feeding to disrupt the host homeostatic balance, as a way to prevent, slow down, and/or evade host defenses (Mulenga et al., 2001). Several tick serpin-encoding cDNAs have been cloned and characterized, including serpins from *Amblyomma americanum* (Mulenga et al., 2007;Mulenga et al., 2013;Porter et al., 2015;Kim et al., 2015), *A. maculatum* (Karim et al., 2011), *Ixodes scapularis* (Ribeiro et al., 2006;Mulenga et al., 2009;Ibelli et al., 2014), *I. ricinus* (Leboulle et al., 2002b;Prevot et al., 2006;Chmelar et al., 2011), *R. microplus* (Jittapalapong et al., 2010;Rodriguez-Valle et al., 2012;Tirloni et al.,

2014b;Rodriguez et al., 2015), *R. appendiculatus* (Mulenga et al., 2003), *R. haemaphysaloides* (Yu et al., 2013), and *Haemaphysalis longicornis* (Sugino et al., 2003;Imamura et al., 2005;Imamura et al., 2006). Additionally, proteomic studies have identified serpins in saliva of blood-fed ticks, such as *R. microplus* (Tirloni, Reck, Terra, Martins, Mulenga, Sherman, Fox, Yates, III, Termignoni, Pinto, and Vaz Ida S Jr ,2014a), *A. americanum* (Radulovic, Kim, Porter, Sze, Lewis, and Mulenga ,2014), *Dermacentor andersoni* (Mudenda, Pierle, Turse, Scoles, Purvine, Nicora, Clauss, Ueti, Brown, and Brayton ,2014), and *H. longicornis* (Tirloni et al., 2015), suggesting that the secretion of serpins is a common biologic strategy adopted by different tick species in order to counteract host's defenses during tick feeding.

Recent evidence shows that some of the tick-encoded serpins are functional inhibitors that are likely associated with tick evasion of host defense. In *A. americanum* two salivary serpins were characterized: serpin 6 (Mulenga, Khumthong, and Blandon ,2007;Chalaire et al., 2011), an inhibitor of papain and trypsin-like proteinases with anti-blood clotting and anti-complement activation functions (Mulenga, Kim, and Ibelli ,2013), and *A. americanum* serpin 19 (AAS19), a conserved serpin among ixodid ticks that acts as a broad spectrum inhibitor of trypsin-like proteases with anti-haemostatic functions (Kim, Tirloni, Radulovic, Lewis, Bakshi, Hill, Da Silva, Jr., Logullo, Termignoni, and Mulenga ,2015). In *I. scapularis*, a blood meal induced serpin inhibited thrombin and reduced platelet aggregation (Ibelli, Kim, Hill, Lewis, Bakshi, Miller, Porter, and Mulenga ,2014). In *I. ricinus* the serpin IRIS is an inhibitor of pro-inflammatory protease elastase and exhibits immunomodulatory properties (Prevot, Adam, Boudjeltia, Brossard, Lins, Cauchie, Brasseur, Vanhaeverbeek, Vanhamme, and Godfroid ,2006;Prevot et al., 2009;Chmelar, Oliveira, Rezacova, Francischetti, Kovarova, Pejler, Kopacek, Ribeiro, Mares, Kopecky,

and Kotsyfakis ,2011). Likewise, *I. ricinus* serpin IRS-2 inhibited pro-inflammatory proteases cathepsin G and chymase, in addition to Th17 differentiation inhibition (Chmelar, Oliveira, Rezacova, Francischetti, Kovarova, Pejler, Kopacek, Ribeiro, Mares, Kopecky, and Kotsyfakis ,2011;Palenikova et al., 2015). In *R. haemaphysaloides* two serpins with inhibitory activity against chymotrypsin were described (Yu, Cao, Zhou, Zhang, and Zhou ,2013).

The objective of this study was to characterize *R. microplus* serpins RmS-3, RmS-6 and RmS-17 that were identified in saliva of ticks during blood feeding (Tirloni, Reck, Terra, Martins, Mulenga, Sherman, Fox, Yates, III, Termignoni, Pinto, and Vaz Ida S Jr ,2014a). Here we report the characterization of these three serpins secreted by *R. microplus* saliva to gain insight into the role(s) these proteins play in the tick-host relationship. The data obtained confirms and builds on previous studies that characterized inhibitor function profiles of RmS-3 and RmS-6 (Rodriguez-Valle, Vance, Moolhuijzen, Tao, and Lew-Tabor ,2012).

2. Materials and Methods

2.1 Ethics statement

Animals used in these experiments were housed at Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil. This study was conducted according to the ethic and methodological aspects preconized by the International and National Directives and Norms by the Animal Experimentation Ethics Committee of the UFRGS. The protocols were approved by the Comissão de Ética no Uso de Animais - CEUA – UFRGS.

2.2 Ticks and animals

R. microplus ticks (Porto Alegre strain), free of pathogens such as *Babesia spp* and *Anaplasma spp* were reared on Hereford calves (*Bos taurus taurus*), which were brought from a naturally tick-free area (Santa Vitória do Palmar, RS, Brazil; 33°32'2'' S, 53°20'59'' W) and maintained in individual sheds. Calves were infested with approximately 20,000 larvae that were 10 days old (from 1 g of *R. microplus* eggs). Twenty-one days later, adult females still attached to the host were manually collected.

2.3 Tick dissections, RNA and protein extractions, and cDNA synthesis

Adult females were collected, weighed, and divided in eight groups according to weight: group 1 (9.9 ± 1.6 mg); group 2 (16 ± 1.2 mg); group 3 (24.2 ± 1.9 mg); group 4 (34.8 ± 2.8 mg); group 5 (53.2 ± 2.3 mg); group 6 (84.2 ± 7.9 mg); group 7 (188.8 ± 15.4 mg); and group 8 (270.2 ± 14.9 mg). Ticks were rinsed with 70% ethanol, followed by dorsal surface dissecting with a scalpel blade to separate salivary glands (SG). Dissected SG from each group were washed with phosphate-buffered solution (PBS) in an RNase-free environment and placed in a tube containing TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). Total RNA and protein were extracted according to the manufacturer's recommendations. The total RNA samples were resuspended in diethylpyrocarbonate (DEPC)-treated water and treated with DNase I (Invitrogen, Carlsbad, CA, USA). Total RNA concentration and purity were determined using a spectrophotometer. Protein samples were resuspended in 1% SDS solution and protein concentration was determined using the bicinchoninic acid method (BCA Protein Assay, Pierce, Rockford, USA) as previously described (Brown et al., 1989). Both total RNA and proteins were stored at –70 °C upon use.

2.4 Saliva collection

Tick saliva was collected as previously described (Clarke and Hewetson ,1971;Tirloni, Reck, Terra, Martins, Mulenga, Sherman, Fox, Yates, III, Termignoni, Pinto, and Vaz Ida S Jr ,2014a). Partially engorged female ticks (varying from 20 to 200 mg) were manually and carefully removed from calves maintained in individual pens. Prior to saliva collection, any host contaminating tissue in tick mouthparts was removed using a scalpel blade and surgical forceps. Ticks were rinsed with sterile distilled water and induced to salivate by dorsal injection of 2-5 μL 2% pilocarpine (in PBS). Ticks were maintained at room temperature in a humid chamber for approximately 3 h, during which time saliva accumulated in the mouthparts was periodically collected using a pipette tip. Saliva protein concentration was determined by BCA and stored at −70 °C upon use.

2.5 Recombinant protein expression and purification of rRmS-3, rRmS-6 and rRmS-17

Serpin-encoding mature protein open reading frames of RmS-3, RmS-6, and RmS-17 (Tirloni, Seixas, Mulenga, Vaz Ida S Jr, and Termignoni ,2014b) were cloned into pPICZαC using a set of primers as described in Table S1. The pPICZαC/RmS expression plasmids were linearized with *Sac*I and electroporated into *Pichia pastoris* X-33 strain according to the manufacturer's recommendations (Life Technologies, Carlsbad, CA, USA). Transformed colonies were selected on yeast extract peptone dextrose medium (YPD) agar plates containing zeocin (100 μg/mL - Life Technologies, Carlsbad, CA, USA) and incubated at 28 °C. After five days, positive grown colonies were inoculated in buffered glycerol-complex medium (BMGY) and grown overnight at 28 °C with shaking (240 rpm).

Subsequently, cells were used to inoculate buffered methanol-complex medium (BMMY) to OD_{600} of 1.0 and grown at 240 rpm at 28 °C during five days. Protein expression was induced daily during that period, by adding methanol to a 0.5 % final concentration. Recombinant proteins in spent culture media were precipitated by ammonium sulfate saturation (525 g/L of media) with stirring at 4 °C overnight. The precipitate was pelleted at 12,000 rpm for 1 h at 4 °C and resuspended in and dialyzed against buffer (20 mM Tris-HCl, 500 mM NaCl, pH 7.4). Expression of rRmS-3, rRmS-6, and rRmS-17 was confirmed resolving samples on a 12.5 % SDS-PAGE. Western blotting analysis was performed using an antibody to the C-terminus hexa histidine tag (Life Technologies, Carlsbad, CA, USA), and positive signal was detected using a metal enhanced DAB chromogenic substrate kit (Thermo Scientific, Waltham, MA, USA). Recombinant proteins were affinity-purified under native conditions using Hi-Trap Chelating HP Columns (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). To evaluate purity, affinity-purified proteins were resolved on a 12.5% SDS-PAGE and stained with Coomassie brilliant blue. Affinity-purified proteins were dialyzed against 20 mM Tris-HCl, NaCl 150 mM buffer pH 7.4, protein concentration was determined by BCA and stored at -80 °C until use.

2.6 Antibody production and characterization

Rabbit anti-sera and murine monoclonal antibodies (MAbs) were raised for each serpin. To raise polyclonal antibodies against the three saliva serpins, rabbits were inoculated subcutaneously with 150 µg of either rRmS-3, rRmS-6 or rRmS-17 emulsified in oil adjuvant (Montanide 888 - Seppic and Marcol 52 – Exxon Mobil Corporation). Following this first administration, two 150-µg boosters of each recombinant serpin in the same adjuvant were applied at 15-day intervals. Antibodies from sera were purified by

affinity chromatography on a protein G Sepharose resin according to the manufacturer's instructions (GE Healthcare, Pittsburgh, PA, USA).

To raise monoclonal antibodies against the three saliva serpins, six-week-old BALB/c mice were injected three times intraperitoneally with 100 µg of either rRmS-6 or rRmS-17 (Freund´s adjuvant, Sigma-Aldrich, St. Louis, MO, USA) at 15-day intervals for monoclonal antibody production (Harlow and Lane ,1988). Indirect ELISA using recombinant protein was used to obtain the sera antibodies titer. Splenic lymphocytes were fused to murine Sp2/O-Ag14 myeloma cells in the presence of PEG 3350 (Sigma-Aldrich, St. Louis, MO, USA) and cultivated in DMEM (Sigma-Aldrich, St. Louis, MO, USA) containing 20% fetal calf serum (Cultilab, Campinas, SP, Brazil) and HAT (Sigma-Aldrich, St. Louis, MO, USA). Hybridomas growing in HAT medium were screened for specific antibodies by indirect ELISA, and those producing positive results were cloned twice using the limiting dilution technique. MAbs were purified by affinity chromatography on a protein G Sepharose resin according to the manufacturer's instructions (GE Healthcare, Pittsburgh, PA, USA) and dialyzed against PBS. Concentrations of purified MAbs and polyclonal antibodies were determined at OD_{280nm}, and the preparations were stored at −20 °C until use.

Anti-sera and MAbs specificity to native RmS targets in tick saliva was tested using western blotting and a sandwich ELISA assay. Purified rabbit IgG against rRmS-6 or rRmS-17 was used to coat 96-well polyestirene plates (310 ng/well) for 1 h at 37 ºC. PBS-Tween (PBS-T) was used as a blocking solution for 1 h at 37 ºC. Saliva from partially engorged *R. microplus* was added to the plate (800 ng of total saliva protein per well diluted in PBS-T) and incubated for 1 h at 37 ºC, when MAbs were added to each well (100 ng/well diluted in PBS-T). After three washes in PBS-T, wells were incubated with a 1:2,000 dilution of

horseradish peroxidase (HRP)-conjugated anti-mouse IgG secondary antibody (Sigma, St. Louis, MO, USA) for 1 h at 37 ºC. After three washes in PBS-T, the reaction was visualized with 100 µL of enzyme substrate/chromogen solution (H_2O_2/ortophenylenediamine) in 100 mM citrate-phosphate buffer, pH 5.0, and the reaction was allowed to take place in the dark for 10 min. Optical density was read at 492 nm using the VersaMax tunable plate reader (Molecular Devices, Sunnyvale, CA, USA).

Purified rabbit IgG and purified MAbs were assayed in an indirect ELISA as above, with modifications to identify cross-reaction with different recombinant serpins. Briefly, 200 ng/well of either rRmS-3, rRmS-6 or rRmS-17 were used to coat 96-well polystyrene plates. After removal of unbound proteins and blocking unspecific binding sites, 1 µg/well of antibody was added to each well and incubated for 1 h at 37 ºC. After three washes in PBS-T, wells were incubated with a 1:2,000 dilution of either horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG secondary antibody (Sigma, St. Louis, MO, USA). The reaction was visualized as described above. Results were expressed as reaction percentage (the optical density from antibody raised to the antigen used as immunogen was taken as 100 %) from triplicates and were expressed as mean ± SD.

2.7 Expression analysis of salivary serpins by RT-PCR and western blotting

Expression profiles of the RmS-3, RmS-6 and RmS-17 genes in salivary glands from eight different adult female developmental stages were determined by RT-PCR and western blotting analysis. OligodT primed cDNA was synthesized from 5 µg of total RNA using the SuperScript III kit (Life Technologies, Carlsbad, CA, USA). Specific forward and reverse primers (Table S1) were used to determine transcription profile in pools of cDNA of SG from those eight groups of ticks (under item 2.3). For a constitutive gene control, forward

and reverse primers targeting the 40S ribosomal protein S3a were used (Table S1). RT-PCR was carried out with 400 ng of cDNA using Taq DNA polymerase (Ludwig Biotecnologia, Porto Alegre, RS, Brazil). The reactions were performed according to the following steps: 5 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 50 °C, and 1 min and 30 s at 72 °C, with a final elongation at 72 °C for 5 min. PCR products were electrophoresed on a 1.0 % agarose gel and visualized by staining with GelRed™ (Uniscience, São Paulo, SP, Brazil). To analyze RmS-3, RmS-6, and RmS-17 protein expression profile in tick SG, total protein extracts were resolved on a 12 % SDS-PAGE (100 µg total protein per lane) and western blotting performed using rabbit pre-immune (1:500) or immune anti-sera raised for each recombinant serpin (1:500) as primary antibody. Subsequently, membranes were incubated with phosphatase-conjugated anti-rabbit IgG (Sigma–Aldrich) as secondary antibody for 1 h in a 1:5000 blocking buffer dilution, and BCIP (5-bromo-4-chloro-3-indolyl-phosphate) and NBT (nitro blue tetrazolium) were used for the colorimetric detection.

2.8 Deglycosylation assay

Amino acid sequence analyses predicted the existence of N-linked glycosylation sites in RmS-3, RmS-6, and RmS-17 (Tirloni, Seixas, Mulenga, Vaz Ida S Jr, and Termignoni ,2014b). To determine if yeast-expressed serpins and native salivary serpins were glycosylated, affinity-purified protein and total saliva proteins were treated with a protein deglycosylation enzyme mix according to the manufacturer's instructions (New England Biolabs, Ipswich, MA, USA). Deglycosylation was confirmed by 12.5 % SDS-PAGE stained with Coomassie brilliant blue and western blotting using antibody to the C-terminus hexa histidine tag or rabbit anti-sera (as described in item 2.5).

2.9 Inter-species cross-reactivity assay

To analyze if recombinant *R. microplus* serpins react with serum from animals infested with *R. microplus* and if they cross-react with serum from animals infested with other ticks species, rRmS-3, rRmS-6, and rRmS-17 (2 μg both glycosylated as well as deglycosylated proteins) were subjected to western blotting analysis using: (i) cattle antibodies generated to replete-fed (allowed to feed to completion) *R. microplus* (Reck, Jr. et al., 2009), and (ii) rabbit antibodies generated to replete-fed adult *I. scapularis*, *A. americanum*, and *R. sanguineus* tick saliva proteins. Antibodies to tick-saliva proteins from replete-fed *I. scapularis* and *A. americanum* were produced as previously published (Chalaire, Kim, Garcia-Rodriguez, and Mulenga ,2011;Ibelli, Kim, Hill, Lewis, Bakshi, Miller, Porter, and Mulenga ,2014). To obtain antibodies to tick saliva proteins from replete-fed *R. sanguineus*, New Zealand White rabbits were repeatedly infested (three times) with 40 adult ticks (20 males and 20 females). Two weeks after the last infestation, immune serum was collected and stored at -70 °C.

2.10 Protease inhibition assay

To get insight into inhibitory activity of rRmS-3, rRmS-6, and rRmS-17, their protease inhibitory activities were evaluated against a panel of 16 mammalian serine proteases, most of which are related to host defense pathways against tick feeding (the amount of protease used is give in parenthesis): bovine thrombin (43 U), pancreatic porcine elastase (21.6 nM), pancreatic bovine trypsin (24.6 nM), pancreatic bovine α-chymotrypsin (96 nM), pancreatic porcine kallikrein (33 U), human chymase (10 U), human tryptase (10 U), human plasmin (10 nM) (Sigma-Aldrich, St. Louis, MO, USA, human neutrophil

cathepsin G (166 nM) (Enzo Life Sciences Inc., Farmingdale, NY, USA), human factor XIa (3.68 nM), bovine factor IXa (306 nM), human factor XIIa (7.6 nM), human t-PA (32 nM), human u-PA (47.2 nM) (Molecular Innovations, Inc., Novi, MI, USA), bovine factor Xa (5.8 nM) (New England Biolabs, Ipswich, MA, USA), and human proteinase-3 (68 U) (EMD Millipore, Billerica, MA, USA). Substrates were used at 0.20 mM final concentration and purchased from Sigma-Aldrich: N α-benzoyl-DL-Arg-pNA for tryptase; N-succinyl-Ala-Ala-Pro-Phe-pNA for chymase, cathepsin G, and chymotrypsin; N-benzoyl-Phe-Val-Arg-pNA for thrombin and trypsin; and N-succinyl-Ala-Ala-Ala-p-nitroanilide for pancreatic elastase. The following substrates were purchased from Chromogenix, a daughter-company of Diapharma Inc. (Philadelphia, PA, USA): Bz-Ile-Glu(γ-OR)-Gly-Arg-pNA for factor Xa; H-D-Val-Leu-Lys-pNA for plasmin; and H-D-Pro-Phe-Arg-pNA for kallikrein, factor XIa, and factor XIIa. The substrate CH_3SO_2-D-CHG-Gly-Arg-pNA was purchased from Enzyme Research and used for factor IXa, u-PA and t-PA. The substrate N-methoxysuccinyl-Ala-Ala-Pro-Val-pNA was purchased from Enzo Life Sciences and used for proteinase-3. Reagents were mixed at room temperature in triplicate. Recombinant proteins (1 μM) were pre-incubated with indicated amounts of the protease for 15 min at 37 °C in 20 mM Tris-HCl, 150 mM NaCl, BSA 0.1%, pH 7.4. The corresponding substrate for each protease was added to a 100-μL final reaction volume. Substrate hydrolysis was measured at OD_{405nm} every 11 s for 30 min at 30 ºC using the Infinite M200 Pro plate reader (Tecan, Männedorf, Switzerland). Acquired OD_{405nm} data were subjected to one phase decay analysis in Prism 6 software (GraphPad Software, La Jolla, CA, USA) to determine plateau values as proxy for initial velocity of substrate hydrolysis. The percent protease activity inhibition level was determined using the formula: 100 - (V_i/V_0) X 100 where, V_i = activity in presence of, and V_0 = activity in absence of

recombinant serpins. Data are presented as mean percent of inhibition from triplicate readings and at least duplicate assays. The unit (U) definition used here is the amount of proteinase necessary for the increase of 0.001 OD_{405nm}/min.

2.11 Stoichiometry of inhibition assay

Stoichiometry of inhibition (SI) indices were determined for each serpin displaying inhibitory activity. The kinetic of substrate hydrolysis in the absence and in the presence of recombinant serpins was evaluated. Preparations were pre-incubated for 1 h with constant concentration of: (i) chymotrypsin (10 nM), cathepsin G (200 nM), and pancreatic elastase (50 nM) for rRmS-3 (molar ratio ranging from 0 to 10); (ii) trypsin (10 nM), chymotrypsin (10 nM), factor Xa (5 nM), factor XIa (5 nM), and plasmin (50 nM) for rRmS-6 (molar ratio ranging from 0 to 10 for trypsin, chymotrypsin and plasmin, and from 0 to 20 for factor Xa and factor XIa); and (iii) trypsin (10 nM), chymotrypsin (10 nM), cathepsin G (200 nM), factor XIa (5 nM), and plasmin (50 nM) for rRmS-17 (molar ratio ranging from 0 to 10 for trypsin, chymotrypsin, cathepsin G and plasmin, and from 0 to 20 for factor XIa). Protease activity was measured using colorimetric substrates specific for each proteinase as described above. Data were plotted as the protease residual activity (V_i/V_0) versus serpin:protease molar ratio. SI or the molar ratio serpin to protease (when protease activity is completely inhibited) was determined by fitting data onto the linear regression line and the SI index was estimated from the X-axis intercept (Kantyka and Potempa ,2011).

2.12 Serpin-protease complex formation

Formation of covalent complexes serpin-protease (Huntington et al., 2000) was evaluated as follows. Affinity-purified (i) rRmS-3 was incubated with chymotrypsin (0.5

μg) and cathepsin G (0.5 μg); (ii) rRmS-6 was incubated with trypsin (1.16 μg); and (iii) rRmS-17 was incubated with trypsin (1.16 μg) and cathepsin G (0.5 μg), all in varying molar ratios (from 0 to 10). These serpin-protease incubations were prepared in buffer (Tris-HCl 20 mM, NaCl 150 mM, pH 7.4) and incubated for 1 h at 37 °C. SDS-PAGE denaturing sample buffer was added to the reaction mix, and incubated for 5 min at 99 °C. Samples were subjected to 12.5 % SDS-PAGE and stained with Coomassie brilliant blue.

2.13 Recalcification time

The recalcification time (RT) assay was performed with 50 μL of universal coagulation reference human plasma Thermo Scientific, Waltham, MA, USA) pre-incubated in presence (10 μM) or absence of recombinant serpins in 90 μL of reaction buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.4) for 15 min at 37 °C. Adding 10 μL of pre-warmed 150 mM calcium chloride ($CaCl_2$) triggered plasma clotting. Plasma clotting was monitored every 20 s for 30 min at OD_{605nm} using the Infinite M200 Pro plate reader (Tecan, Männedorf, Switzerland) (Ciprandi et al., 2006).

2.14 Anti-platelet aggregation activity

Anti-platelet aggregation activity of rRmS-3 and rRmS-17 upon cathepsin G-induced platelet aggregation was determined using platelet-rich plasma (PRP) prepared from citrated (acid citrate dextrose) whole bovine blood as previously described (Berger et al., 2010;Kim, Tirloni, Radulovic, Lewis, Bakshi, Hill, Da Silva, Jr., Logullo, Termignoni, and Mulenga ,2015). To prepare PRP, fresh citrated whole bovine blood was centrifuged at 200 g for 20 min at 18 °C. Subsequently, the PRP (top layer) was transferred into a new tube and centrifuged at 800 g for 20 min at 18 °C. The pellet containing platelets was washed

and diluted with Tyrode solution pH 7.4 (137 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.42 mM Na_2HPO_4, 1 mM $MgCl_2$, 0.1 % glucose, 0.25 % BSA) until OD_{650} = 0.15. To determine anti-platelet aggregation activity, different amounts of rRmS-3 or rRmS-17 (varying from 3 µM to 0 µM) were pre-incubated for 15 min at 37 °C with cathepsin G (0.7 µM) in a 50-µL reaction. Adding 100 µL of pre-warmed PRP triggered platelet aggregation. Platelet aggregation was monitored every 20 s over 30 min at OD_{650nm} using the Infinite M200 Pro plate reader (Tecan, Männedorf, Switzerland). In this assay, higher OD_{650nm} was observed in our blank (platelet only), and increased platelet aggregation was correlated with reduction in OD_{650nm}. Percentage of platelet aggregation inhibition was quantified by calculating the area under the curve and expressed as percent of the negative (only PRP and buffer) and positive control (absence of serpin). Data is presented as mean ± SEM of triplicate platelet aggregation assays.

2.15 Anti-rRmS antibodies effect upon serpin inhibitory activity

Purified rabbit IgG from anti-sera or MAbs raised against serpins were used to check if antibody-binding to serpins could block their protease inhibitory activity. rRmS-3 (55 nM), rRmS-6 (55 nM), and rRmS-17 (88 nM) were pre-incubated with purified rabbit IgG from anti-sera (varying from 0.8 to 3 µM) or purified mouse IgG from MAbs (varying from 0.3 to 1 µM) for 30 min at 37 °C before the addition of chymotrypsin (4.9 nM) for rRmS-3, or trypsin (1.6 nM) for rRmS-6 and rRmS-17, following a new 15- min incubation at 37 °C. Substrate (N-(p-Tosyl)-Gly-Pro-Lys-pNA for trypsin, and N-succinyl-Ala-Ala-Pro-Phe-pNA for chymotrypsin) was added to a 100-µL final reaction volume (final concentration 0.2 mM) and substrate hydrolysis was measured at OD_{405nm} every 11 s for 15 min at 30 ºC

using the VersaMax tunable plate reader (Molecular Devices, Sunnyvale, CA, USA). Data are shown as mean of two assays done in duplicates.

3. Results

3.1 Salivary serpins are glycoproteins

Data about expression and affinity purification of rRmS-3, rRmS-6, and rRmS-17 in *P. pastoris* are summarized in Fig. 1A and 1B. Daily samples of yeast-expressed recombinant proteins were subjected to western blotting analysis using a specific antibody directed to the C-terminus histidine tag (Fig. 1A). Recombinant proteins were purified by affinity chromatography under native conditions and migrated according to molecular weight: ≈40 kDa for rRmS-3 and rRmS-6, and ≈50 kDa for rRmS-17 (Fig. 1B). When treated with deglycosylation enzymes, downward molecular weight shifts were observed (Fig. 1C), demonstrating that rRmS-3, rRmS-6, and rRmS-17 are glycosylated and consistent with sequence-based predictions (Tirloni, Seixas, Mulenga, Vaz Ida S Jr, and Termignoni ,2014b).

3.2 Serum from animals repeatedly infested with different tick species recognize *R. microplus* saliva serpins

Immune serum from bovine repeatedly infested with *R. microplus* recognized all three recombinant salivary serpins (Fig. 2). Both glycosylated and deglycosylated forms of rRmS-3 bound *R. microplus* antibodies, while only glycosylated rRmS-6 and rRmS-17 were recognized by bovine anti-serum to *R. microplus* saliva proteins (Fig. 2). In addition to *R. microplus*-infested bovine serum, serum from *R. sanguineus* and *A. americanum* repeatedly

infested rabbits bound both glycosylated and deglycosylated forms of the three serpins. Immune serum to *I. scapularis* weakly bound to rRmS-6 (Fig. 2).

3.3 Feeding affects salivary serpins expression profile

The temporal transcription analyses show that mRNA of serpins in tick salivary gland was present throughout all SG blood feeding stages investigated (Fig. 3A and Fig. 3B). In order to correlate mRNA expression profiles with protein production, total tick salivary proteins were subjected to western blotting analysis (Fig. 3C). Polyclonal sera recognized all three serpins in the eight different groups of tick SG (Fig. 3C). Serpins appear as a band between 40 and 55 kDa (arrows in Fig. 3C). This molecular size is in accordance as previously published for identification of RmS-3, RmS-6, and RmS-17 in *R. microplus* saliva by GeLC-MS/MS (Tirloni, Reck, Terra, Martins, Mulenga, Sherman, Fox, Yates, III, Termignoni, Pinto, and Vaz Ida S Jr ,2014a). RmS-3 and RmS-17 were expressed mainly during the slow feeding phase (represented by groups 1-6) (Fig. 3C), while RmS-6 was expressed throughout all groups, including the rapid engorgement phase (groups 7 and 8) (Fig. 3C).

3.4 *R. microplus* salivary serpins inhibit trypsin- and chymotrypsin-like proteases

The inhibitory profile of rRmS-3, rRmS-6, and rRmS-17 serpins against a panel of 16 mammalian proteases associated with host defense pathways showed a trypsin- and chymotrypsin-like inhibitory pattern (Table 1). Incubation of serpins with the protease in a molar excess showed that rRmS-3 (1 µM) inhibited the activity of chymotrypsin (96 nM) by 96 %, the activity of cathepsin G (166 nM) by 78 %, the activity of pancreatic elastase (21.6 nM) by 92 %, and the activity of chymase (10 U) by 23 %. Comparatively, rRmS-6

(1 μM) inhibited the activity of trypsin (24.6 nM) by 73 %, the activity of plasmin (10 nM) by 24 %, the activity of factor Xa (5.8 nM) 32 %, the activity of factor XIa (3.68 nM) by 62 %, and the activity of chymotrypsin (96 nM) by 24 %. Also, rRmS-17 (1 μM) inhibited the activity of trypsin (24.6 nM), plasmin (10 nM), cathepsin G (166 nM), chymotrypsin (96 nM), and factor XIa (3.68 nM) by 87 %, 58 %, 78 %, 89 %, and 98 %, in that order (Table 1).

The inhibitory efficiency was evaluated using the SI (stoichiometry of inhibition) index where the kinetics of inhibition by tick serpins upon their sensible protease was tested at different molar ratios. Accordingly, the SI index for rRmS-3 was 2.0 for cathepsin G (Fig. 4A), 1.8 for chymotrypsin (Fig. 4B), and higher than 10 for pancreatic elastase (Fig. 4C). For rRmS-6, SI index was 2.6 for trypsin (Fig. 5A) and higher than 10 for chymotrypsin (Fig. 5B), factor Xa (Fig. 5C), factor XIa (Fig. 5D), and plasmin (Fig. 5E). For rRmS-17, SI index was 1.6 for trypsin (Fig. 6A), 2.7 for chymotrypsin (Fig. 6B), 2.6 for cathepsin G (Fig. 6C), 3.4 for plasmin (Fig. 6D), and higher than 10 for factor XIa (Fig. 6E).

The mechanisms of action of rRmS-3, rRmS-6, and rRmS-17 confirmed that they behave like typical inhibitory serpins (Fig. 7). rRmS-3 is able to form covalent complex with chymotrypsin and cathepsin G (Fig. 7A), rRmS-6 is able to form a stable complex with trypsin (Fig. 7C), and rRmS-17 is able to form stable complexes with trypsin and cathepsin G (Fig. 7B). After incubation of a protease with a serpin, the resulting complex has molecular mass increased to a value corresponding to the sum of the masses of the target protease and the cleaved serpin (complexes indicated by arrows in Fig. 7). These irreversible complexes between serpins and the target proteases were observed at similar molar ratios shown in SI assays (Figs. 4 - 6).

3.5 rRmS-3 and rRmS-17 inhibit cathepsin G-induced platelet aggregation

Cathepsin G released from activated neutrophils induces platelet aggregation via PAR4 activation (Selak et al., 1988;Sambrano et al., 2000). Since rRmS-3 and rRmS-17 were able to inhibit cathepsin G (Table 1, Fig. 4, and Fig. 6), we tested the ability of these serpins to inhibit cathepsin G-induced platelet aggregation. rRmS-3 inhibited cathepsin G-activated platelet aggregation in a dose-responsive manner by 10% at 0.5 μM to 96% at 2.5 μM (Fig. 8A and Fig. 8C). Similar result was observed for rRmS-17, which inhibited cathepsin G-activated platelet aggregation by 18% at 0.5 μM to 94% at 2.5 μM (Fig. 8B and Fig. 8C). *R. microplus* recombinant serpins did not have any effect on thrombin and ADP-induced platelet aggregation (data not shown). When subjected to blood clotting assays, rRmS-17 delayed recalcification time (RT) by 60 s (Fig. 9). However, none of these three serpins delayed plasma clotting time, aPTT, PT, and TT (data not shown).

3.6 Antibodies to RmS-3, -6 and -17 recognize native and recombinant serpins

Five monoclonal antibodies (MAb) were generated from rRmS-6-immunized mice, and one MAb was obtained from an rRmS-17-immunized mouse. MAbs and purified rabbit IgG were tested in an indirect ELISA to detect cross-reactivity among the recombinant serpins (Table 2). MAbs BrBm41 and BrBm42 reacted only with rRmS-6, while MAbs BrBm39 and BrBm43 reacted with all three recombinant proteins. Although clone BrBm40 recognizes all three serpins, it reacts poorly with rRmS-17. Though MAb BrBm44 recognizes all three recombinant serpins, reactions were weak (Table 2). The three polyclonal sera reacted with all three recombinant proteins, showing cross-reactivity ranging from 30% to 55% with two other serpins (Table 2). Two of the MAbs (BrBm39 and BrBm40) were able to recognize native serpins in the saliva, as shown by a sandwich ELISA

(Table 2), although no reaction was observed when MAbs were tested with denatured saliva proteins in a western blotting assay (data not shown). Additionally, all three polyclonal sera were able to recognize serpins in the saliva, whether they were glycosylated or deglycosylated (Fig. 10). The variation in molecular sizes of glycosylated and deglycosylated saliva proteins confirms that native RmS-3, RmS-6, and RmS-17 are secreted as glycosylated proteins (Fig. 10).

3.7 Antibodies abolish serpin inhibitory activity function

Purified IgG from polyclonal serum or MAbs were used for the serpin blocking activity assay. Each serpin was pre-incubated with IgG for 30 min followed by a second incubation with the test protease, when kinetics was monitored for 15 min. Blockage was observed exclusively when purified IgG from polyclonal serum was tested (Fig. 11 and Table 3), since IgG from MAbs was not able to block serpin inhibitory function (Fig. 11). This result shows that polyclonal antibodies were able to block the inhibitory activity of rRmS-3 by approximately 66%, of rRmS-6 by 50% and of rRmS-17 by 85% (Table 3).

4. Discussion

In this work we demonstrate the inhibitory properties of three salivary serpins from the cattle tick *R. microplus*. RmS-3, RmS-6, and RmS-17 are inhibitors of serine proteinases of the serpin superfamily (Tirloni, Reck, Terra, Martins, Mulenga, Sherman, Fox, Yates, III, Termignoni, Pinto, and Vaz Ida S Jr ,2014a). In addition to the confirming that these serpins are clearly present in cattle tick saliva, as found using proteomic approaches in a previous study (Tirloni, Reck, Terra, Martins, Mulenga, Sherman, Fox, Yates, III, Termignoni, Pinto, and Vaz Ida S Jr ,2014a), here it is shown that antibodies raised against these serpins

produced in *P. pastoris* are able to recognize native serpins in tick saliva, proving that these proteins are injected into animals during tick feeding. Consistent with earlier literature findings, according to which some serpins are indeed glycoproteins (Gettins ,2002), the present study shows that RmS-3, RmS-6 and, RmS-17 are secreted as glycoproteins (Fig. 10).

