

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

Enzimas relacionadas ao equilíbrio redox em carrapatos e uso vacinal de uma glutathione S-  
transferase

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Porto Alegre, fevereiro de 2019

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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 de grau de Doutor em Ciências.

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## LISTA DE ABREVIATURAS, SÍMBOLOS E UNIDADES

4-HNE	4-hidroxi-2-nonenal
AP-1	proteína ativadora-1 ( <i>activator protein-1</i> )
ASK1	quinase reguladora do sinal de apoptose ( <i>apoptosis signal-regulating kinase 1</i> )
ATP	adenosina trifosfato
BYC	<i>boophilus yolk pro-cathepsin</i>
CAT	catalase
DNA	ácido desoxirribonucleico
FEF	fêmea totalmente ingurgitada ( <i>fully engorged female</i> )
GPx	glutaciona peroxidase
GSH	glutaciona reduzida
GST	glutaciona S-transferase
GST-HI	glutaciona S-transferase de <i>Haemaphysalis longicornis</i>
HIF-1	fator induzido por hipóxia-1 ( <i>hypoxia-inducible factor-1</i> )
JNK	quinase c-Jun N-terminal ( <i>c-Jun N-terminal kinase</i> )
MDA	malondialdeído
NADPH	nicotinamida adenina dinucleotídeo fosfato reduzida
NF- κB	fator de transcrição nuclear kappa B ( <i>nuclear factor-κB</i> )
NO	óxido nítrico
NO <sub>2</sub> <sup>-</sup>	dióxido de nitrogênio
O <sub>2</sub> <sup>-</sup>	superóxido
O <sub>3</sub>	ozônio
OH	hidroxila
ONOOH/ONOO <sup>-</sup>	peroxinitrito
PEF	fêmea parcialmente ingurgitada ( <i>partially engorged female</i> )
PI3K	fosfoinositídeo 3-quinase ( <i>phosphoinositide 3-kinase</i> )
PTEN	fosfatase homóloga à tensina ( <i>phosphatase and tensin homolog</i> )
rGST	glutaciona S-transferase recombinante

RHBP	<i>Rhodnius heme-binding protein</i>
RNS	espécies reativas de nitrogênio ( <i>reactive nitrogen species</i> )
ROS	espécies reativas de oxigênio ( <i>reactive oxygen species</i> )
SOD	superóxido dismutase
SULT	sulfotransferase
Trx	tiorredoxina

## RESUMO

Carrapatos são parasitas hematófagos mundialmente distribuídos. Além do parasitismo, carrapatos são vetores de bactérias, protozoários e vírus, prejudicando a saúde humana e animal. O carrapato *Rhipicephalus microplus* é o principal parasita transmissor de patógenos entre bovinos, gerando um grande impacto na economia pecuária. Os carrapatos desenvolveram adaptações fisiológicas para transportar, armazenar, metabolizar e secretar componentes tóxicos da sua dieta e do ambiente. Diferentes classes de enzimas estão envolvidas nesses processos, no entanto, o papel de várias dessas enzimas ainda não está caracterizado no carrapato *R. microplus*. Nesse contexto, os objetivos deste trabalho foram analisar a atuação de enzimas antioxidantes e de detoxificação em fêmeas do carrapato *R. microplus* e avaliar o potencial de uma glutatona S-transferase (GST) como antígeno vacinal. Os dados demonstram que a grande maioria dos níveis transcricionais e de atividade enzimática no ovário e no corpo gorduroso é maior em fêmeas parcialmente ingurgitadas (PEF- *partially engorged female*) do que em fêmeas totalmente ingurgitadas (FEF- *fully engorged females*), com exceção da atividade de glutatona peroxidase, que foi a única enzima com maior atividade no ovário em FEF. Esses resultados sugerem que o carrapato necessita de maior controle de oxidantes nos momentos iniciais de alimentação do que na fase final, em que se prepara para iniciar a postura. No intestino, no entanto, existe uma maior variação sobre os níveis transcricionais e de atividade enzimática entre PEF e FEF. De acordo com a capacidade do carrapato em manter a homeostase redox sem causar estresse oxidativo, os níveis de NADPH mostraram-se inalterados entre PEF e FEF. No entanto, os níveis de glutatona total variaram nos órgãos analisados comparando PEF e FEF, assim como também foi observado no nível transcricional e de atividade enzimática da maioria das enzimas analisadas. O silenciamento de catalase e tiorredoxina redutase não foram capazes de prejudicar a capacidade de alimentação e de postura das fêmeas e de eclosão das larvas, sugerindo a manutenção da função enzimática pelas enzimas remanescentes ao silenciamento, ou ainda, a ativação de mecanismos compensatórios. Finalmente, a rGST-HI (GST recombinante de *Haemaphysalis longicornis*) foi capaz de gerar anticorpos que reconhecem as GSTs nativas de *Rhipicephalus appendiculatus* e *Rhipicephalus sanguineus*, no entanto, sua utilização como antígeno vacinal mostrou ser eficiente apenas contra *R. appendiculatus* e não contra *R. sanguineus*, demonstrando que mesmo espécies evolutivamente próximas podem possuir diferentes mecanismos de evasão da resposta imune do hospedeiro para esse antígeno. Em conjunto, os dados demonstram a complexidade do sistema enzimático antioxidante e de detoxificação no carrapato e sua relação com diversos mecanismos fisiológicos além da alimentação.

## ABSTRACT

Ticks are globally distributed hematophagous parasites. In addition to parasitism, ticks are vectors of bacteria, protozoa and viruses, which damage human and animal health. The *Rhipicephalus microplus* tick is the major pathogen of cattle, causing a great impact on the livestock economy. Ticks have developed physiological adaptations to transport, store, metabolize and secrete toxic components from the diet and environment. Different classes of enzymes are involved in these processes, however, the role of several of these enzymes is not yet characterized in *R. microplus*. In this context, the goals of this work were to analyze the performance of antioxidant and detoxification enzymes in partially and fully engorged *R. microplus* females and to evaluate the vaccine potential of a glutathione S-transferase (GST). Results demonstrate that transcriptional levels and enzymatic activity from ovary and fat body are predominantly higher partially engorged female in (PEF) than in fully engorged females (FEF), except to GPx activity in ovary, the only enzyme with highest enzymatic activity in the FEF stage. These results suggest a fundamental need of the antioxidant potential at the initial feeding moments than in the final phase, when ticks start the laying. In midgut, however, there is a wider variation on the transcriptional levels and enzymatic activity between the PEF and FEF phases. According to the great ability of the tick to maintain redox homeostasis without causing oxidative stress, NADPH levels were unaffected between the PEF and FEF. However, total glutathione levels fluctuate between the PEF and FEF phases according to what was observed at the transcriptional level and the enzymatic activity of most of the enzymes analyzed. The silencing of CAT and TRx reductase did not impair the females feeding and laying capacity, and larvae hatching, indicating the high capacity of remaining enzyme activity, or the activation of compensatory mechanisms. Finally, rGST-H1 (recombinant GST from *Haemaphysalis longicornis*) was able to induce antibodies that recognize the native GSTs of *Rhipicephalus appendiculatus* and *Rhipicephalus sanguineus*, however, their use as a vaccine antigen proved to be efficient only against *R. appendiculatus* but not against *R. sanguineus*. This suggests that evolutionarily close species could have different mechanisms of evasion of the immune response. Together, results demonstrate the complexity of the antioxidant enzyme system and detoxification in the tick and its relation with several physiological mechanisms.



## 1. INTRODUÇÃO

Carrapatos são artrópodes hematófagos pertencentes a ordem Acari, dentro da classe Arachnida. Aproximadamente 900 espécies de carrapatos já foram identificadas dentro de três famílias: Ixodidae (692 espécies), Argasidae (186 espécies) e Nuttallielidae (1 espécie) (SONENSHINE e ROE, 2013).

Durante a hematofagia, carrapatos Ixodidae podem ingerir até 3 mL de sangue, provocando lesões na pele e, dependendo da carga parasitária, levando o hospedeiro a anemia (PFAFFLE *et al.*, 2009) . Além dos danos diretos gerados pelo parasitismo, os carrapatos atuam como vetores de bactérias, protozoários e vírus, causando doenças em humanos e animais (DE LA FUENTE *et al.*, 2017; ISMAIL e MCBRIDE, 2017). Os carrapatos da família Argasidae permanecem relativamente pouco tempo fixados no hospedeiro, geralmente algumas horas ou menos, ingerindo pouca quantidade de sangue, enquanto que os carrapatos da família Ixodidae necessitam de um tempo de hematofagia mais prolongado, durante dias, permanecendo fixados geralmente em um único hospedeiro, hábitos que favorecem a transmissão de agentes infecciosos (SONENSHINE e ROE, 2013). A maioria dos carrapatos da família Ixodidae está classificada em um dos cinco gêneros: *Ixodes*, *Amblyomma*, *Dermacentor*, *Rhipicephalus* e *Haemaphysalis*, sendo esses responsáveis pela maior parte dos patógenos transmitidos por carrapatos (DANTAS-TORRES *et al.*, 2012).

Os carrapatos possuem uma ampla distribuição geográfica, podem ser encontrados em todos os continentes. São capazes de parasitar uma grande variedade de hospedeiros vertebrados, incluindo mamíferos, répteis, pássaros e anfíbios (SONENSHINE e ROE, 2013). Depois dos mosquitos, os carrapatos são considerados os maiores artrópodes vetores de patógenos para humanos e animais. Entre as doenças causadas por esses patógenos, foram

descritas 17 para humanos e 19 para animais domésticos e de produção (DANTAS-TORRES, *et al.*, 2012). Deste modo, carrapatos geram significativos prejuízos econômicos na pecuária, principalmente nas zonas tropicais e subtropicais do mundo, e um importante impacto na saúde pública predominantemente no hemisfério norte, devido a prevalência da doença de Lyme e outras zoonoses transmitidas por carrapatos. Além disso, animais domésticos também são acometidos por patógenos transmitidos por carrapatos, gerando problemas em centros urbanos por todo o mundo (MAGNARELLI, 2009).