The protease inhibitory profile of *R. microplus* salivary serpins shows that rRmS-3 has anti-chymotrypsin activity, rRmS-6 has anti-trypsin activity, and rRmS-17 has both anti-trypsin and anti-chymotrypsin activity. Taking all data together, it becomes clear that rRmS-3 and rRmS-6 have a more strict inhibitory profile, while RmS-17 has a broad-spectrum of inhibition. rRmS-3 action upon chymotrypsin and pancreatic elastase is in accordance with previous work (Rodriguez, Xu, Kurscheid, and Lew-Tabor ,2015). However, in that study, the authors showed that rRmS-6 has only anti-chymotrypsin activity (Rodriguez, Xu, Kurscheid, and Lew-Tabor ,2015).

Aiming to compare the inhibitory efficiency of these serpins, stoichiometry of inhibition (SI) indices were determined. This index reflects the number of serpin molecules required to form a stable inhibitory complex with a given protease. Accordingly, rRmS-3 is an efficient inhibitor of chymotrypsin and cathepsin G. Although it also inhibits pancreatic elastase at a high excess molar ratio, SI data suggests rRmS-3 is not an efficient pancreatic elastase inhibitor. As pancreatic elastase (used in this study) and neutrophil elastase have different biochemical properties (Sinha et al., 1987;Bode et al., 1989), the inhibition of neutrophil elastase cannot be ruled out, and needs further characterization. Differences in elastase inhibition by some parasitic inhibitors was shown to be dependent on the source of elastase (Jin et al., 2011). rRmS-6 efficiently inhibits trypsin, while rRmS-17 has a broad spectrum of efficient inhibition, as demonstrated using trypsin, chymotrypsin, cathepsin G.

However, it is less efficient upon plasmin and factor XIa. The high SI indices observed for some serpin-protease pairs point to the possibility that some serpins need a co-factor to enhance inhibition, as demonstrated for some mammalian serpins (Gettins ,2002;McCoy et al., 2003).

Tertiary serpin structure contains typically three β-sheets, eight or nine α-helices, and a reactive center loop (RCL). RCL is a solvent exposed flexible stretch of 21 amino acid residues positioned between β-sheets sA and sC and that acts as a suicide-substrate of the specific target protease (Gettins ,2002). RCL contains the peptide bond (between residues P_1 and $P_{1'}$) that is cleaved by the target protease, while the P_1 residue in RCL is critical to define the specificity of a serpin for a particular protease (Hopkins et al., 1993;Hopkins and Stone ,1995). The predicted P_1-$P_{1'}$ residues of *R. microplus* salivary serpins are Leu-Ser for RmS-3, Arg-Ile for RmS-6, and Lys-Ser for RmS-17 (Tirloni, Seixas, Mulenga, Vaz Ida S Jr, and Termignoni ,2014b), which were predicted to inhibit chymotrypsin-like (RmS-3) and trypsin-like proteinases (RmS-6 and RmS-17) (Keil ,1992). This prediction is in accordance with the inhibitory profile described here. However, the finding that rRmS-17 was able to inhibit α-chymotrypsin is intriguing, since a serpin possessing a Lys at P_1 has not been reported to inhibit chymotrypsin-like proteases, which have preference for large hydrophobic amino acids such as Phe, Tyr, or, to a lesser extent, Leu, Met, and His at the P_1 position (Keil ,1992). It is possible that RmS-17 has two close reactive sites in RCL, allowing the interaction with both trypsin-like and chymotrypsin-like proteases, as yet demonstrated for another serpin (Liu et al., 2014). The P_4-$P_{3'}$ region of RmS-17 is comprised of Phe-Tyr-Thr-Lys-Ser-Ala-Val (Tirloni, Seixas, Mulenga, Vaz Ida S Jr, and Termignoni ,2014b), thus the Phe or Tyr at P_4 and P_3 position could be the real reactive site in RmS-17 for chymotrypsin inhibition.

rRmS-3 and rRmS-17 were able to inhibit cathepsin G. This protease has both trypsin- and chymotrypsin-like specificity, being able to accommodate both large, hydrophobic P_1 residues (Phe, Leu, Met) as well as positive ones (Lys or Arg) (Hof et al., 1996). Modeling studies suggest that Ser40 in cathepsin G forms hydrogen bonds with $P_{1'}$ Ser, providing a structural basis for the inhibition by natural cathepsin G inhibitors such as α_1-antichymotrypsin, α_1-antitrypsin, and squamous cell carcinoma antigen 2 (SCCA-2) (Korkmaz, Moreau, and Gauthier ,2008). This is in accordance with both RmS-3 and RmS-17 RCL, where the P_1-$P_{1'}$ residues for RmS-3 and RmS-17 are Leu-Ser and Lys-Ser, respectively. Interestingly, the RCL of RmS-3 resembles the chymotrypsin and cathepsin G cleavage site, as present in α_1-antichymotrypsin and SCCA-2 serpins (Schick et al., 1997). Since serpins are natural inhibitors of proteases with a role in host defenses against tick feeding (Rau, Beaulieu, Huntington, and Church ,2007), we hypothesize that this finding is the result of a parasite convergent evolution process that, in ticks, selected a serpin with biologically equivalent recognition sites present in the host's serpin, allowing tick saliva to target both cathepsin G and chymotrypsin-like proteases, mimicking proteins from the host itself.

After RCL P_1-$P_{1'}$ peptide bond cleavage, serpin covalently traps and distorts the target protease, resulting in a covalent complex serpin-protease, and therefore serpins are classified as suicide inhibitors (Huntington, Read, and Carrell ,2000). Data presented here showed that *R. microplus* salivary serpins interact with proteases via this classical suicide inhibition, as demonstrated by the formation of heat- and SDS-stable complex between serpin and protease (Fig. 8). Altogether, these results demonstrate that RmS-3, RmS-6 and RmS-17 are classical serine protease inhibitors injected in the host during tick feeding, and

that they target different host proteases. However, what could be the role of these protease inhibitory profiles regarding host defense modulation?

It was demonstrated that rRmS-6 and rRmS-17 are effective inhibitors of trypsin. Although they are produced predominantly by the pancreas as a means to degrade dietary proteins, trypsins are also expressed in the nervous system and in epithelial tissues, where they have diverse actions that could be mediated by protease-activated receptors (PARs). Trypsins are the most powerful PAR2 activator, and have been proved to be key factors in neurogenic inflammation and pain in the skin (Steinhoff et al., 2000;Cattaruzza, Amadesi, Carlsson, Murphy, Lyo, Kirkwood, Cottrell, Bogyo, Knecht, and Bunnett ,2014). Recently, it was demonstrated that several pro-inflammatory and algesic agents activate trypsin-like activity in a mouse paw model of inflammation (Cattaruzza, Amadesi, Carlsson, Murphy, Lyo, Kirkwood, Cottrell, Bogyo, Knecht, and Bunnett ,2014). These proteases generated cleaved PAR-derived peptides and signal cells by a PAR2-dependent mechanism. It was demonstrated that a specific inhibitor of trypsin IV is able to inhibit the pro-inflammatory and algesic action of these agents (Cattaruzza, Amadesi, Carlsson, Murphy, Lyo, Kirkwood, Cottrell, Bogyo, Knecht, and Bunnett ,2014). Thus, injection of trypsin-like inhibitors such as RmS-6 and RmS-17 in the feeding site could interfere in pro-inflammatory and algesic responses during tick feeding.

Neutrophils play an essential part as triggers of an immune response against tick feeding (Heinze et al., 2012a;Heinze et al., 2012b). It was demonstrated that *I. scapularis and I. dammini* saliva inhibits neutrophil function, pointing to its importance in the tick feeding process as well as in pathogen transmission (Ribeiro et al., 1990;Guo et al., 2009). rRmS-3 and rRmS-17 effectively inhibit neutrophil cathepsin G (Table 1 and Fig. 5 and Fig. 7). Cathepsin G along with neutrophil elastase and proteinase 3 are the most abundant

molecules present in neutrophil azurophil granules. Cathepsin G has a role in tissue remodeling during inflammation and significantly influences chemotaxis by cleaving the N-terminal residues of chemokine ligand 5 (CXCL5) and chemokine ligand 15 (CCL15) to generate more potent chemotactic factors for neutrophils and monocytes, respectively (Nufer et al., 1999;Richter et al., 2005). Indeed, cathepsin G (and neutrophil elastase) converts prochemerin to chemerin, a chemoattractant for APCs (Wittamer et al., 2005). The secretion of the chemotactic factor CXCL2 by neutrophils also depends on the action of cathepsin G (Raptis et al., 2005). Secreted cathepsin G also cleaves CD43 from the neutrophil cell surface, which may be important for cell migration (Mambole et al., 2008). In addition to the specific immune modulatory properties described above, cathepsin G participates in cell signaling by cleaving PAR4, which has been identified as an important signaling receptor in inflammation and platelet activation (Sambrano, Huang, Faruqi, Mahrus, Craik, and Coughlin ,2000). rRmS-3 and rRmS-17 were able to inhibit platelet aggregation induced by cathepsin G. The ability of neutrophils to enhance aggregation of human platelets *in vitro* is linked to cathepsin G activity. Also, tail bleeding time in a mouse model was prolonged by a cathepsin G inhibitor (Faraday et al., 2013). Altogether, inhibition of cathepsin G is potentially associated with blockage of several relevant pathways that may result in diminished neutrophil-mediated inflammation and thrombosis. Based on the cathepsin G inhibitory profile of RmS-3 and RmS-17 we suggest that these serpins play a role in neutrophil function, modulating pro-inflammatory and pro-coagulant activity of cathepsin G during tick feeding.

Another subset of cells with an important function in tick feeding are eosinophils. These cells spend a brief time in the peripheral blood, and the bulk of eosinophil cell population is found in tissues, predominantly on the body surfaces interacting with the

external environment, such as skin and mucosae (Rosenberg et al., 2013). Eosinophils contain many cytotoxic mediators, including eosinophil peroxidase (EPO), major basic protein (MBP), eosinophil cationic protein (ECP), and eosinophil-derived neurotoxin (EDN) in their granules (Blanchard and Rothenberg ,2009). Release of these mediators during degranulation produces severe tissue damage. It was demonstrated that eosinophils have significant chymotrypsin-like activity, and that an inhibitor of the chymotrypsin-like protease markedly inhibited EPO release from eosinophils (Matsunaga, Kido, Kawaji, Kamoshita, Katunuma, and Ogura ,1994). This result indicates that chymotrypsin-like protease has a pivotal role in eosinophil degranulation. Since rRmS-3 and rRmS-17 have anti-chymotrypsin activity, these serpins could inhibit chymotrypsin-like proteases from cattle eosinophils, enabling EPO release and impairing eosinophil function. The importance of eosinophils for tick feeding was highlighted in studies showing hosts upon repeated tick infestation present eosinophil accumulation in the attachment site, which has been linked to tick resistance (Schleger et al., 1981;Brown et al., 1982;Ushio et al., 1993;Carvalho et al., 2010).

Mast cells are typically associated with allergic reactions and auto-immune disorders, and contribute to host defenses against bacteria and parasites (Urb and Sheppard ,2012). When mast cells are challenged by an external stimulus, they may respond by degranulation, releasing a number of powerful preformed inflammatory mediators, which include several specific proteases with trypsin- and chymotrypsin-like activity (such as chymases and tryptases) and have pro-inflammatory functions (Wernersson and Pejler ,2014). rRmS-3 inhibits human chymase by a low rate (≈23 %), and *R. microplus* serpins did not inhibit human lung tryptase (Table 1). The cattle chymase locus contains two α-chymase functional genes (Gallwitz et al., 2006). Immunohistological studies revealed that

chymase-containing mast cells are predominant in connective tissues, including the skin, and more than one chymase subset of mast cells are present in different tissues (Jolly et al., 2000). The chymase and the tryptase used in this study were from human source and, since inhibition of specific bovine chymases and/or tryptases proteases could not be ruled out, they need further characterization. In mice, activation of mast cells may contribute to the expulsion of nematodes based on the release of the chymase mMCP-1 (Knight et al., 2000). Also, mast cells have been found to be crucial for the cutaneous resistance against larval *H. longicornis* ticks (Matsuda et al., 1990) and *D. variabilis* nymphs (Steeves and Allen ,1990). Therefore, inhibition of cattle chymase and tryptase by tick serpins could modulate function of mast cell during tick feeding.

Classically described as a protease related to fibrinolytic system, plasmin has been reported to participate also in pro-inflammatory processes (Syrovets et al., 1997;Burysek et al., 2002;Li et al., 2007;Li et al., 2010;Li et al., 2012). Plasmin inhibition by rRmS-6 and rRmS-17 could impair these plasmin immune functions. To investigate whether tick serpins could alter the classical coagulation parameters, standard coagulation tests in the presence of tick serpins were conducted. Given that rRmS-17 interferes with recalcification time, this feature seems to have a relationship with the inhibition of factor XIa. Serpins did not significantly alter plasma clotting time, TT, or aPTT (data not shown).

As serpins are secreted into host during tick feeding, and in order to investigate the specificity of host immune response against serpins during tick infestation, sera of repeated tick-infested hosts were used to probe glycosylated as well as deglycosylated serpins. Data showed that sera from tick-infested animals recognized either glycosylated or deglycosylated serpins. Interestingly, *R. microplus* serpins were also recognized by serum from hosts infested with *A. americanum*, *R. sanguineus*, and *I. scapularis* (Fig. 2),

confirming that serpins are naturally secreted during tick feeding, and that hosts are able to produce a humoral immune response against these proteins after sequential tick infestations. These data are in accordance with a previous study in which RmS-3 was recognized by serum from tick-resistant cattle (Rodriguez-Valle, Vance, Moolhuijzen, Tao, and Lew-Tabor ,2012). Furthermore, data showing cross-reactivity among sera from hosts infested with different tick species suggest secretion of similar serpins into saliva of other tick species, which highlight a potential use of salivary serpins as antigens in a universal anti-tick vaccine (Parizi et al., 2012). Serpins have been identified in saliva of other tick species, including *D. andersoni, H. longicornis*, and *A. americanum* (Chalaire, Kim, Garcia-Rodriguez, and Mulenga ,2011;Mudenda, Pierle, Turse, Scoles, Purvine, Nicora, Clauss, Ueti, Brown, and Brayton ,2014;Kim, Tirloni, Radulovic, Lewis, Bakshi, Hill, Da Silva, Jr., Logullo, Termignoni, and Mulenga ,2015;Tirloni, Islam, Kim, Diedrich, Yates, III, Pinto, Mulenga, You, and Da Silva, Jr. 2015), lending strength to the hypothesis that different tick species secrete these proteins in their tick saliva, and pointing to a role in the evasion from host defenses during tick feeding (Leboulle et al., 2002a;Prevot, Adam, Boudjeltia, Brossard, Lins, Cauchie, Brasseur, Vanhaeverbeek, Vanhamme, and Godfroid ,2006;Chmelar, Oliveira, Rezacova, Francischetti, Kovarova, Pejler, Kopacek, Ribeiro, Mares, Kopecky, and Kotsyfakis ,2011;Mulenga, Kim, and Ibelli ,2013;Kim, Tirloni, Radulovic, Lewis, Bakshi, Hill, Da Silva, Jr., Logullo, Termignoni, and Mulenga ,2015). Weak reactivity of sera from *I. scapularis*-infested animals against *R. microplus* serpins was observed (Fig. 2). Such an observation comes as no surprise, since *I. scapularis* is a prostriata tick while *R. microplus*, *R. sanguineus*, and *A. americanum* are metastriata ticks. The presence of specific either prostriata as well as metastriata specific-protein families in salivary glands has been clearly demonstrated (Francischetti, Sa-Nunes, Mans, Santos, and

Ribeiro ,2009), suggesting differences in salivary gland contents between these groups of ticks. According to western blotting results, the anti-*R. microplus* sera reactivity was lower with deglycosylated as compared with glycosylated serpins. These results suggest that the carbohydrate moieties of the serpin proteins participate in but are not essential for eliciting an immune response in the host, which could also be variable. Admittedly, the real importance of oligosaccharides concerning the immunogenicity of a particular glycoprotein cannot be determined without immunization experiments (Gavrilov et al., 2011). Many recombinant non-glycosylated proteins have been used as vaccine antigen, showing that glycosylation is not essential for the induction of a protective response (Sugino, Imamura, Mulenga, Nakajima, Tsuda, Ohashi, and Onuma ,2003;Imamura, da Silva, I, Sugino, Ohashi, and Onuma ,2005).

MAbs and polyclonal sera raised against tick serpins crossreacted, though at different levels, with recombinant and native salivary serpins, suggesting that similar epitopes are present in different serpins. When poly- and monoclonal antibodies were used to assay the effect of antibodies against serpin activity, polyclonal antibodies abolished serpin inhibitory activity (Fig. 11). This is of major importance, when serpins are considered antigens in an anti-tick vaccine development. Host functional antibodies raised during immunization could block serpin inhibitory activity during tick feeding and interfere with modulatory functions of serpins, impairing blood meal acquisition and tick development. The different inhibition capacities of the MAbs and polyclonal antibodies can be explained based on the possibility that polyclonal antibodies recognize more than one epitope, while monoclonal antibodies can be recognized as a structure with unstable or non-functional epitopes (Saunders et al., 1998;Kummer et al., 2004;Parra-Lopez et al., 2014).

Analyzed together, our results and previous findings show that serpins are inoculated during tick infestation and play the role of immunogenic inductors of humoral responses. Indeed, serpins are involved in host-parasite interactions and help the parasite to evade the host immune system. In previous studies, proteins from *I. ricinus* (Prevot *et al.*, 2007), *H. longicornis* (Sugino *et al.*, 2003), and *R. appendiculatus* (Imamura *et al*., 2006; Imamura *et al*., 2008) were shown to induce the production of antibodies and to be associated with protection against tick infestation. Inducing an efficacious immune response against tick serpins could help hosts to limit and control parasite infections and, therefore, serpins may be included as suitable antigen candidates in an anti-tick vaccine.

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Figure legends

Figure 1. Recombinant expression of *R. microplus* salivary serpins in *Pichia pastoris*. (A) Daily expression levels of rRmS-3, rRmS-6, and rRmS-17 throughout five days (1-5). Recombinant proteins in spent culture media were precipitated by ammonium sulfate and verification of protein expression was performed using an antibody to the C-terminus hexa histidine tag. (B) Total expression (1) and affinity purified- (2) rRmS-3, rRmS-6, and rRmS-17 were resolved on a 12.5 % SDS-PAGE following Coomassie brilliant blue staining. (C) Recombinant serpins treated with deglycosylation enzyme mix (D+) or without treatment (D-) were resolved on a 12.5 % SDS-PAGE following Coomassie brilliant blue staining and western blotting using anti-C-terminus hexa histidine tag antibody.

Figure 2. **Inter-species cross-reactivity assay.** Purified rRmS-3, rRmS-6 and rRmS-17 treated with deglycosylation enzyme mix (D+) or without treatment (D-) were resolved on a 12.5 % SDS-PAGE following western blotting analysis with: cattle serum generated by *R. microplus* tick infestation (dilution 1:50), rabbit serum generated by adult *R. sanguineus* infestations (dilution 1:50), *A. americanum* infestation (dilution 1:50), and adult *I. scapularis* infestation (dilution 1:25). Anti-C-terminus hexa histidine tag antibodies (dilution 1:5000) and rabbit pre-immune serum (dilution 1:50) were used as control.

Figure 3. Transcription and expression profile of salivary serpins. (A) Ticks were divided in 8 different groups. (B) Total RNA was extracted from ticks and subjected to RT-PCR to amplify RmS-3, RmS-6, and RmS-17 fragments. Tick ribosomal protein S3a was used as reference. (C) Total protein extracts of SG from eight groups of ticks were subjected

to western blotting analyses using anti-rRmS-3, anti-rRmS-6, rRmS-17 sera as well as pre-immune serum. Arrows indicate the position of the native protein.

Figure 4. rRmS-3 stoichiometry inhibition (SI) assay. Residual protease activity in the presence and absence of rRmS-3 was evaluated pre-incubating serpin for 1 h at 37 °C with (A) cathepsin G (200 nM), (B) chymotrypsin (10 nM), and (C) pancreatic elastase (50 nM), resulting in molar ratios (serpin:protease) ranging from 0 to 10. Protease activity was measured using specific colorimetric substrate for each protease as described in Materials and Methods. The data were plotted as residual protease activity (V_i/V_0) versus molar ratio (serpin:protease).

Figure 5. rRmS-6 stoichiometry inhibition (SI) assay. Residual protease activity in the presence and absence of rRmS-6 was evaluated pre-incubating serpin for 1 h at 37 °C with (A) trypsin (10 nM), (B) chymotrypsin (10 nM), (C) factor Xa (5 nM), (D) factor XIa (5 nM), and (E) plasmin (50 nM), resulting in a molar ratio (serpin:protease) ranging from 0 to 10 for trypsin, chymotrypsin, and plasmin, and from 0 to 20 for factor Xa and factor XIa. Protease activity was measured using specific colorimetric substrate for each protease as described in Materials and Methods. The data were plotted as residual protease activity (V_i/V_0) versus molar ratio (serpin:protease).

Figure 6. rRmS-17 stoichiometry inhibition (SI) assay. Residual protease activity in the presence and absence of rRmS-17 was evaluated pre-incubating serpin for 1 h at 37 °C with (A) trypsin (10 nM), (B) chymotrypsin (10 nM), (C) cathepsin G (200 nM), (D) factor XIa (5 nM), and (E) plasmin (50 nM), resulting in a molar ratio (serpin:protease) ranging 0 to

10 (for factor XIa molar ratio ranges between 0 to 20). Protease activity was measured using specific colorimetric substrate for each protease as described in Materials and Methods. The data were plotted as residual protease activity (V_i/V_0) versus molar ratio (serpin:protease).

Figure 7. Heat and SDS-stable complex formation assay. Increasing amounts of recombinant serpins were pre-incubated for 1 h at 37 °C with a constant concentration of (A) chymotrypsin and cathepsin G for rRmS-3, (B) trypsin and cathepsin G for rRmS-17, and (C) trypsin for rRmS-6, resulting in molar ratios varying from 0.6:1 to 10:1 (serpin:protease). Samples were resolved on 12.5% SDS–PAGE and Coomassie blue-stained to identify SDS-stable complexes (indicated by arrows).

Figure 8. rRmS-3 and rRmS-17 effect upon cathepsin G-induced platelet aggregation. Platelet aggregation function induced by cathepsin G assay was done using bovine platelet rich plasma (PRP) as described in Materials and Methods. (A) Tyrode solution with varying amounts of rRmS-3 or rRmS-17 (3 μM, 2.5 μM, 2 μM, 1.5 μM, 1 μM, and 0.5 μM) were pre-incubated with cathepsin G (0.7 μM) in a 50-μL reaction for 15 min at 37 °C. Platelet aggregation was initiated with addition of 100 μL pre-warmed PRP and monitored at 20-s intervals over 30 min at OD_{650nm}. (B) Percent of reduction of cathepsin G-induced platelet aggregation inhibited by rRmS-3 and rRmS-17.

Figure 9. Effect of *R. microplus* serpins upon plasma recalcification time. Human reference plasma (50 μL) was incubated with rRmS-3 (10 μM), rRmS-6 (10 μM), and rRmS-17 (10 μM) in 90 μL of Tris–HCl reaction buffer for 15 min at 37 °C followed by the addition of 150 mM $CaCl_2$ (10 μL). Clotting was measured every 20 s for 30 min.

Figure 10. Reactivity of anti-rRmS antibodies with RmS-3, RmS-6 and RmS-17 from tick saliva. Pilocarpine-induced tick saliva were harvested as described in Material and Methods. Saliva total protein treated with deglycosylation enzyme mix (D+) or non-treated (D-) were resolved on a 12 % SDS-PAGE following western blotting using polyclonal sera (A) anti-rRmS-3, (B) anti-rRmS-6, and (C) anti-rRmS-17.

Figure 11. Effect of antibodies upon serpin inhibitory activity. Purified IgG from (A) rabbit polyclonal serum or (B) from mice MAbs (anti-rRmS-3, anti-rRmS-6, and rRmS-17) were incubated with rRmS-3 (55 nM), rRmS-6 (55 nM) or rRmS-17 (88 nM) at 37 °C for 30 min before the addition of chymotrypsin (4.9 nM) for rRmS-3, or trypsin (1.6 nM) for rRmS-6 and rRmS-17, following a new 15-min incubation. Protease kinetics was monitored for 15 min every 11 s.

Figure 1

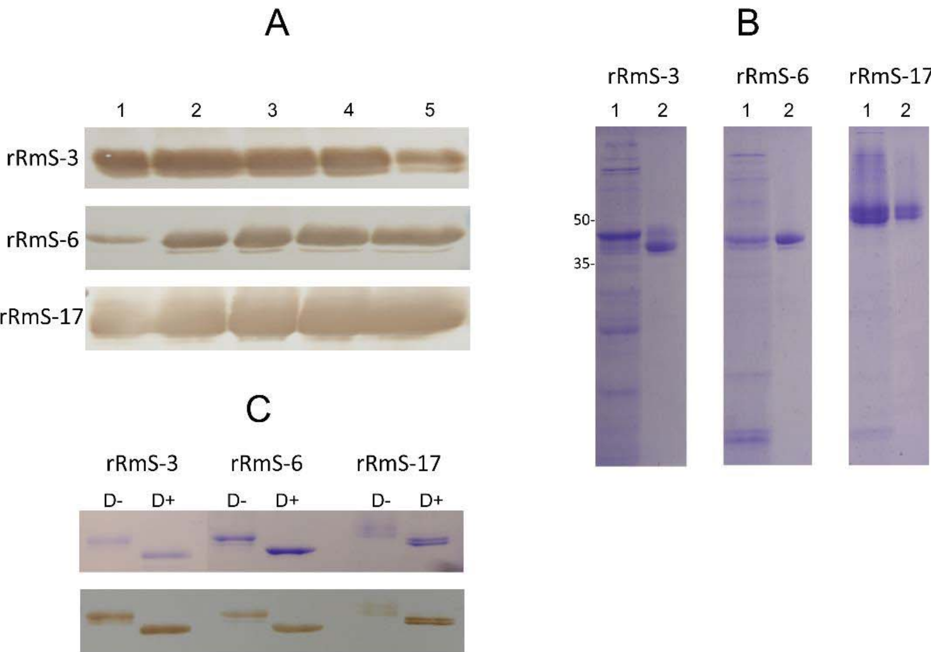
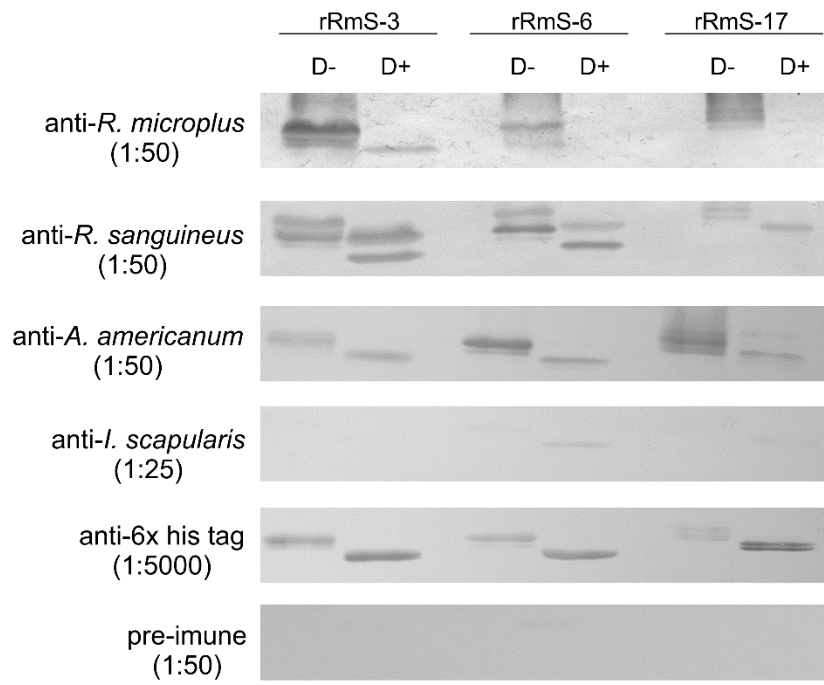


Figure 2



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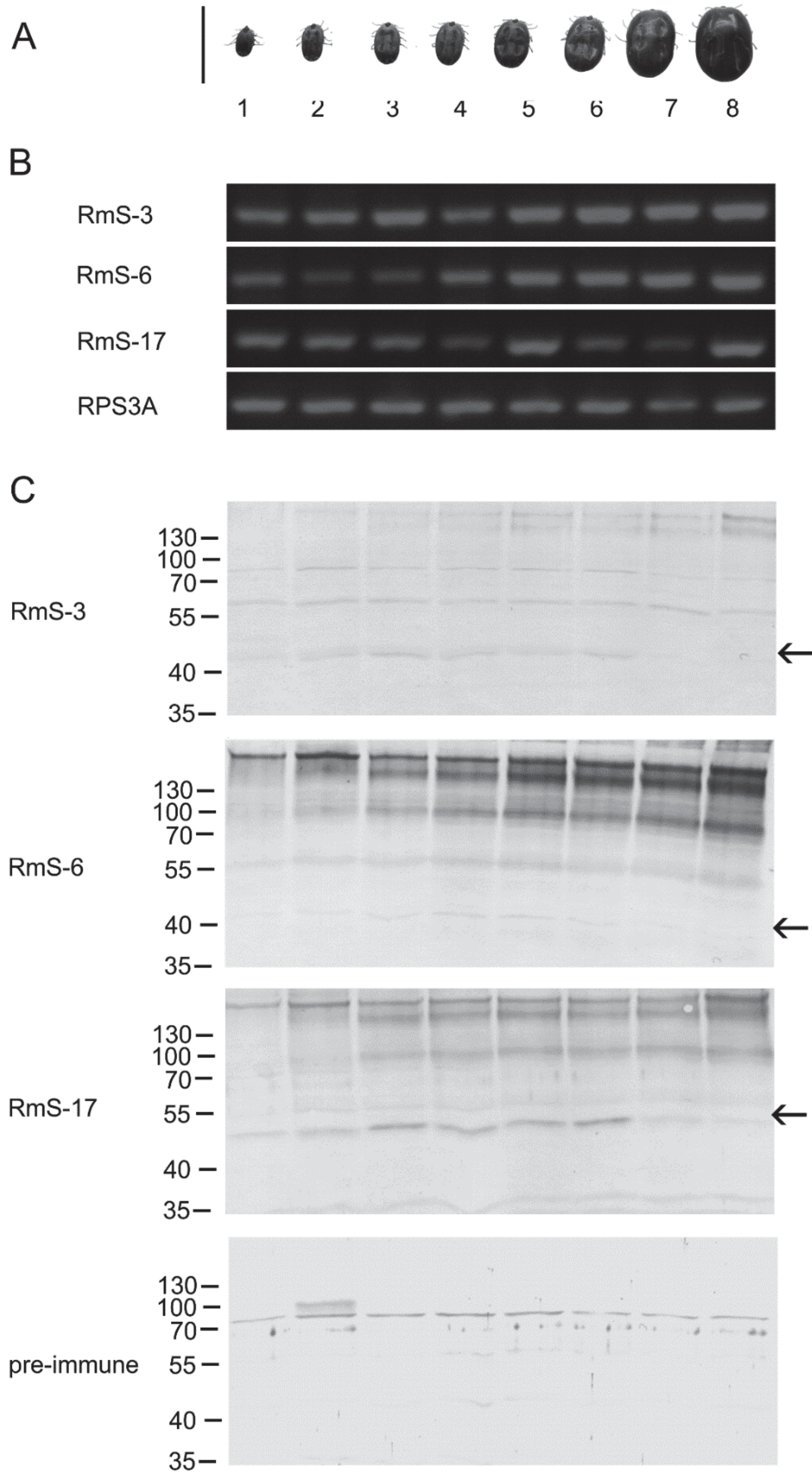

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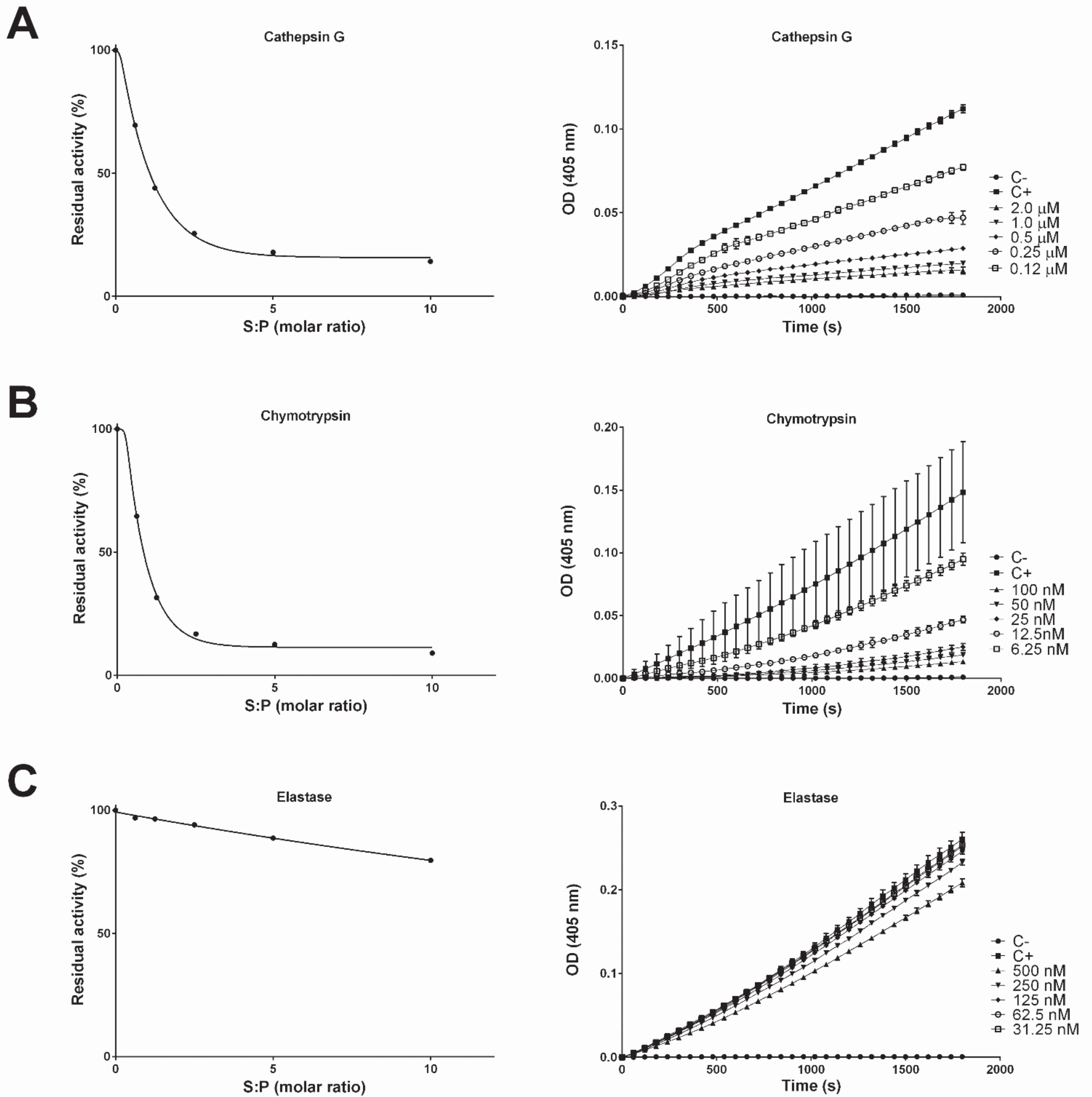

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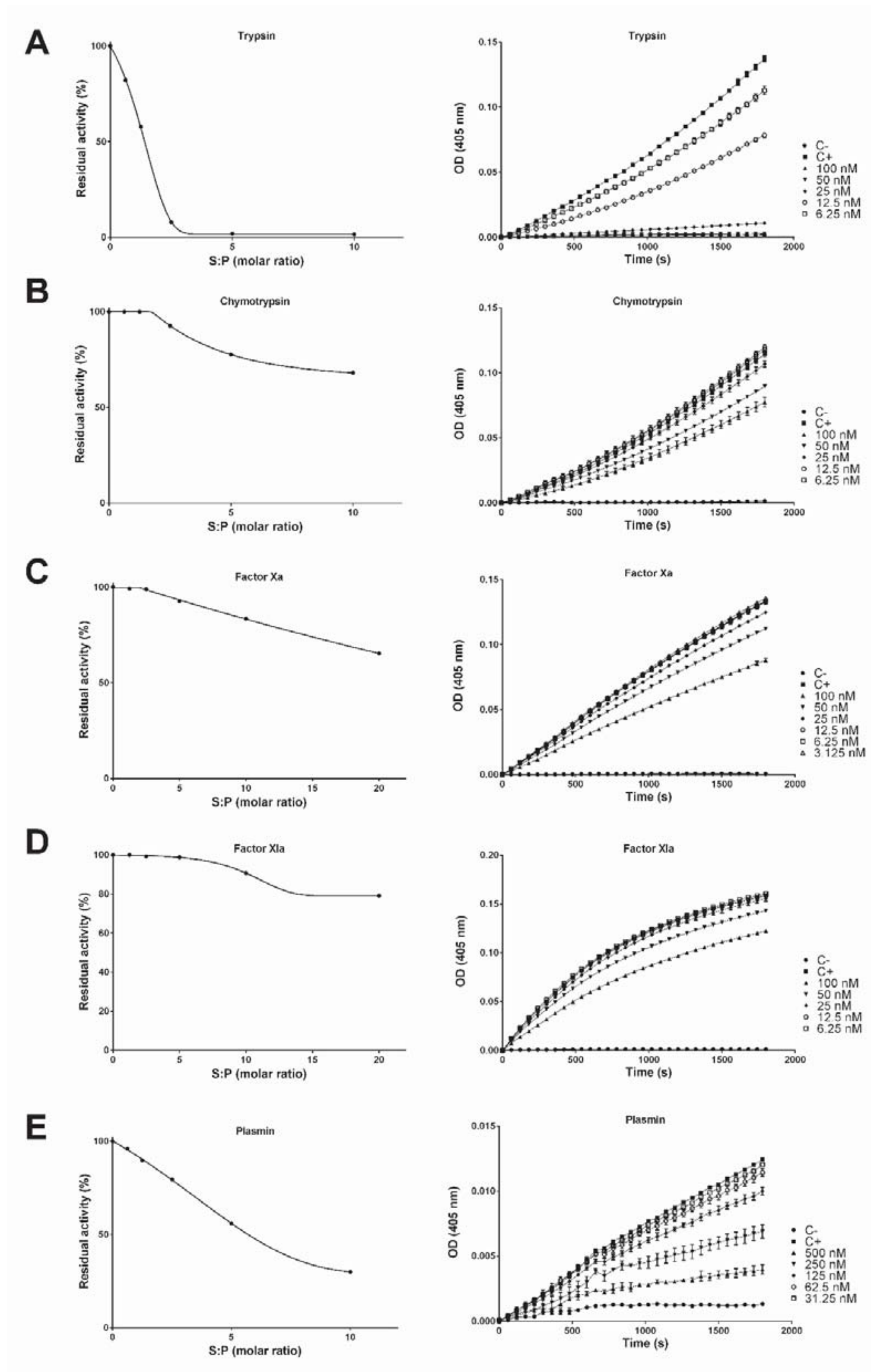
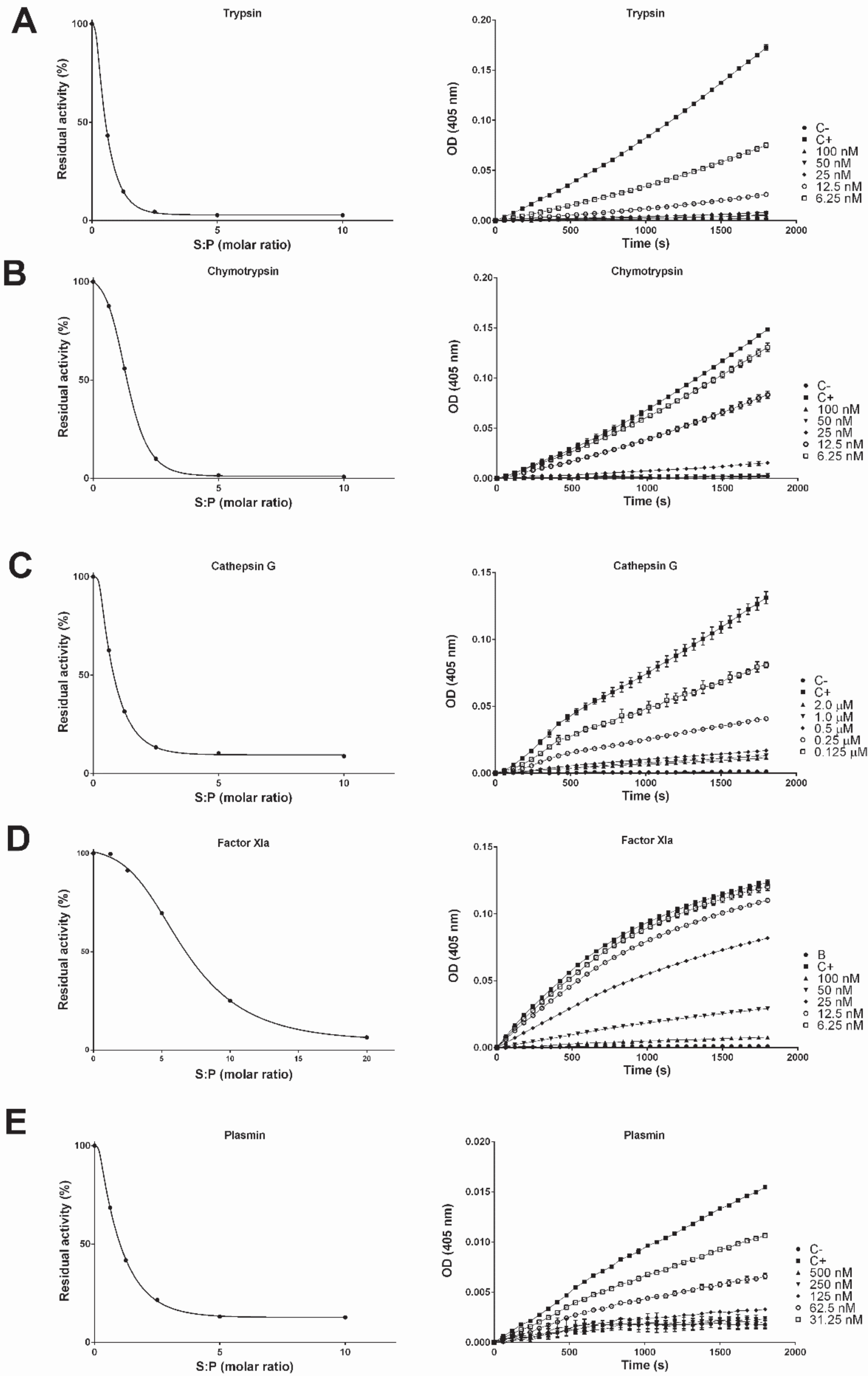


Figure 6



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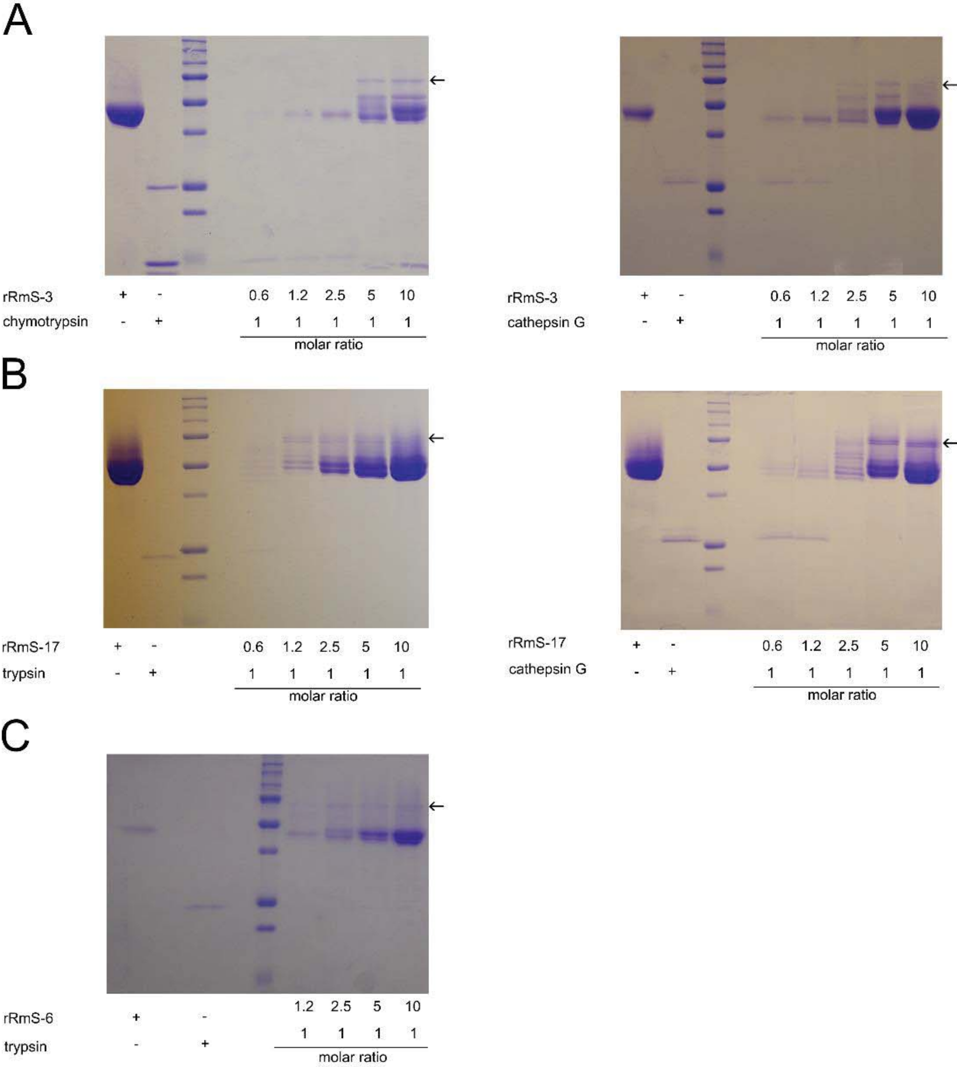

Figure 8

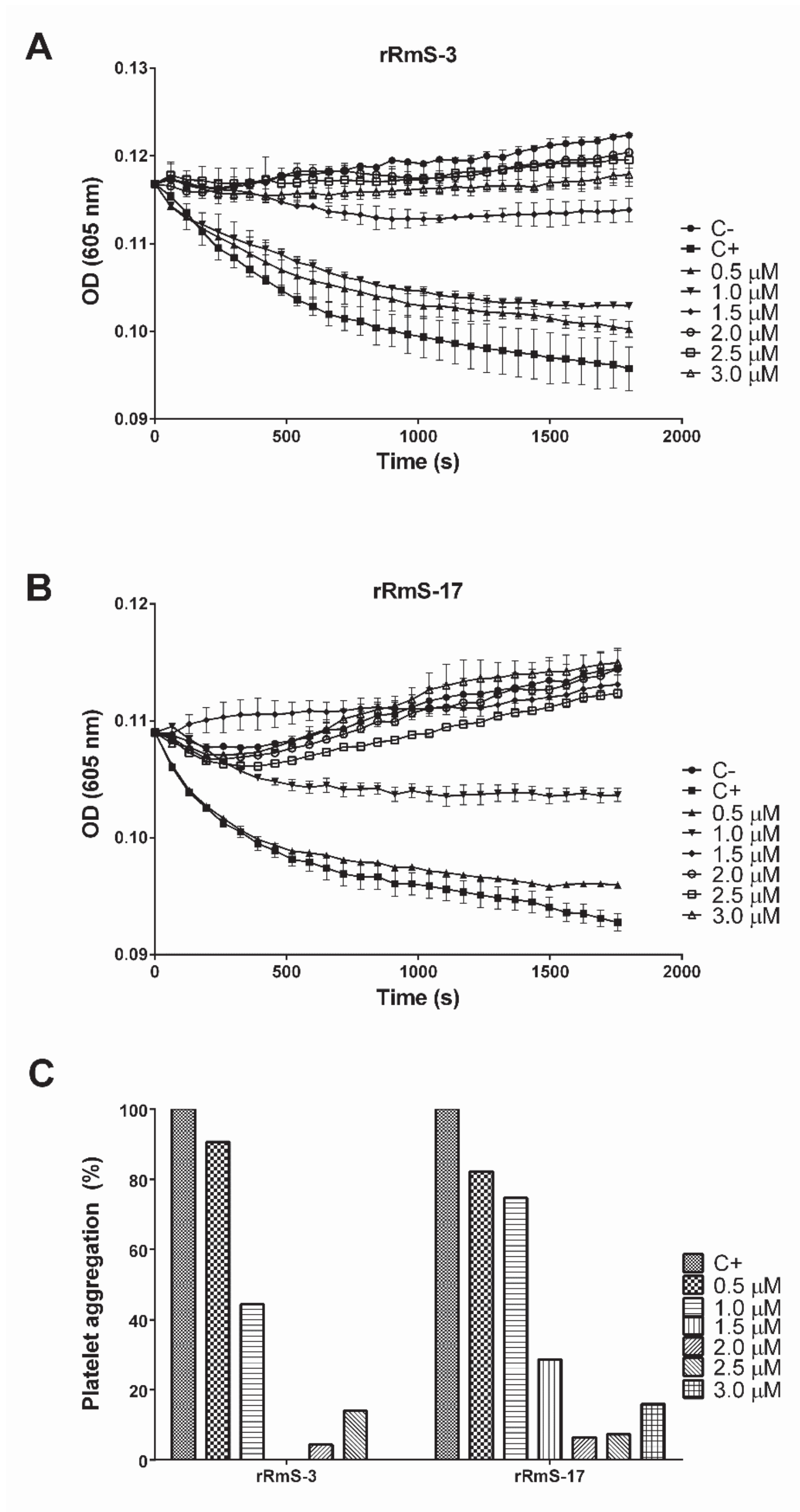

Figure 9

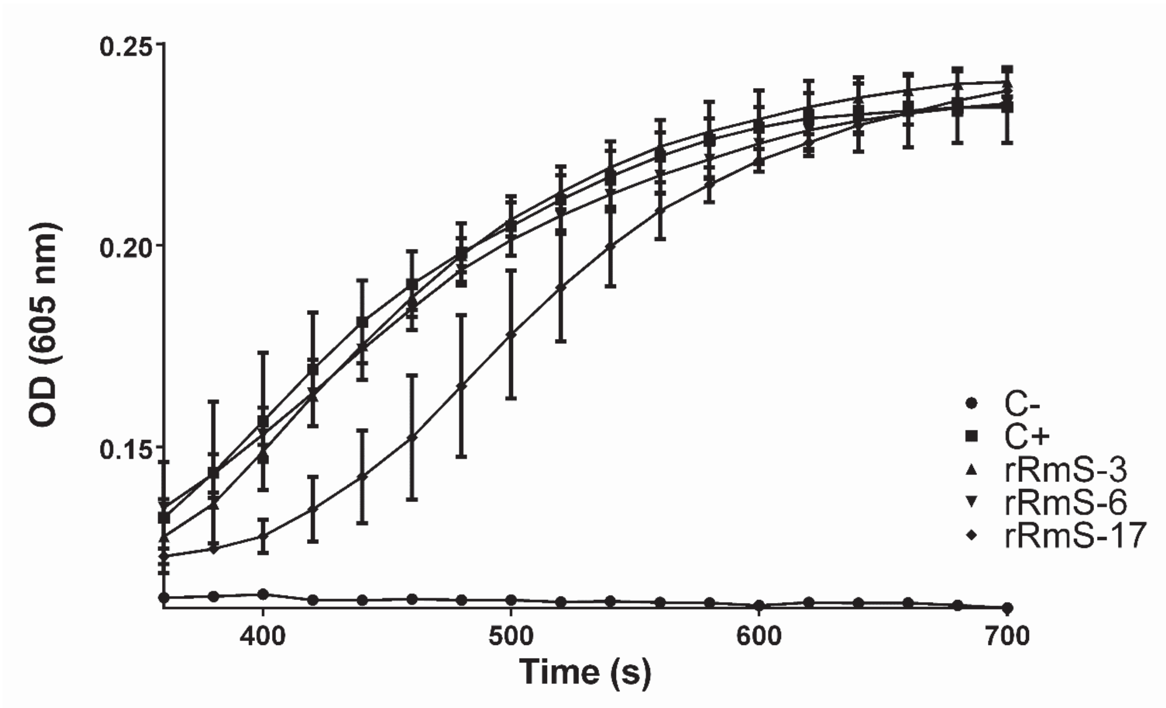

Figure 10

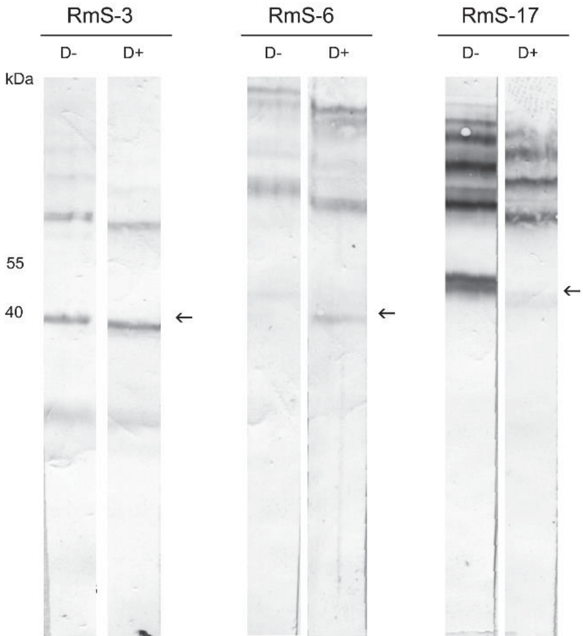

Figure 11

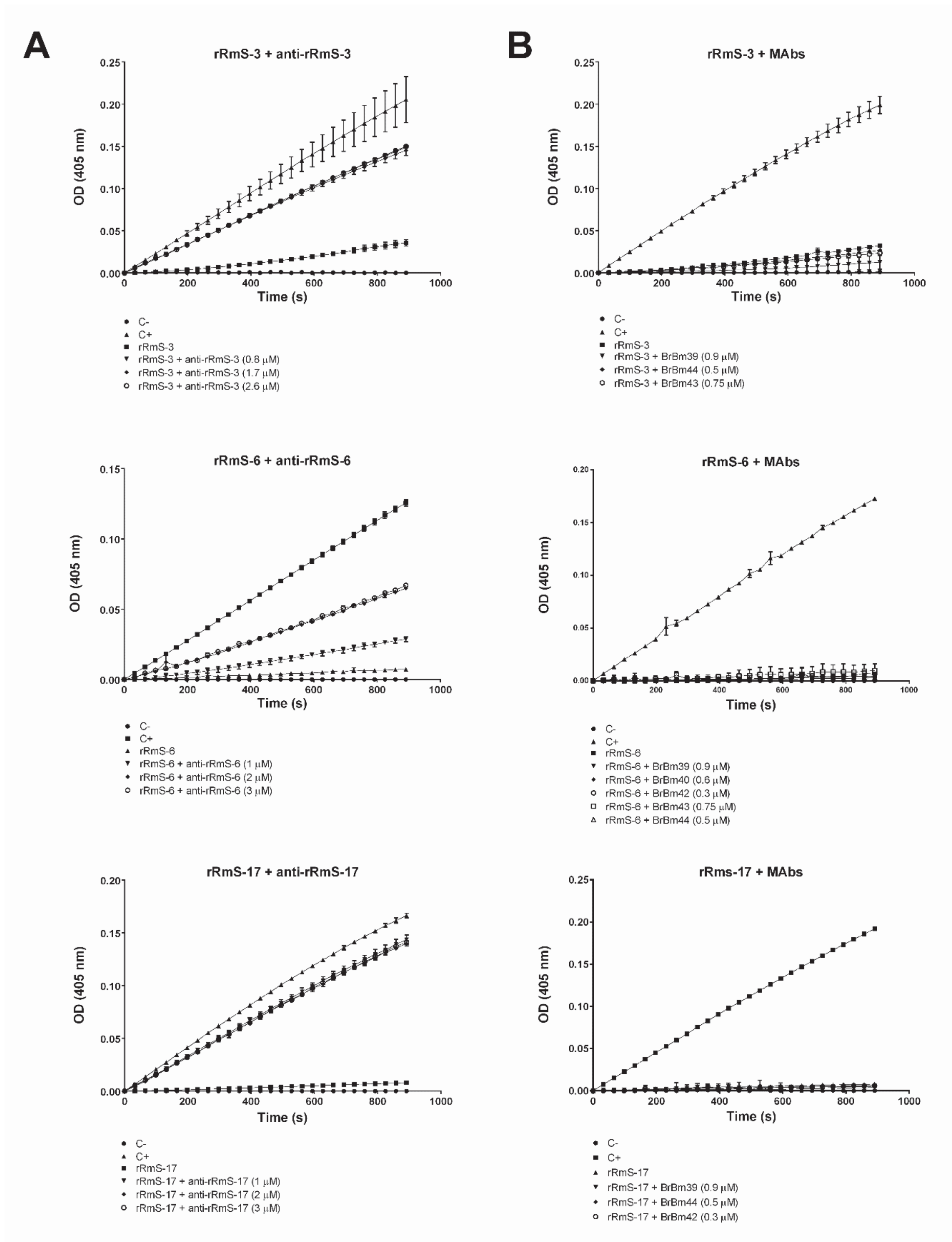

Table 1. *R. microplus* salivary serpins inhibitory profile.

Protease	Serpin		
	RmS-3 (1 μM)	RmS-6 (1 μM)	RmS-17 (1 μM)
Chymotrypsin (96 nM)	96.94 ± 1.07	24.29 ± 7.39	89.76 ± 2.97
Factor IXa (300 nM)	n.i	n.i	n.i
Cathepsin G (166 nM)	78.74 ± 0.20	n.i	78.84 ± 0.29
Factor XIa (3.68 nM)	n.i	62.86 ± 11.10	98.02 ± 1.24
Trypsin (24.6 nM)	n.i	73.52 ± 11.91	87.03 ± 4.80
Factor Xa (5.8 nM)	n.i	32.76 ± 10.68	n.i
Elastase (21.6 nM)	92.62 ± 2.40	n.i	n.i
Plasmin (10 nM)	n.i	24.13 ± 4.98	58.39 ± 13.52
Chymase (10 U)	23.36 ± 4.64	n.i	n.i
Factor XIIa (7.6 nM)	n.i	n.i	n.i
Thrombin (43 U)	n.i	n.i	n.i
u-PA (47.2 nM)	n.i	n.i	n.i
Tryptase (10 U)	n.i	n.i	n.i
t-PA (32 nM)	n.i	n.i	n.i
Proteinase-3 (68 U)	n.i	n.i	n.i
Kallikrein (33 U)	n.i	n.i	n.i

n.i: not inhibited or inhibition rate lower than 20%.

Table 2. Antibodies reactivity with native salivary and recombinant serpins.

Antibody	Immunogen	Cross-reactivity (%)*			
		Recombinant			Native
Monoclonal		rRmS-3	rRmS-6	rRmS-17	Saliva
BrBm39	rRmS-6	79 (±8)	100	69 (±20)	91 (±5.5)
BrBm40	rRmS-6	51	100	30	106 (±19)
BrBm41	rRmS-6	NR**	100	NR**	NR**
BrBm42	rRmS-6	NR**	100	NR**	NR**
BrBm43	rRmS-6	76 (±22)	100	49 (±11)	NR**
BrBm44	rRmS-17	Weak**	Weak**	Weak**	NR**
Polyclonal					
Anti-RmS-3	rRmS-3	100	47 (±10)	40 (±9)	+***
Anti-RmS-6	rRmS-6	35 (±10)	100	31 (±6)	+***
Anti-RmS-17	rRmS-17	46 (±10)	56 (±14)	100	+***

* Compared to primary reactivity. Results are shown as mean (±SD)

__NR__ = No reaction; **Weak = low reaction with all three serpins tested

***+ : please refer to Figure 10 for western blotting reactions

Table 3. Antibodies effect on serpin inhibitory activity.

Antibody	Serpin activity inhibition (%)*
rRmS-3	
rRmS-3	0
rRmS-3 + anti-rRmS-3 (0.8 µM)	66.7
rRmS-3 + anti-rRmS-3 (1.7 µM)	64.2
rRmS-3 + anti-rRmS-3 (2.6 µM)	66.8
rRmS-6	
rRmS-6	0
rRmS-6 + anti-rRmS-6 (1 µM)	18.9
rRmS-6 + anti-rRmS-6 (2 µM)	48.8
rRmS-6 + anti-rRmS-6 (3 µM)	50.4
rRmS-17	
rRmS-17	0
rRmS-17 + anti-rRmS-17 (1 µM)	85.6
rRmS-17 + anti-rRmS-17 (2 µM)	84.1
rRmS-17 + anti-rRmS-17 (3 µM)	84.3

Table S1: Primers used to RT-PCR and cloning *R. microplus* salivary serpins.

Gene	Primer	Sequence 5'-3'	Tm (°C)	Amplicon (bp)
RmS-3	Sense (pPIC)	TTTTTTTATCGATG GAGACCGACGACTCCACGCTTC	59.7	1149
	Antisense (pPIC)	AAGCGGCCGCTCAGTGGTGGTGGTGGTG GTGTAGTG TGTTGACCTCTCCAATGAAAAGAA	59.5	
	Sense (PCR)	GACTTGAGGTCGGCAAACTT	50.9	144
	Antisense (PCR)	GTTCAGGATGAAGAGCACGA	50	
RmS-6	Sense (pPIC)	TTTTTTTATCGATG CAGACGGAGCAAGAACACAAGCTC	59.1	1137
	Antisense (pPIC)	AAGCGGCCGCTCAGTGGTGGTGGTGGTG GTG AAGGACG TTCACTTGTCCGGC	57.7	
	Sense (PCR)	CTCTCTCAGCGAAAGTGACG	50	
	Antisense (PCR)	TCAGGGCGTCGTAGTAAGTG	50.4	149
RmS-17	Sense (pPIC)	TTTTTTTATCGATG CAAGAGGAACACAAGGTGACCGC	59.5	1128
	Antisense (pPIC)	AAGCGGCCGCTCAGTGGTGGTGGTGGTG GTG AAGGTG GTTCACTTGACCCGCG	61.8	
	Sense (PCR)	CTTTCGATGCGAGAGGAAC	49.7	
	Antisense (PCR)	GTAAGGCAGGTCCAGAACATC	50.3	143
RPS3a	Sense (PCR)	CGCCAATCAACTCAAGAAGA	50.4	
	Antisense (PCR)	TGCTTCCAGGAATGAGTTTG	50.2	140

4 DISCUSSÃO

O impacto causado por carrapatos na saúde humana e na produção animal é notável, acarretando prejuízos para diferentes setores da sociedade. O estudo da hematofagia pode fornecer dados importantes para a melhor compreensão dos processos que regem a relação parasito-hospedeiro, possibilitando o desenvolvimento de novas abordagens para o controle desses parasitos. Sendo assim, a identificação e caracterização de proteínas que desempemham papéis no processo hematofágico podem auxiliar no desenvolvimento racional de novos métodos de controle.

No primeiro capítulo desta teste, comparamos o proteoma da saliva de fêmeas parcialmente ingurgitadas e fêmeas totalmente ingurgitadas. Já em um segundo momento, caracterizamos o proteoma da saliva em vários estágios de ingurgitamento. Nestes trabalhos, além de identificar a composição protéica da saliva, objetivamos também a evidenciar funções e mecanismos dessas proteínas na relação parasito-hospedeiro. Buscamos sempre contextualizar a presença de certas classes de proteínas com o seu papel na modulação das defesas do hospedeiro, ou no processo hematofágico. Os resultados aqui apresentados demostram que a saliva de *R. microplus* é uma amostra protéica complexa, contendo diferentes classes de proteínas que variam ao longo da alimentação. Entre as classes de proteínas identificadas, podemos citar proteínas relacionadas com metabolismo de heme e ferro, sendo a glicoproteína ligadora de heme (HeLp) a proteína mais abundante na saliva. Dentre as demais classes de proteínas, identificamos proteínas relacionadas com processos de oxidação e detoxificação; enzimas como fosfolipases, proteases pertencentes à família das serino-proteases, cisteíno-proteases, metalo-proteases; inibidores de proteases da família das serpinas, cistatinas, TIL, Kunitz; peptídeos antimicrobianos; e famílias de proteínas específicas e conservados em carrapatos, como as lipocalinas (*tick histamine-*

binding proteins) e o grupo das proteínas conservadas que não possuem função descrita. Um exemplo desse grupo de proteínas conservadas com função desconhecida é a proteína AV422 de *A. americanum*. Esta proteína foi descrita pela primeira vez como uma proteína com expressão regulada em resposta à exposição ao hospedeiro ou a estímulos de alimentação em coelhos (MULENGA *et al*., 2007). Posteriormente foi demonstrado que a AV422 é uma proteína secretada no hospedeiro e possui atividade anticoagulante (interferindo no tempo de coagulação do plasma), além de inibir o sistema complemento (MULENGA *et al*., 2013). A presença de classes de proteínas conservadas em carrapatos e com função desconhecida na saliva abrem novas perspectivas para a caracterização funcional dessas proteínas (como no caso da AV422), podendo levar à descoberta de novos antígenos ou de novos compostos que possam ter outras aplicações biotecnológicas.

Nos estudos proteômicos da saliva do carrapato observou-se a presença de diversas proteínas do hospedeiro. Muitas vezes essa observação é interpretada como contaminação, e acaba sendo um tópico negligenciado no estudo da biologia do carrapato. Essas observações levantam novas hipóteses que podem ser exploradas no que concerne à utilização de proteínas do hospedeiro na fisiologia dos carrapatos. Uma questão bastante interessante é: essas proteínas podem exercer funções biológicas no hospedeiro? Recentemente foi demonstrado em *H. longicornis* que a transferrina do hospedeiro é utilizada como transportador ativo de ferro, transportando esse metal do intestino para o ovário via hemolinfa (MORI *et al*., 2014). Também já foi relatado que a hidrólise da hemoglobina do hospedeiro durante a hematofagia é capaz de gerar peptídeos com função antimicrobiana (FOGAÇA *et al*., 1999).

Diversas moléculas da saliva do carrapato já foram caracterizadas como detentoras de propriedades farmacológicas (VALENZUELA, 2004; MARITZ-OLIVIER *et al*., 2007).

As serpinas têm sido descritas em diferentes espécies de carrapatos, possuindo funções anticoagulantes, anti-inflamatórias, e imunomodulatórias. Nas análises proteômicas descritas nesta teste foi possível observar que o *R. microplus* secreta de forma majoritária as serpinas RmS-3, RmS-6 e RmS-17. Compatíveis com os resultados obtidos na caracterização funcional dessas proteínas, mostrou-se que essas serpinas salivares são inibidores de protease pró-inflamatórias e pró-coagulantes, o que sugere que essas serpinas possam atuar modulando proteases dos sistemas de defesa do hospedeiro. Essa modulação das respostas do hospedeiro permite que o parasito permaneça fixado no hospedeiro durante a fase parasitária, completando assim o seu ciclo de desenvolvimento. Ainda que esta modulação permita o sucesso no desenvolvimento dos parasitos, já foi mostrado que ao longo de repetidas infestações os animais são capazes desenvolver resistência ao parasitismo. Mostrou-se que o soro de animais repetidamente infestados pelo *R. microplus* é capaz de neutralizar a ativade farmacológica de alguns componentes salivares, como as atividades anticoagulantes da saliva (RECK, JR. *et al*., 2009). Isso evidencia que a resposta imune humoral possui papel na resistência adquirida. Do ponto de vista do controle imunológico, pudemos observar que anticorpos policlonais produzidos contra as serpinas foram capazes de bloquear as funções inibitórias desses inibidores. Essa observação reforça a ideia de que serpinas podem ser utilizadas como antígenos vacinais. Uma vez usadas como antígeno, os anticorpos produzidos pelos animais podem bloquear a atividade da serpina, podendo impactar no processo hematofágico, e consequentemente no desenvolvimento do parasita. No mesmo sentido, outra observação interessante foi a reatividade cruzada com anticorpos gerados contra infestações experimentais com outras espécies de carrapatos, como *A. americanum*, *I. scapularis* e *R. sanguineus*. Esses dados sugerem que, além de serem secretadas em diferentes espécies de carrapatos, as serpinas são proteínas com certo

grau de conservação em diferentes espécies, o que as tornam antígenos potenciais para o desenvolvimento de vacinas anti-carrapato universais.

A saliva dos carrapatos, como em outros artrópodes hematófagos, representa o principal componente do parasito que entra em contato com o hospedeiro. A exposição de antígenos através da secreção da saliva durante o parasitismo pode ser utilizada como uma aliada em protocolos de imunização para carrapatos. Um vez que os hospedeiros fossem imunizados com antígenos salivares, durante uma infestão natural por carrapatos, a inoculação da saliva atuaria como um *booster* natural re-ativando a memória imunológica do hospedeiro.

Assim sendo, espera-se que o conjunto de dados gerados nesta tese possam contribuir e expandir significativamente o conhecimento atual sobre a fisiologia desses parasitos, fornecendo subsídeos para novos estudos dos aspectos da biologia, assim como para o desenvolvimento de novas formas de controle.

5 PERSPECTIVAS

- Estudos de caracterização funcional de outras classes de proteínas salivares;
- Estudos explorando as funções anti-inflamatórias e imunomodulatórias das serpinas, atrevés de ensaios *in vitro* e *in vivo*;
- Estudos do potencial imunoprotetor dessas serpinas salivares, podendo tornar-se um produto biotecnológico compondo uma vacina contra o carrapato bovino.

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ANEXOS

Anexo A: pedido de patente depositado junto ao Instituto Nacional de Propriedade Intelectual.

Anexos B e C: artigos publicados como primeiro autor no período de doutorado. Os temas desses trabalhos estão diretamente vinculados ao tema da tese.