### **1.1. O carrapato *Rhipicephalus microplus***

O carrapato da espécie *Rhipicephalus microplus* pertence à família dos Ixodidae, é monoxeno e tem o bovino como hospedeiro preferencial, podendo, eventualmente, parasitar outros animais (MCCOY *et al.*, 2013). Esta espécie está amplamente distribuída em toda a região tropical e subtropical do mundo. Estudos sugerem que o *R. microplus* teve origem no sudoeste asiático, co-evoluindo com raças zebuínas e foi transportado para outros continentes e regiões devido à ampla migração de bovinos para a produção de leite durante os séculos XVII-XIX (BARRE e UILENBERG, 2010; ESTRADA-PENA *et al.*, 2006).

O ciclo de vida do *R. microplus* desenvolve-se em duas fases de vida: livre e parasitária. Durante a fase de vida livre, as larvas podem sobreviver durante um período de 3 a 4 meses sem se alimentar, podendo alcançar até 6 meses em períodos de frio até que encontre o hospedeiro. As larvas possuem geotropismo negativo, ou seja, quando estão no solo, se aglomeram em regiões mais altas das gramíneas. São estimuladas na presença de dióxido de carbono, alguns odores, vibrações e mudanças luminosas, hábitos que favorecem o encontro do hospedeiro (OSTERKAMP *et al.*, 1999; SONENSHINE e ROE, 2013). Na fase de vida parasitária, as larvas realizam a fixação no bovino ingerindo inicialmente exsudatos celulares e fluidos linfáticos (SUTHERST *et al.*, 1978), após 6 dias,

aproximadamente, larvas sofrem muda e tornam-se ninfas, quando é iniciada a ingestão de sangue por mais 6 dias, até que se tornem adultos. Os adultos completam o dimorfismo sexual. As fêmeas parcialmente ingurgitadas, denominadas de partenóginas, tornam-se atrativas para o macho, momento em que ocorre o acasalamento, dando origem às fêmeas fecundadas e iniciando a fase de rápido ingurgitamento, próximo ao 21º dia de alimentação. Somente as fêmeas fecundadas são capazes de ingerir as maiores quantidades de sangue do hospedeiro. No total, são necessárias aproximadamente três semanas desde o início da alimentação até o completo ingurgitamento. A fêmea completamente ingurgitada é denominada teleóquina. Nesta fase, a fêmea desprende-se do hospedeiro e cai ao solo, momento em que se inicia a fase de vida livre. A fêmea procura um local úmido e protegido do sol para a postura dos ovos, que dura cerca de 14 dias. A eclosão das larvas começa aproximadamente 7 dias após o final da postura, iniciando um novo ciclo de vida do parasita (SONENSHINE e ROE, 2013; SUTHERST, *et al.*, 1978).

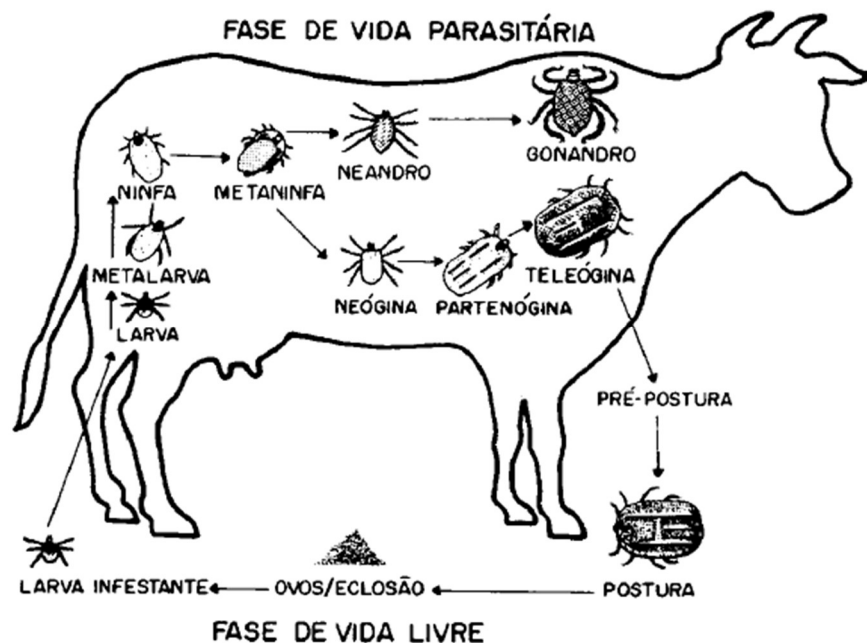


Figura 1 - Ciclo de vida do carrapato *R. microplus* (Fonte: GONZALES, 1975).

Com especial relevância na saúde animal, o carrapato *R. microplus* é o principal ectoparasita em rebanhos bovinos (TABOR *et al.*, 2017). Uma elevada carga parasitária debilita a saúde do animal, causando anemia e consequente perda de peso e produção de leite. No local da picada um processo inflamatório é estimulado, causando danos ao couro e inviabilizando sua comercialização (SUTHERST, *et al.*, 1978). Além dos danos diretos provocados pelo parasitismo, o carrapato *R. microplus* é o principal transmissor dos protozoários *Babesia bovis* e *Babesia bigemia* e das bactérias *Anaplasma marginale* e *Anaplasma centrale*. Esses patógenos causam as doenças babesiose e a anaplasmoze, que podem causar a tristeza parasitária bovina, uma doença que causa anemia hemolítica progressiva, icterícia, febre, apatia, inapetência e taquipnéia, podendo levar o animal à morte (SUTHERST, *et al.*, 1978; TABOR, *et al.*, 2017). Segundo o IBGE, em 2016 a carne bovina esteve entre os quatro principais produtos industriais do país, gerando juntos 9,5% da receita das vendas industriais, que foi de R\$ 2,17 trilhões (IBGE, 2016). No 1º trimestre de 2018, foram abatidas 7,72 milhões de cabeças de bovinos sob algum tipo de serviço de inspeção sanitária (IBGE, 2018), destinando um faturamento de 3,5 bilhões de dólares somente na exportação (IBGE, 2018). Diante da importância da pecuária no Brasil, é fundamental ressaltar as significativas perdas econômicas geradas pelo *R. microplus* na pecuária. Essas perdas podem ser associadas, portanto, à diminuição do rendimento dos produtos derivados do gado, à mortalidade de rebanhos causada pelos patógenos transmitidos por carrapatos, aos custos com tratamentos veterinários e com métodos de controle do carrapato (DE CASTRO, 1997; JONSSON *et al.*, 2008). Considerando esses parâmetros, estimou-se que, anualmente, as perdas econômicas geradas pelo carrapato podem atingir mais de 3,2 bilhões de dólares americanos no Brasil (GRISI *et al.*, 2014).

A utilização de acaricidas químicos é o principal método de controle do *R. microplus*. A grande maioria desses acaricidas consiste em neurotoxinas que atuam especificamente sobre o sistema nervoso do carrapato (TAYLOR, 2001). As principais classes de drogas utilizadas são os organoclorados, piretróides sintéticos, organofosforados, amidinas, fenilpirazóis, reguladores de crescimento e lactonas macrocíclicas (RODRIGUEZ-VIVAS *et al.*, 2018). Este método é capaz de eliminar grande parte dos parasitas nas regiões em que é aplicado, no entanto, a constante utilização do mesmo acaricida gera uma pressão seletiva sobre as populações de carrapatos sensíveis, selecionando carrapatos resistentes e gerando populações resistentes que sobrevivem aos compostos, o que torna o método ineficaz a longo prazo (RODRIGUEZ-VIVAS *et al.*, 2014). Além disso, acaricidas químicos são espalhados no ambiente com pouco controle, contaminando o solo e ambientes aquáticos, intoxicando outras espécies de animais e eliminando artrópodes que pertencem ao ecossistema. A potencial contaminação dos produtos derivados do bovino como a carne e o leite e a exposição de trabalhadores aos compostos são outras desvantagens do uso de acaricidas (KUNZ e KEMP, 1994).

Tendo em vista os diversos problemas que limitam o uso de acaricidas para o controle efetivo do carrapato, outros métodos surgem como alternativa. Foi observado que após sofrerem repetidas infestações por *R. microplus*, bovinos tornaram-se resistentes ao parasita, o que indica a atuação da resposta imune contra o carrapato (ALLEN, 1994; TATCHELL e MOORHOUSE, 1968). A partir desse conhecimento, o desenvolvimento de vacinas contra o carrapato tornou-se uma alternativa interessante, visto que elimina, tanto os problemas de seleção de carrapatos resistentes quanto a contaminação ambiental observados na utilização de acaricidas. No início dos anos 90 surgem as primeiras formulações vacinais comerciais contra o carrapato *R. microplus*. Essas vacinas contêm o antígeno recombinante Bm86, uma

proteína da membrana celular do intestino do carrapato (WILLADSEN *et al.*, 1995; WILLADSEN *et al.*, 1989). Esta vacina foi capaz de reduzir o número de fêmeas ingurgitadas, seu peso e sua capacidade reprodutiva, reduzindo significativamente as subsequentes infestações por larva (WILLADSEN, *et al.*, 1989). A utilização desta vacina já foi realizada em diversos países como Cuba, Austrália, México, Colômbia e Brasil, no entanto, não possui a mesma eficácia em todas as regiões. Foram identificadas variações na sequência de aminoácidos de Bm86 entre diferentes populações de carrapato (COBON *et al.*, 1995; COBON *et al.*, 1997; RODRIGUEZ *et al.*, 1994), o que foi posteriormente relacionado com a variação da eficiência dessa vacina entre diferentes populações de carrapato (GARCIA-GARCIA *et al.*, 1999).

Diversas proteínas do carrapato vêm sendo caracterizadas com o intuito de desenvolver antígenos vacinais capazes de induzir uma resposta imune protetora (BLECHA *et al.*, 2018; PARIZI *et al.*, 2012; RODRIGUEZ-MALLON, 2016). Ainda que muito conhecimento tenha sido gerado nesse sentido, contribuindo cada vez mais para a compreensão desse organismo, o estudo de proteínas quanto ao seu papel na fisiologia, genética e comportamento do carrapato ainda se faz necessário para o progresso das vacinas e de outros métodos de controle do carrapato.

## **1.2. O processo de hematofagia e o controle do estresse oxidativo em carrapatos e outros artrópodes hematófagos**

Entre as mais de 800 mil espécies de artrópodes descritas, aproximadamente 15 mil possuem hábitos hematófagos. Estima-se que a hematofagia tenha surgido entre 145-65 milhões de anos atrás, nas eras jurássica e cretácea, uma estratégia que surgiu independentemente pelo menos seis vezes durante o curso evolutivo dos artrópodes (MANS *et al.*, 2002). O sangue dos hospedeiros consiste em uma rica fonte de nutrientes: além de

proteínas, contém açúcares, sais, lipídeos, aminoácidos e hormônios, suprindo as necessidades nutricionais do artrópode e de seus embriões (HOCKING, 1971). O carrapato é capaz de ingerir grandes quantidades de sangue, por exemplo, fêmeas da família Íxodidae ingerem entre 50-200 vezes o seu peso inicial em sangue, um volume entre 1 e 3 mL aproximadamente (MEHLHORN, 2016). Adaptações morfológicas, comportamentais e fisiológicas desses artrópodes no curso da evolução se tornaram essenciais para o sucesso da hematofagia (GRACA-SOUZA *et al.*, 2006; RIBEIRO, 1987; SANDERS *et al.*, 2003).

A hemoglobina é uma das proteínas mais abundantes no sangue de mamíferos (COTTER, 2001), junto com a albumina compreendem cerca de 80% das proteínas do sangue (SOJKA *et al.*, 2016). A digestão da hemoglobina em hematófagos resulta na liberação de grandes quantidades de heme (HORN *et al.*, 2009; LARA *et al.*, 2003), o grupo prostético da hemoglobina. O heme é uma molécula essencial para os organismos, pois é o grupo prostético das hemo enzimas (MENSE e ZHANG, 2006). Consiste em um átomo de ferro (Fe) ligado a quatro átomos de nitrogênio de um anel porfirínico. O Fe é um metal de transição, que pode existir na forma ferrosa ( $Fe^{2+}$ ) ou férrica ( $Fe^{3+}$ ), essa propriedade transitória é o que permite a catálise óxido-redutora pelas hemo enzimas (POULOS, 2014). Por outro lado, esse grupo possui uma natureza potencialmente tóxica: por ser uma molécula naturalmente lipofílica, quando livre tende a interagir com membranas de células e organelas, desestabilizando o citoesqueleto e a permeabilidade das membranas (SCHMITT *et al.*, 1993). Experimentos em cultivo celular de *Plasmodium falciparum* revelam a lise das células após a adição de 10 mM de heme ao meio de cultivo (ORJIH *et al.*, 1981). Além disso, devido a sua natureza reativa, o Fe livre pode reagir facilmente com ROS fisiologicamente produzidas durante processos metabólicos, desencadeando a reação de Fenton, uma reação em cadeia que leva a peroxidação lipídica e a formação de mais ROS

(KEHRER, 2000), que interagem com estruturas celulares danificando proteínas (AFT e MUELLER, 1984), lipídeos (TAPPEL, 1955) e DNA (AFT e MUELLER, 1983). Para evitar esses danos, artrópodes hematófagos possuem mecanismos moleculares que impedem o desencadeamento dessas reações e o consequente estresse oxidativo.

Uma das primeiras linhas de defesa contra a toxicidade do heme em hematófagos consiste em isolar fisicamente esta molécula das demais moléculas celulares, reduzindo a sua potencial reatividade (OLIVEIRA *et al.*, 2002). Para isso, durante a digestão da hemoglobina, as moléculas de heme liberadas são agregadas em organelas que separam fisicamente esses agregados de várias moléculas celulares. Esse mecanismo de agregação já foi descrito em diversos organismos. Inicialmente em *P. falciparum*, o vetor da malária, esse agregado foi denominado pigmento malárico (PAGOLA *et al.*, 2000; SLATER *et al.*, 1991). Em *Rhodnius prolixus* a maioria das moléculas de heme livre derivadas da digestão da hemoglobina são acumuladas em hemozoínas, agregados de heme organizados na forma de cristais, que resultam na redução da formação de radicais livres (OLIVEIRA *et al.*, 1999; OLIVEIRA, *et al.*, 2002). Mecanismo também observado no helminto *Schistosoma mansoni* (OLIVEIRA *et al.*, 2000), e no protozoário *Haemoproteus columbae* (CHEN *et al.*, 2001). No carrapato *R. microplus*, onde a digestão é intracelular e ocorre em vesículas digestivas, a hemoglobina é internalizada por endocitose através de receptores e após a digestão da hemoglobina o heme é acumulado em organelas denominadas hemossomos, agregados também organizados, mas não na forma de cristais (LARA, *et al.*, 2003). No mosquito *Aedes aegypti* o heme também é agregado em vesículas não-cristalinas chamadas hemozoínas, e estas estruturas estão associadas à matriz peritrófica, uma camada extracelular do intestino composta por proteínas e polissacarídeos (PASCOA *et al.*, 2002).



Proteínas carreadoras de heme possuem outro mecanismo que impede essa molécula de reagir livremente com outras moléculas da célula. Diversos artrópodes como carrapatos, mosquitos e hemípteros expressam proteínas carreadoras de heme (DONOHUE *et al.*, 2009; MAYA-MONTEIRO *et al.*, 2000; OLIVEIRA *et al.*, 1995; PASCOA, *et al.*, 2002). Duas dessas proteínas foram bem caracterizadas em *R. prolixus* e *R. microplus* e denominadas RHBP (DANSA-PETRETSKI *et al.*, 1995) e HeLp (MAYA-MONTEIRO, *et al.*, 2000), respectivamente. Apesar de exercerem funções análogas, estas proteínas apresentam diferentes mecanismos de ação, estrutura e composição, sugerindo que evoluíram independentemente entre esses organismos (GRACA-SOUZA, *et al.*, 2006). Além dessas proteínas atuarem como um mecanismo de defesa contra o estresse oxidativo, atuam também como transportadores e reservatórios de heme. Em *R. prolixus* RHBP é sintetizada no corpo gorduroso em todos os estágios do desenvolvimento. Essa proteína é secretada na hemolinfa, onde se liga ao heme e o transporta até os oócitos, desempenhando um papel fundamental para o desenvolvimento do embrião (MACHADO *et al.*, 1998). Em *R. microplus*, foi demonstrado que a aquisição de heme através da dieta é essencial para que o embrião complete seu desenvolvimento e que proteínas carreadoras e armazenadoras de heme também são essenciais nesse processo (PERNER *et al.*, 2016b; THOMPSON *et al.*, 2007). Ao contrário da maioria dos organismos, o carrapato não possui a via de síntese do heme, adquirindo todo o heme necessário através da dieta (PERNER, *et al.*, 2016b). Por esse motivo, proteínas carreadoras de heme desempenham funções especialmente importantes para o desenvolvimento desse parasita. Em vertebrados, a hemopexina, uma proteína com grande afinidade ao heme, inibe reações de oxidação provocadas pelo heme, desempenhando uma função antioxidante (GUTTERIDGE e SMITH, 1988; SEERY e MULLER-

EBERHARD, 1973; SMITH e MORGAN, 1984), mostrando que esse é um mecanismo necessário mesmo em organismos com baixa relação evolutiva.

Os organismos possuem sistemas naturais para o controle de radicais livres. Os antioxidantes não enzimáticos podem ser produzidos pelo próprio organismo ou adquiridos através da dieta e também controlam o estresse oxidativo em hematófagos. Estas moléculas atuam tanto como cofatores enzimáticos, como é o caso da GSH (DICKINSON e FORMAN, 2002), quanto de maneira autônoma como as vitaminas. Sua propriedade antioxidante se dá através da capacidade de doar elétrons a sistemas oxidativos, estabilizando espécies reativas e impedindo a continuidade dessas reações descontroladas. Devido a suas propriedades eletroquímicas esses antioxidantes não são tão reativas mesmo após terem doado elétrons. Em alguns artrópodes esses componentes são mais facilmente adquiridos através da alimentação da seiva das plantas, que contém antioxidantes como o tocoferol, caroteno e flavonoides (PISOSCHI *et al.*, 2016). O urato é descrito como um potente antioxidante em vertebrados (MAPLES e MASON, 1988) e insetos (BARRETT e FRIEND, 1970) pois inativa a reatividade de metais de transição. Em insetos, é produzido pelo corpo gorduroso, exportado para a hemolinfa e depois para os túbulos de Malpighi. Sua ação antioxidante foi demonstrada em *R. prolixus* (SOUZA *et al.*, 1997) e *Drosophila melanogaster* (HILLIKER *et al.*, 1992).

A degradação do heme compreende outro mecanismo de controle descrito em hematófagos (SPENCER *et al.*, 2018; WIGGLESWORTH, 1943). Essa degradação é realizada pela enzima heme oxigenase (HO) que catalisa a oxidação do heme em biliverdina, CO e  $Fe^{2+}$ , reação que depende de oxigênio e NADPH (KIKUCHI *et al.*, 2005). Neste sistema, a participação da enzima ferritina é descrita como importante para a remoção do  $Fe^{2+}$  liberado dessa reação, evitando a formação de ROS através da reação de Fenton

(RYTER e TYRRELL, 2000). Não é possível definir exatamente qual o papel da degradação do heme exclusivamente relacionado com uma defesa direta contra a reatividade do heme, pois observa-se que a biliverdina gerada a partir dessa degradação possui uma natureza antioxidante (JANSEN e DAIBER, 2012). Inclusive *D. melanogaster*, um artrópode não hematófago, o catabolismo do heme pela heme oxigenase desempenha um papel fundamental para a formação de tecidos (CUI *et al.*, 2008). Em contrapartida, foi observada a ausência do gene da enzima em diversos genomas e transcriptomas de carrapatos (BRAZ *et al.*, 1999; CRAMARO *et al.*, 2015; PAIVA-SILVA *et al.*, 2006).