Anexo D: *Curriculum Vitae* resumido.

Anexo A

Uso de serpinas de carrapato ou peptídeos derivados como vacina contra o carrapato

Pedido de patente depositado junto ao **Instituto Nacional de Propriedade Intelectual**

< Uso exclusivo do INPI >

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Espaço reservado para o protocolo | Espaço reservado para a etiqueta | Espaço reservado para o código QR

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DIRPA	Tipo de Documento: Formulário	DIRPA	Página: 1/3
Título do Documento: **Depósito de Pedido de Patente**		Código: FQ001	Versão: 2
		Procedimento: DIRPA-PQ006	

Ao Instituto Nacional da Propriedade Industrial:
O requerente solicita a concessão de um privilégio na natureza e nas condições abaixo indicadas:

1. **Depositante (71):**

1.1 Nome: UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

1.2 Qualificação: INST. PÚBLICA DE ENSINO SUP

1.3 CNPJ/CPF: 92969856000198

1.4 Endereço Completo: AV. PAULO GAMA, 110 - CENTRO, PORTO ALEGRE - RS, Brasil

1.5 CEP: 90040-060

1.6 Telefone: 51 3308 3800 1.7 Fax: 51 3308 4237

1.8 E-mail: sedetec@ufrgs.br

[x] continua em folha anexa

2. **Natureza:** [x] Invenção [ ] Modelo de Utilidade [ ] Certificado de Adição

3. **Título da Invenção ou Modelo de Utilidade (54):**

USO DE SERPINAS DE CARRAPATO OU PEPTÍDEOS DERIVADOS COMO VACINA CONTRA O CARRAPATO

[ ] continua em folha anexa

4. **Pedido de Divisão:** do pedido Nº **Data de Depósito:**

5. **Prioridade:** [ ] Interna (66) [ ] Unionista (30)

O depositante reivindica a(s) seguinte(s):

País ou Organização do depósito	Número do depósito (se disponível)	Data de depósito

[ ] continua em folha anexa

Anexo B

Saliva from nymph and adult females of *Haemaphysalis longicornis*: a proteomic study

Artigo científico publicado no periódico **Parasites & Vectors**

Lucas Tirloni, Mohammad Saiful Islam, Tae Kwon Kim, Jolene K. Diedrich, John R. Yates III, Antônio F. M. Pinto, Albert Mulenga, Myung-Jo You and Itabajara Da Silva Vaz Jr. (2015). Saliva from nymph and adult females of *Haemaphysalis longicornis*: a proteomic study. Parasites & Vectors, 8:338

Contribuição dos autores

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RESEARCH Open Access

Saliva from nymph and adult females of *Haemaphysalis longicornis*: a proteomic study

Lucas Tirloni[1], Mohammad Saiful Islam[2,3], Tae Kwon Kim[6], Jolene K. Diedrich[4], John R. Yates III[4], Antônio F. M. Pinto[4,5], Albert Mulenga[6], Myung-Jo You[2] and Itabajara Da Silva Vaz Jr.[1,7*]

Abstract

Background: *Haemaphysalis longicornis* is a major vector of *Theileria* spp., *Anaplasma phagocytophilum, Babesia* spp. and *Coxiella burnetti* in East Asian countries. All life stages of ixodid ticks have a destructive pool-feeding style in which they create a pool-feeding site by lacerating host tissue and secreting a variety of biologically active compounds that allows the tick to evade host responses, enabling the uptake of a blood meal. The identification and functional characterization of tick saliva proteins can be useful to elucidate the molecular mechanisms involved in tick development and to conceive new anti-tick control methods.

Methods: *H. longicornis* tick saliva was collected from fully engorged nymphs and fully engorged adults induced by dopamine or pilocarpine, respectively. Saliva was digested with trypsin for LC-MS/MS sequencing and peptides were searched against tick and rabbit sequences.

Results: A total of 275 proteins were identified, of which 135 were tick and 100 were rabbit proteins. Of the tick proteins, 30 proteins were identified exclusively in fully engorged nymph saliva, 74 in fully engorged adult females, and 31 were detected in both stages. The identified tick proteins include heme/iron metabolism-related proteins, oxidation/detoxification proteins, enzymes, proteinase inhibitors, tick-specific protein families, and cytoskeletal proteins. Proteins involved in signal transduction, transport and metabolism of carbohydrate, energy, nucleotide, amino acids and lipids were also detected. Of the rabbit proteins, 13 were present in nymph saliva, 48 in adult saliva, and 30 were present in both. The host proteins include immunoglobulins, complement system proteins, antimicrobial proteins, serum albumin, peroxiredoxin, serotransferrin, apolipoprotein, hemopexin, proteinase inhibitors, and hemoglobin/red blood cells-related products.

Conclusions: This study allows the identification of *H. longicornis* saliva proteins. In spontaneously detached tick saliva various proteins were identified, although results obtained with saliva of fully engorged ticks need to be carefully interpreted. However, it is interesting to note that proteins identified in this study were also described in other tick saliva proteomes using partially engorged tick saliva, including hemelipoprotein, proteases, protease inhibitors, proteins related to structural functions, transporter activity, metabolic processes, and others. In conclusion, these data can provide a deeper understanding to the biology of *H. longicornis*.

Keywords: Tick, Proteomic, Saliva, Tick-host relationship

* Correspondence: itabajara.vaz@ufrgs.br
[1]Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
[7]Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
Full list of author information is available at the end of the article

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Background

The hard tick *Haemaphysalis longicornis* is a medically and veterinary important vector of many tick-borne disease (TBD), transmitting pathogens such as *Ehrlichia chaffeensis* [1], *Anaplasma bovis* [2], *A. phagocytophilum* [3], *Coxiella burnetii* [4], and Spotted fever group rickettsiae [5]. Of significant veterinary importance, this tick species is considered the primary vector of theileriosis caused by *Theileria* spp. and of babesiosis caused by *Babesia* spp. in both sheep and cattle in East Asia [6, 7]. As a three-host tick, it has a wide range of hosts, from birds and lagomorphs (as immature ticks), and large domestic and wild mammals (as adult parasites). This tick is vastly distributed throughout Eastern Asian countries such as China, Korea, Japan, New Zealand, and Australia [8].

The tick feeding process is initiated when the tick engages and attaches onto its host. As a pool feeder, upon attachment the tick determines a suitable feeding site, and prepares it by lacerating small blood vessels. Feeding occurs by sucking up the blood that flows to the pool. This feeding style triggers host defense mechanisms such as pain or itching, hemostasis, inflammatory reactions, tissue repair, and immune rejection [9–12]. To control the feeding site and counteract the host defenses, ticks secrete and inject saliva into its host, of which contains hundreds of different proteins [7, 13–16] and other pharmacologically active molecules that confer anti-hemostatic, anti-inflammatory and immunomodulatory properties, supporting blood feeding [17–20].

During the feeding process, infected ticks may transmit TBD-causing pathogens. Besides being a critical component of the feeding process, saliva has also been shown to play a role in pathogen transmission [21]. Therefore, the identification and characterization of novel *H. longicornis* saliva proteins could point to candidates for the development of anti-tick and transmission-blocking vaccines [22–26] and of new pharmacological active molecules for medical application [18, 20, 27–29].

Currently the saliva proteome of *Amblyomma americanum*, *Ixodes scapularis*, *Ornithodoros moubata*, *Rhipicephalus sanguineus* sensu lato, *R. microplus*, and *Dermacentor andersoni* [13–16, 30–33] tick species have been analyzed. However, *H. longicornis* tick saliva proteome has not been the object of any analysis. The objectives of this study were to identify secreted proteins in the saliva of fully engorged nymphs (nymphs) and fully engorged adult females (adults) of *H. longicornis* ticks, comparing the protein profile of these developmental tick stages to evaluate the variation in tick saliva during feeding of different life stages. This affords to identify tick saliva proteins shared by the two developmental stages. Such proteins may play an important role in the success of both developmental stages in their feeding cycle. The novel catalog of tick saliva proteins identified in this study provides a deeper understanding to the biology of *H. longicornis*.

Methods

Ethics statement ethical approval

All animals used in these experiments were housed in Laboratory of Veterinary Parasitology, College of Veterinary Medicine and Bio-Safety Research Institute in Chonbuk National University, Jeonju 561–756, Republic of Korea. All animal studies and protocols are in agreement with the ethical principles for animal research and approved by the Chonbuk Animal Care and Use Committee (CBNU 2015–003).

Ticks and saliva collection

The Jeju strain of the hard tick *H. longicornis* has been maintained on rabbits in our laboratory since 2003. To feed, *H. longicornis* ticks were placed onto the ears of specific pathogen-free (SPF) New Zealand White rabbits. Ticks were restricted to the ear using cloth pocket-like socks attached on ear ending with tape. Approximately 150 nymphs and 150 adults were placed in their respective feeding apparatuses and allowed to feed upon full engorgement and spontaneous detachment. Saliva was collected from 90 nymphs and 50 adults that were fully engorged and that detached from the rabbits spontaneously. Ticks were rinsed with sterile distilled water and induced to salivate by dorsal injection (posterior to fourth coxae in the region of epimeral and anal plates) of 5.0 to 7.0 μL 0.2 % dopamine or 1.5 to 3.0 μl 2 % pilocarpine (in 10 mM Tris-EDTA buffer) for nymphs and adults, respectively. Injections were applied using a micro-fine especially homemade glass needle. Then, ticks were maintained at 37 °C in an 85 % relative humidity chamber, and saliva was periodically collected for approximately 4 h using a pipette tip. Tick saliva was lyophilized and stored at −80 °C for LC-MS/MS analysis. Saliva protein concentrations were determined by Qubit-fluorometer (Life Technologies, Carlsbad, CA, USA).

Protein digestion and sample preparation

Saliva of *H. longicornis* nymphs and adult females was digested in solution with trypsin. Lyophilized salivary proteins were dissolved in 8 M urea/0.1 M Tris, pH 8.5, reduced with 5 mMTris(2-carboxyethyl)phosphine hydrochloride (TCEP, Sigma-Aldrich, St Louis, MO, USA) and alkylated with 25 mM iodoaceamide (Sigma-Aldrich). Proteins were digested overnight at 37 °C in 2 M urea/0.1 M Tris pH 8.5, 1 mM $CaCl_2$ with trypsin (Promega, Madison, WI, USA) at a final 1:20 ratio (enzyme:substrate). Digestions were stopped with formic acid (5 % final concentration) and centrifuged for debris removal.

Precolumns and analytical columns

Reversed phase pre-columns were prepared by first creating a Kasil frit at one end of a deactivated 250-μm ID/360-μm OD capillary (Agilent Technologies, Santa Clara, CA, USA). Kasil frits were prepared by dipping 20 cm capillary in 300 μL Kasil 1624 (PQ Corporation, Malvern, PA, USA) and 100 μL formamide solution, curing at 100 °C for 3 h, and cutting the frit to a length of 2 mm. Precolumns were packed in-house with 5 μm ODS-AQ C18 (YMC America, INC., Allentown, PA, USA) particles from particle slurries in methanol upon reaching the height of 2 cm. Analytical reversed phase columns were assembled by pulling a 100-μm ID/360-μm OD (Molex Polymicro Technologies™, Austin, TX, USA) silica capillary to a 5 μm ID tip. The same packing material was packed directly into the pulled column until the length of 20 cm was reached. Reversed phase precolumns and analytical columns were connected using a zero-dead volume union (IDEX Corp., Upchurch Scientific, Oak Harbor, WA, USA).

LC-MS/MS

Peptide mixtures were analyzed by nanoflow liquid chromatography mass spectrometry using an Easy NanoLC II and a Q Exactive mass spectrometer (Thermo Scientific, Waltham, MA, USA). Peptides eluted from the analytical column were electrosprayed directly into the mass spectrometer. Buffer A and B consisted of 5 % acetonitrile/0.1 % formic acid and 80 % acetonitrile/0.1 % formic acid, respectively. The flow rate was set to 400 nL/min. *H. longicornis* digested saliva samples (1.5 μg per injection) were separated in 155 min chromatographic runs, with linear gradient from 1 to 10 % of buffer B for 10 min followed by an increase to 40 % of buffer B in 100 min, an increase to 50 % of buffer B in 10 min and finally an increase to 90 % of buffer B for additional 10 min. Column was held at 90 % of buffer B for 10 min, reduced to 1 % of buffer B and re-equilibrated prior to the next injection.

The mass spectrometer was operated in a data dependent mode, collecting a full MS scan from 400 to 1200 m/z at 70,000 resolution and an AGC target of 1×10^6. The 10 most abundant ions per scan were selected for MS/MS at 17,500 resolution and AGC target of 2×10^5 and an underfill ratio of 0.1 %. Maximum fill times were 20 and 120 ms for MS and MS/MS scans, respectively, with dynamic exclusion of 15 s. Normalized collision energy was set to 25.

Data analysis

Tandem mass spectra were extracted from raw files using RawExtract 1.9.9.2 [34] and searched with ProLuCID [35] against a combined non-redundant database containing (i) Ixodidae database from NCBI; (ii) *R. microplus* transcriptome database (Rm-INCT-EM, containing 22,009 sequences produced by our research group using Illumina Sequencing technology – BioProject ID PRJNA 232001 at Transcriptome Shotgun Assembly (TSA) database – GenBank); (iii) *Oryctolagus cuniculus* database from SwissProt and (iv) reverse sequences of all database entries. Searches were done using an Integrated Proteomics Pipeline – IP2 (Integrated Proteomics Applications) for The Scripps Institute (La Jolla, CA, USA). The search space included all fully-tryptic and half-tryptic peptide candidates. Carbamidomethylation on cysteine was used as static modification. Data was searched with 50-ppm precursor ion tolerance and 20-ppm fragment ion tolerance.

The validity of the peptide spectrum matches (PSMs) generated by ProLuCID was assessed using Search Engine Processor (SEPro) [36]. Identifications were grouped by charge state and tryptic status, resulting in four distinct subgroups. For each group, ProLuCIDXCorr, DeltaCN, DeltaMass, ZScore, number of peaks matched and Spec Count Score values were used to generate a Bayesian discriminator. The identifications were sorted in a nondecreasing order according to the discriminator score. A cutoff score was established to accept a false discovery rate (FDR) of 1 % based on the number of decoys. This procedure was independently performed on each data subset, resulting in a false-positive rate that was independent of tryptic status or charge state. Additionally, a minimum sequence length of six residues per peptide was required. Results were post-processed to only accept PSMs with < 10 ppm precursor mass error.

A Volcano plot was generated by a pairwise comparison between nymphs and fully engorged female *H. longicornis* tick saliva using the TFold module from PatternLab for Proteomics platform [37]. The following parameters were used to select differentially expressed proteins: proteins were grouped by maximum parsimony, spectral count data was normalized using normalized spectral abundance factor (NSAF) [38], and two nonzero replicate values were required for each condition (at least two out of four replicates). A BH q-value was set at 0.02 (2 % FDR). A variable fold-change cutoff for each individual protein was calculated according to the *t*-test *p*-value using an F-Stringency value automatically optimized by the TFold software. Low abundant proteins were removed using an L-stringency value of 0.4.

Venn diagrams were manually generated from the output of PatternLab's Birds Eye view report. Proteins were grouped by maximum parsimony and the presence of proteins in at least two out of four replicates was required for each condition.

Functional annotation and classification

To gain insight on the nature of the identified protein sequences, BLASTp searches against several databases

were performed. To check the identity of tick saliva proteins detected, several databases were used for screening: non-redundant (NR), Acari and refseq-invertebrate from NCBI; Acari from Swissprot; the GeneOntology (GO) FASTA subset [39]; MEROPS database; and the conserved domains database of NCBI [40] containing the KOG [41], PFAM [42], and SMART motifs [43]. To check rabbit proteins identity, we used *Oryctolagus cuniculus* and refseq-vertebrates databases from NCBI; the conserved domains database of NCBI [40] containing the KOG [41], PFAM [42], and SMART motifs [43]; and the GeneOntology (GO) FASTA subset [39]. To functionally classify the protein sequences, a program written and provided by Dr. José M. C. Ribeiro in Visual Basic 6.0 (Microsoft, Redmond, Washington, USA) was used [44]. The functionally annotated catalog for each dataset was manually curated and plotted in a hyperlinked Excel spreadsheet (Additional file 1: Table S1 and Additional file 2: Table S2).

Results and discussion

H. longicornis is the primary tick known to transmit disease pathogens to humans and animals in East Asian countries [45]. Like several other blood-sucking parasites, its saliva secretion is a mixture of proteins produced in different salivary gland acinar cells [46]. Tick salivation can be studied *in vivo* by injection of dopamine, a neurotransmitter that stimulates fluid secretion by salivary gland, or by using pilocarpine, a cholinomimetic agent that induces the release of dopamine from the salivary nerves, resulting in salivation [47–49]. In *Ornithodoros moubata*, comparative analysis by SDS-PAGE of several pilocarpine- and dopamine-induced saliva batches demonstrated reproducibility between both protocols used to induce tick salivation [13]. We have tested the use of pilocarpine and/or dopamine in both adult and nymph saliva collection. However, using pilocarpine for nymph saliva collection did not result in a substantial amount of saliva. When dopamine was used in adult ticks, the saliva acquired a darker color (data not shown). This observation using dopamine for adult saliva stimulation is in accordance with results described for *R. sanguineus* s.l. [15]. However, differently from data obtained for *R. sanguineus* s.l. adult tick saliva, we successfully collected and identified proteins from *H. longicornis* nymph saliva induced by dopamine. These differences could be associated to stage and/or species of ticks used in the salivation induction. Few tick saliva proteomes have been published, most of which using adult ticks and, therefore, knowledge about nymph saliva collection is not available.

At the time study was carried out, *H. longicornis* genome and transcriptome sequences were not available, therefore we screened against available Ixodidae databases from NCBI and an in-house *R. microplus* transcriptome database to identify tick salivary proteins by shotgun proteomics. Additionally, saliva was screened for host proteins using available rabbit protein sequences from Uniprot. The comparison in proteomic content between samples from different life cycle stages may show unique or increased levels of particular proteins that are important for tick biology. Moreover, it is possible to provide information about semi-quantitative variations on the levels of the specific proteins. Comparative proteomics of spontaneously detached fully engorged nymph and fully engorged adult females *H. longicornis* have shown alterations in protein salivary content through different life cycle stages, generating new insights into tick physiology. The main objective in this study was to evaluate the *H. longicornis* saliva proteome, showing saliva protein content in nymphs, as an immature stage, and in fully engorged adult females, as an experimental end-point, evaluating not only differences, but also the similarities in saliva contents at different stages of tick development. It is important to observe that analyses of saliva of fully engorged ticks have to be carefully interpreted, since they are at the end of the feeding process. However, this analysis can provide useful information, since various categories of proteins identified in this study were also described in other tick saliva proteomes using partially engorged tick saliva [13–16], including: proteins related to heme/iron metabolism (hemelipoprotein, ferritin), proteases (cathepsin, trypsin-like, metalloprotease), protease inhibitors (serpin, cystatin, alpha-2-macroglobulin, TIL), proteins related to structural functions, transporter activity, metabolic processes, protein modification machinery (heat shock proteins), and others. In addition, other proteins from fully engorged ticks have been characterized as anticoagulant molecules, like microphilin [27], BmAP [28], and haemalin [29]. Therefore, despite the limitation in using fully engorged adult saliva, the biological interpretation of these data can provide a deeper understanding to the biology of *H. longicornis*.

An overview of identified saliva proteins

Tick saliva was obtained by dopamine and pilocarpine stimulations from *H. longicornis* nymphs and adult females, respectively. The saliva accumulated in the mouthparts was periodically collected from the ticks using a pipette tip (Fig. 1a and b). Crystal particles formed around the nymph mouthpart were also collected (Fig. 1c). Collected saliva was subjected to tryptic digestion and analyzed by shotgun proteomics in quadruplicate. A total of 135 proteins were identified matching tick databases, and 100 proteins matching rabbit database (Fig. 2 and Tables 1, 2 and 3). The identified tick proteins were classified and divided into groups according to their putative functions (Fig. 3 and Tables 1, 2 and 3), consistent with previously

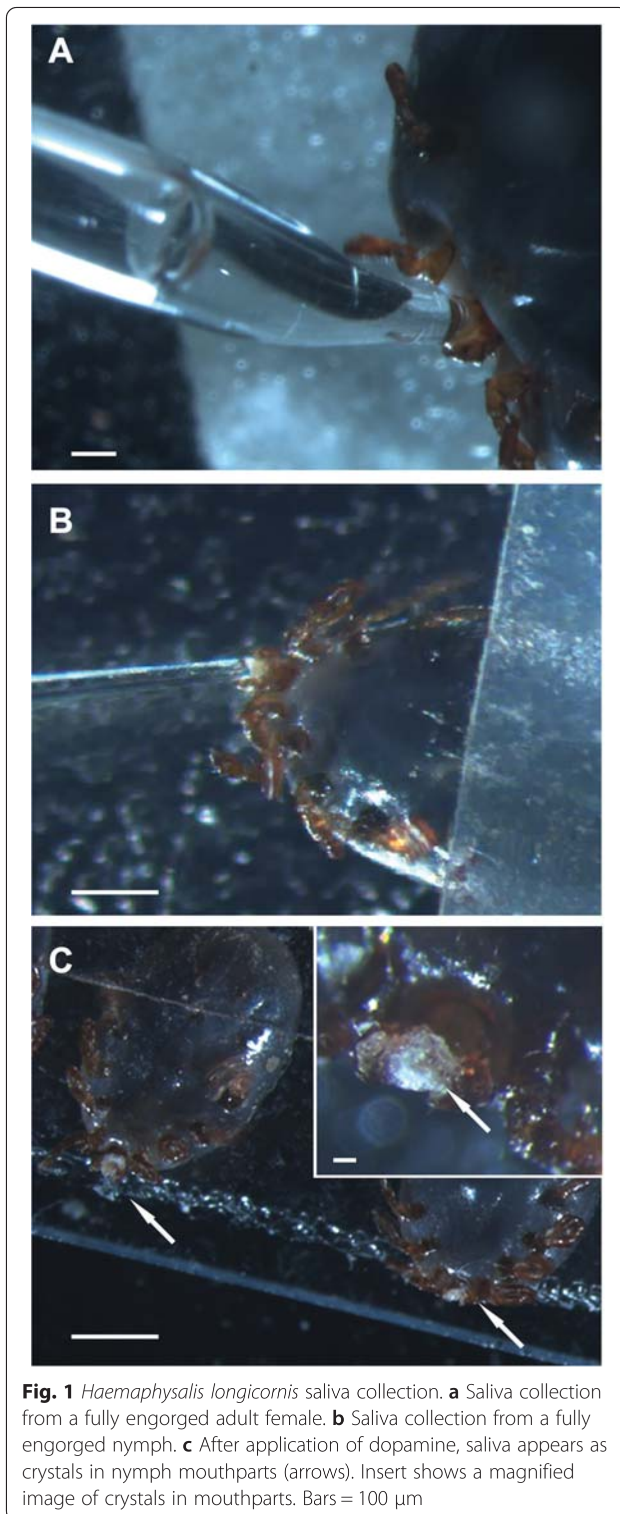

Fig. 1 *Haemaphysalis longicornis* saliva collection. **a** Saliva collection from a fully engorged adult female. **b** Saliva collection from a fully engorged nymph. **c** After application of dopamine, saliva appears as crystals in nymph mouthparts (arrows). Insert shows a magnified image of crystals in mouthparts. Bars = 100 μm

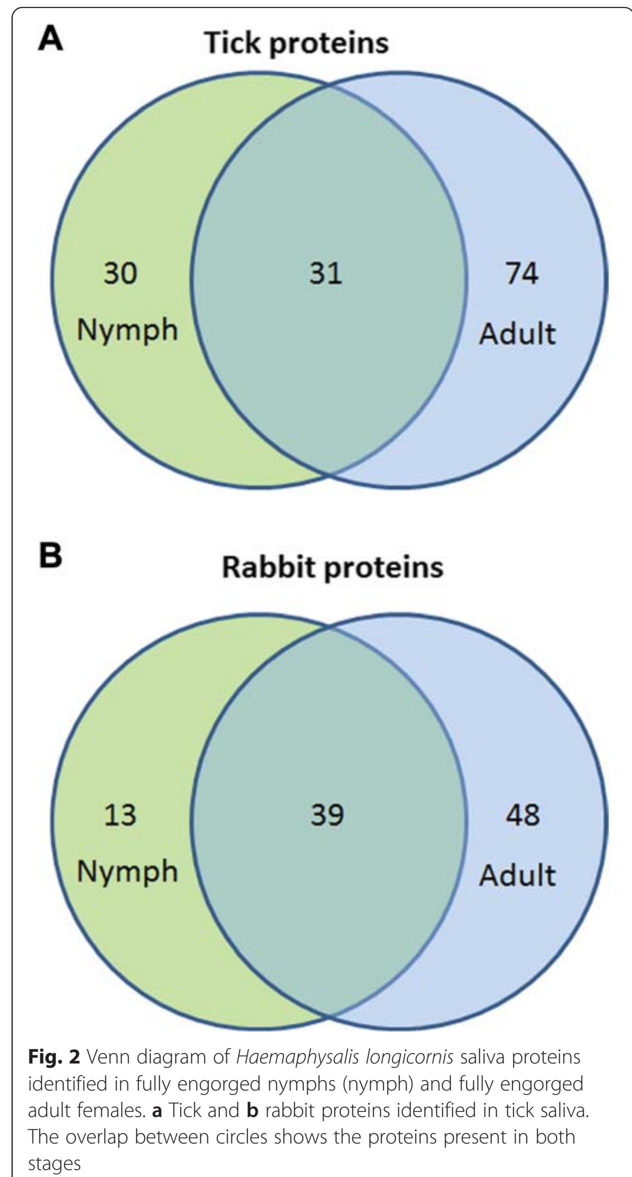

Fig. 2 Venn diagram of *Haemaphysalis longicornis* saliva proteins identified in fully engorged nymphs (nymph) and fully engorged adult females. **a** Tick and **b** rabbit proteins identified in tick saliva. The overlap between circles shows the proteins present in both stages

published tick sialomes [9]. In the set of tick specific proteins, 30 proteins were identified exclusively in nymph saliva, 74 proteins were identified exclusively in adult saliva, and 31 proteins were detected in both stages (Fig. 2). Of those 31 identified tick proteins detected in both stages, 11 had statistical differential expression confirmed (Fig. 4 and Table 4). This finding is discussed below. These proteins identified in nymph saliva as well as in adult saliva can be secreted through developmental stages throughout the tick feeding process, since proteomic studies using saliva from other tick species collected during feeding process have described the presence of similar classes of proteins as identified in this study [14–16]. Moreover, some of these proteins have been used in anti-tick vaccination experiments, e.g. glutathione S-transferase [50, 51], cystatin [52], ferritin [53, 54], serpins [23, 55–59], and hemelipoproteins [60, 61].

The identified tick proteins include (i) heme/iron metabolism-related proteins, including hemelipoproteins and ferritin; (ii) proteins related to oxidation/detoxification functions, including glutathione S-transferase, aldehyde

Table 1 Tick and host proteins identified exclusively in nymph saliva

Contig number[a]	Annotation	Class	Spec count
TICK			
BAF36722	glycine-rich cell wall structural protein	Glycine-rich protein	1.00
Rm-6837	glycine proline-rich secreted protein	Glycine-rich protein	5.25
Rm-21891	actin-depolymerizing factor	Cytoskeletal	3.00
JAA63693	substrate adhesion-dependent cell spreading	Extracellular matrix/cell adhesion	2.50
JAA55046	toll-like receptor 5	Immunity	5.00
ACX33152	catalytically inactive chitinase-like lectins	Metabolism, carbohydrate	5.00
JAA63210	lysosomal alpha-mannosidase-like isoform *X*2	Metabolism, carbohydrate	2.00
JAA54599	phospholipase A2	Metabolism, lipid	6.00
Rm-32843	lysosomal & prostatic acid phosphatase	Protein modification machinery	4.66
BAF31119	secreted protein	Secreted conserved protein	7.75
AEO33293	antigen 5/SCP	Secreted conserved protein	5.75
AEO36255	secreted protein	Secreted conserved protein	1.66
JAA60619	secreted protein	Secreted conserved protein	5.50
Rm-9069	secreted protein	Secreted conserved protein	1.50
BAE02551	lipocalin	Lipocalin	5.50
BAF43801	longipain	Proteinase	5.75
DAA34687	cathepsin L-like	Proteinase	1.50
JAA62284	tick serine protease	Proteinase	1.50
Rm-44814	serine carboxypeptidase	Proteinase	2.00
ACJ26770	alpha-2-macroglobulin	Proteinase inhibitor	27.75
EEC05896	serpin	Proteinase inhibitor	1.00
AEO34218	serpin	Proteinase inhibitor	2.33
AEO34349	serpin	Proteinase inhibitor	4.75
JAB70612	alpha-2-macroglobulin	Proteinase inhibitor	1.25
Rm-12491	serpin	Proteinase inhibitor	4.00
AEO35364	cystatin	Proteinase inhibitor	1.25
EEC02492	TGF-beta-induced protein ig-h3	Signal transduction	5.25
JAA58175	chorion peroxidase	Heme/iron metabolism	5.50
BAN13552	ferritin	Heme/iron metabolism	5.00
JAA54618	metabotropic glutamate receptor	Transporters/receptors	2.25
RABBIT			
G1T4V7	myosin tail	Cytoskeletal	6.25
G1T4A5	collagen alpha-1 (I) chain-like	Extracellular matrix/cell adhesion	4.00
P25227	alpha-1-acid glycoprotein 1	Immunity	1.66
G1TJG6	glyceraldehyde-3-phosphate dehydrogenase	Metabolism, energy	1.50
G1TFX2	alpha-1-antiproteinase	Proteinase inhibitor	10.00
Q28665	alpha-1-antiproteinase	Proteinase inhibitor	10.00
G1SDN2	keratin 17	Keratin	35.50
G1SHY2	keratin 75 - extracellular vesicular exosome	Keratin	71.00
G1SHZ4	keratin type II cytoskeletal 7	Keratin	48.50
G1SPP3	keratin type II cytoskeletal 4	Keratin	59.50

Table 1 Tick and host proteins identified exclusively in nymph saliva *(Continued)*

G1SUH1	keratin type II cytoskeletal 72 isoform X1	Keratin	7.66
G1T8T1	keratin type I cytoskeletal 28	Keratin	13.25
G1U758	keratin type I cytoskeletal 18	Keratin	16.25

[a]Protein and spectral count of host and tick proteins identified in nymph *Haemaphysalis longicornis* saliva. Annotation and accession numbers of best match identities obtained using BLASTP against several protein databases can be checked with more details in Additional file 1: Table S1 and Additional file 2: Table S2

dehydrogenase, glutathione peroxidase, and catalase; (iii) enzymes such as serine proteinases, cysteine proteinases, metalloexopeptidases, carboxypeptidases, and aspartic proteases; (iv) proteinase inhibitors of the serpin, cystatin, alpha-2-macroglobulin, trypsin inhibitor-like (TIL), and Kunitz families; (v) tick-specific protein families including lipocalin (histamine-binding proteins), glycine-rich proteins, and the group of secreted conserved proteins [9]. In addition, proteins related to cytoskeletal functions, protein modification machinery, signal transduction, transporters and receptors, metabolism of carbohydrate, energy, nucleotide, amino acids, and lipid were identified (Fig. 3 and Tables 1, 2 and 3).

Heme/iron metabolism-related proteins

The most abundant proteins identified in *H. longicornis* saliva are hemelipoproteins, which are associated with heme/iron metabolic processes. This finding is in agreement with previous studies describing hemelipoproteins in proteomic saliva of *Ornithodoros moubata*, *Rhipicephalus microplus*, *Ixodes scapularis* [13, 16, 30, 31], as well as with a study about *A. americanum* immunoproteome [32], which showed that hemelipoproteins are the major proteins in tick saliva.

Hemelipoproteins were first described as heme-binding proteins from tick hemolymph, being able to transport cholesterol, phospholipids, and free fatty acids, in addition to heme [62, 63]. These proteins are the most abundant proteins in spontaneously detached fully engorged *H. longicornis* saliva, which were relatively highly expressed in nymph saliva (Fig. 3 and Table 4). This data is in accordance with the findings observed for *R. microplus*, where hemelipoproteins were highly expressed in partially engorged adult ticks, showing a higher expression in the early developmental stages of tick feeding [16]. The physiological role of hemelipoproteins in blood meal acquisition is not completely understood. Tissue and vessel dilaceration produced by style of tick feeding and the presence of hemoglobin digestive enzymes (such as cathepsin and legumain) in tick saliva cause hemolysis and heme release in the feeding site. It is known that heme activates innate immune cells such as macrophages and neutrophils through activation of innate immune receptors [64–66], thus a role of hemelipoproteins being injected into the feeding site to prevent heme-induced inflammation is suggested. In addition, these proteins could be involved in a heme-excretory system, removing heme excess from tick and re-injecting it into the host. However, the presence of heme in tick saliva needs further investigation.

Proteinases

Several proteinases classes were identified in *H. longicornis* saliva: (i) serine proteinases; (ii) cysteine proteinases (including longipain, legumain and cathepsin L); (iii) aspartic proteinases; (iv) serine carboxypeptidases; and (v) proteins that belong to peptidase family M20 from MEROPS (including dipeptidases, and metalloexopeptidases). This set of different proteins present in tick saliva could have multiple modes of action during blood feeding. Serine proteinases may interfere with host inflammation and blood clotting. As shown by the presence of *I. scapularis* saliva protein C activator, a protein acting in the production of activated protein C, a potent anticoagulant that also regulates a myriad of inflammatory responses through protease activated receptors activation [67].

Cysteine proteinases, aspartic proteinases, serine carboxypeptidases and legumains have been described mainly as digestive enzymes with a role in hemoglobin digestion [68, 69] and pathogen transmission in ticks [70]. Thus, the presence of these enzymes in the feeding site may indicate that they act as digestive enzymes secreted into the host, digesting blood components at the tick attachment site and facilitating pathogen transmission during tick feeding. The presence of putative digestive enzymes in tick saliva was also observed in *R. microplus* [16].

In general, dipeptidases hydrolyze the late products of protein degradation to complete the conversion of proteins to free amino acids. In ticks, dipeptidases were reported to be responsible for the destruction of bradykinin, a potent pain inductor [71]. These enzymes are related to the kininase activity found in *I. scapularis* saliva, which may be responsible for the lack of host pain response subsequent to attachment and feeding [72].