As enzimas antioxidantes são oxidoreduases com diferentes mecanismos de ação, estrategicamente localizadas em diferentes tecidos e microambientes celulares e degradam determinadas espécies reativas. Elas desempenham um papel fundamental no combate a ROS e na homeostase redox da célula (SEN, 2001). Visto que são enzimas encontradas em todos os reinos, supõe-se que possuam um papel fundamental também em organismos não hematófagos (PERNER *et al.*, 2016a). Uma variedade de enzimas antioxidantes já foi descrita nesses organismos (BUDACHETRI e KARIM, 2015; DIAS *et al.*, 2016; OLIVEIRA *et al.*, 2017), e a sua relação com a hematofagia também foi observada. No carrapato *Ixodes ricinus*, foi relatado que há um maior número de transcritos da enzima sulfotransferase no intestino de carrapatos após a alimentação com sangue do que antes da alimentação (PERNER, *et al.*, 2016a). O mesmo padrão se repete com catalase em *Aedes aegypti* (OLIVEIRA, *et al.*, 2017). Foi observado também em *Dermacentor variabilis* que a hematofagia promove o aumento da transcrição GSTs no intestino e no ovário (DREHER-LESNICK *et al.*, 2006). Em *R. prolixus* foi descrita uma intensa produção de peróxido de hidrogênio após a alimentação com sangue e um aumento da expressão das enzimas superóxido dismutase e catalase (PAES *et al.*, 2001). A inibição de catalase e da síntese de

GSH levam a um aumento nos níveis de peróxido de hidrogênio no intestino (PAES, *et al.*, 2001). Em *A. aegypti* ocorre aumento da transcrição do gene de uma GST apenas após a alimentação com sangue, o que não ocorre após a alimentação com açúcar (BOTTINO-ROJAS *et al.*, 2015). Esses resultados sugerem que enzimas antioxidantes atuam em conjunto, cooperando para controlar espécies reativas e prevenir a geração de estresse oxidativo que poderia ser causada pela grande quantidade de sangue ingerida.

### **1.3. Os radicais livres na homeostase redox e no controle do estresse oxidativo**

O planeta Terra formou-se há cerca de 4,6 bilhões de anos. Evidências sugerem que as primeiras formas de vida surgiram há 3,7 bilhões de anos (UREY, 1952), mas o oxigênio apareceu em significativas quantidades na atmosfera terrestre somente há 2,3 bilhões de anos, principalmente devido à fotossíntese por cianobactérias (LUO *et al.*, 2016). A formação da camada de ozônio (O<sub>3</sub>) e o acúmulo de oxigênio atmosférico favoreceram a disseminação de organismos vivos devido ao bloqueio de radiação ultravioleta (GRENFELL *et al.*, 2010). Sugere-se que as primeiras formas de vida eucariota surgiram há cerca de 1,8 bilhões de anos (KNOLL *et al.*, 2006), um salto evolutivo que também pode ser atribuído ao uso do oxigênio como o acceptor final de elétrons no metabolismo energético, o que aumenta a eficiência na produção de ATP (KAISER, 2001). Devido a sua alta eletronegatividade, entretanto, o oxigênio é capaz de formar espécies altamente reativas (ROS), que podem atacar estruturas celulares de organismos que até então não tinham evoluído com esse metabolismo, por isso, muitas formas de vida primitivas foram extintas e atualmente os organismos anaeróbios, que sobrevivem em ambientes com restrição de oxigênio, são supostamente descendentes desses ancestrais. Outros organismos começaram a desenvolver sistemas de defesas antioxidantes, promovendo a homeostase de um sistema oxidativo de

eficiente produção de energia sem causar toxicidade, dando origem aos organismos complexos que existem atualmente (ARUOMA *et al.*, 2006).

Os radicais livres são bioprodutos da respiração aeróbia e fazem parte de processos fisiológicos e patológicos dos organismos. Uma ideia comum sobre os radicais livres é a de que eles levam os sistemas biológicos ao estresse oxidativo. Um aumento na produção de ROS ou RNS, ou uma diminuição na capacidade de eliminação desses elementos devido a interferências exógenas ou alterações metabólicas endógenas pode levar à instabilidade desse sistema, aumentando os danos às biomoléculas e gerando o estresse oxidativo/nitrosativo. No entanto, além do potencial dano aos sistemas biológicos, os radicais livres também possuem importantes funções na sinalização e sobrevivência celular (SEN, 2001).

Um radical livre é definido como uma molécula que contém um ou mais elétrons desemparelhados (um número ímpar de elétrons) geralmente na sua última camada de eletrônica. Os radicais livres incluem ROS e RNS. Os ROS são formados pela redução parcial do oxigênio: o ânion  $O_2^-$ , o  $H_2O_2$  e os radicais OH. Os RNS são formados por NO e moléculas derivadas, como o ONOOH/ONOO<sup>-</sup> e o  $NO_2^-$ . Essas espécies reativas são formadas por oxigênio ou nitrogênio, ambos são átomos extremamente eletronegativos, o que confere a sua propriedade reativa. Além do NO, as RNS são produtos que surgem da reação entre o NO e o radical  $O_2^-$ . Na célula, uma das principais fontes de RNS é a óxido nítrico sintase, enzima constitutivamente expressa nos neurônios e células endoteliais, que produzem NO para manter a vasodilatação (BREDT *et al.*, 1991; MARSDEN *et al.*, 1992). As espécies reativas NO,  $O_2^-$  e  $H_2O_2$  são menos reativas, por isso, mais relacionadas com a sinalização celular do que com reações destrutivas. O  $H_2O_2$  é formado pela redução do  $O_2$  através da adição de dois hidrogênios, é gerado em condições fisiológicas majoritariamente

pelos neutrófilos e macrófagos durante uma resposta do sistema imune, pela atividade da superóxido dismutase sobre o superóxido produzido pela mitocôndria e pela NADPH oxidase (BRAND, 2010). Nas proteínas, os resíduos de cisteína estão na forma de tiolato (Cys-S) em pH fisiológico e são mais suscetíveis à oxidação em comparação com o tiol reduzido (Cys-SH). Na presença de  $H_2O_2$  os ânions tiolato são oxidados para sua forma sulfênica (Cys-SOH), causando alterações alostéricas que alteram a atividade da proteína. A forma sulfênica pode ser novamente reduzida pelas dissulfeto redutases (tioredoxina e glutathione redutases), retomando a conformação original da proteína (WINTERBOURN e HAMPTON, 2008). Dessa forma, em um nível oxidativo basal, os resíduos de cisteína das proteínas servem como um mecanismo reversível de transdução de sinal. Estima-se que a oxidação de tiolato nas células ocorra em uma concentração de  $H_2O_2$  na faixa dos nanomolar, enquanto níveis mais elevados dessa espécie reativa podem oxidar os ânions tiolato em espécies sulfínicas (Cys-SO<sub>2</sub>H) ou sulfônicas (Cys-SO<sub>3</sub>H), essas formas, ao contrário da forma sulfênica, podem ser irreversíveis, resultando em danos permanentes à proteína. Além disso, na presença de metais de transição o  $H_2O_2$  pode ser rapidamente transformado em  $OH^\bullet$ , o radical mais reativo. Por isso, o controle dos níveis de  $H_2O_2$  deve ser regulado pelas peroxidases.

A mitocôndria é a principal fonte de radicais livres na célula, onde a maior parte do oxigênio é reduzido a água. Uma pequena parte dessas moléculas de  $O_2$  é convertida em  $O_2^-$  pelos complexos I e III da cadeia transportadora de elétrons (LE BRAS *et al.*, 2005). O  $O_2^-$  resulta da redução do  $O_2$  por um único elétron e pode danificar proteínas que contém grupamentos constituídos pelos metais ferro e enxofre. Níveis muito elevados de  $O_2^-$  estão mais associados ao estresse oxidativo do que à sinalização celular (SCHIEBER e CHANDEL, 2014). É importante observar, no entanto, que o  $O_2^-$  não danifica proteínas

indiscriminadamente: um conjunto específico de metaloproteínas são sensíveis à inativação pelo  $O_2^-$ , que promovem a ativação de mecanismos adaptados a níveis elevados de  $O_2^-$  ou iniciam a morte celular (CHEN *et al.*, 2009).

O radical OH é o elemento mais reativo entre todos os radicais livres, oxida indiscriminadamente lipídeos, proteínas e DNA, resultando em danos nas biomoléculas ou instabilidade genômica (DIZDAROGLU e JARUGA, 2012). Esse radical normalmente é gerado a partir de  $H_2O_2$  na presença de íons  $Fe^{+2}$  ou  $Fe^{+3}$ , desencadeando a reação de Fenton. Por isso, além do controle sobre os radicais livres, as células também possuem mecanismos para manter a homeostase do Fe, evitando a formação desse radical super-reativo.

Os lipídeos são as biomoléculas mais susceptíveis à oxidação, principalmente os poli-insaturados, que possuem elétrons mais expostos e vulneráveis ao ataque de radicais livres. Ao serem atacados, os fosfolipídeos propagam uma reação em cadeia oxidativa nos demais fosfolipídeos da membrana plasmática, em que os produtos gerados são diferentes espécies reativas capazes de atacar especificamente proteínas e DNA. Os danos oxidativos a esses fosfolipídeos, portanto, podem levar à morte celular não somente devido a alterações na membrana plasmática, mas a alterações funcionais das proteínas e DNA (TRACHOOTHAM *et al.*, 2008). O MDA (MARNETT, 1999), por exemplo pode reagir com o DNA, causando mutações gênicas. Já o 4-HNE reage mais especificamente com proteínas, inclusive as quinases JNK e caspase-3, causando alterações funcionais e ativação de mecanismos de morte celular induzido pela peroxidação lipídica (AWASTHI *et al.*, 2003; BREITZIG *et al.*, 2016).

As proteínas podem ser alteradas por espécies reativas de modo reversível ou irreversível, dependendo do alvo e do radical que reage. Apesar de virtualmente todos os aminoácidos serem susceptíveis ao ataque de radicais livres, alguns são particularmente

vulneráveis devido a suas propriedades como os aminoácidos lisina, arginina, histidina, prolina, treonina e cisteína (TRACHOOTHAM, *et al.*, 2008). Um estresse oxidativo elevado pode induzir a formação de ligações dissulfeto entre proteínas, ou modificações oxidativas secundárias como a conjugação de proteínas oxidadas (adutos proteicos), podendo gerar agregação de complexos proteicos que inativam proteossomas, levando ao acúmulo de proteínas danificadas e morte celular (JUNG *et al.*, 2014).