Proteinase inhibitors

Host defense responses triggered by tick feeding are mainly dependent on the action of several proteinases, such as procoagulant (thrombin, factor Xa and other coagulation factors), pro-inflammatory (neutrophil elastase, proteinase-3, chymase, tryptase, kallikrein, cathepsin L, cathepsin B, cathespin S, cathepsin C, and cathepsin G) and complement enzymes (factors B, C, D, and component 2)

Table 2 Tick and host proteins identified *exclusively* in adult saliva

Contig number[a]	Annotation	Class	Spec count
TICK			
JAA54320	glycine-rich protein	Glycine-rich protein	1.50
EEC14126	radixin/ezrin/moesin	Cytoskeletal	2.00
ACX53929	putative beta thymosin	Cytoskeletal	1.33
DAA34555	microtubule-binding protein	Cytoskeletal	1.00
AEO32824	actin depolymerizing factor	Cytoskeletal	1.50
AEO33976	beta tubulin	Cytoskeletal	6.50
AFR32950	paramyosin	Cytoskeletal	5.33
JAB76162	dynein light chain	Cytoskeletal	1.00
JAB80373	myosin class i heavy chain	Cytoskeletal	12.25
AAY42205	troponin T	Cytoskeletal	1.00
Rm-1533	beta tubulin partial	Cytoskeletal	6.33
Rm-80704	myosin class ii heavy chain	Cytoskeletal	4.67
ACF35539	glutathione S-transferase	Oxidant metabolism/detoxification	6.50
ACG76272	aldehyde dehydrogenase	Oxidant metabolism/detoxification	1.00
AAQ74441	glutathione S-transferase	Oxidant metabolism/detoxification	1.50
AEO33057	sulfotransferase	Oxidant metabolism/detoxification	2.00
AEO35358	catalase	Oxidant metabolism/detoxification	8.00
JAA63098	putative aldehyde dehydrogenase	Oxidant metabolism/detoxification	3.00
Rm-14504	aldehyde dehydrogenase	Oxidant metabolism/detoxification	14.75
Rm-46289	aldehyde dehydrogenase	Oxidant metabolism/detoxification	2.00
ADN34303	cuticular protein	Extracellular matrix/cell adhesion	4.75
AEO34631	phosphoserine aminotransferase	Metabolism, amino acid	1.50
Rm-73466	homocysteine S-methyltransferase	Metabolism, amino acid	2.50
AEO32930	transketolase	Metabolism, carbohydrate	2.33
ACH88101	glyceraldehyde-3-phosphate dehydrogenase	Metabolism, energy	2.67
AEO32901	ATP synthase subunit beta	Metabolism, energy	7.75
AEO34579	3-phosphoglycerate dehydrogenase	Metabolism, energy	3.75
AEO35473	Isocitrate dehydrogenase NADP	Metabolism, energy	6.00
JAA60091	3-phosphoglycerate dehydrogenase	Metabolism, energy	3.33
JAA60302	Isocitrate dehydrogenase NADP	Metabolism, energy	7.00
JAA62224	imp cyclohydrolase/methylglyoxal synthase	Metabolism, energy	8.75
JAA66712	C-1-tetrahydrofolate synthase	Metabolism, energy	2.00
JAA68969	glyceraldehyde-3-phosphate dehydrogenase	Metabolism, energy	2.50
JAB74970	cytochrome B5	Metabolism, energy	1.33
Rm-26196	Malate dehydrogenase	Metabolism, energy	2.67
Rm-18758	15-hydroxyprostaglandin dehydrogenase	Metabolism, lipid	1.50
Rm-73635	farnesoic acid o-methyltransferase	Metabolism, lipid	1.67
JAA59537	3′(2′)5′-bisphosphate nucleotidase	Metabolism, nucleotide	1.00
JAA60240	GDP dissociation inhibitor	Metabolism, nucleotide	1.50
	Inosine-5′-monophosphate dehydrogenase	Metabolism, nucleotide	4.75
Rm-32846	purine nucleoside phosphorylase transferase	Metabolism, nucleotide	2.00
Rm-6587	SAICAR synthase	Metabolism, nucleotide	2.00
AEO35504	annexin	Protein export machinery	1.00

Table 2 Tick and host proteins identified *exclusively* in adult saliva *(Continued)*

BAF63673	protein disulfide-isomerase	Protein modification machinery	2.50
EEC14106	multifunctional chaperone	Protein modification machinery	2.50
JAA60036	heat shock protein	Protein modification machinery	1.50
JAA67522	heat shock protein hsp 90-alpha isoform	Protein modification machinery	2.50
JAB73342	methyltransferase	Protein modification machinery	1.50
JAB75807	cysteine s-methyltransferase	Protein modification machinery	2.00
Rm-24571	heat shock protein 70 cognate	Protein modification machinery	6.50
Rm-62470	heat shock-related protein	Protein modification machinery	3.75
JAA62377	gmp synthase	Protein modification machinery	2.50
AEO32591	elongation factor 1-alpha	Protein synthesis machinery	8.33
AEO32633	AAA+ ATPase	Protein synthesis machinery	1.00
Rm-50382	elongation factor 2-like	Protein synthesis machinery	4.00
AEO34773	secreted protein	Secreted conserved protein	3.00
AEO32884	lipocalin	Lipocalin	7.25
Rm-26539	lipocalin	Lipocalin	5.00
BAF51711	tick legumain	Proteinase	8.50
BAH86062	cathepsin L-like	Proteinase	2.33
JAA60171	metalloexopeptidase	Proteinase	3.00
JAA65860	CNDP dipeptidase	Proteinase	2.33
EEC20000	alpha-2-macroglobulin	Proteinase inhibitor	6.00
BAH02683	haemalin	Proteinase inhibitor	4.50
AEO35673	TIL	Proteinase inhibitor	5.00
BAG41813	cyclophilin 1	Signal transduction	3.50
AEO32842	regulatory protein mlp	Signal transduction	2.00
JAB75372	calmodulin	Signal transduction	1.67
Rm-69447	neuropeptide-like protein 31	Signal transduction	1.00
Rm-79606	Regucalcin	Signal transduction	2.50
Rm-154439	hemelipoprotein	Heme/iron metabolism	1.67
JAA62029	zinc finger protein	Transcription machinery	1.00
EEC05877	apolipophorin	Transporters/receptors	2.75
JAB78095	glutamate receptor 1-like	Transporters/receptors	1.67
RABBIT			
G1U9R8	gelsolin	Cytoskeletal	2.50
P27170	serum paraoxonase/arylesterase 1	Oxidant metabolism/detoxification	1.00
G1SR65	myeloperoxidase	Oxidant metabolism/detoxification	1.50
G1T049	collagen alpha-6(IV) chain	Extracellular matrix/cell adhesion	1.00
G1TZA1	type X collagen alpha 1-like	Extracellular matrix/cell adhesion	2.25
O77791	protein S100-A12	Immunity	4.50
P01840	Ig kappa-b4 chain C region	Immunity	10.25
P01870	Ig gamma-1 chain C region	Immunity	48.50
P04221	Ig mu chain C region	Immunity	4.00
G1SS91	complement C4-A	Immunity	3.75
G1T3X1	complement component C9	Immunity	2.00
G1TEC1	Ig kappa chain V-I	Immunity	4.75
G1TFU1	Ig kappa chain V-I	Immunity	5.25

Table 2 Tick and host proteins identified *exclusively* in adult saliva *(Continued)*

G1TKP3	immunoglobulin lambda-like	Immunity	4.00
G1TM51	Ig kappa chain V-I	Immunity	4.00
G1TPF2	T-cell surface glycoprotein CD8 -like	Immunity	5.00
G1TPZ1	galectin-1	Immunity	2.33
G1TRW8	neutrophil gelatinase-associated lipocalin	Immunity	8.50
G1TUX5	C1q and TNF related protein 7-like	Immunity	2.50
G1TVN7	Ig kappa chain V-I	Immunity	5.75
G1TVZ5	T-cell surface glycoprotein CD8 -like	Immunity	1.50
Q9GK63	mammaglobin-B	Immunity	2.00
Q9GK67	secretoglobin	Immunity	3.67
U3KM01	Ig kappa chain V-I	Immunity	4.00
G1SQA8	ATP synthase mitochondrial precursor	Metabolism, energy	3.50
G1TS29	triosephosphate isomerase	Metabolism, energy	1.33
G1TYA7	L-lactate dehydrogenase B chain	Metabolism, energy	2.67
G1TB24	peroxysomal Fatty Acyl CoA Transporter	Metabolism, lipid	1.50
G1SN21	purine nucleoside phosphorylase	Metabolism, nucleotide	2.00
G1TXI1	nucleophosmin	Metabolism, nucleotide	3.33
G1U9T4	nucleoside diphosphate kinase B	Metabolism, nucleotide	3.33
G1U155	histone H2B type 1-like	Nuclear regulation	3.00
P51662	annexin A1	Protein export machinery	5.50
G1TDI4	transthyretin	Protein export machinery	3.00
G1SJZ9	thioredoxin	Protein modification machinery	1.33
G1SEK8	metalloendopeptidase inhibitor	Proteinase inhibitor	6.75
G1SIK0	antithrombin III	Proteinase inhibitor	8.67
G1SZA4	inter-alpha-trypsin inhibitor heavy chain H1	Proteinase inhibitor	2.00
G1TM88	alpha-1-antitrypsin	Proteinase inhibitor	2.33
G1U6R8	negative regulation of endopeptidase	Proteinase inhibitor	8.25
Q45GR2	alpha-2-antiplasmin	Proteinase inhibitor	3.50
G1SNP6	serine/threonine-protein kinase	Signal transduction	1.50
P09809	apolipoprotein A-I	Heme/iron metabolism	4.25
G1TFW8	lactotransferrin	Heme/iron metabolism	5.75
G1SU82	vitamin D-binding protein	Transporters/receptors	4.75
G1T4K1	sodium channel protein type 3 isoform X1	Transporters/receptors	2.00
G1TP66	keratin type II cuticular Hb3	Keratin	1.50
P00919	carbonic anhydrase 2	Hemoglobin/RBC products	3.50

[a]Protein and spectral count of host and tick proteins identified in adult *Haemaphysalis longicornis* saliva. Annotation and accession numbers of best match identities obtained using BLASTP against the several protein databases can be checked with more details in Additional file 1: Table S1 and Additional file 2: Table S2

[9–12]. Proteinases from these pathways are regulated by specific endogenous inhibitors, maintaining homeostasis. From this perspective, proteinase inhibitors secreted into the feeding site act by disrupting host defenses, facilitating blood meal acquisition. Several families of protease inhibitor domains were found in saliva of *H. longicornis* (Tables 1, 2 and 3).

Serpins are a superfamily of serine proteinase inhibitors involved in blood coagulation, fibrinolysis, inflammation, and complement activation in mammals [73, 74]. These proteins were found mostly in nymph saliva (Table 1). Tick serpins are secreted into the feeding site to disrupt host defenses against tick feeding, including anticoagulant [75–77] and immunomodulatory responses [78–80]. The potential effect of these proteins on host systems is supported by several studies that demonstrate the mortality and reduced feeding efficiency when several tick species were fed on host immunized with tick recombinant

Table 3 Tick and host proteins identified in nymph and adult saliva

Contig number	Annotation	Class	Spec count	
			Nymph	Adult
TICK				
ABQ96858	tropomyosin	Cytoskeletal	3.00	5.25
BAF98180	actin	Cytoskeletal	21.75	18.75
AEO32669	alpha tubulin	Cytoskeletal	3.25	11.00
AGC13075	glutathione peroxidase	Oxidant metabolism/detoxification	10.50	4.33
AEO34612	enolase	Metabolism, carbohydrate	2.00	4.25
Rm-10851	ATP synthase subunit alpha	Metabolism, energy	1.50	4.67
AEO34838	histone H4	Nuclear regulation	5.75	7.33
AEO34879	histone H2A	Nuclear regulation	4.00	10.00
AEO32095	heat shock 70 kDa protein	Protein modification machinery	5.50	5.67
AEO32791	heat shock protein	Protein modification machinery	9.75	11.00
AEO34048	protein disulfide-isomerase A6-like	Protein modification machinery	1.50	1.00
JAA62581	heat shock protein	Protein modification machinery	3.00	3.50
ADG86641	lysosomal acid phosphatase	Protein modification machinery	9.50	26.75
JAA73257	ubiquitin/40s ribosomal protein s27a	Proteasome machinery	17.25	4.75
ADK47399	secreted protein	Secreted conserved protein	2.67	3.00
AGH08176	AV422	Secreted conserved protein	4.75	4.25
BAE53722	aspartic protease	Proteinase	4.00	3.25
BAD11156|	serpin	Proteinase inhibitor	15.50	3.50
JAA60430	alpha-2-macroglobulin	Proteinase inhibitor	41.00	12.33
JAA64973	alpha-2-acroglobulin	Proteinase inhibitor	17.50	3.50
Rm-7619	alpha-2-macroglobulin	Proteinase inhibitor	26.25	9.00
ABZ89554.	cystatin	Proteinase inhibitor	6.33	2.67
Rm-69112	14–3–3 zeta	Signal transduction	4.33	2.33
BAG12081	hemelipoprotein	Heme/iron metabolism	796.25	323.25
BAJ21514	hemelipoprotein	Heme/iron metabolism	405.00	224.00
BAJ21515	hemelipoprotein	Heme/iron metabolism	98.50	6.25
BAL42280	hemelipoprotein	Heme/iron metabolism	141.75	60.75
JAA59652	hemelipoprotein	Heme/iron metabolism	19.67	14.25
ABD83654	hemelipoprotein	Heme/iron metabolism	12.50	12.25
Rm-72548	hemelipoprotein	Heme/iron metabolism	32.25	14.75
JAA61676	plexins functional semaphorin receptor	Transporters/receptors	12.50	2.50
RABBIT				
G1T229	filaggrin-2	Cytoskeletal	17.00	1.50
G1T6W7	catalase	Oxidant metabolism/detoxification	5.25	7.50
P16973	lysozyme C	Immunity	3.00	7.50
P25230	antimicrobial protein CAP18	Immunity	1.50	5.33
P50117	protein S100-A9	Immunity	3.75	3.00
G1SUZ1	complement C3	Immunity	5.25	15.00
G1SYM4	alpha-1B-glycoprotein	Immunity	3.75	3.00
G1THZ6	Ig gamma-1 chain C region	Immunity	15.00	62.00
G1T0Z2	histone H2A type 1-A	Nuclear regulation	4.00	5.67
G1T9M9	heat shock cognate 71 kDa protein	Protein modification machinery	9.00	10.00

Table 3 Tick and host proteins identified in nymph and adult saliva *(Continued)*

G1SQ70	alpha-2-macroglobulin	Proteinase inhibitor	10.75	32.50
G1TFV7	alpha-1-antiproteinase	Proteinase inhibitor	10.25	37.25
Q07298	alpha-1-antiproteinase	Proteinase inhibitor	10.00	32.67
P19134	serotransferrin	Heme/iron metabolism	33.75	95.25
P20058	hemopexin	Heme/iron metabolism	12.00	36.50
G1SQ02	peroxiredoxin-1	Heme/iron metabolism	3.00	3.25
G1SWF6	haptoglobin - hemoglobin binding	Heme/iron metabolism	7.50	20.00
G1TVS4	hemopexin	Heme/iron metabolism	12.00	38.25
G1U9S2	serum albumin	Heme/iron metabolism	156.00	665.50
G1SGQ5	alpha-2-HS-glycoprotein	Transporters/receptors	3.00	1.50
G1SLY0	HCO3- transporter family	Transporters/receptors	1.50	3.00
U3KMC6	ceruloplasmin	Transporters/receptors	3.67	8.50
G1SKE3	keratin type II cytoskeletal 6A	Keratin	84.50	20.00
G1SUH8	keratin 2	Keratin	228.75	52.00
G1SWB8	keratin type I cytoskeletal 27	Keratin	23.67	14.00
G1SY72	keratin type II cytoskeletal	Keratin	68.50	17.33
G1T1V0	keratin type I cytoskeletal 10	Keratin	108.50	58.50
G1T1Y7	keratin type I cytoskeletal 14 isoform X1	Keratin	56.00	12.50
G1T4R6	keratin type I cytoskeletal 16	Keratin	57.50	13.25
G1T4S1	keratin 15	Keratin	51.75	17.00
G1TDN6	keratin type II cytoskeletal 5	Keratin	99.75	14.75
G1U754	histidine-rich glycoprotein	Heme/iron metabolism	11.75	25.25
G1U9I8	keratin type II cytoskeletal 1	Keratin	104.25	39.75
G1T0W8	fibrinogen beta chain	Fibrinogen	5.25	26.50
G1T0X2	fibrinogen alpha chain	Fibrinogen	9.00	13.50
G1TKX3	fibrinogen gamma chain	Fibrinogen	6.25	34.75
P01948	hemoglobin subunit alpha-1/2	Hemoglobin/RBC products	87.00	544.00
P02057	hemoglobin subunit beta	Hemoglobin/RBC products	100.75	517.50
P07452	carbonic anhydrase 1	Hemoglobin/RBC products	5.00	11.00

[a]Protein and spectral count of host and tick proteins identified both in nymph and adult *Haemaphysalis longicornis* saliva. Annotation and accession numbers of best match identities obtained using BLASTP against several protein databases can be checked with more details in Additional file 1: Table S1 and Additional file 2: Table S2

serpins [55–59]. Additionally, serpins in *Aedes aegypti* and *Anopheles stephensi* have been shown to play a role in pathogen transmission [81, 82].

Cystatins form a large superfamily of reversible and tight-binding inhibitors that interact with papain-like cysteine proteases and legumains [83]. Tick salivary cystatins have been described as immunosuppressive and anti-inflammatory proteins [84–87]. Moreover, the importance of cystatins in tick physiology was observed in studies that showed that neutralization of cystatin reduces tick feeding ability [52, 85, 88, 89].

Haemalin is a member of Kunitz-type inhibitors identified in *H. longicornis* saliva. This protein has been described as a thrombin inhibitor, delaying bovine plasma clotting time and inhibiting both thrombin-induced fibrinogen clotting and thrombin-induced platelet aggregation. This protein was described as a midgut protein [29], and this is the first time it is described in *H. longicornis* saliva. Taking into account haemalin function, we suggested that this protein acts as an anticoagulant salivary protein during tick feeding.

TIL (trypsin inhibitor-like) proteins have been reported in several tick sialomes [9, 10] and are described as elastase inhibitors, which also have antimicrobial activity [90, 91]. Alpha-2-macroglobulin are a group of proteins that have been found to inhibit several serum proteinases in vertebrates, including thrombin, factor Xa and kallikreins [77, 92–94], mediating T-cell proliferation and activating macrophages [95, 96]. Thus, as these proteins are secreted both in nymph and in adult tick

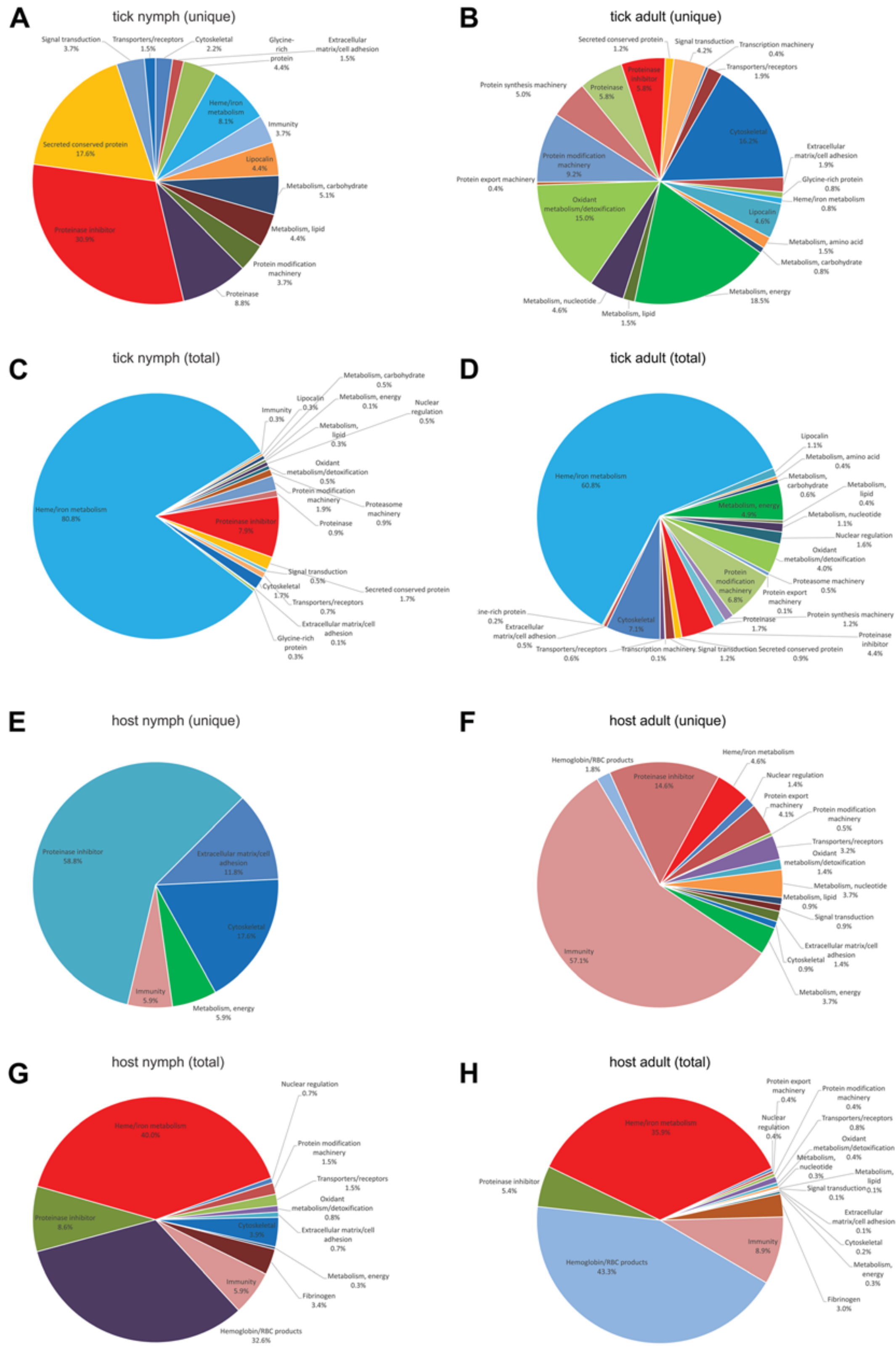

Fig. 3 (See legend on next page.)

(See figure on previous page.)
Fig. 3 Functional classification of *Haemaphysalis longicornis* salivary proteins identified in fully engorged nymph (nymph) and fully engorged adult females (adult). **a** Tick proteins detected in nymph (**a** and **c**) and adult (**b** and **d**). Proteins were divided as detected both in nymph (**c**) and adult (**d**) and proteins detected exclusively in nymph (**a**) or adult (**b**) and classified in groups according to their function and/or protein family. Pie charts represent the percentage of proteins found in each group with respect to normalized spectral counting for each sample. **b** Host proteins detected in nymph (**e** and **g**) and adult (**f** and **h**). Proteins were divided as detected both in nymph (**g**) and adult (**h**) and proteins detected exclusively in nymph (**e**) or adult (**f**) and classified in groups according to their function and/or protein family. Pie charts represent the percentage of proteins found in each group with respect to normalized spectral counting for each sample. Keratin was removed before data interpretation. (Additional file 3: Figure S1 shows data with keratin)

saliva, they could act as anticoagulant and/or immunomodulatory proteins during blood feeding.

Tick-specific protein families

Advances in transcriptomic and proteomic studies of tick salivary gland have created new opportunities to identify the variety of tick salivary transcripts and proteins. Many proteins are described to have no similarities to non-tick proteins from the NCBI database [9]. The secreted conserved protein group is composed mainly of tick proteins containing a signal peptide predicted and with similarities to proteins identified in other ticks [9]. Most of proteins included in this group have unknown functions and were described only in gene or protein sequencing projects, having their expression up-regulated after blood acquisition [97, 98]. A functionally described member of tick secreted conserved protein group is *A. americanum* AV422 protein. This protein was first described as an up-regulated protein in response to tick host exposure and/or to feeding stimuli in rabbits [99]. AV422 is secreted into the host during tick feeding, acting as an anticoagulant and anti-complement protein [100, 101]. In *H. longicornis*, this protein is identified in both the spontaneously detached fully engorged nymph and adult saliva (Table 3). Additionally, an AV422-like protein was identified in partially and fully engorged *R. microplus* female saliva proteome [16]. Based on the high amino acid conservation and on its presence in other tick proteomes, it will be interesting to determine whether or not *H. longicornis* AV422-like protein is functionally similar to that of *A. americanum*.

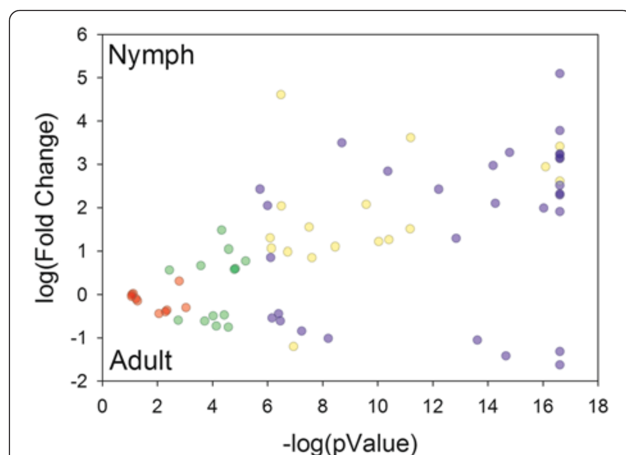

Fig. 4 Volcano plot abundance changes analysis of *Haemaphysalis longicornis* saliva proteins identified both in fully engorged nymph (nymph) and fully engorged adult females (adult). Each point represents the difference in expression (log2 fold difference) between nymph and adult plotted against the level of statistical significance. Proteins represented by (blue dot) had an identification that satisfied both fold and statistical criteria; (yellow dot) had an identifications that was filtered out by the L-stringency; (green dot) had an identification satisfied the fold criteria but, most likely, this happened by chance; and (red dot) had identification did not meet the fold and *p*-value criteria

The antigen 5 protein family is a group of cysteine-rich secreted proteins [102]. This group is described in the salivary glands of blood-sucking insects and ticks, with functions that remain mostly unknown [10, 103, 104], being identified exclusively in nymph saliva (Table 1). Glycine-rich proteins are extracellular matrix proteins and/or structural proteins with an important role in attachment to the host, since it they are present in cement material secreted by salivary glands during feeding process [105, 106]. The expression levels of these proteins are associated with size of mouthparts and the number of hosts used by tick during its life cycle. Ticks with short mouthparts and one-host ticks express more glycine-rich protein. It could be implied that one-host ticks are more consistently pressed to sustain attachment on the host's skin [107]. In this sense, glycine-rich proteins were identified mostly in nymph saliva (Table 1). These proteins are also involved in the defense against pathogens, since they are inducible antibacterial proteins predominantly active by Gram-negative bacteria presents in insect and tick hemolymph and salivary gland [108].

Tick proteins of the lipocalin family are classified based on their homology with tick proteins containing the characteristic tick histamine-binding domain (PF02098) [109, 110]. They are a group of multifunctional secreted proteins that bind several types of small hydrophobic ligands. The role as transport proteins is well studied, however it is clear that the arthropod lipocalin family is involved in various other physiological functions such as cell growth and metabolism, regulation of the immune response, and tissue repair [111]. Lipocalins were detected in several tick saliva proteomes [13, 16], and *H. longicornis*

Table 4 Differential abundance of nymph and adult saliva proteins (determined using the TFold)

Annotation	Contig number[a]	Class	Fold change[b]	*p* value
TICK				
Actin	BAF98180	Cytoskeletal	2.485	0.0001
glutathione peroxidase	AGC13075	Oxidant metabolism/detoxification	5.464	0.0002
histone H4	AEO34838	Nuclear regulation	1.845	0.0145
ubiquitin/40s ribosomal protein s27a	JAA73257.1	Proteasome machinery	7.980	0.0000
serpin	BAD11156	Proteinase inhibitor	8.991	0.0000
cystatin	ABZ89554	Proteinase inhibitor	5.470	0.0190
hemelipoprotein	BAG12081	Heme/iron metabolism	5.089	0.0000
hemelipoprotein	BAJ21514	Heme/iron metabolism	3.823	0.0000
hemelipoprotein	BAJ21515	Heme/iron metabolism	34.697	0.0000
hemelipoprotein	BAL42280	Heme/iron metabolism	4.997	0.0000
hemelipoprotein	Rm-72548	Heme/iron metabolism	4.371	0.0000
RABBIT				
Ig gamma-1 chain C region	G1THZ6	Immunity	-1.975	0.0034
alpha-1-antiproteinase	G1TFV7	Proteinase inhibitor	-1.757	0.0066
serotransferrin	P19134	Heme/iron metabolism	-1.328	0.0119
hemopexin	P20058	Heme/iron metabolism	-1.423	0.0140
hemopexin	G1TVS4	Heme/iron metabolism	-1.493	0.0113
serum albumin	G1U9S2	Heme/iron metabolism	-2.028	0.0000
keratin type II cytoskeletal 6A	G1SKE3	Keratin	8.889	0.0000
keratin 2	G1SUH8	Keratin	9.480	0.0000
keratin type I cytoskeletal 27	G1SWB8	Keratin	4.210	0.0157
keratin type II cytoskeletal	G1SY72	Keratin	9.613	0.0000
keratin type I cytoskeletal 10 -	G1T1V0	Keratin	4.046	0.0000
keratin type I cytoskeletal 14	G1T1Y7	Keratin	11.478	0.0024
keratin type I cytoskeletal 16	G1T4R6	Keratin	9.827	0.0000
keratin 15	G1T4S1	Keratin	7.271	0.0007
keratin type II cytoskeletal 5	G1TDN6	Keratin	13.976	0.0000
keratin type II cytoskeletal 1	G1U9I8	Keratin	5.796	0.0000
fibrinogen gamma chain	G1TKX3	Fibrinogen	-2.612	0.0000
hemoglobin subunit alpha-1/2	P01948	Hemoglobin/RBC products	-3.021	0.0000
hemoglobin subunit beta	P02057	Hemoglobin/RBC products	-2.440	0.0000
TICK				
heat shock protein	AEO32791	Protein modification machinery	1.839	0.0051
secreted protein	ADK47399	Secreted conserved protein	2.127	0.0143
AV422	AGH08176	Secreted conserved protein	2.434	0.0007
aspartic protease	BAE53722	Proteinase	2.506	0.0147
alpha-2-macroglobulin	JAA60430	Proteinase inhibitor	7.808	0.0000
alpha-2-acroglobulin	JAA64973	Proteinase inhibitor	12.469	0.0004
alpha-2-macroglobulin	Rm-7619	Proteinase inhibitor	6.210	0.0000
14-3-3 zeta	Rm-69112	Signal transduction	4.292	0.0013
hemelipoprotein	JAA59652	Heme/iron metabolism	2.899	0.0004
hemelipoprotein	ABD83654	Heme/iron metabolism	2.186	0.0028
plexins functional semaphorin	JAA61676	Transporters/receptors	10.845	0.0000

Table 4 Differential abundance of nymph and adult saliva proteins (determined using the TFold) *(Continued)*

RABBIT				
filaggrin-2	G1T229	Cytoskeletal	24.880	0.0112
protein S100-A9	P50117	Immunity	2.361	0.0009
alpha-1B-glycoprotein	G1SYM4	Immunity	2.983	0.0055
peroxiredoxin-1	G1SQ02	Heme/iron metabolism	2.017	0.0094
alpha-2-HS-glycoprotein	G1SGQ5	Transporters/receptors	4.184	0.0111
fibrinogen beta chain	G1T0W8	Fibrinogen	-2.248	0.0081
TICK				
heat shock 70 kDa protein	AEO32095	Protein modification machinery	2.101	0.0418
alpha tubulin	AEO32669	Cytoskeletal	-1.649	0.0422
protein disulfide-isomerase	AEO34048	Protein modification machinery	2.844	0.0498
heat shock protein	JAA62581	Protein modification machinery	1.620	0.0842
ATP synthase subunit alpha	Rm-10851	Metabolism energy	-1.475	0.1486
RABBIT				
antimicrobial protein CAP18	P25230	Immunity	-1.493	0.0764
alpha-2-macroglobulin	G1SQ70	Proteinase inhibitor	-1.357	0.0467
complement C3	G1SUZ1	Immunity	-1.377	0.0617
fibrinogen alpha chain	G1T0X2	Fibrinogen	1.546	0.0353
histone H2A type 1-A	G1T0Z2	Nuclear regulation	1.509	0.1858
catalase	G1T6W7	Oxidant metabolism/detoxification	1.525	0.0362
heat shock cognate 71 kDa	G1T9M9	Protein modification machinery	1.747	0.0272
alpha-1-antiproteinase	Q07298	Proteinase inhibitor	-1.618	0.0571
TICK				
Tropomyosin	ABQ96858	Cytoskeletal	1.264	0.1452
lysosomal acid phosphatase	ADG86641	Protein modification machinery	-1.256	0.1979
Enolase	AEO34612	Metabolism, carbohydrate	1.035	0.4617
histone H2A	AEO34879	Nuclear regulation	-1.327	0.2417
RABBIT				
carbonic anhydrase 1	P07452	Hemoglobin/RBC products	-1.048	0.4302
lysozyme C	P16973	Immunity	-1.287	0.2053
HCO3- transporter family	G1SLY0	Transporters/receptors	1.020	0.4774
haptoglobin - hemoglobin binding	G1SWF6	Heme/iron metabolism	-1.205	0.1225
histidine-rich glycoprotein	G1U754	Heme/iron metabolism	-1.006	0.4825
Ceruloplasmin	U3KMC6	Transporters/receptors	-1.081	0.4154

Blue: identifications that satisfied both fold and statistical criteria
Yellow: identifications were filtered out by the L-stringency
Green: identifications satisfied the fold criteria but, most likely, this happened by chance
Red: identifications did not meet the fold and *p*-value criteria
[a]Accession numbers of best matches identities obtained using BLASTP against the non-redundant protein database in GenBank
[b]Positive number means the fold increased expression in nymph (relation between nymph and adult). Negative number means the fold increased expression in adults (relation between adult and nymph)

saliva contains at least three proteins described as lipocalin. The presence of lipocalins in tick saliva is related to control of inflammatory processes and interference with host hemostatic functions [112–114]. Furthermore, the importance of lipocalins in nymph and adult saliva has been demonstrated, since the expression of lipocalins in

insects and ticks is up-regulated in response to injury [115], as well as to viral [116] or bacterial infections [117], enhancing tick immune responses and resilience to infection.

Detoxification/oxidation

In nymph and adult saliva, various proteins involved in processes of detoxification and redox buffering were detected, including glutathione S-transferase, aldehyde dehydrogenase, sulfotransferase, and catalase. These proteins could detoxify oxidants generated during blood meal acquisition and/or host oxidants associated with inflammation. The antioxidant expression levels differ throughout arthropod development [118]. In *H. longicornis* saliva, the abundance of antioxidant proteins is higher in adults, when compared to nymphs (Tables 1, 2 and 3). A glutathione peroxidase was the only protein of this class identified in nymph saliva. This protein has been well characterized for its antioxidant and anti-inflammatory activity in mammals [119], and could be related as an immunomodulatory protein from *H. longicornis* saliva. Sulfotransferase may inactivate dopamine, the secretagogue found in the salivary gland of ticks [120]. Glutathione S-transferase expression has been associated with resistance to acaricides and insecticides in many species [121, 122]. In addition, it has been proposed that GST secreted by parasites has immunomodulatory activity due to the alteration of the cytokine gene expression profile, modulation of immune cell proliferation, and decrease in oxidative ability of phagocytes [123, 124]. The roles of GST and other proteins in the detoxification of endogenous toxin preventing and repairing the damage of ROS generated by hemoglobin degradation has been described [125], and the induction of the expression of these proteins in response to oxidative stress have been observed, supporting the antioxidant physiological role [126, 127]. Furthermore, immunization experiments showed the potential use of tick GST to protect hosts against tick infestation [50, 51].

Cytoskeletal

Proteins in *H. longicornis* saliva associated with cytoskeletal and structural cellular function were identified, including actin, tubulin, paramyosin, among others, which are fundamental to intracellular transport and cellular division. Notably, these proteins were abundantly expressed in adult saliva, when compared to nymph saliva, suggesting that the presence of such proteins has a physiological explanation, as opposed to tick/host tissue contamination during saliva collection (Table 4). Since these are intracellular proteins, and considering that most lack signal peptides, it may be hypothesized that they are released as consequence of damage, degeneration, or apoptosis of salivary gland acines [128, 129]. Furthermore, the presence of apocrine secretion in tick salivary gland is described [130]. Another structural protein has been identified in *R. microplus* saliva is paramyosin. This protein is secreted in saliva and recognized during the tick infestation, further suggesting that it may possess additional, non-muscle functions in the tick-host relationship [131]. Troponin I-like molecule was detected in *H. longicornis* saliva, with angiogenesis inhibitor activity impairing host tissue repair and helping the tick feeding process [132]. These observations suggested that these proteins could have roles other than structural functions.

Metabolism

A wide variety of enzymes and proteins related to carbohydrate, lipid and amino acid metabolism and to the energetic pathways was observed in *H. longicornis* saliva. This finding is in accordance with other tick saliva proteomes. Similarly to cytoskeletal proteins, proteins that belong to this class are predominant in adult tick saliva (Table 1, 2 and 3). Although functional activity of these proteins remains unknown concerning tick feeding, the activity of some proteins related to metabolism has been characterized.

A salivary enolase from *O. moubata* was described acting as a plasminogen receptor, and may play a role stimulating host fibrinolysis and maintaining blood fluidity during tick feeding [133]. Similar activity was described for enolases from other parasites [134, 135].

Another group of metabolism-related proteins with a possible role in tick feeding is formed by chitinases. Chitinases are either active or inactive, based on the functional domain. Active chitinases are mostly described as responsible for the hydrolytic cleavage of the β-glycosidic linkages between GlcNAc residues of chitin, involved in molting and growth of arthropods [136]. On the other hand, inactive chitinases were suggested to be involved in the maintenance of a stable feeding site and in the activity of a potential immunoglobulin G binding protein in *A. americanum* [137]. It is interesting to note that we show the presence of a putative inactive chitinase only in nymphs (Table 1). However, previous studies showed the presence of an inactive chitinase in adults, as a secreted saliva protein [16, 138].

Enzymes of the o phospholipase A2 family play important roles in phospholipid digestion, rearrangement of cellular membrane phospholipid structures, inflammatory responses, defense and predation mechanisms, and signal transduction [139]. These enzymatic activities have been identified in tick saliva, and are speculated to stimulate tick prostaglandin E2 production [140]. Tick saliva proteomic studies have identified these proteins [16], pointing to their presence in tick saliva. A phospholipase A2 was identified exclusively in nymph saliva (Table 1). These proteins could provide the anti-

inflammatory, anti-hemostatic, and vasodilator activity required for long-term blood feeding.

It should be noted that these metabolism-related proteins have been identified in tick saliva, and may be classified as moonlighting proteins, so they can have other distinct functions [141, 142]. Several studies on moonlighting proteins are being carried out, and the discovery of new functions could afford deeper insights into metabolism-related proteins in tick feeding physiology.

Differential expression between nymph and adult female saliva proteins

Ixodid ticks begin attachment by cutting into the host skin, followed by secretion of cement, a process that may take from one to two days. When completely affixed to the wound site, these ticks feed slowly from the pooled blood formed, for several days [143–145]. During feeding, the salivary gland of adult ixodid ticks undergoes remarkable growth and differentiation, which is accompanied by significant increases in the rate of proteins synthesis [46, 146]. It has been proposed that different tick feeding conditions might affect salivary gland transcription of hard ticks. This feature is related to different vertebrate host exposure and distinct developmental stages, leading to changes in salivary transcription dynamics, as shown previously [14, 16, 32, 99, 147–150].

As shown in other tick saliva proteomes [14, 16], the protein content of tick saliva in different developmental stages varies, such as in this case from nymph to adult female ticks in *H. longicornis*. Of the 135 proteins detected in *H. longicornis* saliva, 30 proteins were identified in nymph saliva, 74 proteins were identified in fully engorged adult females, and 31 were detected in both stages (Fig. 2, Tables 1, 2 and 3). Nymph-specific proteins are represented mostly by proteinase inhibitors and a secreted conserved proteins group (Fig. 3 and Table 1), while adult specific proteins are represented primarily by proteins related to energy metabolism, oxidant/detoxification metabolism, and cytoskeleton (Fig. 3 and Table 2). This finding in adult saliva may be related to salivary gland degeneration starting after adult ticks detach from the host [128, 151].

Changes in expression levels were observed for 11 of the 31 identified tick proteins detected in both stages. The changes in the protein secretion were determined by pairwise comparison between nymphs and fully engorged *H. longicornis* female tick saliva using the TFold module from PatternLab for Proteomics platform [37], and were shown to be statistically significant (Fig. 4 and Table 4). The range in fold change was shown to be greater in the specific-tick proteins than in host protein secreted in nymph saliva. The most significantly affected nymph up-regulated proteins were a hemelipoprotein (up-regulated 34.69-fold), a serpin (up-regulated 8.99-fold), a ribosomal protein s27a (up-regulated 7.98-fold), a cystatin (up-regulated 5.47-fold), and a glutathione peroxidase (up-regulated 5.46-fold). The physiological meaning of these differences is not clear. However, as discussed previously, hemelipoproteins are known by their heme-binding function [63], and expression in early developmental stages of tick feeding observed here is in accordance with that found in *R. microplus* [16]. Proteinase inhibitors were described as early stage secreted saliva proteins in tick, with anticoagulant and immunomodulatory properties, modulating host defense systems trigged against tick feeding [14, 16, 75, 76, 78, 87]. This observation can be related to the nature of the blood feeding process, throughout the different stages of tick as they have to use unique proteins to counteract host defenses, especially for fully engorged nymphs, which need to molt into adults and prepare to feed on another host.

Host proteins

As shown previously for other tick species [13, 15, 16, 30, 31], a large number of host proteins were identified both in nymph and adult *H. longicornis* saliva (Table 1, 2 and 3). It was demonstrated that ticks transport intact proteins across the digestive system to the hemolymph. After blood ingestion, host blood proteins such as albumin and immunoglobulin cross the midgut epithelium of ticks, and are detected in tick tissues, including secretion into saliva [15, 152, 153]. This finding suggests that the presence of host proteins in tick saliva may be a real and common recycling system present in ticks, not a result of contamination during saliva collection.

In *H. longicornis* saliva, 100 proteins matched the rabbit database. Host identified proteins in tick saliva included proteins related to (i) immunity, such as immunoglobulins, complement system proteins, and antimicrobial proteins; (ii) heme/iron metabolism-related proteins, like serum albumin, peroxiredoxin, serotransferrin, apolipoprotein, and hemopexin; (iii) proteinase inhibitors of the serpin and alpha-2-macroglobulin superfamilies; and (iv) hemoglobin/red blood cells-related products. A set of 13 rabbit proteins was found only in nymph saliva samples, mostly rabbit keratin (Table 4, Fig. 3, and Additional file 3: Figure S1), suggesting that host keratin from tick mouthparts reached saliva during collection, since cleaning of host tissue in nymphs' mouthparts is more critical than in adults, due to its smaller size. Forty-eight rabbit proteins were only found in this adult saliva, and 30 rabbit proteins were present in both samples (Fig. 2).

The presence of different classes of host proteins in the saliva of the two tick developmental stages suggests the existence of a selective uptake process for host proteins (Fig. 3 and Additional file 3: Figure S1, Tables 1, 2, and 3) as observed in other studies [15, 16]. Furthermore,

the relationship between concentrations of these proteins in saliva is different from that observed in host blood. This data is similar to findings observed in other tick species [30, 31, 153, 154]. An explanation for the presence of host proteins in tick saliva is that ticks recycle pivotal host proteins in order to subvert their role in the host and/or using host proteins in specific tick physiologic systems. Utilization of host hemoglobin as substrate to generate antimicrobial peptides against microorganisms was observed in *R. microplus* [155] and *O. moubata* [156]. As observed in *R. microplus*, here we found the same profile for proteins related to heme/iron metabolism. While the major tick heme-binding protein is secreted into saliva from nymphs, its expression decreases in adults. Reduction of heme-binding proteins in adults was accompanied by an increase in the host heme-binding proteins serum albumin, hemopexin, apolipoprotein, and peroxiredoxin (Fig. 3, Table 1, 2 and 3). These observations could suggest that the tick replaces hemelipoproteins by host derived heme-binding proteins, since hemelipoproteins are used for vitellogenesis at the end of the feeding process [62]. The host-derived transferrin was described in the hemolymph of *D. variabilis* and in whole nymphal ticks of *A. americanum* [157, 158]. Recently, a study showed the movement of host-derived transferrin *H. longicornis*, particularly from the midgut to the ovary, via hemolymph [159]. In the same way, *R. microplus* re-use heme from blood meal to synthetize heme proteins during protein synthesis [160].

Mammalian serpins are described endogenous regulators of host defenses against tick feeding [161, 162]. Host proteins of the serpin superfamily were identified in saliva, including alpha-1-antiprotease, antithrombin III, and alpha-2-antiplasmin. These proteins regulate enzymes such as neutrophil elastase, thrombin, and plasmin. It is important to find out whether these host proteins have the potential to inhibit their own serine proteinases. The presence of immunoglobulin chains could be explained as a tick self-defense system, since antibodies remain in an active form in tick hemolymph [152].

These observations suggest that the use of host proteins in tick physiology is not an unusual occurrence, and that these proteins may have an important physiology role in tick feeding process.

Conclusions

H. longicornis tick saliva has not been previously studied due to the considerable difficulty to collect saliva. Previously, researchers used salivary gland extract instead of saliva for protein analysis. This study describes the first proteome analysis of saliva of nymph and adult *H. longicornis*. Despite the use of saliva of fully engorged ticks, we could identify several tick proteins that can provide useful information for basic and applied aspects of the host-parasite interaction. The role of saliva proteins in the contact between a tick and the host is crucial during feeding process, and the knowledge about salivary components may improve the understanding of tick physiology, aiding the identification of a new target for tick control.

Additional files

Additional file 1: Table S1. Tick proteins in *H. longicornis* saliva identified by mass spectrometry. Name, classification based on function and/or protein family and annotation and accession numbers of best match identities obtained using BLASTP against the several protein databases are provided in the accompanying spreadsheet (Hyperlinked Excel spreadsheet, zipped).

Additional file 2: Table S2. Host proteins in *H. longicornis* saliva identified by mass spectrometry. Name, classification based in function and/or protein family and annotation and accession numbers of best match identities obtained using BLASTP against the several protein databases are provided in the accompanying spreadsheet (Hyperlinked Excel spreadsheet, zipped).

Additional file 3: Figure S1. Functional classification of *Haemaphysalis longicornis* salivary proteins identified in fully engorged nymph (nymph) and fully engorged adult females (adult). Host proteins detected in nymph (A and C) and adult (B and D). Proteins divided as detected both in nymph (C) and adult (D) or detected exclusively in nymph (A) or adult (B) and classified in groups according to their function and/or protein family. Pie charts represent the percentage of proteins found in each group with respect to normalized spectral counting for each sample.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
LT, MSI, MY, and ISV designed the study. LT, MSI, JKD, and AFMP carried out the experiments. LT, MSI, TK, JKD, JRY, AFMP, AM, MY, and ISV analyzed the data. LT, MSI, TK, AFMP, AM, MY, and ISV drafted the manuscript. All authors read and approved the final manuscript.

Authors' information
Lucas Tirloni and Mohammad Saiful Islam are co-first authors.
Myung-Jo You and Itabajara da Silva Vaz Jr are co-senior authors.

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Author details
[1]Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. [2]Department of Veterinary Parasitology, College of Veterinary Medicine and Bio-safety Research Centre, Chonbuk National University, Jeonju, Republic of Korea. [3]Department of Medicine, Surgery and Obstetrics, Faculty of Veterinary and Animal Science, Hajee Mohammad Danesh Science and Technology University, Dinajpur, Bangladesh. [4]Department of Chemical Physiology, The Scripps Research Institute, La Jolla, CA, USA. [5]Centro de Pesquisas em Biologia Molecular e Funcional, Instituto Nacional de Ciência e Tecnologia em Tuberculose (INCT-TB), Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Porto Alegre, RS, Brazil. [6]Department of Veterinary Pathobiology, College of Veterinary Medicine, Texas A&M University, College Station, TX, USA. [7]Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

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Anexo C

Conserved *Amblyomma americanum* tick serpin 19, an inhibitor of blood clotting factors Xa and XIa, trypsin and plasmin, has anti-haemostatic functions

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Conserved *Amblyomma americanum* tick Serpin19, an inhibitor of blood clotting factors Xa and XIa, trypsin and plasmin, has anti-haemostatic functions

Tae Kwon Kim [a,1], Lucas Tirloni [a,b,1], Zeljko Radulovic [a], Lauren Lewis [a], Mariam Bakshi [a], Creston Hill [a], Itabajara da Silva Vaz Jr. [b,c], Carlos Logullo [d], Carlos Termignoni [b,e], Albert Mulenga [a,*]

[a] *Department of Veterinary Pathobiology, College of Veterinary Medicine, Texas A&M University, College Station, TX, USA*
[b] *Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil*
[c] *Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil*
[d] *Laboratório de Sanidade Animal, Laboratório de Química e Função de Proteínas e Peptídeos and Unidade de Experimentação Animal, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes, RJ, Brazil*
[e] *Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil*

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ABSTRACT

Tick saliva serine protease inhibitors (serpins) facilitate tick blood meal feeding through inhibition of protease mediators of host defense pathways. We previously identified a highly conserved *Amblyomma americanum* serpin 19 that is characterised by its reactive center loop being 100% conserved in ixodid ticks. In this study, biochemical characterisation reveals that the ubiquitously transcribed *A. americanum* serpin 19 is an anti-coagulant protein, inhibiting the activity of five of the eight serine protease blood clotting factors. *Pichia pastoris*-expressed recombinant (r) *A. americanum* serpin 19 inhibits the enzyme activity of trypsin, plasmin and blood clotting factors (f) Xa and XIa, with stoichiometry of inhibition estimated at 5.1, 9.4, 23.8 and 28, respectively. Similar to typical inhibitory serpins, recombinant *A. americanum* serpin 19 forms irreversible complexes with trypsin, fXa and fXIa. At a higher molar excess of recombinant *A. americanum* serpin 19, fXIIa is inhibited by 82.5%, and thrombin (fIIa), fIXa, chymotrypsin and tryptase are inhibited moderately by 14–29%. In anti-hemostatic functional assays, recombinant *A. americanum* serpin 19 inhibits thrombin but not ADP and cathepsin G activated platelet aggregation, delays clotting in recalcification and thrombin time assays by up to 250 s, and up to 40 s in the activated partial thromboplastin time assay. Given *A. americanum* serpin 19 high cross-tick species conservation, and specific reactivity of recombinant *A. americanum* serpin 19 with antibodies to *A. americanum* tick saliva proteins, we conclude that recombinant *A. americanum* serpin 19 is a potential candidate for development of a universal tick vaccine.

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1. Introduction

Ticks and tick borne diseases (TBDs) have a global veterinary and public health impact that in livestock production amounts to large monetary losses worldwide (Jongejan and Uilenberg, 2004; Grisi et al., 2014). For many years, ticks and TBDs were mostly a veterinary problem. However, following the description of *Borrelia burgdorferi*, the causative agent of Lyme disease, in the 1980s (Burgdorfer et al., 1982), the impact of human TBDs has increased. Recently, 17 human TBD agents were listed in a study advocating for One Health solutions (Dantas-Torres et al., 2012). Long considered a nuisance tick (Childs and Paddock, 2003), *Amblyomma americanum* is the principal vector of *Ehrlichia chaffeensis* and *Ehrlichia ewingii*, the causative agents of human monocytic ehrlichiosis (Anderson et al., 1993; Wolf et al., 2000). It is also associated with epidemiology of *Francisella tularensis* (Taylor et al., 1991); a yet to be described causative agent of the southern rash illness (STARI) (James et al., 2001); and a newly identified heartland virus (Savage et al., 2013). In veterinary health, *A. americanum* transmits *Theileria cervi*, a parasite of white tailed deer (Laird et al., 1988), and heavy infestations caused mortality in white tailed deer

* Corresponding author at: Department of Veterinary Pathobiology, College of Veterinary Medicine, Texas A&M University, 422 Raymond Stotzer Parkway, College Station, TX 77845, USA. Tel.: +1 979 458 4300.
E-mail address: amulenga@cvm.tamu.edu (A. Mulenga).
[1] These authors contributed equally.

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fawns (Yabsley et al., 2005) as well as production losses in cattle (Barnard, 1985, 1990).

Ticks feed by disrupting host tissue and sucking up blood that pools in the feeding lesion, which provokes host defense responses including pain, hemostasis (to limit blood loss), inflammation, complement activation (to protect against invading microbial organisms) and tissue repair responses (to heal the feeding lesion) (Francischetti et al., 2009). To date, many proteins derived from tick saliva have been identified which have a direct role at the feeding site, allowing the tick to successfully acquire its blood meal (Steen et al., 2006; Maritz-Olivier et al., 2007). Following the blood meal acquisition, it is also necessary that blood remains fluid for subsequent digestion, in which different tick proteins have been described to act as anti-coagulant molecules in the tick midgut (Ricci et al., 2007; Anderson et al., 2008; Liao et al., 2009). Serine proteases mediate some host defense pathways to tick feeding and are controlled in some pathways by inhibitors belonging to the serine protease inhibitors (serpins) family (Gettins, 2002; Huntington, 2006; Rau et al., 2007). From this perspective, it is proposed that ticks inject serpins into the host to mediate evasion of host defenses and thus they could be suitable targets for tick vaccines (Mulenga et al., 2001). Several tick serpin-encoding cDNAs have been cloned and characterised, including serpins of *A. americanum* (Mulenga et al., 2007; Porter et al., 2015), *Amblyomma maculatum* (Karim et al., 2011), *Ixodes scapularis* (Ribeiro et al., 2006; Mulenga et al., 2009), *Ixodes ricinus* (Leboulle et al., 2002; Prevot et al., 2006; Chmelar et al., 2011), *Rhipicephalus microplus* (Rodriguez-Valle et al., 2012; Tirloni et al., 2014b), *Rhipicephalus appendiculatus* (Mulenga et al., 2003a), *Rhipicephalus haemaphysaloides* (Yu et al., 2013), and *Haemaphysalis longicornis* (Sugino et al., 2003; Imamura et al., 2005, 2006). As of January 2015, more than 200 tick serpin-encoding cDNAs were available in public databases (Porter et al., 2015).

The concept that ticks utilise serpins to evade host defense mechanisms assumes that ticks inject inhibitory serpins into the host during feeding. Indeed, the presence of serpins in tick saliva was well demonstrated though saliva proteomic studies in *Dermacentor andersoni* (Mudenda et al., 2014) and *R. microplus* (Tirloni et al., 2014a) as well as being inferred from transcriptional analysis of salivary glands from *A. americanum* (Mulenga et al., 2007; Porter et al., 2015), *A. maculatum* (Karim et al., 2011), *Amblyomma triste*, *Amblyomma parvum*, *Amblyomma cajennense* (Garcia et al., 2014), *Amblyomma variegatum* (Ribeiro et al., 2011), *Hyalomma marginatum rufipes* (Francischetti et al., 2011), *I. scapularis* (Valenzuela et al., 2002; Ribeiro et al., 2006; Mulenga et al., 2009), *I. ricinus* (Leboulle et al., 2002; Schwarz et al., 2013, 2014), *R. appendiculatus* (Mulenga et al., 2003a,b), *R. microplus* (Tirloni et al., 2014b), *R. haemaphysaloides* (Yu et al., 2013), *H. longicornis* (Sugino et al., 2003; Imamura et al., 2005), and *Antricola delacruzi* (Ribeiro et al., 2012). Accordingly, inhibitory tick serpins have been found and characterised in tick saliva, including *A. americanum* salivary serpin (AAS) 6 (Mulenga et al., 2007; Chalaire et al., 2011), a cross-class inhibitor of papain and trypsin-like proteases able to inhibit blood clotting and complement activation (Mulenga et al., 2013). A blood meal-induced salivary *I. scapularis* serpin has been shown to act upon thrombin and platelet aggregation (Ibelli et al., 2014). In related studies, an inhibitor of pro-inflammation proteases, elastase, cathepsin G and chymase was found in *I. ricinus* (Prevot et al., 2006, 2009; Chmelar et al., 2011). Similarly, *I. ricinus* serpin Iris2 inhibited inflammation by inhibiting cathepsin G and chymase (Chmelar et al., 2011). *Rhipicephalus haemaphysaloides* has two serpins which are able to inhibit chymotrypsin (Yu et al., 2013). In another study, Rodriguez-Valle et al. (2012) reported a characterisation of *R. microplus* serpin-3, an inhibitor of trypsin and thrombin that is recognised by naturally tick-infested bovine serum, and antibodies against an epitope of this protein impairs tick fertility. Indeed, tick vaccine efficacy studies showed tick-feeding efficiency is reduced when *H. longicornis* (Sugino et al., 2003; Imamura et al., 2005), *R. appendiculatus* (Imamura et al., 2006, 2008), *R. microplus* (Jittapalapong et al., 2010), and *I. ricinus* (Prevot et al., 2007) serpins are used as antigen. The goal of the present study was to characterise the role(s) of *A. americanum* tick serpin-19 (AAS19) in tick feeding success. This study demonstrates that AAS19 is a potential target for development of a universal tick vaccine that is effective against more than one tick species.

2. Materials and methods

2.1. Ethics statement

All animal work was conducted and approved according to the Texas A&M University, USA, Institutional Animal Care and Use Committee (AUP 2011-0207).

2.2. Tick feeding, dissections, total RNA extractions and cDNA synthesis

Amblyomma americanum ticks were purchased from the tick laboratory at Oklahoma State University (Stillwater, OK, USA). Routinely, ticks were fed on rabbits according to animal use protocols approved by the Texas A&M University Institutional Animal Care and Use Committee. Feeding was performed as previously described (Mulenga et al., 2013; Kim et al., 2014a). *Amblyomma americanum* ticks were restricted to feed on the outer part of the ear of New Zealand rabbits with orthopedic stockinets glued with Kamar Adhesive (Kamar Products Inc., Zionsville, IN, USA). Six male ticks were pre-fed for 3 days prior to introducing 15 female ticks in each of the ear stockinets (total of 30 female ticks per rabbit).

Ticks were collected and dissected as previously described (Mulenga et al., 2013). Five ticks were manually detached every 24 h for 5 days (24–120 h). Within the first hour of detachment, tick mouthparts were inspected to remove remnant tissue and washed in RNase inhibitor diethylpyrocarbonate (DEPC)-treated water to prepare for dissection. Dissected tick organs, salivary glands (SG), midgut (MG), ovary (OV), synganglion (SYN), Malpighian tubules (MT) and carcass (CA, the remnants after removal of other organs) were placed in RNA Later (Life Technologies, Carlsbad, CA, USA) or 1 mL of Trizol total RNA extraction reagent (Life Technologies) and stored at −80 °C until total RNA extraction.

Total RNA was extracted using the Trizol reagent according to the manufacturer's instructions (Life Technologies) and re-suspended in DEPC-treated water. Total RNA was quantified using a UV-VIS Spectrophotometer DU-640B (Beckman Coulter, Brea, CA, USA) or the Infinite M200 Pro plate reader (Tecan, Männedorf, Switzerland). Up to 1 μg of total RNA was used to synthesise cDNA using the Verso cDNA Synthesis Kit following the manufacturer's instructions (Thermo Scientific, Waltham, MA, USA).

2.3. Structural alignment, amino acid motif scanning and comparative modeling

To gain an insight into the relationship of AAS19 (GenBank accession number: GAYW01000076; Porter et al., 2015) protein from the *A. americanum* tertiary structure compared with its homologs in other tick species, amino acid sequences from UniProt in other tick species (*Rhipicephalus pulchellus* (L7LRY7), *I. ricinus* (V5IHU3), *A. maculatum* (G3ML49 and G3ML50), *R. microplus* (V9VP22), *I. scapularis* (B7QJF1), *A. triste* (A0A023GPF9) and *A. cajennense* (A0A023FM57)) were subjected to structure-based

ClustalW alignment using the AAS19 tertiary structure as a template. The alignment sequences were subsequently viewed using GeneDoc software (http://www.nrbsc.org/gfx/genedoc/ebinet.htm). Additionally, the AAS19 amino acid sequence was manually inspected for annotated glycosaminoglycan (GAG) binding motifs as previously reviewed (Hileman et al., 1998). To determine potential N- or O-linked glycosylation sites, AAS19 amino acid was scanned using NetNGlyc 1.0 and NetOGlyc 4.0 servers (www.cbs.dtu.dk).

The three-dimensional (3-D) structure of AAS19 was predicted using a comparative modeling approach. The serpin protein C inhibitor structure (2OL2) (Li and Huntington, 2008) was retrieved from the Protein Data Bank (PDB) (http://www.rcsb.org) and used as a molecular template for AAS19 modeling based on 30% and 53% sequence identity and similarity, respectively. Sequence alignments were generated using the ClustalW algorithm (Larkin et al., 2007) and used as input in the Modeller 9v14 program (Webb and Sali, 2014). Models generated were evaluated using QMEAN4 and PROCHECK to estimate model reliability and predict quality (Morris et al., 1992; Benkert et al., 2008). The electrostatic potential of AAS19 and protein C inhibitor (2OL2, positive control template) was calculated using two approaches. First, the Adaptive Poisson–Boltzmann Solver (APBS) was used and protonation states were assigned using the parameters for solvation energy (PARSE) force field for each structure by PDB2PQR (Unni et al., 2011). Execution of APBS and visualisation of resulting electrostatic potentials were performed by using the Visual Molecular Dynamics (VMD) program (Humphrey et al., 1996) at ±5 kT/e of positive and negative contour fields. In the second approach, electrostatic potential was computed using APBS and visualised in the SWISS PDB viewer (Guex and Peitsch, 1997) (http://www.expasy.org/spdbv/) set to default parameters.

2.4. Temporal and spatial quantitative reverse transcription (qRT)-PCR transcription analyses of AAS19

Transcription analysis was done using a two-step quantitative (q) reverse transcription (RT)-PCR using the Applied Biosystems 7300 Real Time PCR System (Life Technologies) as described (Kim et al., 2014a). AAS19-specific qRT-PCR forward ($^{5\prime}$GACAAGACGCACGGCAAAA$^{3\prime}$) and reverse ($^{5\prime}$GAAGTCCGGCGGCTCAT$^{3\prime}$) primers were used to determine transcript abundance in triplicate pools of cDNA of dissected SG, MG and remnant tissues as CA of unfed, 24 and 48 h fed (*n* = 15 ticks per pool), 72 and 96 h fed (*n* = 10 ticks per pool) and 120 h fed ticks (*n* = five ticks). Cycling conditions were as follows: stage one at 50 °C for 2 min, stage two at 95 °C for 10 min, and stage three contained two steps with 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Reaction volumes, in triplicate, contained 10-fold diluted cDNAs that were originally synthesised from 1 μg of total RNA, 350 nM of forward and reverse AAS19 primers, and 2× SYBR Green Master Mix (Life Technologies). For an internal reference control, forward ($^{5\prime}$GGCGCCGAGGTGAAGAA$^{3\prime}$) and reverse ($^{5\prime}$CCTTGCCGTCCACCTTGAT$^{3\prime}$) primers targeting the 40S ribosomal protein S4 (RPS4; accession number GAGD01011247.1), which is stably expressed in *I. scapularis* during feeding (Koci et al., 2013), was used. Relative quantification (RQ) of AAS19 transcript was determined using the comparative C_T ($2^{-\Delta\Delta Ct}$) method as previously described (Livak and Schmittgen, 2001) and adapted in Kim et al. (2014a). The data was presented as the percent mean (M) transcript abundance ± S.E.M. per tissue type.

2.5. Expression and affinity purification of recombinant AAS19

Recombinant (r) AAS19 protein was expressed using the *Pichia pastoris* and pPICZα plasmid expression system (Life Technologies) as described previously (Mulenga et al., 2013; Kim et al., 2014b). A mature AAS19 protein open reading frame (Porter et al., 2015) was sub-cloned into pPICZαA *Eco*RI and *Not*I restriction sites using forward ($^{5\prime}$GAATTCGCAGAGCCCGACGAAGATGGC$^{3\prime}$) and reverse ($^{5\prime}$GCGGCCGCGAGGGCGTTAATTTCGCCCAG$^{3\prime}$) primers with added restriction enzyme sites in bold. The pPICZαA-AAS19 expression plasmid was linearised with *PmeI* and electroporated into the *P. pastoris* X-33 strain (Life Technologies) using an ECM600 electroporator (BTX Harvard Apparatus Inc., Holliston, MA, USA) with parameters set to 1.5 kV, 25 uF and 186 Ω. Transformed colonies were selected on Yeast Extract Peptone Dextrose Medium with Sorbitol (YPDS) agar plates with zeocin (100 μg/μl), and methanol utilisation on Minimal Methanol (MM) agar plates, both incubated at 28 °C. Positive transformants were inoculated in buffered glycerol-complex medium (BMGY) and grown overnight at 28 °C with shaking (230–250 rpm). Subsequently, the cells were used to inoculate buffered methanol-complex medium (BMMY) to A_{600} of 1 after which protein expression was induced by adding methanol to 0.5% final concentration every 24 h for 5 days. rAAS19 in spent culture media was precipitated by ammonium sulfate saturation (525 g/L of media) with stirring overnight at 4 °C. The precipitate was pelleted at 11,200 *g* for 1 h at 4 °C and re-suspended in, and dialysed against, 20 mM Tris–HCl buffer pH 7.4. To verify expression of rAAS19, western blotting analysis was performed using the horseradish peroxidase (HRP)-labeled antibody to the C-terminal hexahistidine tag (Life Technologies) diluted to 1:5000 in 5% blocking buffer (5% skim milk powder in PBS with Tween-20). The positive signal was detected using a metal-enhanced DAB chromogenic substrate kit (Thermo Scientific). Subsequently, rAAS19 was affinity purified under native conditions using Hi-Trap Chelating HP Columns (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). Affinity purified putative rAAS19 was dialysed against 20 mM Tris–HCl buffer pH 7.4 for downstream assays. To verify purity and background contamination, affinity purified rAAS19 was resolved on a 10% SDS–PAGE gel and silver stained. Samples with the least background were selected and concentrated by either ammonium sulfate precipitation or by centrifugation using MicroSep Centrifugal Concentration Devices (Pall Corporation, Port Washington, NY, USA).

2.6. N- and O-linked deglycosylation assay

Preliminary amino acid sequence analyses predicted N- and O-linked glycosylation sites in AAS19. To determine whether rAAS19 was N-glycosylated and/or O-glycosylated, 5 μg of affinity purified rAAS19 were treated with protein deglycosylation enzyme mix according to the manufacturer's instructions (New England Biolabs, Ipswich, MA, USA). Deglycosylation was verified by western blotting analysis using the antibody to the C-terminal hexahistidine tag (Life Technologies) and the positive signal detected using HRP chromogenic substrate (Thermo Scientific).

2.7. Determining whether native AAS19 is injected into the host during tick feeding

To determine whether native AAS19 is injected into the host during feeding, glycosylated and deglycosylated affinity-purified rAAS19 was subjected to routine western blotting analyses using antibodies to replete-fed *A. americanum* tick saliva proteins. The antibodies to replete-fed tick saliva proteins were produced as previously described (Mulenga et al., 2013).

2.8. Protease inhibitor (PI) profiling

Inhibitory activity of rAAS19 was tested against a panel of 16 mammalian serine proteases related to host defense pathways against tick feeding. Mammalian proteases (per reaction) tested were: bovine thrombin (43 U), pancreatic porcine elastase (21.6 nM), pancreatic bovine trypsin (24.6 nM), pancreatic bovine α-chymotrypsin (96 nM), pancreatic porcine kallikrein (33 U), human chymase (10 U), human tryptase (10 U), human plasmin (10 nM) (Sigma–Aldrich, St. Louis, MO, USA), human neutrophil cathepsin G (166 nM) (Enzo Life Sciences Inc., Farmingdale, NY, USA), human factor (f) XIa (3.68 nM), bovine fIXa (306 nM), human fXIIa (7.6 nM), human t-PA (32 nM), human u-PA (47.2 nM) (Molecular Innovations, Inc., Novi, MI, USA), fXa (5.8 nM) (New England Biolabs) and proteinase-3 (68 U) (EMD Millipore, Billerica, MA, USA). Substrates were used at 0.20 mM final concentration and purchased from Sigma-Aldrich: N α-benzoyl-DL-Arg-pNA for tryptase, N-Succinyl-Ala-Ala-Pro-Phe-pNA for chymase, cathepsin G and chymotrypsin, N-Benzoyl-Phe-Val-Arg-pNA for thrombin and trypsin, N-Succinyl-Ala-Ala-Al a-p-nitroanilide for elastase. The following substrates were purchased from Diapharma Inc. (Philadelphia, PA, USA): Bz-Ile-Glu(γ-OR)-Gly-Arg-pNA for fXa, H-D-Val-Leu-Lys-pNA for plasmin, and H-D-Pro-Phe-Arg-pNA for kallikrein, fXIa and fXIIa. The substrate CH_3SO_2-D-CHG-Gly-Arg-pNA was purchased from Molecular Innovations and used for fIXa, u-PA and t-PA. The substrate N-Methoxysuccinyl-Ala-Ala-Pro-Val-pNA was purchased from Enzo Life Sciences and used for proteinase-3.

Reagents were mixed at room temperature in triplicate. One μM of rAAS19 was pre-incubated with amounts of the enzyme listed above for 15 min at 37 °C in 20 mM Tris–HCl, 150 mM NaCl, BSA 0.1%, pH 7.4 buffer. The corresponding substrate for each enzyme was added in a 100 μL final reaction volume and substrate hydrolysis was measured at A_{405nm} every 11 s for 30 min at 30 °C using the Infinite M200 Pro plate reader (Tecan). Acquired A_{405nm} data were subjected to one-phase decay analysis in Prism 6 software (GraphPad Software, La Jolla, CA, USA) to determine plateau values as proxies for initial velocity of substrate hydrolysis (V_{max}) or residual enzyme activity. The percent enzyme activity inhibition level was determined using the formula: $100 - V_{max}(V_i)/V_{max}\ (V_0) \times 100$ where $V_{max}\ (V_i)$ = activity in presence of, and $V_{max}\ (V_0)$ = activity in absence of rAAS19. Data are presented as mean ± S.E.M. of triplicate readings.