Em comparação com proteínas e lipídeos, o DNA é menos susceptível a modificações oxidativas devido à sua estrutura dupla hélice compartimentalizada, a proteínas histonas e outras proteínas ligadas ao DNA que protegem seus centros nucleofílicos. O DNA está sujeito a danos nas purinas e pirimidinas nos seus átomos de O e N, que são altamente eletronegativos. Além disso, as ligações duplas dentro das bases são os alvos primários do radical OH. As reações são principalmente direcionadas aos C5 e C6 nas pirimidinas e C4 e C8 nas purinas. Danos oxidativos através da subtração de H causam a ruptura da dupla fita (BREEN e MURPHY, 1995). Os danos oxidativos no DNA mitocondrial são mais frequentes, uma vez que esse DNA está mais próximo a ROS produzidos na mitocôndria, e não possui mecanismos de reparo tão efetivos quanto o DNA nuclear (INOUE *et al.*, 2003).

Apesar de serem altamente reativos, esses radicais possuem certa especificidade, como citado anteriormente. Além disso, a compartimentalização da produção de ROS na célula é um importante determinante da sinalização celular ou geração de estresse oxidativo (KALUDERCIC *et al.*, 2014). Proteínas que participam de vias de sinalização redox precisam estar próximas de onde o radical é produzido. Por exemplo, os alvos do H<sub>2</sub>O<sub>2</sub> gerado a partir de NADPH oxidases da membrana plasmática também estão localizados na membrana plasmática, permitindo que essas vias de sinalização sejam ativadas, o mesmo pode ser observado nas mitocôndrias (AL-MEHDI *et al.*, 2012).



Alterações nos níveis de ROS podem modular os sistemas biológicos através da ativação e inativação de vias de sinalização modificando as proteínas através da regulação da sua expressão (oxidando fatores de transcrição), modificações pós-traducionais (oxidando proteínas quinases) e diretamente através da oxidação de seus aminoácidos (TRACHOOTHAM, *et al.*, 2008). ROS estão envolvidas em diversas vias de proliferação, diferenciação, sobrevivência e morte celular. Foi observado que ROS interferem na ativação de fatores de crescimento celular (TRACHOOTHAM, *et al.*, 2008) e na inativação da fosfatase homóloga à tensina (PTEN), uma proteína que atua como supressor tumoral e regulador negativo da fosfatidilinositol 3-quinases (PI3K) (LEE *et al.*, 2002). Diversos fatores transcricionais possuem cisteínas em sítios de ligação ao DNA sensíveis à oxidação como o NF- $\kappa$ B (SCHRECK *et al.*, 1991), AP-1 (RAHMAN *et al.*, 2002), HIF-1 (CALLAPINA *et al.*, 2005) e P53 (CHEN *et al.*, 2003). As modificações oxidativas diretas como a S-glutationação, formação de pontes dissulfeto e a S-nitrosilação regulam diversas proteínas como a TRX (KONDO *et al.*, 2004), P53 (HAFSI e HAINAUT, 2011), e AKT (ZHAO *et al.*, 2017). A dissociação de proteínas conjugadas sensíveis à sinalização redox também corresponde a um mecanismo de regulação por ROS. A modificação oxidativa de conjugados proteicos pode levar à dissociação desse complexo, permitindo a ativação da proteína livre, como é observado no caso dos complexos TRX-ASK1 (HSIEH e PAPACONSTANTINO, 2006) e JNK-GST (ADLER *et al.*, 1999).

#### **1.4. O papel das enzimas antioxidantes na regulação da homeostase redox**

Embora inicialmente tenha sido observado que os radicais livres são responsáveis por danos à célula, trabalhos mostraram que concentrações moderadas dessas moléculas estão envolvidas em respostas fisiológicas como parte de mecanismos de sinalização celular e resposta imune (MARTIN e BARRETT, 2002; SCHIEBER e CHANDEL, 2014). De fato,

atualmente uma nova perspectiva mostra que um equilíbrio anti/pró-oxidante é necessário para a homeostase celular. Como parte desse sistema, uma série de mecanismos evoluíram para regular a ação das espécies reativas (ESPINOSA-DIEZ *et al.*, 2015). Nesse sentido, enzimas antioxidantes atuam como um dos principais sistemas de redução dessas moléculas nos organismos, controlando danos e regulando a sinalização.

A ação dos radicais livres está relacionada com a sua natureza química. Por serem mais eletronegativos, o oxigênio e o nitrogênio atraem com mais força elétrons de um microambiente, ou de moléculas próximas. Uma vez que moléculas com elétrons não emparelhados tornam-se espécies reativas, sua natureza tende a sequestrar elétrons de outros compostos para que se torne estável, produzindo radicais livres capazes de desencadear reações oxidativas em cadeia. Os antioxidantes são moléculas que atuam doando os elétrons necessários para estabilizar essas espécies reativas, sem que o próprio antioxidante se torne tão reativo. As enzimas antioxidantes atuam através de reações de oxi-redução (redox), que reduzem a espécie reativa através da oxidação de outro composto, mantendo, ainda, esse composto em uma forma não reativa.

A catalase, uma das enzimas envolvidas nessa regulação, é uma hemo-peroxidase (consome  $H_2O_2$ ) tetramérica majoritariamente presente nos peroxissomos (EMERIT e MICHELSON, 1982), organelas celulares envolvidas em várias funções, como a oxidação de ácidos graxos, a  $\beta$ -oxidação de ácidos graxos de cadeia longa, o catabolismo de purinas e a biossíntese de glicerolipídeos, esteroides e ácidos biliares (FRANSEN *et al.*, 2017). Essas atividades metabólicas são fontes de ROS, e por isso a atividade de enzimas antioxidantes nessa organela é importante. A catalase realiza uma reação de dismutação de duas moléculas  $H_2O_2$  em duas moléculas de água e uma de  $O_2$ . A atividade da enzima depende de três componentes: a porção heme do sítio ativo, um NADPH reduzido ligado ao domínio de

ligação a NADPH e uma estrutura secundária complexa formada por longas alças de peptídeo que interagem durante tetramerização (ZAMOCKY *et al.*, 2008). Sua atividade catalítica ocorre por meio de um mecanismo de dois estágios. No primeiro estágio da reação o  $\text{Fe}^{+3}$  ligado ao grupo heme atua como agente redutor, reduzindo uma molécula de  $\text{H}_2\text{O}_2$  em água e gerando  $\text{Fe}^{+4}$  ligado a um átomo de oxigênio. Esse produto intermediário é chamado de composto I. No segundo estágio, outra molécula de  $\text{H}_2\text{O}_2$  é usada como agente redutor para regenerar o  $\text{Fe}^{+3}$  do centro catalítico a partir de  $\text{Fe}^{+4}$ , produzindo também outra molécula de água e uma de oxigênio (ANDERSSON e DAWSON, 1990). O papel do NADPH ainda não está completamente elucidado, no entanto, algumas das hipóteses propostas estão relacionadas com a inibição da formação de alguns produtos intermediários de catalase inativos (GOYAL e BASAK, 2010; HILLAR e NICHOLLS, 1992).

A superóxido dismutase (SOD), outra enzima que participa na regulação redox, é uma metaloenzima que realiza a dismutação de  $\text{O}_2^-$  em  $\text{H}_2\text{O}_2$  e  $\text{O}_2$ , produtos menos reativos que o primeiro. O  $\text{O}_2^-$  é produto de algumas enzimas de sinalização, bem como o subproduto de vários processos metabólicos, incluindo a respiração mitocondrial. Todos os organismos aeróbicos possuem diferentes tipos de SOD situadas em variadas localizações celulares e subcelulares, reflexo das múltiplas fontes de  $\text{O}_2^-$  na célula. Quatro classes de SOD com diferentes íons metálicos são encontradas nos organismos: Cu/Zn-SOD, Mn-SOD, Fe-SOD e a mais recentemente descoberta Ni-SOD em bactérias (MILLER, 2012; YOUN *et al.*, 1996). Os organismos eucariotos possuem Cu/Zn-SOD no citoplasma e também secretam para o meio extracelular. Mn/Fe-SODs está presente tipicamente nas mitocôndrias, uma das maiores fontes de  $\text{O}_2^-$  na célula (WEISIGER e FRIDOVICH, 1973). A evolução dessas enzimas contendo diferentes metais em seus centros catalíticos tem sido associada à disponibilidade desses metais de transição na atmosfera durante as diferentes eras geológicas

(CASE, 2017). Todas elas desempenham um mecanismo semelhante em que uma molécula de  $O_2^-$  atua inicialmente reduzindo o metal de transição, formando  $O_2$ , e em seguida, outro  $O_2^-$  oxida o metal de transição, formando  $H_2O_2$ . Nessa reação uma fonte de hidrogênio advinda de uma molécula de água adjacente é necessária para que o  $O_2$  seja reduzido a peróxido (ABREU e CABELLI, 2010).

As peroxidases compreendem um grupo heterogêneo de enzimas que atuam na redução de  $H_2O_2$  e possuem mecanismos catalíticos baseados em princípios diferentes. Duas peroxidases que atuam na homeostase redox são as glutathiona e tioredoxina peroxidases. Essas enzimas utilizam, respectivamente, GSH e Trx como cofatores enzimáticos fontes de poder redutor. Elétrons são transferidos desses cofatores para a espécie reativa através da catálise da peroxidase e voltam a ser restaurados por redutases que utilizam elétrons do NADPH. Nesses casos, enzimas atuam aos pares de forma acoplada (peroxidase-redutase), mantendo um sistema ativo capaz de se auto reciclar (FLOHE e URSINI, 2008). A GPx possui um resíduo de selenocisteína ativo em seu centro catalítico, que pode reduzir tanto  $H_2O_2$  em  $H_2O$  e  $O_2$ , quanto hidroperóxidos de lipídeos (LOOH) em  $H_2O_2$  e álcoois lipídicos (LOH), utilizando duas moléculas de GSH como doadoras de elétrons. Nessa reação forma-se glutathiona oxidada (GSSG) que pode ser regenerada à GSH através da atividade da glutathiona redutase ou exportada para o meio extracelular (HIRRLINGER *et al.*, 2001). A GPx foi localizada em diversos compartimentos: citosol, núcleo (MUSE *et al.*, 1994) e mitocôndria (UTSUNOMIYA *et al.*, 1991), o que sugere sua ampla distribuição celular. Ela possui uma especial função relacionada à redução de hidroperóxidos de lipídeos (CAVAS e TARHAN, 2003; URSINI *et al.*, 1982), devido a sua conformação e natureza monomérica, que conferem um fácil acesso a hidroperóxidos maiores não catalisados por outras peroxidases tetraméricas. Nesse sentido, possui um papel fundamental na manutenção da

integridade das membranas, evitando a peroxidação lipídica (COZZA *et al.*, 2017; SEILER *et al.*, 2008).

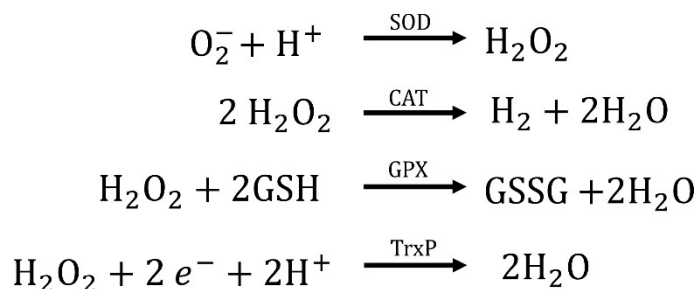


Figura 2 - Algumas reações enzimáticas do sistema antioxidante. Superóxido dismutase (SOD); catalase (CAT); glutathione peroxidase (GPX); tioredoxina peroxidase (TrxP).

Outra classe de enzimas que atua na eliminação de xenobióticos, drogas e componentes endógenos são algumas transferases. As glutathione S-transferases e as sulfotransferases atuam na conjugação de GSH (ANGELUCCI *et al.*, 2005) e sulfato (DONG *et al.*, 2012), respectivamente, a compostos tóxicos. Possuem, portanto, uma variedade indefinida de substratos, facilitando sua excreção pelo metabolismo devido a essa modificação na molécula inicial. Apesar de também catalisarem reações com substratos endógenos (BALOGH e ATKINS, 2011; HARTLEY *et al.*, 1995), a grande maioria dos estudos com GSTs relaciona sua atividade com a resistência a drogas em diversos organismos (MORROW e COWAN, 1990). Outros trabalhos ainda revelam uma atividade não clássica como peroxidase (ORTELLI *et al.*, 2003; SINGH *et al.*, 2001; ZIEGLER e IBRAHIM, 2001). As sulfotransferases estão, ainda, relacionadas com a inativação de hormônios em mamíferos (MUELLER *et al.*, 2015; SUZUKI-ANEKOJI *et al.*, 2013), um mecanismo comumente encontrado também em outros eucariotos.

É possível observar que as enzimas antioxidantes possuem certa especificidade em relação ao substrato, no entanto, a grande variedade de enzimas antioxidantes, incluindo suas

inúmeras isoformas encontradas nos proteomas e transcriptomas, sugere que elas atuam sobre vários substratos e fazem parte de processos celulares fundamentais, atuando como sistemas compensatórios ou complementares dentro dos sistemas biológicos.

### **1.5. O estudo de proteínas como antígenos vacinais contra o carrapato**

A partir das primeiras observações de que hospedeiros vertebrados após repetidas infestações eram capazes de desenvolver uma resposta imune protetora contra carrapatos (ALLEN, 1994; TATCHELL e MOORHOUSE, 1968), o estudo e desenvolvimento de vacinas vem sendo ampliado. Esse método de controle não apresenta riscos de intoxicação para o trabalhador, é ambientalmente adequada e diminui drasticamente os riscos de selecionar carrapatos resistentes em comparação com os acaricidas químicos (WILLADSEN, 2004). Em 1986, uma proteína de *R. microplus* denominada Bm86 foi descoberta como antígeno vacinal, tornando-se o primeiro antígeno a compor uma vacina comercial contra um ectoparasita (WILLADSEN *et al.*, 1988). Embora as formulações vacinais contendo o antígeno Bm86 sejam efetivas contra *R. microplus*, o nível de proteção pode ser maior ou menor dependendo da variabilidade genética das populações de carrapatos (ANDREOTTI *et al.*, 2008; FREEMAN *et al.*, 2010; PENICHET *et al.*, 1994). Por isso, o estudo desses organismos e a identificação de nova proteínas e a caracterização do seu papel na fisiologia do carrapato ainda é necessário.

Extratos proteicos de órgãos de carrapato como a glândula salivar e o intestino foram inicialmente testados como antígenos vacinais, produzindo efeitos variados sobre as infestações. A vacinação de coelhos com extrato de intestino de *R. appendiculatus* foi capaz de reduzir o número de larvas infestantes (JONGEJAN *et al.*, 1989). A imunização de cães com extrato de intestino de *R. sanguineus* reduziu a capacidade de oviposição das fêmeas do carrapato que se alimentaram nesse hospedeiro (SZABO e BECHARA, 1997). Bezerras

foram imunizados com extrato de glândula salivar e intestino de *Hyalomma marginatum*, apresentando níveis de proteção variados em parâmetros relacionados à fixação, ingurgitamento, oviposição e eclosão (SAHIBI *et al.*, 1997). O potencial e interferência dos adjuvantes nas formulações vacinais contra carrapatos também foram relatados nesses estudos (SAHIBI, *et al.*, 1997; SZABO e BECHARA, 1997).

Com o avanço da tecnologia do DNA recombinante, muitas proteínas foram caracterizadas quanto a sua atividade e ao seu potencial como antígeno vacinal (CSORDAS *et al.*, 2018; DA SILVA VAZ *et al.*, 2004; SABADIN *et al.*, 2017; YOU, 2004). Nesse sentido, surgiram outras abordagens como a utilização de múltiplos antígenos contra uma espécie, como uma tentativa de ampliar os alvos fisiológicos do carrapato atingidos pelos anticorpos induzidos por essas proteínas (PARIZI, *et al.*, 2012). Por exemplo, as proteínas recombinantes BYC, VTDCE e GST-HI foram utilizadas em conjunto em um experimento de vacinação contra *R. microplus*, mostrando uma diminuição no nível de infestação (PARIZI, *et al.*, 2012). Um “coquetel” vacinal elaborado com três inibidores de serinoproteases (serpinas) recombinantes foi capaz de induzir uma resposta imune protetora contra *R. appendiculatus* (IMAMURA *et al.*, 2008).

Outro conceito utilizado para o desenvolvimento de vacinas foi de que antígenos conservados entre espécies poderiam ser utilizados para proteger contra mais de uma espécie (PARIZI *et al.*, 2011), facilitando o controle do carrapato em regiões onde houvesse infestações por diferentes espécies. Nesse sentido, a proteína 64TRP da saliva de *R. appendiculatus* envolvida na formação do cemento mostrou ser eficaz contra *R. sanguineus* e *Ixodes ricinus* (HAVLIKOVA *et al.*, 2009). Uma ferritina recombinante de *R. microplus* (RmFER2) utilizada na imunização de bovinos foi capaz de proteger o hospedeiro contra infestações por *R. microplus* e *Rhipicephalus annulatus* (HAJDUSEK *et al.*, 2010).

Também, foi observado que uma GST de *H. longicornis* (GST-HI) utilizada como antígeno vacinal foi capaz de induzir resposta imune protetora contra *R. microplus* (PARIZI, *et al.*, 2011). Outro trabalho mostrou que esse antígeno protegia também contra *R. appendiculatus* (SABADIN, *et al.*, 2017). Outros antígenos como a subolesina (ALMAZAN *et al.*, 2005), uma proteína altamente conservada em artrópodes, envolvida com a regulação da expressão gênica (CANALES *et al.*, 2009b), e a própria Bm86 (CANALES *et al.*, 2009a; DE VOS *et al.*, 2001; PIPANO *et al.*, 2003) são capazes de gerar proteção contra múltiplas espécies de carrapato.

A ação integrada entre vacinas e acaricidas demonstram resultados interessantes, no sentido de diminuir a quantidade de acaricida necessário para uma eliminação satisfatória de carrapatos de uma determinada região. Utilizando a vacina comercial com o antígeno Bm86 em uma população de bovinos foi observado que o número de tratamentos com acaricida poderia ser reduzido em 87% durante o período de 8 anos (1995-2003), além de reduzir as infecções por babesia (VALLE *et al.*, 2004). Outro estudo demonstrou a eliminação de quase 100% de *R. microplus* resistentes à acaricidas de uma região através da utilização integrada entre vacinas e acaricidas (REDONDO *et al.*, 1999), demonstrando que esse método possui perspectivas positivas na eliminação de infestações por carrapatos.



## 2. OBJETIVOS

O objetivo deste trabalho foi analisar o papel de enzimas antioxidantes e de detoxificação em carrapatos.

Objetivos específicos

- 1) Determinar a relação entre o nível transcricional do gene de enzimas antioxidantes e de detoxificação e a alimentação em diferentes tecidos de fêmeas de *R. microplus*;
- 2) Determinar a relação entre a atividade de enzimas antioxidantes e de detoxificação e a alimentação em diferentes tecidos de fêmeas de *R. microplus*;
- 3) Determinar a relação entre o nível de potenciais redutores celulares e a alimentação em diferentes tecidos de fêmeas de *R. microplus*;
- 4) Avaliar o efeito do silenciamento de enzimas antioxidantes sobre o desenvolvimento do carrapato *R. microplus*.
- 5) Avaliar os efeitos da vacinação com o antígeno rGST-HI sobre os carrapatos *R. appendiculatus* e *R. sanguineus*

### 3. EXPERIMENTOS E RESULTADOS

Esta tese é composta por dois capítulos que contém os artigos científicos que descrevem o trabalho experimental elaborado. O primeiro capítulo apresentado como artigo foram avaliados os efeitos da vacinação utilizando o antígeno rGST-HI sobre os tecidos e o desenvolvimento de carrapatos das espécies *R. sanguineus* e *R. appendiculatus*. Esses resultados foram publicados em um artigo científico na revista científica *Vaccine*. Além disso, no segundo capítulo apresentado como artigo, analisa as principais enzimas antioxidantes e de detoxificação, além de potenciais redutores em dois diferentes estágios de alimentação em fêmeas do carrapato *R. microplus*. Esse artigo ainda será submetido para publicação.