2.9. Stoichiometry of inhibition (SI)

We determined stoichiometry of inhibition (SI) indices against five proteases (trypsin, plasmin, fXa, fXIa and fXIIa) that were inhibited by more than 80% in the PI profiling assay described in Section 2.8. Different molar ratios (serpin:protease) of rAAS19 were pre-incubated for 1 h with a constant concentration of trypsin (10 nM), fXa (5 nM), fXIa (5 nM) and plasmin (10 nM). The residual enzyme activity was measured using colorimetric substrates specific for each enzyme as described in Section 2.8. The data were plotted as the residual activity (V_i/V_0) versus the inhibitor to enzyme molar ratio. SI, or the molar ratio of rAAS19 to protease when enzyme activity is completely inhibited, was determined by fitting data onto the linear regression line in PRISM.

2.10. rAAS19 and protease complex formation

In varying molar ratios, affinity purified rAAS19 was incubated with trypsin, plasmin, fXa and fXIa, in Tris–HCl reaction buffer (20 mM Tris–HCl, 150 mM, NaCl, pH 7.4) for 1 h at 37 °C. Denaturing sample buffer was added to the reaction mix and incubated at 99.9 °C for 5 min in a thermocycler. Samples were subjected to SDS–PAGE electrophoresis on a 12.5% acrylamide gel and stained with Coomassie brilliant blue using routine protocols.

2.11. Anti-platelet aggregation function of rAAS19

Anti-platelet aggregation function(s) of rAAS19 was determined using platelet rich plasma (PRP) isolated from citrated (acid citrate dextrose) whole bovine blood (WBBL) as previously described (Horn et al., 2000; Berger et al., 2010). To prepare PRP, freshly citrated WBBL was centrifuged at 200*g* for 20 min at 18 °C. Subsequently, the PRP (top layer) was transferred into a new tube and centrifuged at 800*g* for 20 min at 18 °C. The pellet containing platelets was washed and diluted with Tyrode buffer, pH 7.4 (137 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.42 mM Na_2HPO_4, 1 mM $MgCl_2$, 0.1% glucose, 0.25% BSA), until A_{650} = 0.15. To determine anti-platelet aggregation function, various amounts of rAAS19 (1, 0.5, 0.25 and 0 μM) were pre-incubated for 15 min at 37 °C with agonist 10 NIH-U thrombin, 20 μM ADP or 0.7 μM cathepsin G in a 50 μL reaction. Adding 100 μL of pre-warmed PRP triggered platelet aggregation. Vice versa, PRP was pre-incubated with various amounts of rAAS19 prior to addition of the agonist. Platelet aggregation was monitored every 20 s for 30 min at A_{650nm} using the Infinite M200 Pro plate reader (Tecan). In this assay, a higher O.D. was observed in our blank control (platelet only), and increased platelet aggregation was correlated with a reduction in O.D. To determine the percentage of platelet aggregation inhibition, O.D. data was fitted to the one-phase decay equation in PRISM (GraphPad) to determine the plateau O.D. The percent reduction in platelet aggregation was calculated using this formula: $Y = 100 - (\Delta Pn)/\Delta PC) * 100$, where Y = % reduction in platelet aggregation, ΔPn and ΔPC = a respective rAAS19-treated and positive control plateau O.D. subtracted from the blank plateau O.D. Data are presented as mean ± S.E.M. of duplicate platelet aggregation assays.

2.12. Anti-blood clotting function of rAAS19

The effect of rAAS19 against blood clotting was assessed using modified recalcification time (RCT), activated partial thromboplastin time (aPTT), thrombin time (TT) and prothrombin time (PT) to, respectively, measure the effect on the entire blood cascade, the intrinsic, extrinsic and common blood clotting activation pathways, as described (Mulenga et al., 2013). All assays were done in duplicate, with clotting time monitored at A_{650nm} using the Infinite M200Pro plate reader (Tecan) set to 37 °C. In these assays, clot formation was directly proportional to an increase in O.D., and results presented as mean ± S.E.M.

In the RCT assay, 50 μL of universal coagulation reference human plasma (UCRP) (Thermo Scientific) were pre-incubated with 10, 5, 1.25, 0.625, 0 μM rAAS19 in 90 μL of Tris–HCl reaction buffer (20 mM Tris–HCl, 150 mM NaCl, pH 7.4) for 15 min at 37 °C. Adding 10 μL of pre-warmed 150 mM calcium chloride ($CaCl_2$) triggered plasma clotting. Plasma clotting was monitored at every 20 s for 30 min.

In the aPTT assay, various amounts of rAAS19 (indicated above) were pre-incubated for 15 min at 37 °C with 50 μL of UCRP diluted to 100 μL with 20 mM Tris–HCl, 150 mM NaCl, pH 7.4. Subsequently, 50 μL of the aPTT reagent (Thermo Scientific) was added to plasma and incubated at 37 °C for an additional 5 min to activate the reaction. Addition of 50 μL of 25 mM $CaCl_2$ to the reaction triggered clotting, and clot formation was monitored every 10 s for 5 min.

In the TT assay, various amounts of rAAS19 (indicated above) were pre-incubated at 37 °C for 15 min with 25 μL of the TT reagent (Thermo Scientific) containing $CaCl_2$ in 50 μL of Tris–HCl reaction buffer with 20 mM Tris–HCl, 150 mM NaCl, pH 7.4.

Adding 50 μL of pre-warmed UCRP started plasma clotting and clotting time was monitored every 10 s for 5 min.

In the PT assay various rAAS19 amounts as above were pre-incubated at 37 °C for 15 min with 100 μL of PT reagent (Thermo Scientific) containing $CaCl_2$ diluted up to 150 μL with 20 mM Tris–HCl, 150 mM NaCl, pH 7.4 reaction buffer. Adding 50 μL of the pre-warmed UCRP (Thermo Scientific) triggered plasma clotting and clotting time was monitored every 10 s for 5 min.

2.13. Statistical analysis

Statistical software packages in PRISM version 6 (GraphPad Software Inc.) were used.

3. Results

3.1. AAS19 tertiary structure retains features of a typical serpin

AAS19 amino acid sequence is homologous to serpins from other tick species, being that its functional domain reactive center loop (RCL) is 100% conserved among the majority of ixodid tick species (Porter et al., 2015). In this study we conducted comparative amino acid motif and secondary structure analyses to determine the relationship of AAS19 to its homologues in other tick species (Fig. 1). Comparative modeling using protein C inhibitor tertiary structure (2OL2) as a template showed that AAS19 predicted tertiary structure retains the typical serpin fold formed by nine α-helices, three β sheets (five strands each in βsA and βsB, and three strands in βsC) (Figs. 1 and 2) (Gettins, 2002). Amino acid motif scanning analyses showed conservation of the "RGD" motif (positions 40–42, Fig. 1), which is the binding site to integrin GPIIb-IIIa (Srinivasan et al., 2010; Nurden, 2014). Additionally, two N-glycosylation sites at positions 97–99 and 198–200 in AAS19 and in the majority of its homologues are conserved as noted with "#" symbol below the sequence (Fig. 1). Visual inspection of the alignment in Fig. 1 revealed seven clusters of basic amino acid residues that show similarity to annotated GAG binding sites (BS) (reviewed in Hileman et al., 1998). The seven clusters of basic residues (basic residues in bold, amino acid positions based on structural alignment in Fig. 1) in AAS19 are:

[^{59}**K**VLALF**R**EQLDAS**R**^{72}],
[^{117}**KK**GEEAVE**K**INNWVSD**K**T**H**G**K**I**RR**^{140}],
[^{151}**R**LILLNAVYY**K**GTWLYEFN**K**A**R**T**K**P**R**^{176}],
[^{185}**K**VLVPMM**K**M**K**^{194}],
[^{229}**R**NGLE**H**L**K**SVLTTQTLN**R**AIS**R**MYP**K**DM
KF**R**MP**K**L**K**LDT**K**YTL**K**^{272}],
[^{281}**KKK**IFSADADLSGISGA**K**NLYVSDVL**HK**^{301}] and
[^{343}**K**VYVD**H**PFIFLI**R**^{355}].

To gain further insight regarding the possibility that clusters of basic amino acid residues in AAS19 form basic patches, we calculated surface electrostatic potentials on the AAS19 model (Fig. 2B). This analysis predicts that [K^{137}-R^{139}-R^{140}-K^{161}-K^{264}-K^{268}-K^{308}], [K^{8}-K^{236}-R^{246}-R^{250}], [R^{172}-K^{174}-R^{176}-K^{185}-K^{192}-K^{194}-K^{343}], and [K^{117}-K^{118}-R^{151}-K^{272}-K^{298}], respectively, interact to form putative GAG binding sites (GAGBS) 1–4 on the AAS19 surface (Fig. 2B). This methodology was able to recover the GAGBS in PCI, a protein whose GAGBS was previously described (Fig. 2A) (Li and Huntington, 2008).

3.2. AAS19 mRNA is expressed in several tick tissues during feeding

To determine the relationship of AAS19 transcription with the *A. americanum* tick-feeding phase, we determined its spatial and temporal transcription profile. Fig. 3 shows AAS19 mRNA is expressed during blood meal and in unfed ticks. AAS19 is transcribed in SG (Fig. 3A), MG (Fig. 3B) and other tissues (eg. CA) (Fig. 3C). AAS19 transcription in the SG does not change from unfed to 120 fed ticks. Contrarily in MG and CA, AAS19 transcription increases (RPS4 was used for normalisation) with feeding (Fig. 3B, C).

3.3. AAS19 protein is injected into the host during feeding

Fig. 4A and B summarise expression and affinity purification of rAAS19 in *P. pastoris*. To verify expression, daily samples of 1 mL of yeast spent media were concentrated by ammonium sulfate saturation and subjected to western blotting analysis using a specific antibody to the C-terminal histidine tag (as summarised in Fig. 4A). For large-scale expression, ammonium sulfate-precipitated rAAS19 was affinity purified under native conditions and purity checked by SDS–PAGE with silver staining (Fig. 4B). Fractions W2, E1, E2 and E4 in Fig. 4B, the fractions that showed the smallest amount of contaminating protein, were pooled, concentrated and dialysed in appropriate buffer by spin columns and used for further assays. Fraction E3 was used in western blotting analysis assays summarised in Fig. 4C–E. As shown in Fig. 4C, a 1:50 dilution of rabbit pre-immune serum did not recognise rAAS19, while a 1:250 dilution of antibodies against saliva proteins of fully engorged *A. americanum* (Fig. 4B), and against the C-terminal histidine tag (Fig. 4E) recognise both glycosylated (lane 1) and de-glycosylated (lane 2) rAAS19. In Fig. 4D and E, there is smearing in lane 1 but not in lane 2. Additionally, there is a downward molecular weight shift in lane 2. This shift in molecular weight and disappearance of smears following de-glycosylation validates that rAAS19 is glycosylated.

3.4. rAAS19 is a broad spectrum inhibitor of trypsin-like proteases

Protease inhibition profiling was done against 16 mammalian proteases that regulate host defenses against tick feeding such as hemostasis, wound healing/tissue remodeling and inflammatory response (Table 1). Molar excess of rAAS19 inhibited the activity of trypsin and trypsin-like proteases associated with hemostasis (Fig. 5). Pre-incubation of 1 μM rAAS19 inhibited, respectively, approximately 80%, 82%, 86%, 95% and 98% the activity of plasmin (34.8 nM), fXIIa (7.62 nM), fXa (5.81 nM), fXIa (3.68 nM) and trypsin (24.6 nM) (Fig. 5A, B). Additionally, rAAS19 inhibits thrombin (43 U), tryptase (10 U), fIXa (30.6 nM) and chymotrypsin (96 nM) by approximately 13%, 16%, 20% and 28%, respectively.

To evaluate rAAS19 inhibitory efficiency, we determined the SI of proteases that were inhibited by more than 80% by rAAS19. Fig. 6A, C, E and G show the kinetics of the hydrolysis of specific substrates by these enzymes in the absence (a) and in the presence (b–g) of rAAS19 at various concentrations. These data were fitted to a linear regression, as shown in Fig. 6B, D, F and H. The rAAS19 SI index was estimated from the *X*-axis intercept ($Y = 0$) for trypsin, plasmin, fXa and fXIa at 5, 9, 23 and 28, respectively. The SI index for fXIIa was estimated at 103 (not shown). The rAAS19 mechanism of action as a typical inhibitory serpin was confirmed by the inability of heating and SDS (Huntington et al., 2000) to dissociate it from trypsin (Fig. 7A), fXa (Fig. 7B) and fXIa (Fig. 7C). Irreversible complex between rAAS19 and the target enzyme was observed only at inhibitor enzyme molar ratios (Fig. 7A lanes 1–3, Fig. 7B lanes 1–2 and Fig. 7C lanes 1–6) where enzyme activity inhibition was observed (Fig. 6A, E, G).