Capítulo I: Effect of recombinant glutathione S-transferase as vaccine antigen against *Rhipicephalus appendiculatus* and *Rhipicephalus sanguineus* infestation.

Capítulo II: An insight into the functional role of antioxidant and detoxification enzymes in partially and fully engorged females of *Rhipicephalus microplus* tick.

### 3.1. Capítulo I

#### **Effect of recombinant glutathione S-transferase as vaccine antigen against *Rhipicephalus appendiculatus* and *Rhipicephalus sanguineus* infestation**

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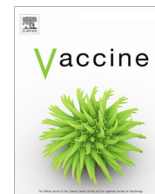
Execução dos experimentos: G.A.S., L.F.P., M.A.X, R.d.S.M.

Contribuição de reagentes/materiais/ferramentas de análise: I.d.S.V., M.I.C., N.W., G.V.N.

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## Effect of recombinant glutathione S-transferase as vaccine antigen against *Rhipicephalus appendiculatus* and *Rhipicephalus sanguineus* infestation



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### ABSTRACT

The ticks *Rhipicephalus appendiculatus* and *Rhipicephalus sanguineus* are the main vectors of *Theileria parva* and *Babesia* spp. in cattle and dogs, respectively. Due to their impact in veterinary care and industry, improved methods against *R. appendiculatus* and *R. sanguineus* parasitism are under development, including vaccines. We have previously demonstrated the induction of a cross-protective humoral response against *Rhipicephalus microplus* following vaccination with recombinant glutathione S-transferase from *Haemaphysalis longicornis* tick (rGST-HI), suggesting that this protein could control tick infestations. In the present work, we investigated the effect of rGST-HI vaccine against *R. appendiculatus* and *R. sanguineus* infestation in rabbits. *In silico* analysis revealed that GST from *H. longicornis*, *R. appendiculatus* and *R. sanguineus* have >80% protein sequence similarity, and multiple conserved antigenic sites. After the second vaccine dose, rGST-HI-immunized rabbits showed elevated antibody levels which persisted until the end of experiment (75 and 60 days for *R. appendiculatus* and *R. sanguineus*, respectively). Western blot assays demonstrated cross-reactivity between anti-rGST-HI antibodies and native *R. appendiculatus* and *R. sanguineus* GST extracts from ticks at different life stages. Vaccination with rGST-HI decreased the number, weight, and fertility of engorged *R. appendiculatus* adults, leading to an overall vaccine efficacy of 67%. Interestingly, histological analysis of organ morphology showed damage to salivary glands and ovaries of *R. appendiculatus* adult females fed on vaccinated animals. In contrast, rGST-HI vaccination did not affect *R. appendiculatus* nymphs, and it was ineffective against *R. sanguineus* across the stages of nymph and adult. Taken together, our results show the potential application of rGST-HI as an antigen in anti-tick vaccine development, however indicating a broad difference in efficacy among tick species.

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### 1. Introduction

Ticks are hematophagous ectoparasites that, in addition to the direct harm caused to their hosts, are often responsible for patho-

gen transmission [1]. Among these species, *Rhipicephalus appendiculatus* and *Rhipicephalus sanguineus* cause great impact on animal health, resulting mainly from disease transmission [2,3]. *R. appendiculatus* occurs in eastern, central and southern Africa region [4], while *R. sanguineus* has a worldwide distribution; however, the taxonomical status of *R. sanguineus* is under revision since the name *R. sanguineus* has been applied for different *Rhipicephalus* spp. tick populations [3,5].

Tick feeding induces host immune responses that build resistance against future tick infestations [6], and this observation led to the hypothesis that a strategy to modulate host immunity could be used to control tick infestation. There is currently a broad

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consensus that effective anti-tick vaccines could constitute a sustainable approach for the control of ticks and tick borne diseases [7], avoiding the deleterious effects associated with acaricide application. Due to the presence, under field conditions, of multiple tick species, and the need to simplify commercial production of an effective vaccine, there is a pressing demand to identify and evaluate antigens offering heterologous protection [8–10]. For some candidate antigens, however, very low or absent effectiveness against other tick species were observed [11,12].

Glutathione S-transferases (GSTs) are proteins found in all eukaryotes; they are responsible for elimination of toxic substances by catalyzing a nucleophilic conjugation reaction of reduced glutathione with the toxic compound [13]. In the ticks *R. sanguineus* and *Rhipicephalus microplus*, GSTs are involved in permethrin detoxification [14] and resistance to synthetic pyrethroid [15], respectively. Furthermore, *R. microplus* GST plays a role in egg antioxidant defenses, as indicated by its effects on redox balance of these cells, and by a positive correlation with lipid peroxidation [16]. Recombinant *Haemaphysalis longicornis* GST (GST-HI), used in vaccination trials as single- [17] or multi-antigenic vaccines [18], induced a partial protection against *R. microplus* infestation. The protective capacity of GST-HI was also tested against *R. microplus* using GST-HI-expressing *Babesia bovis* as a live vector vaccine [19]. Moreover, a multi-antigenic vaccine composed of rGST-HI, recombinant *R. microplus* VTDCE (vitellin degrading cysteine endopeptidase), and recombinant BYC (*Boophilus* yolk cathepsin), reduced *R. microplus* survival in 35–60%, and improved cattle body weight gain in 14% [18]. These data support the use of rGST-HI in cross-protection vaccination trials against other tick species. In this work, we evaluated the potential of rGST-HI as a vaccine antigen against *R. appendiculatus* and *R. sanguineus* infestations.

## 2. Methods

### 2.1. Ticks and rabbits

The standard *R. appendiculatus* Muguga stocks were maintained at the tick unit of the International Livestock Research Institute (ILRI) for at least forty years [20]. *R. sanguineus* tropical lineage were collected in Rio de Janeiro, Brazil [21], and kept at the Universidade Federal do Rio Grande do Sul (UFRGS). Ticks were fed by experimental infestation on 4-months old New Zealand rabbits (2 kg) in isolated cages, and kept at 28 °C and 85% relative humidity. The experiments were approved and conducted following the guidelines of the Ethics Committee on Animal Experimentation of UFRGS and ILRI.

### 2.2. In silico analyses

Amino acid sequence predictions, alignment using ClustalW algorithm, and the identity matrix of GSTs amino acid sequences (*R. appendiculatus* AY298732, *R. sanguineus* AGK29895, and *H. longicornis* AAQ74441), were performed using BioEdit software version 7.2.5 [22]. Immunogenic regions of tick GSTs were predicted by Jameson Wolf algorithm [23], using the software LaserGene version 7.0.0.

### 2.3. Expression and purification of rGST-HI

rGST-HI was expressed and purified as previously described [24] in *Escherichia coli* strain D494 (DE). Purification of the soluble fraction containing rGST-HI was performed by affinity chromatography using GSTrap (GE Healthcare). Protein concentration was determined by the Bradford method [25].

### 2.4. Tick tissues extraction

Ovaries, salivary glands and gut of partially and fully engorged *R. appendiculatus* and *R. sanguineus* female ticks were dissected using a stereomicroscope (Stemi DRC; Zeiss) as previously described [17] and lysed by sonication (Qsonica-model Q125) or by maceration, for *R. sanguineus* or *R. appendiculatus* respectively. Ten-days old eggs, whole larvae, and nymphs of both tick species were also macerated in PBS. Tissue homogenates were centrifuged at 16,000g for 15 min at 4 °C. For purification of native GST from tissues, 60 µl of glutathione-Sepharose 4B resin were mixed with tissue extracts aliquots (300 µg) and incubated for 10 min at room temperature. Fractions were separated by centrifugation at 10,000g for 10 min at 4 °C. The soluble fractions were collected and the resin containing bound native GST was washed 10 times with PBS.

### 2.5. In vitro cross-reactivity evaluation

The cross-reactivity of anti-rGST-HI antibodies with native GSTs from *R. appendiculatus* and *R. sanguineus* was evaluated by western blotting. The total amount of native GST obtained by purification from each of the different tissues of *R. appendiculatus* or *R. sanguineus* (see Section 2.4) was resolved by 14% SDS-PAGE and transferred to nitrocellulose membranes [26]. Membranes were blocked with 5% non-fat dry milk in PBS for 1 h at room temperature and incubated with anti-rGST-HI rabbit sera (1:100) for 16 h at 4 °C, followed by incubation with anti-rabbit IgG alkaline phosphatase conjugate (1:5000) for 2 h at room temperature. Detection was performed with NBT (nitro blue tetrazolium, Thermo Scientific; 0.3 mg/mL) and BCIP (5-bromo-4-chloro-3-indolyl phosphate, Fermentas; 0.15 mg/mL) in alkaline phosphatase buffer (tris-HCl 100 mM, NaCl 100 mM, and MgCl<sub>2</sub> 5 mM).