3.5. rAAS19 inhibits thrombin-triggered platelet aggregation

PRP was used in platelet aggregation assays using three agonists: thrombin (0.03 U/μL), ADP (20 μM) and cathepsin G

```
                   hA                      hB                 hC                    hD
              HHHHHHHHHHHH        HHHHHHHHHHHHHH  HHHHHHHHHHH        HHHHHHHHHHHHHH
GAYW01000076: FLGLNLLKALPSNANTNVFFSPYSVATAMGMAYAGARGDTLEQLTLNFGYAADELNEQKVLALFREQLDA :  70
L7LRY7      : YLGLNLLKALPSNANTNVFFSPFSVSAALGMAYIGARGDTLQQLTLNFGYTADELNEMKILALFKEQLEA :  70
V5IHU3      : YLGLNLLKQFPCNDKTNVFFSPFSVSTAMGMAYAGARGDTLEQLTLNFGYAADELNEGKVLALFKEQLQS :  70
G3ML49      : FLGLNLLKALPSNAKTNIFFSPYSVATAMGMAYAGARGDTLEQLTLNFGYTADELNDQKVLALFKEQLDA :  70
G3ML50      : FLGLNLLKALPSNAKTNIFFSPYSVATAMGMAYAGARGDTLEQLTLNFGYTADELNDQKVLALFKEQLDA :  70
V9VP22      : YLGLNLLKALPSNANTNVFFSPFSVSAALGMAYIGARGDTLEQLTLNFGYTAEELNEQKILALFKEQLEA :  70
B7QJF1      : YLGLNLLKQLPSNDKTNVFFSPFSVSTAMGMAYAGARGDTLEQLTLNFGYAADELNEGKVLALFKEQLQS :  70
A0A023GPF9  : FLGLNLLKALPSNAKTNIFFSPYSVATAMGMAYAGARGDTLEQLTLNFGYTADELNDQKVLALFKEQLDA :  70
A0A023FM57  : FLGLNLLKALPGNAKTNVFFSPYSVATAMGMAYAGARGDTLEQLTLNFGYAADELNDRKILALFKEQLDA :  70
                                                     &&&                 *     *

                   s2A                 hE     s1A          hF
              HH    EEEEEEEEEE        HHHHHHHHHHH EEEE    HHHHHHHHHHHHHHHH
GAYW01000076: SRELPHEYALDVANAAVAQEGYGVLPNYTDALMSSFGAEYIEADSSKKGEEAVEKINNWVSDKTHGKIRR : 140
L7LRY7      : AHELPHEYTLDIANAAVAQEGYGVLPNYTEALMSAFGAEYLEADFSKKGQEAIDKINKWVSEKTHGKVRS : 140
V5IHU3      : TNDLPHDYTLNIANAAVAQEGYGILPEYTDALTSSFGAEYIEADFQKRGQEAIQKINTWVSNRTHGKVQS : 140
G3ML49      : SRELPHEYTLDIANAAVAQEGYGVLLNYTSALMSSFGAEYLEADFSKKGQEAVEKINKWVSEKTHGKIRT : 140
G3ML50      : SRELPHEYTLDIANAAVAQEGYGVLPNYTSALMSSFGAEYLEADFSKKGQEAVEKINKWVSEKTHGKIRT : 140
V9VP22      : ARDLPHEYTLDIANAAVAQEGYGVLPNYTEALVSAFGAEYLKADFSKKGQEAIDNINKWVREKTHGKIRS : 140
B7QJF1      : TNDLPHDYTLNIANAAVAQEGYGILPEYTDALTSSFGAEYIEADFQKRGQEAIQKINAWVSNRTHGKVQS : 140
A0A023GPF9  : SRDLPHEYTLDIANAAVAQEGYGVLPNYTSALMSSFGAEYLEADFSKKGQEAVEKINKWVSEKTHGKIRT : 140
A0A023FM57  : SRELPHEYTLDVANAAVAQEGYGVLPNYTDALMSSFGAEYIEADFSKKGEEAIEKINKWVSEKTHGKIRS : 140
               *                         ###                 **     *       * * * **

                         s3A                s4C            s3C                s1B
                    EEEEEEEEEEE EEE        EEEEEE     EEEEEEEEEEEEEEEEEE      EEEEE
GAYW01000076: LFDEPPDFSTRLILLNAVYYKGTWLYEFNKARTKPRSFYNGGVTKVLVPMMKMKSTLNHTFDATLNADVV : 210
L7LRY7      : LFEEPPDFSTRLILLNAVYYKGTWLYEFNKLRTKPRSFYNGGVTKVLIPMMKMKSTLNHTFDAMLNADVV : 210
V5IHU3      : LFDEPPDFSTRLILLNAIYYKGTWLYEFDKTKTKPRSFYNGGVEKVQVPMMRLKSTLNHTYNAILNADLV : 210
G3ML49      : LFEEPPDFSTRLILLNAVYYKGTWLYEFNKAKTKPRSFYNGGVTKVIVPTMKMKSTLNHTFDATLNADIV : 210
G3ML50      : LFEEPPDFSTRLILLNAVYYKGTWLYEFNKAKTKPRSFYNGGVTKVIVPTMKMKSTLNHTFDATLNADIV : 210
V9VP22      : LFDMPPDTSTRLILLNAVYFKGTWLYEFNKLKTKPRSFYNGGVTKVLIPTMKMKSTLNHTFDAMLNADVV : 210
B7QJF1      : LFDEPPDFSTRLILLNAIYYKGTWLYEFDKTKTKPRSFYNGGVEKVQVPMMRLKSTLNHTYNAILNADLV : 210
A0A023GPF9  : LFEEPPDFSTRLILLNAVYYKGTWLYEFNKAKTKPRSFYNGGVTKVLVPTMKMKSTLNHTFDATLNADIV : 210
A0A023FM57  : LFDEPPDFSTRLILLNAVYYKGTWLYEFNKAKTKPRSFYNGGVTKVLVPMMKMKSTLNHTFDATLNADIV : 210
                       *        *        * * * *         *       * *   ###

                  s2B         hG         hH         s2C       s6A      hI
              EEE EEEEE     HHHHHHHH   HHHHHHHHHH EEEEEEEEEE EEEEEEEE   HHHHH
GAYW01000076: DLPYVGIDFAMTILLPSERNGLEHLKSVLTTQTLNRAISRMYPKDMKFRMPKLKLDTKYTLKPTLETMGI : 280
L7LRY7      : DLPYVGNDIAMTILLPSERNGIEHLKSALTTQNLNKAIARMYPKDMKLKLPKFKLDTKYTLKPTLEALGI : 280
V5IHU3      : DLPYVGNDFSMTIILPREKTGLASLKSVLTSQTLNLALQNMYPKDMKLKLPKFKLDTKYTLKPTLEAMGI : 280
G3ML49      : DLPYVGIDFSMTVLLPSERNGLEHLKSVLTPQTLNRAIAKMYPKDMKFRMPKLKMETKYTLKPSLETLGI : 280
G3ML50      : DLPYVGIDFSMTVLLPSERNGLEHLKSVLTPQTLNRAIAKMYPKDMKFRMPKLKMETKYTLKPSLETLGI : 280
V9VP22      : DLPYVGKDIAMTILLPSERNGIEHLKSALTTQNLNKAVARMYPKDMKVRLPKFKLDNKYTLKPTLETLGI : 280
B7QJF1      : DLPYVGNDFSMTIILPREKTGLASLKSVLTSQTLNLALQNMYPKDMKLKLPKFKLDTKYTLKPTLEAMGI : 280
A0A023GPF9  : DLPYVGIDFSMTVLLPSERNGLEHLKSVLTPQTLNRAIAKMYPKDMKFRMPKLKMETKYTLKPSLETLGI : 280
A0A023FM57  : DLPYVGIDFSMTILLPSERNGLEHLKSALTTQTLNRAIARMYPKDMKFKMPKLKMDTKYTLKPTLETLGI : 280
                                *    * *        *   *   *  * *  * *  *   *

                                 s5A             RCL               s1C
                            EEEEEEEEEE ---------------------       EEE   E
GAYW01000076: KKKIFSADADLSGISGAKNLYVSDVLHKAVLEVNEEGSEAAAVTGFVIQLRTAAFVTPPPLPKVYVDHPF : 350
L7LRY7      : K-KIFSADADLSGISGAKNLYVSDVLHKAVLEVNEEGSEAAAVTGFVIQLRTAAFVTPPPLPKVYVDHPF : 349
V5IHU3      : T-KIFSADADLSGISGSRNLYVSDVLHKAVLEVNEEGSEAAAVTGFVIQLRTAAFVTPXXXXXVYVDHPF : 349
G3ML49      : K-KIFSADADLSGISGAKNLYVSDVLHKAVLEVNEEGSEAAAVTGFVIQLRTAAFVTPPPLPKVYVDHPF : 349
G3ML50      : R-KIFSADADLSGISGAKNLYVSDVLHKAVLEVNEEGSEAAAVTGFVIQLRTAAFVTPPPLPKVYVDHPF : 349
V9VP22      : T-KIFSADADLSGISGAKNLYVSDVLHKAVLEVNEEGSEAAAVTGFVIQLRTAAFVTPPPLPKVYVDHPF : 349
B7QJF1      : T-KIFSADADLSGISGSRNLYVSDVLHKAVLEVNEEGSEAAAVTGFVIQLRTAAFVTPPPLPKVYVDHPF : 349
A0A023GPF9  : K-KIFSADADLSGISGAKNLYVSDVLHKAVLEVNEEGSEAAAVTGFVIQLRTAAFVTPPPLPKVYVDHPF : 349
A0A023FM57  : K-KIFSADADLSGISGAKNLYVSDVLHKAVLEVNEEGSEAAAVTGFVIQLRTAAFVTPPPLPKVYVDHPF : 349
              ***             *        **                                   *    *

               s4B       s5B
              EEEEE     EEEEE
GAYW01000076: IFLIRNVNTNCIMFLGEINAL : 371
L7LRY7      : IFLIRNVNTNTIMFLGEVNAL : 370
V5IHU3      : IFLIRNSKTNTIMFLGEINAL : 370
G3ML49      : LFLIRNVNTNAIMFLGEINAL : 370
G3ML50      : LFLIRNVNTNAIMFLGEINAL : 370
V9VP22      : IFLIRNVHTNTIMFLGEVNAL : 370
B7QJF1      : IFLIRNSRTNTIMFLGEINAL : 370
A0A023GPF9  : LFLIRNVNTNAIMFLGEINAL : 370
A0A023FM57  : IFLIRNVNTNCIMFLGEINAL : 370
                  *
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Fig. 1. Structure-based sequence alignment of *Amblyomma americanum* serpin-19 (AAS19; GenBank accession number: GAYW01000076) protein sequence with its homologs in other tick species from the Uniprot database: *Rhipicephalus pulchellus* (L7LRY7), *Ixodes ricinus* (V5IHU3), *Amblyomma maculatum* (G3ML49 and G3ML50), *Rhipicephalus microplus* (V9VP22), *Ixodes scapularis* (B7QJF1), *Amblyomma triste* (A0A023GPF9) and *Amblyomma cajennense* (A0A023FM57). Secondary structures, assigned based on AAS19 comparative tertiary structure, are labeled "H" for α-helix and "E" for β-strand. Helices are labeled from "hA" to "hI" and β-strands that constitute β-sheets A–C are labeled "s1–6A", "s1–5B" and "s1–4C", respectively. The "RGD" motif is noted by the "&" symbol and the two putative N-glycosylation sites are noted by the "#" symbols below the sequence. The residues that correspond to the basic patches are indicated by an asterisk (*). The high and low conserved residues are highlighted in black and gray, respectively. The 100% conserved reactive center loop (RCL) region is denoted with black broken lines (---) above the sequence.

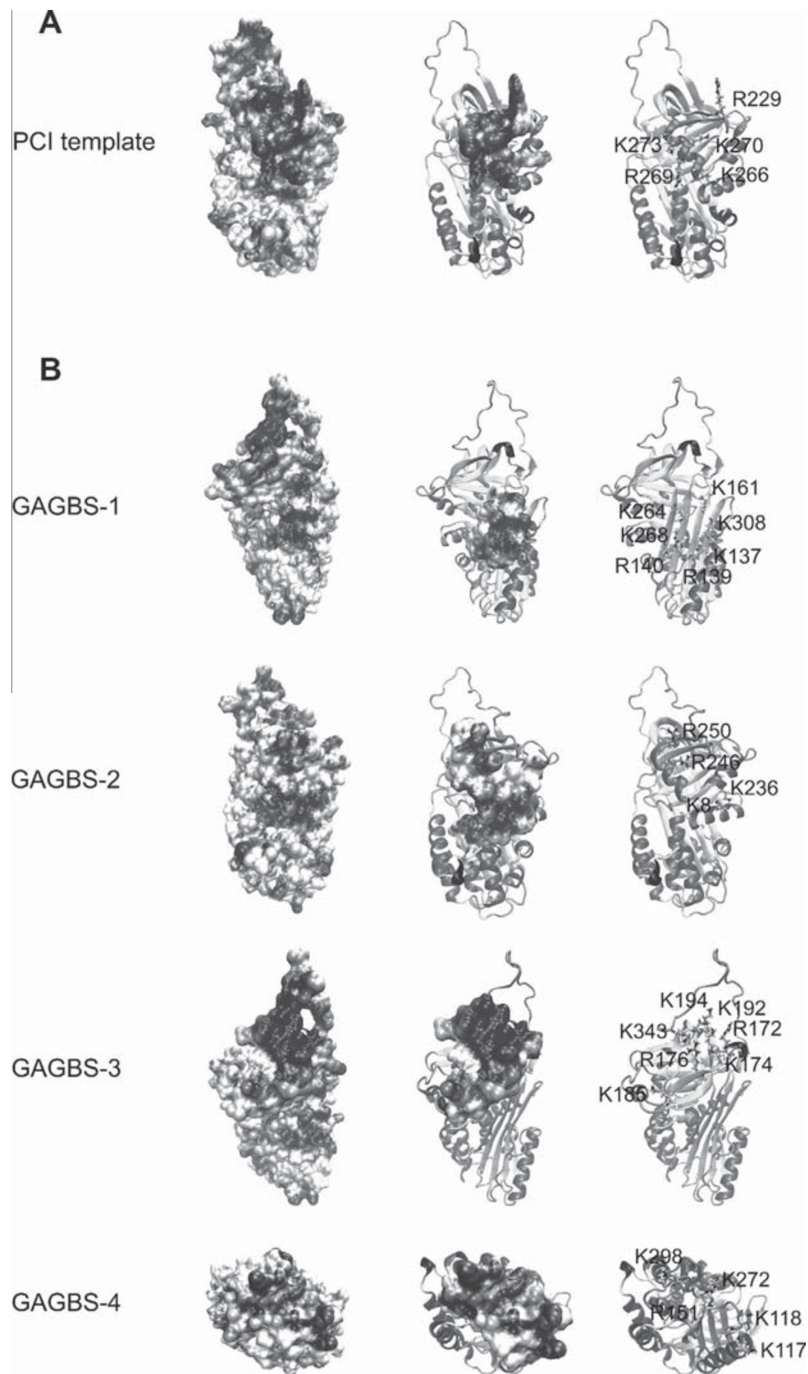

Fig. 2. Structure and putative glycosaminoglycan-binding sites of *Amblyomma americanum* serpin-19 (AAS19). The AAS19 model was constructed using the coordinates generated with the Modeler 9v14 program and protein C inhibitor as template (PDB 2OL2). Calculation of the electrostatic potential surface map was generated using the Adaptive Poisson–Boltzmann Solver (APBS) tool in the Visual Molecular Dynamics (VMD) program. (A) Electrostatic surface potential (indicated by a dark surface for positively, and light surface for negatively, charged regions) and ribbon diagram of the native protein C inhibitor (template) showing the glycosaminoglycan-binding along the helix-H. Residues responsible for glycosaminoglycan-binding activity are shown as sticks and labeled (Li and Huntington, 2008). (B) Electrostatic surface potential and ribbon diagram of the AAS19 model showing putative glycosaminoglycan-binding sites (GAGBS 1–4). Clusters of basic amino acid residues potentially involved in formation of GAGBS 1–4 [K^{137}-R^{139}-R^{140}-K^{161}-K^{264}-K^{268}-K^{308}], [K^{8}-K^{236}-R^{246}-R^{250}], [R^{172}-K^{174}-R^{176}-K^{185}-K^{192}-K^{194}-K^{343}] and [K^{117}-K^{118}-R^{151}-K^{272}-K^{298}] are shown as sticks and labeled.

(0.7 μM). rAAS19 inhibited thrombin activated platelet aggregation (Fig. 8). In data shown in Fig. 8A, we did not observe platelet aggregation without addition of the agonist (a). However with platelets activated by thrombin that was pre-incubated with 1 (b), 0.5 (c), 0.25 (d) and 0 μM (e), rAAS19 showed variable levels of platelet aggregation. Pre-incubating 3U of thrombin with 1 μM and 0.5 μM rAAS19, platelet aggregation was reduced by 56% and 27%, respectively, while at 0.25 μM rAAS19 did not have any effect (Fig. 8B). The rAAS19 did not have any effects on ADP and cathepsin G activated platelet aggregation. On the other hand, pre-incubation of rAAS19 with platelets prior to addition of the agonist did not affect platelet aggregation.

3.6. rAAS19 delays plasma clotting

Plasma clotting time (CT), RCT, activated aPTT and TT were delayed by rAAS19 in a dose-response manner (Fig. 9). However, rAAS19 did not affect PT. In the RCT assay, to measure the effect

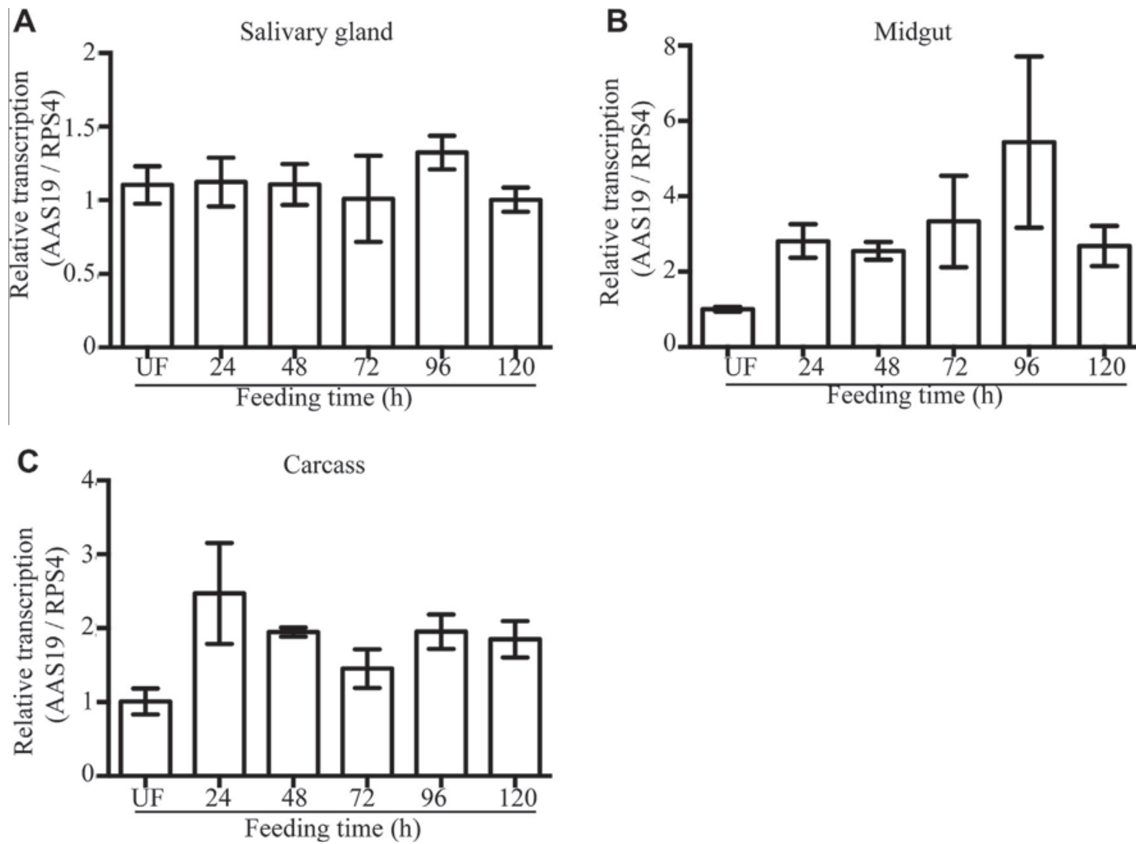

Fig. 3. Temporal and spatial expression analysis of *Amblyomma americanum* serpin-19 (AAS19) mRNA through 120 h post-attachment. *Amblyomma americanum* serpin-19 mRNA relative expression analysis in unfed (UF), 24, 48, 72, 96, and 120 h fed tick dissected tissues: (A) salivary gland, (B) midgut and (C) carcass analysed by quantitative reverse transcription-PCR. To determine the *A. americanum* serpin-19 mRNA relative expression (*X*-axis), data were analysed using the $2^{-\Delta\Delta Ct}$ method comparing with ribosomal protein S4 (RPS4) in three biological replicates of different tissues in relation to time of feeding (*X*-axis). The lowest expressed time point was used as a calibrator for each tissue.

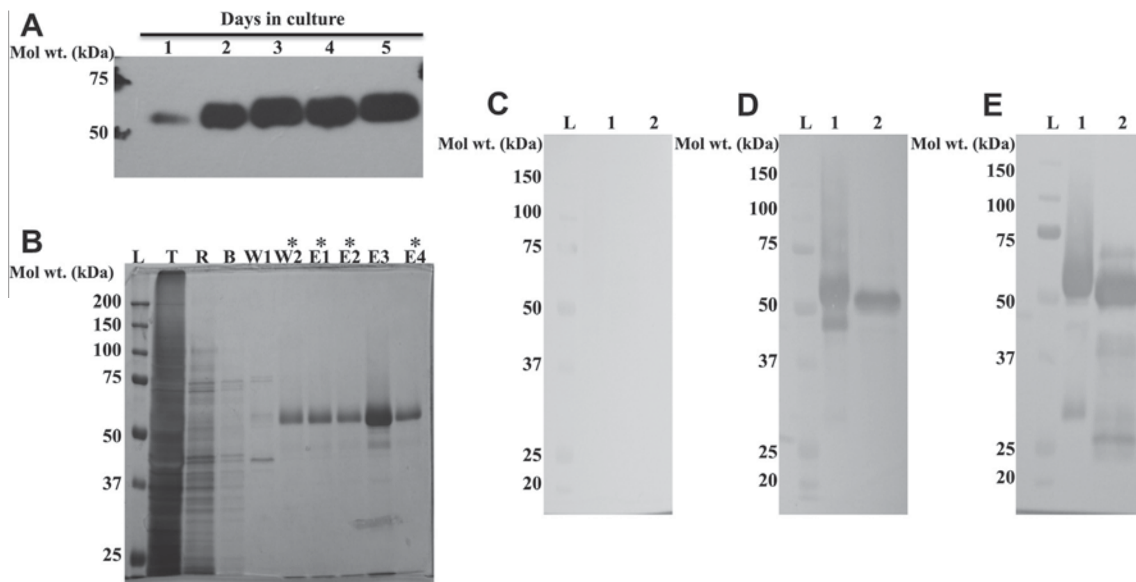

Fig. 4. Expression and affinity purification of recombinant (r) *Amblyomma americanum* serpin-19 (AAS19) in *Pichia pastoris*. Construction of expression plasmid, induction and validation of expression levels using the antibody to the C-terminal hexahistidine tag and affinity purification of rAAS19 were performed as described in Section 2.5. (A) Daily expression levels of rAAS19 through 5 days. (B) Affinity purified rAAS19 resolved on a 10% SDS–PAGE and silver staining (L, molecular weight ladder; T, total protein loaded onto column; R, column run through; B, binding buffer wash; W1 and W2, wash buffers; E1–E4, eluted rAAS19 fractions). Purified rAAS19 fractions used for assays in Figs. 5 – 9 are noted with asterisks (*). Western blotting analysis with (C) 1:50 dilution pre-immune sera, (D) 1:250 dilution immune sera to replete-fed *A. americanum* tick saliva proteins, and (E) antibody to C-terminal hexahistidine tag. Lanes L, 1 and 2 in C, D and E show a molecular weight ladder, glycosylated and de-glycosylated rAAS19.

Table 1
Proteases used in recombinant *Amblyomma americanum* serpin-19 (rAAS19) protease inhibitor profiling.

Proteases	Biological function	References
Thrombin	Common, intrinsic and extrinsic coagulation pathways; fibrinolysis pathway; platelet activation and aggregation pathways via activation of protease-activated receptors (PARs); wound healing/tissue remodeling; inflammation	Reimers et al. (1976), Gartner et al. (1978), Davie et al. (1991), Hoffman (2003) and Olszewska-Pazdrak et al. (2010)
Trypsin	Digestion; wound healing/tissue remodeling; inflammation and pain via activation of PAR2	Walsh et al. (1964), White et al. (2013) and Cattaruzza et al. (2014)
Cathepsin G	Platelet activation; wound healing/tissue remodeling; inflammation; antimicrobial properties	Renesto and Chignard (1993), Mak et al. (2003) and Korkmaz et al. (2008)
Kallikrein	Wound healing/tissue remodeling; inflammation; intrinsic coagulation pathway	Li et al. (2007) and Chao et al. (2010)
Elastase	Wound healing/tissue remodeling; inflammation; digestion of elastin; activation of platelets and lymphocytes; antimicrobial properties	Renesto and Chignard (1993), Belaaouaj (2002), Korkmaz et al. (2008) and Kessenbrock et al. (2008, 2011)
Plasmin	Fibrinolysis pathway; inflammation	Collen (1999), Li et al. (2012), Carmo et al. (2014) and Mancek-Keber (2014)
Factor IXa	Intrinsic coagulation pathway; platelet amyloid precursor protein pathway	Davie et al. (1991) and Hoffman (2003)
Factor Xa	Common, intrinsic and extrinsic coagulation pathways	Davie et al. (1991) and Hoffman (2003)
Factor XIa	Intrinsic coagulation pathway; platelet amyloid precursor protein pathway	Davie et al. (1991) and Hoffman (2003)
Factor XIIa	Intrinsic coagulation pathway	Davie et al. (1991) and Hoffman (2003)
Tissue-type plasminogen activator (t-PA)	Fibrinolysis pathway; platelet amyloid precursor protein pathway	Angles-Cano (1994), Angles-Cano et al. (1994) and Collen (1999)
Urokinase plasminogen activator (u-PA)	Fibrinolysis pathway; platelet amyloid precursor protein pathway	Angles-Cano (1994), Angles-Cano et al. (1994) and Collen (1999)
α-chymotrypsin	Wound healing/tissue remodeling; inflammation; digestion; regulation of proteases: chymotrypsin, cathepsin G, mast cell chymase	Algermissen et al. (1999a), Matsunaga et al. (1994, 2000) and Korkmaz et al. (2008)
Chymase	Wound healing/tissue remodeling; inflammation	Algermissen et al. (1999a,b) and Caughey (2007)
Tryptase	Wound healing/tissue remodeling; inflammation	Algermissen et al. (1999a), Hallgren and Pejler (2006) and Caughey (2007)
Proteinase-3	Wound healing/tissue remodeling; inflammation; platelet activation. Antimicrobial properties	Renesto et al. (1994), Algermissen et al. (1999a), Kessenbrock et al. (2008) and Korkmaz et al. (2008)

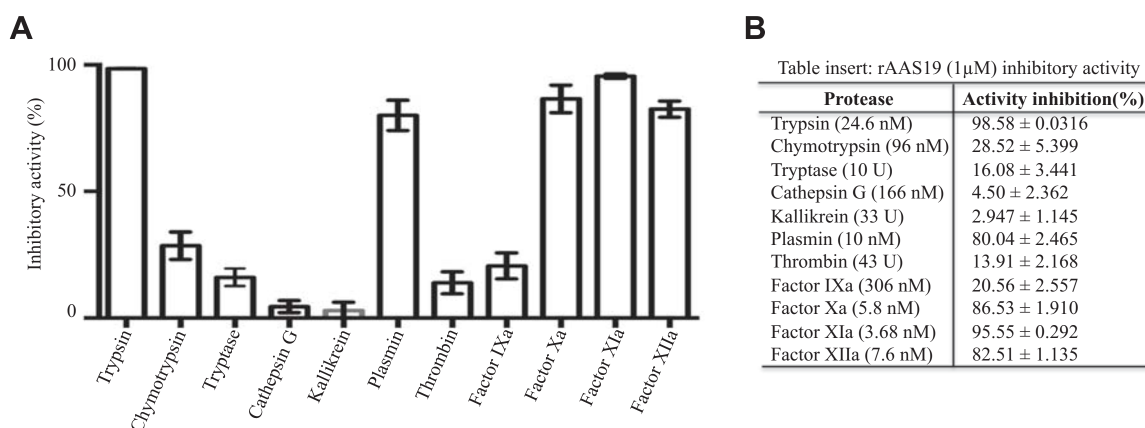

Table insert: rAAS19 (1 μM) inhibitory activity

Protease	Activity inhibition(%)
Trypsin (24.6 nM)	98.58 ± 0.0316
Chymotrypsin (96 nM)	28.52 ± 5.399
Tryptase (10 U)	16.08 ± 3.441
Cathepsin G (166 nM)	4.50 ± 2.362
Kallikrein (33 U)	2.947 ± 1.145
Plasmin (10 nM)	80.04 ± 2.465
Thrombin (43 U)	13.91 ± 2.168
Factor IXa (306 nM)	20.56 ± 2.557
Factor Xa (5.8 nM)	86.53 ± 1.910
Factor XIa (3.68 nM)	95.55 ± 0.292
Factor XIIa (7.6 nM)	82.51 ± 1.135

Fig. 5. Protease inhibitor function profiling. Enzymes, at concentrations indicated in Section 2.8, were pre-incubated with recombinant *Amblyomma americanum* serpin-19 (rAAS19) (1 μM) at 37 °C for 15 min. Subsequently, specific colorimetric substrates were added and hydrolysis monitored at A_{450nm} over 30 min at 30 °C. The percent enzyme activity inhibition level was determined using the formula: $100 - V_{max}(V_i)/V_{max}(V_0) \times 100$ where $V_{max}(V_i)$ = activity in presence of, and $V_{max}(V_0)$ = activity in absence of, rAAS19. Data are presented as mean ± S.E.M. of triplicate readings.

of on the entire blood clotting activation system, 0.625, 1.25 and 5 μM (arrowhead R-CT2), and 10 μM (arrowhead R-CT3) rAAS19 dose responsively delayed RCT-CT by ~100 and 250 s compared with CT in the absence of rAAS19 (arrowhead R-CT1, 0 μM) (Fig. 9A). In the aPTT assay to measure the effect on the intrinsic blood clotting activation pathway (Fig. 9B), 0.625 and 1.25 μM rAAS19 did not have any effect (arrowhead A-CT1), while 5 and 10 μM rAAS19 delayed aPTT-CT by ~20 (A-CT2) and 40 (A-CT3) s, respectively. In the TT assay (Fig. 9C) 0.625 μM rAAS19 was not different from the control (arrowhead T-CT1), while 1.25 and 5 μM rAAS19 (arrowhead T-CT2), and 10 μM rAAS19 (arrowhead T-CT3) delayed clotting by ~180 and 250 s, respectively.

4. Discussion

This study was prompted by the characteristic high conservation of AAS19 in all ixodid tick species, for which data are available (Porter et al., 2015). We were particularly interested in AAS19 due to its functional domain RCL being 100% conserved in other ixodid ticks, suggesting this serpin has a role in regulating proteolytic pathways crucial to all ixodid tick species. The observation that AAS19 motifs and secondary structure are conserved in AAS19 homologues in several tick species further suggests this protein has the potential to regulate processes that are important to ixodid ticks.

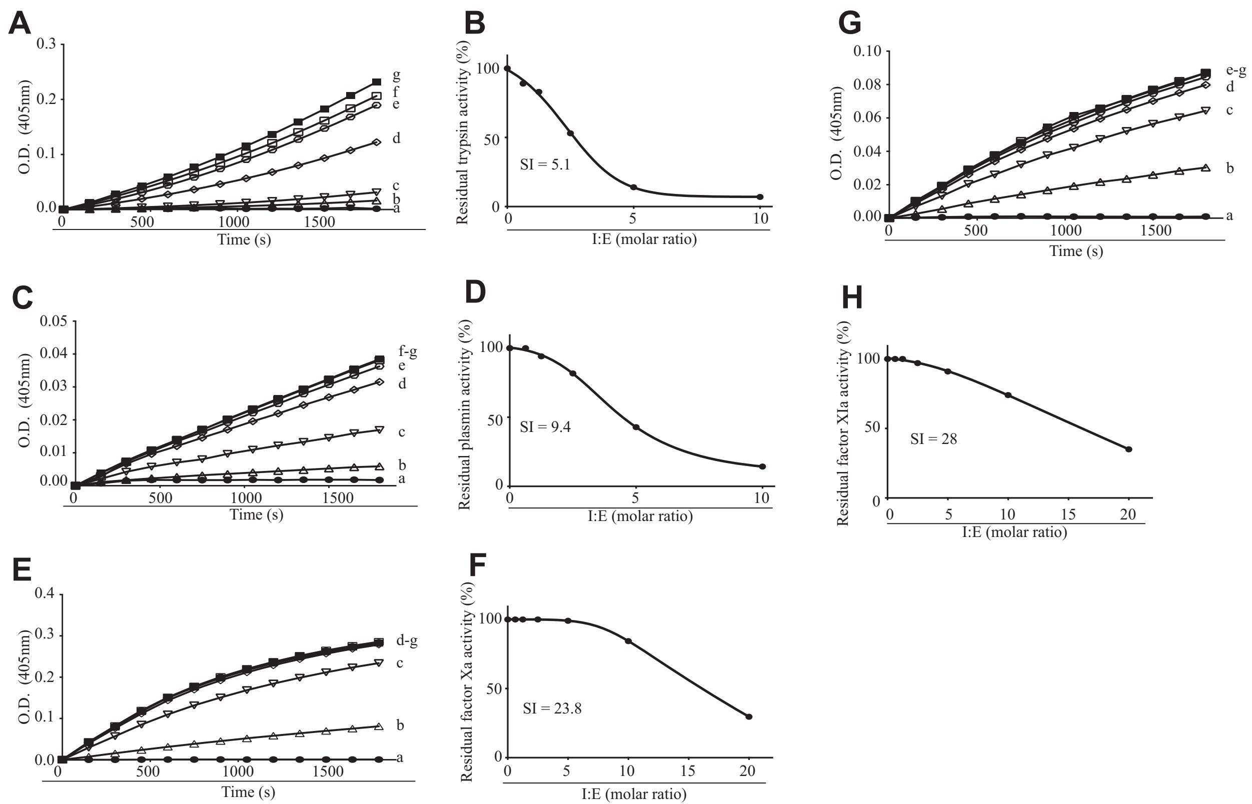

Fig. 6. Stoichiometry inhibition assay for *Amblyomma americanum* serpin-19 (AAS19). Residual enzyme activity without (a) and with presence of increasing (b–g) amounts of rAAS19 were pre-incubated for 1 h at 37 °C with a constant concentration of trypsin (A,B: 10 nM), plasmin (C,D: 10 nM), factor Xa (E,F: 5 nM), and factor XIa (G,H: 5 nM), resulting in rAAS19 inhibitor: enzyme protease (I:E) molar ratios varying from 0 to 10 (for plasmin and trypsin) or 0–20 (for factor Xa and factor XIa). Residual enzymatic activity was measured using specific colorimetric substrate for each enzyme noted in Section 2.9. The data were plotted as enzymatic residual activity (V_i/V_0) versus molar ratio (rAAS19: enzyme protease). The stoichiometry inhibition was determined by fitting data onto a linear regression line.

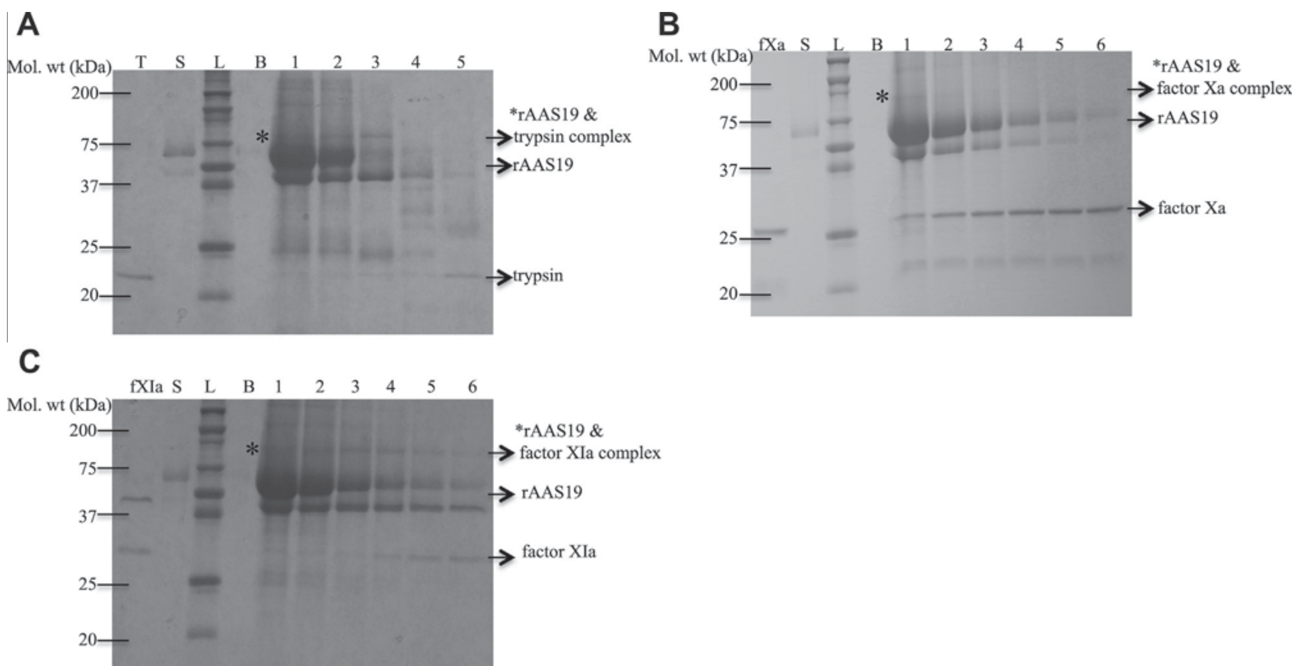

Fig. 7. SDS-stable complex formation assay. Increasing amounts of recombinant *Amblyomma americanum* serpin-19 (rAAS19) were pre-incubated for 1 h at 37 °C with a constant concentration of trypsin (A), factor Xa (B) and factor XIa (C), resulting in molar ratios varying from 0.625:1 to 10:1 (rAAS19: enzyme protease). Samples were resolved on 12.5% SDS–PAGE and Coomassie blue-stained to identify SDS-stable complexes. The rAAS19: enzyme protease complex formation is denoted with an asterisk.

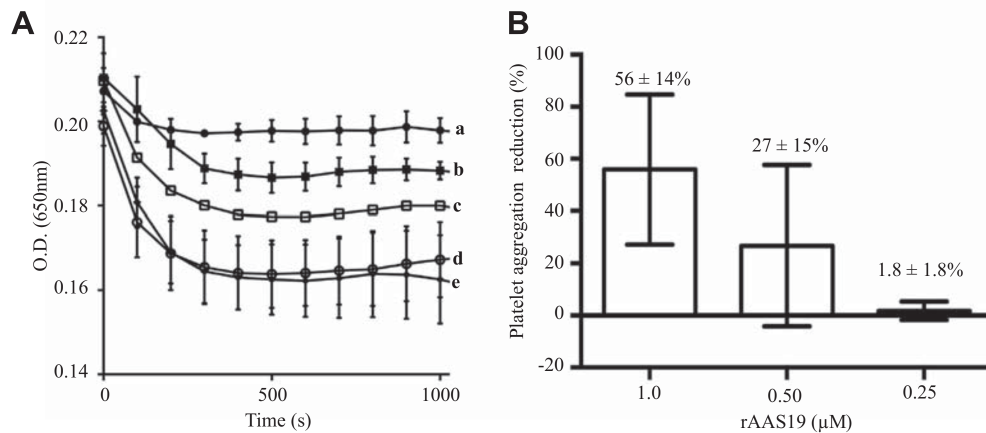

Fig. 8. Effect of recombinant *Amblyomma americanum* serpin-19 (rAAS19) on thrombin activated platelet aggregation. (A) The platelet aggregation function assay was done using the platelet rich plasma approach described in Section 2.11. Tyrode buffer without thrombin (a), various amounts of rAAS19 with 1 μM (b), 0.5 μM (c), and 0.25 μM (d) rAAS19 and thrombin only (e) were pre-incubated with thrombin (3 U) in a 50 μL reaction for 15 min at 37 °C. Platelet aggregation was initiated with the addition of 100 μL of pre-warmed platelet rich plasma and monitored at intervals of 20 s over 30 min at A_{650nm}. (B) Percent reduction of thrombin-induced platelet aggregation inhibition by rAAS19. In the assay used here, platelet aggregation was directly proportional to reduced O.D. (A_{650nm}). Data are presented as mean ± S.E.M. of duplicate platelet aggregation assays.

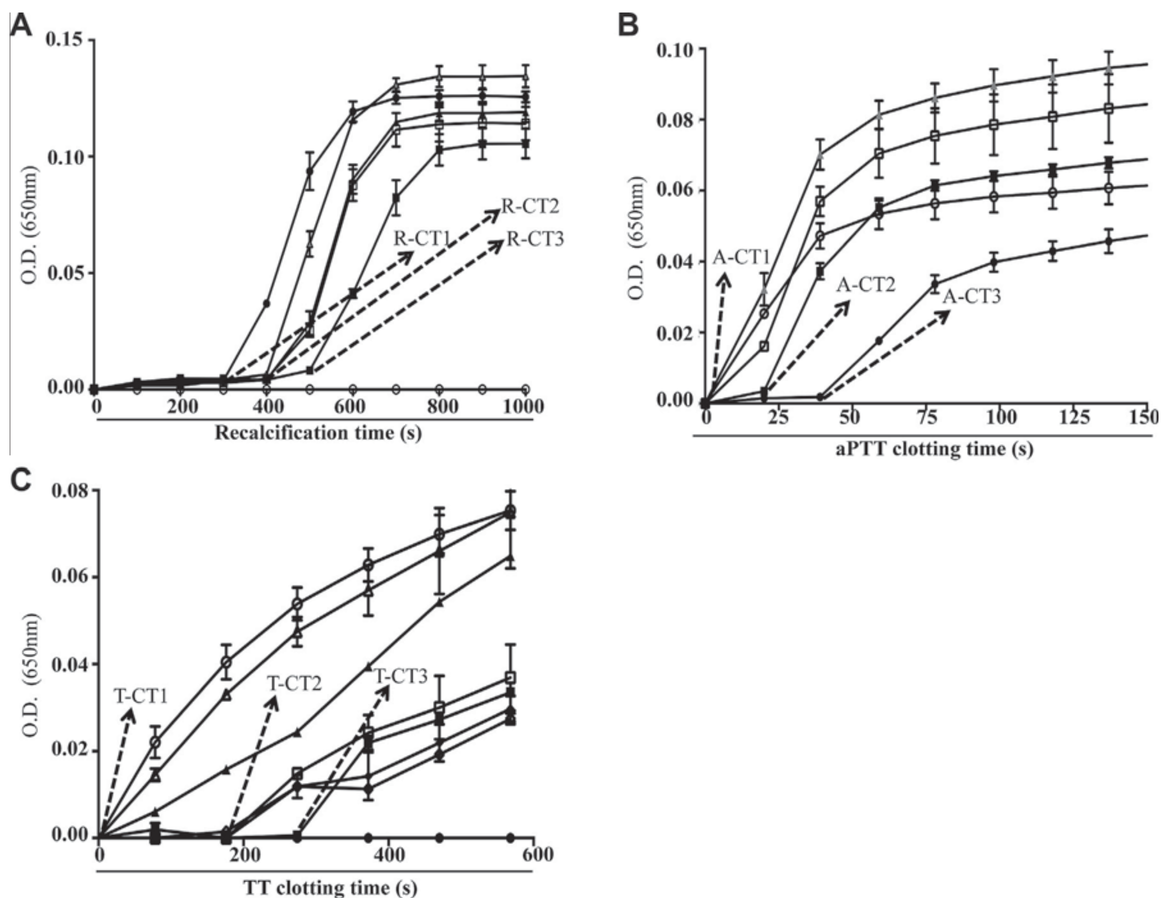

Fig. 9. The effect of recombinant *Amblyomma americanum* serpin-19 (rAAS19) on plasma clotting time in the recalcification time (RCT), activated partial thromboplastin time and thrombin time assays. (A) Universal coagulation reference human plasma (50 μL) was incubated with 0 (arrowhead R-CT1), 0.625, 1.25 and 5 (arrowhead R-CT2), and 10 (arrowhead R-CT3) μM rAAS19 in 90 μL of Tris–HCl reaction buffer for 15 min at 37 °C followed by the addition of 150 mM $CaCl_2$ (10 μL) thereafter clotting was measured every 20 s for 30 min. (B) Universal coagulation reference human plasma (50 μL) was incubated with 0, 0.625, 1.25 (arrowhead A-CT1), 5 (arrowhead A-CT2), and 10 (arrowhead A-CT3) μM rAAS19 up to 100 μL of Tris–HCl reaction buffer for 15 min at 37 °C, followed by the addition of activated partial thromboplastin time reagent and incubation for 5 min at 37 °C before the addition of 25 mM $CaCl_2$ (10 μL); clotting time was monitored every 10 s for 5 min. (C) rAAS19 with 0, 0.625 (arrowhead T-CT1), 1.25 and 5 (arrowhead T-CT2), and 10 (arrowhead T-CT3) μM up to 25 μL of Tris–HCl reaction buffer were incubated with 25 μL of the thrombin time reagent containing $CaCl_2$ for 15 min at 37 °C before addition of 50 μL of pre-warmed Universal coagulation reference human plasma with clotting time monitored every 10 s for 5 min. All assays were done in duplicate, with clotting time monitored at A_{650nm}.

In proposing that ticks use serpins to evade host defense mechanisms, the assumption is that ticks inject serpins into the host during feeding. Although several serpin cDNAs have been cloned, only a few reports confirm that saliva actually contains serpins (Mulenga et al., 2013; Ibelli et al., 2014; Mudenda et al., 2014; Tirloni et al., 2014a). Thus, observations in this study demonstrating native AAS19 is injected into the host during tick feeding and its activity inhibiting proteases participating in host defenses reinforces the idea that inhibitory serpins have a role in the tick-host relationship. Based on the rAAS19 inhibitory activity against trypsin and trypsin-like proteases including plasmin and blood clotting factors Xa and XIa, we conclude this serpin inhibits proteases involved in host defense mechanisms against tick feeding. Albeit at a low rate, it is notable that rAAS19 also inhibits other blood clotting enzymes including fXIIa, fIIa (thrombin) and fIXa, as well as chymotrypsin and tryptase, further confirming that AAS19 has a broad spectrum of inhibitory functions. Although AAS19 inhibits these enzymes, we are cautious about these enzymes being physiological targets for AAS19 because the SI indices estimated in this study were not near 1:1, as would be expected for an inhibition by serpins (Irving et al., 2000; Silverman et al., 2001). However, we note here that the observed high SI indices are not unique for rAAS19. For instance, a SI index of 10 was estimated for *Drosophila melanogaster* Spn1, which inhibits trypsin activity by 96% (Fullaondo et al., 2011). A high SI index could also be explained by the possibility that AAS19 requires a co-factor to enhance inhibition. Indeed, nearly all serpin acting upon the blood clotting activation cascade and fibrinolysis (antithrombin III, heparin-cofactor II, plasminogen activator inhibitor 1, protein C inhibitor and protease nexin inhibitor) need to bind GAGs in order to accelerate the inhibition rate, which can be up to 10,000-fold higher compared with the unbound native serpin (Rein et al., 2011). Accordingly, Z-protein-dependent inhibitor (ZPI) has been shown to accelerate the inhibitory effect of ZPI upon fXa by 1000-fold (Broze, 2001; Munoz and Linhardt, 2004). Our demonstration that AAS19 has four putative GAGBSs suggests its activity could be modulated by heparin or another GAG. Studies to investigate the effects of heparin on rAAS19 function are underway.

From the perspective of tick feeding physiology, the AAS19 inhibition activity upon trypsin, fXa and fXIa could be rationally explained. Trypsin, mostly known for its role as a digestive enzyme (Rawlings and Barrett, 1994), is also associated with inflammation in the skin (Cattaruzza et al., 2014; Meyer-Hoffert et al., 2004), while blood clotting factors Xa and XIa are critical proteases of the blood clotting activation cascade (Monroe and Hoffman, 2006; Roberts et al., 2006). On the other hand, AAS19 inhibition of plasmin can, at a glance, be viewed as contradictory since plasmin is mostly known for its role in digesting blood clots (fibrinolysis) (Angles-Cano, 1994; Angles-Cano et al., 1994), an activity that seems beneficial to tick feeding. Similarly, plasmin degrades and inactivates blood-clotting factors V, VIII, IX and X in vitro, suggesting plasmin has anti-coagulant functions (Hoover-Plow, 2010) and is thus beneficial to tick feeding. On the other hand, plasmin has been reported to participate in several processes such as pro-inflammatory cytokine release (Syrovets et al., 2001), inducing monocyte and dendritic cell chemotaxis (Li et al., 2010), modifying IL-8 and producing a potent attractant of neutrophils (Mortier et al., 2011), tissue remodeling and wound healing (Shen et al., 2012), all of which can negatively impact tick-feeding success. From this perspective, AAS19 inhibition of plasmin seems to contribute to feeding on blood.

Given that AAS19 is injected into the host during tick feeding and due to its inhibitory activity on blood clotting factors, we investigated its action on platelet aggregation and plasma clotting. Platelet aggregation is important to stop bleeding in injured small blood vessels, as observed in tick feeding (Francischetti et al., 2009). Platelet aggregation is activated by multiple agonists including thrombin, collagen, ADP and cathepsin G (Selak et al., 1988; Ohlmann et al., 2000; Brass, 2003). Since thrombin is considered the most efficient platelet aggregation agonist (Furman et al., 1998; Davi and Patrono, 2007), the observation that rAAS19 reduced thrombin-activated platelet aggregation by up to 56 ± 14% suggests that AAS19 may play a crucial role in tick modulation of platelet function. Thrombin activates platelet aggregation through activation of Protease Activated Receptors (PARs) 1, 3 and 4 (Kahn et al., 1999; Asokananthan et al., 2002), and thus it is plausible that native AAS19 may contribute to binding and inhibiting thrombin at the tick feeding site, preventing the formation of the platelet plug and thus impairing the integrity of the blood clot. The observation that pre-incubating rAAS19 with platelets for 15 min prior to addition of the agonist had minimal effect suggests that the observed rAAS19 anti-platelet aggregation effects were mediated through inhibition of thrombin activity. However, it is interesting to note that the "RGD" motif, which is known to interfere with platelet aggregation binding the integrin GPIIb-IIIa on activated platelets (Varon et al., 1993; Katada et al., 1997) is conserved in AAS19 and its homologues. If this motif was functional, pre-incubation with platelets should interfere with platelet aggregation function.

Platelet aggregation is followed by fibrin clot formation, which reinforces the platelet plug (Clemetson, 2012). Blood clotting can be activated via the extrinsic pathway initiated upon release of tissue factor from injured tissue or via the intrinsic pathway when factors XII, XI, IX and VIII are converted into their active forms. These pathways converge into the common pathway when fXa activates prothrombin to thrombin, which in turn catalyzes formation of the fibrin clot (Hoffman and Monroe, 2001; Hoffman, 2003; Monroe and Hoffman, 2006). The effects of anti-blood clotting agents are routinely addressed by using RCT assays, while effects on the extrinsic, intrinsic and common blood clotting activation pathways are examined using PT, aPTT, and TT assays (Davie et al., 1991), as were done in this study. Eight of the 11 blood clotting factors are serine proteases (Walsh and Ahmad, 2002). Thus, although empirical evidence is needed, it is conceivable that the observed rAAS19 anti-coagulant function could be attributed to its inhibitory activity against five (fIIa, fIXa, fXa, fXIa and fXIIa) of the eight serine protease mediators of the blood clotting activation cascade. Except for fVIII, the other four blood clotting factors (IXa, Xa, XIa, and XIIa) in the intrinsic pathway (aPTT assay) are serine proteases, all of which were inhibited by rAAS19, and this could explain the observation of up to 40 s plasma clotting time delay in the aPTT assay. Similarly, in the TT (common pathway) assay, the observed 250 s delay in plasma clotting time could be explained by rAAS19 inhibitory activity against thrombin. The discrepancy of 250 s delay in the TT assay and ~14% inhibition of thrombin activity in substrate hydrolysis is interesting and raises questions for further investigation. There is a possibility that the observed significant clotting time delay in the TT assay could be explained by a possibility of a yet unknown factor in host serum that potentiates AAS19 anti-thrombin activity. Given that AAS19 has four putative heparin binding sites, it is conceivable that GAGs in serum binding to AAS19 could potentiate its activity against thrombin. Experiments to resolve this question are underway.

We would like to note here that arthropods do have hemolymph-clotting cascades that are also controlled by serpins (Agarwala et al., 1996; Iwanaga et al., 1998; Kanost, 1999). Thus, there is a possibility that native AAS19 could be a well-conserved regulator of the tick's hemolymph clotting in itself and the observed anti-coagulant function in this study could be an artifact. However, the observation in this study that native AAS19 was injected into the host during tick feeding strongly suggests the role

of this protein at the tick-feeding site. Alternatively, AAS19 may function both in the tick and at the tick-feeding site. It is important to note here that based on transcript abundance, AAS19 is predominant in the tick MG, which could strongly indicate a role for this protein in the MG. Tick digestion of the blood meal nutrients is intracellular (Lara et al., 2005; Franta et al., 2010), and for this to happen, host blood must remain in the unclotted state. Could it be that AAS19 anti-coagulant function is part of the mechanism in the MG to prevent host blood from clotting before digestion commences?

In conclusion, data in this study contribute towards our understanding of the molecular basis of tick feeding physiology. Data here contribute to the growing list of tick saliva proteins that potentially regulate tick evasion of the mammalian host's defense mechanisms to tick feeding as well as having role(s) in other tick physiological systems. On the basis of AAS19 being highly conserved, coupled with the fact that its RCL is 100% conserved, we conclude that tick physiological processes regulated by AAS19 are evolutionary conserved in all tick species. From the perspective of our long-term interest to find target tick vaccine antigens, AAS19 represents an interesting candidate. Human and animal tick borne diseases are transmitted by different species of ticks, and thus developing universal tick vaccines based on highly conserved proteins such as AAS19 has been advocated (Fragoso et al., 1998; Mulenga et al., 2001, 2013; de la Fuente and Kocan, 2006; Azhahianambi et al., 2009; Parizi et al., 2012). Work toward validating the tick vaccine efficacy of rAAS19 is warranted.

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Anexo D

CURRICULUM VITAE

TIRLONI, L.

1. DADOS PESSOAIS

Nome: Lucas Tirloni
Local de nascimento: Ibirubá/RS - Brasil
Data de Nascimento: 16/12/1986

Endereço profissional: Laboratório de Imunologia Aplicada à Sanidade Animal
Centro de Biotecnologia
Universidade Federal do Rio Grande do Sul (UFRGS)
Av Bento Gonçalves, 9500 – Campus do Vale
Setor IV – Prédio 43.421 – Lab-205
CEP 91501-970
Bairro Agronomia – Porto Alegre – RS - Brasil

Telefone profissional: (51) 3308-6078

Endereço eletrônico: ltirloni@gmail.com

2. FORMAÇÃO

2005 - 2009 Graduação em Biomedicina.
Universidade de Cruz Alta, UNICRUZ, Cruz Alta, RS, Brasil.
Monografia:Princípios ativos provenientes de venenos de serpentes: uma perspectiva na terapia anticoagulante.
Orientador: Giovanna Grunewald Vietta

2010 - 2012 Mestrado em Biologia Celular e Molecular.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, RS, Brasil.
Dissertação: Identificação e caracterização de inibidores de serino-endopeptidases (serpinas) em *Rhipicephalus* (*Boophilus*) *microplus*
Orientação: Carlos Termignoni
Bolsista: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq)

2012 - atual Doutorado em Biologia Celular e Molecular.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil
Orientação: Itabajara da Silva Vaz Jr., Carlos Termignoni e Albert Mulenga
Bolsista: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)

3. FORMAÇÃO COMPLEMENTAR

2014 - 2014 Transcriptomas de artrópodes vetores por RNAseq.
Universidade de São Paulo, USP, São Paulo, Brasil

2013 - 2013 Bioinformática estrutural e análise do proteoma.
Universidade Federal de Minas Gerais, UFMG, Belo Horizonte, Brasil

2011 - 2011 Determinação de Estrutura e Dinâmica de Proteínas.
Sociedade Brasileira de Bioquímica e Biologia Molecular, SBBQ, São Paulo, Brasil

2010 - 2010 Topics in Tick and anti-ticks Vaccines.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil

4. ARTIGOS COMPLETOS PUBLICADOS

KIM, TAE KWON, **TIRLONI, LUCAS**, RADULOVIC, ZELJKO, LEWIS, LAUREN, BAKSHI, MARIAM, HILL, CRESTON, DA SILVA VAZ, ITABAJARA, LOGULLO, CARLOS, TERMIGNONI, CARLOS, MULENGA, ALBERT. Conserved *Amblyomma americanum* tick Serpin19, an inhibitor of blood clotting factors Xa and XIa, trypsin and plasmin, has anti-haemostatic functions. International Journal for Parasitology. , v.45, p.613 - 627, 2015.

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7. ORIENTAÇÕES E SUPERVISÕES

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