### 2.6. Vaccination trial

Fourteen New Zealand rabbits were divided in 4 groups for immunization experiments and tick infestation challenge as follows: *R. appendiculatus* vaccine group (rabbits GST-1Ra, GST-2Ra, and GST-3Ra) and control group (rabbits PBS-1Ra, PBS-2Ra, and PBS-3Ra); and *R. sanguineus* vaccine group (rabbits GST-4Rs, GST-5Rs, GST-6Rs and GST-7Rs) and control group (rabbits PBS-4Rs, PBS-5Rs, PBS-6Rs, and PBS-7Rs). Rabbits in vaccine groups were subcutaneously immunized (needle length of 2.5 cm) in the back with 200 µg of rGST-HI (0.5 mL) emulsified with Marcol/Montanide (0.5 mL) in 3 doses at 14-day intervals, a protocol similar to bovine vaccination experiment reported by Parizi et al. [17]. Control groups were injected with PBS (0.5 mL) plus Marcol/Montanide (0.5 mL). For *R. appendiculatus* challenge, each rabbit was infested with 190 nymphs placed in a chamber on one ear, and 60 adults (30 males and 30 females) in another chamber on the other ear. For *R. sanguineus* challenge, two control and two immunized rabbits were infested with 40 adults (20 male and 20 female) each, while the other two control and two immunized rabbits were infested with 80 nymphs each. Ticks of each instar were placed in separate chambers fixed on the back of the rabbits. Blood samples were collected at each inoculation and post-infestation days. Serum was separated by centrifugation at 5000g for 10 min and stored at –20 °C for further analysis. Tick number, weight, molt, egg laying, and hatch rates were analyzed.

### 2.7. Serological assays

Rabbit humoral immune responses were evaluated using an ELISA protocol previously published by Ali et al. [27], with slightly modified washing and blocking steps, which in our study were

performed in 0.05% PBS-tween. Sera from experimental and control groups were used as primary antibodies. Anti-rabbit IgG alkaline peroxidase conjugate (1:5000) was used as secondary antibody. Absorbance at 492 nm was measured in a microplate spectrophotometer (Spectramax Microplate Reader, Molecular Devices Corporation). Serum titration of vaccinated rabbits was evaluated by the same ELISA protocol. The titer was defined as the dilution of post-immunization serum that showed an optical density (OD) more than two times the OD of pre-immune serum.

## 2.8. Histology

Tissues of fully engorged *R. appendiculatus* females fed on rabbits during vaccination trial were collected for histology. A total of 9 ticks per group (3 ticks from each rabbit of vaccine and control groups) were dissected and tissues fixed in 4% paraformaldehyde/10% formalin and dehydrated in ethanol series at 15 min intervals. The resin blocks were sectioned at 3  $\mu$ m on Leica RM2255 microtome, followed by staining with haematoxylin and eosin (HE), according to [28]. Slides were air-dried, mounted in Canada balsam, and photodocumented.

## 2.9. Statistical analysis

Vaccine efficacy was estimated based on the difference in number and weight of fully engorged females, egg laying capacity, egg fertility, and molting, between ticks fed on vaccinated or non-vaccinated rabbits. The overall protection was calculated as previously described, taking into account female numbers [29],

or alternatively using female weight as parameter, since engorgement was affected by vaccination. Significant differences were identified using *t*-test, both for physiological parameters of ticks fed in vaccinated versus control groups, and for ELISA assays.

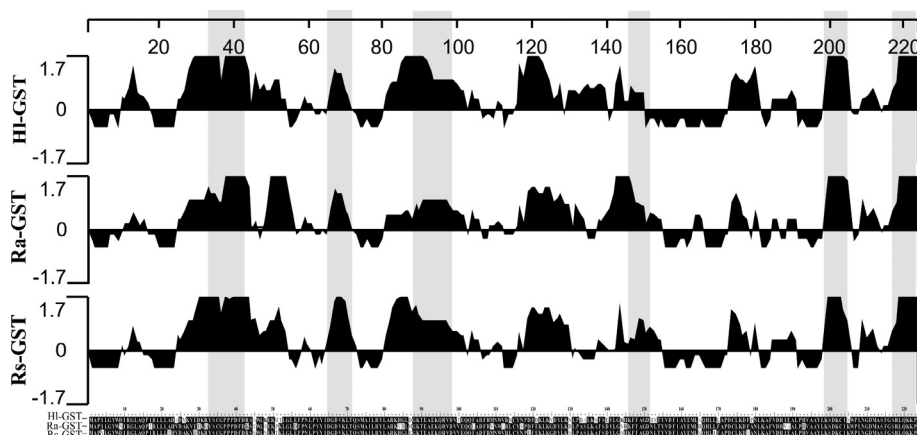
## 3. Results

### 3.1. Identity, antigenicity and cross-recognition of GSTs

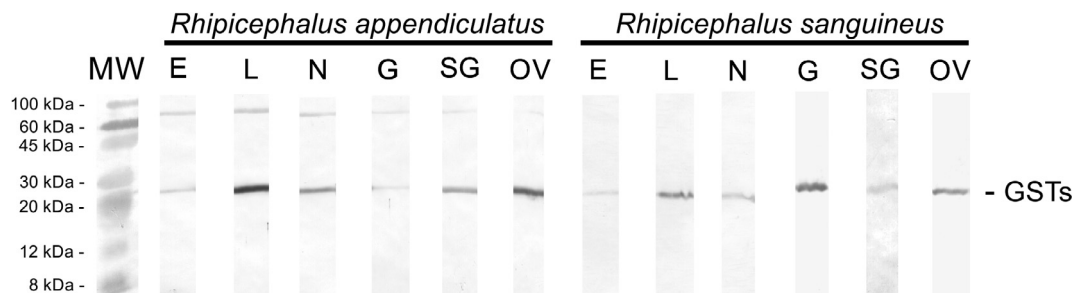
The predicted amino acid sequences of *R. appendiculatus* and *R. sanguineus* GSTs were found to be 82.5% and 86.9% identical to *H. longicornis* GST, respectively (Suppl. Fig. 1). Protein alignment showed that the three GST sequences possess conserved regions within the primary structures, and *in silico* epitope mapping analysis revealed that most of the conserved regions are also antigenic (Fig. 1). Sera raised against rGST-HI was able to cross-react with native GSTs purified from *R. appendiculatus* and *R. sanguineus* tissues (Fig. 2); no recognition was observed with pre-immune sera (data not shown). The western blot also revealed that *R. appendiculatus* and *R. sanguineus* GST are expressed in adults in different organs (ovary, salivary glands and gut), and across all different developmental stages: embryonic (egg), larvae, nymph, and adult ticks (Fig. 2).

### 3.2. Vaccination and challenge trials

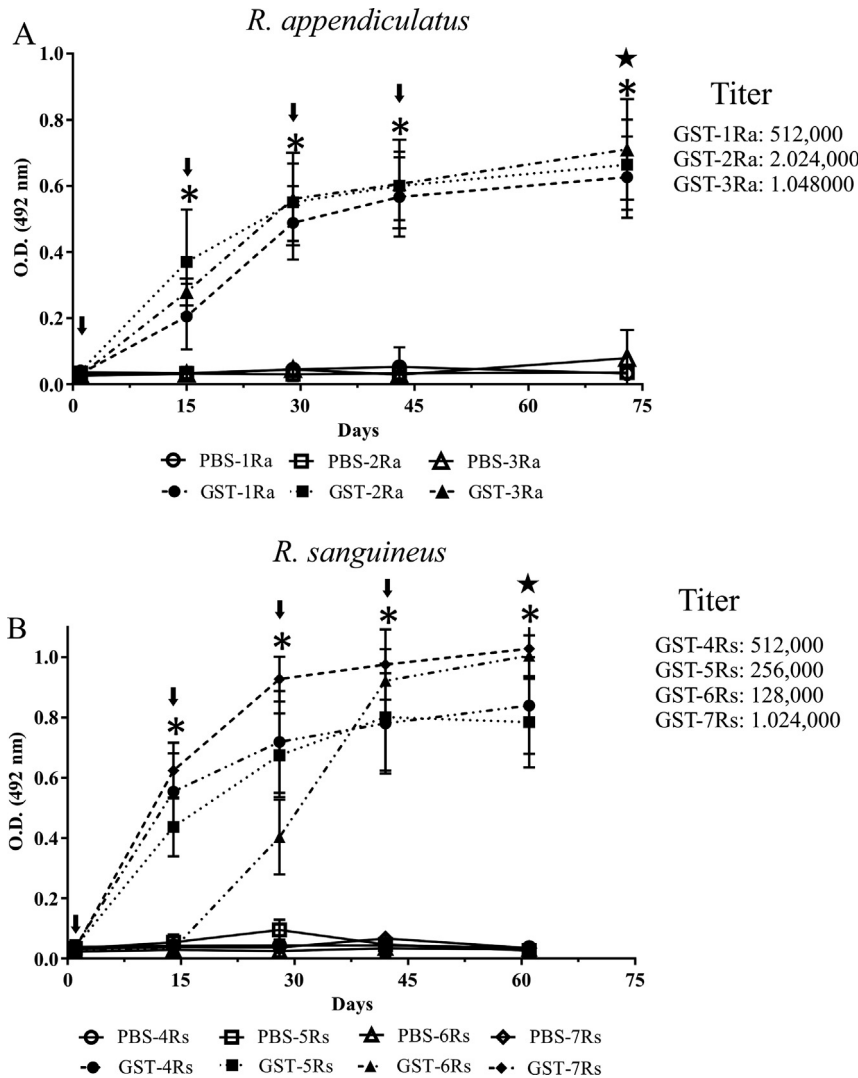
IgG levels in rabbit sera from rGST-HI immunized and control groups were evaluated by ELISA (Fig. 3). After two inoculations, sera from immunized rabbits were able to recognize rGST-HI,



**Fig. 1.** Comparative analysis of conserved and antigenic regions of *H. longicornis*, *R. sanguineus* and *R. appendiculatus* GSTs. Antigenicity index plots for tick GSTs were predicted using the Jameson–Wolf algorithm. Graphic increased positivity shows predicted antigenic sites. The alignment shows conserved regions among GST-HI, *R. sanguineus* and *R. appendiculatus* GST. Regions with high antigenicity and identity are highlighted in gray boxes.



**Fig. 2.** *In vitro* cross-reactivity between *H. longicornis* and *R. sanguineus* GSTs. Immune sera from rabbit vaccinated against rGST-HI cross-react with native GST from *R. appendiculatus* and *R. sanguineus* tissues: egg (E), larvae (L), nymph (N), midgut (G), salivary glands (SG) and ovary (OV). The recognition of native GST is indicated by the protein band of molecular weight (MW) 25 kDa.



**Fig. 3.** Humoral immune response of rabbits from vaccinated (GST) and control (PBS) groups analyzed by ELISA. Sera from rabbits infested with (A) *R. appendiculatus* (Ra) and (B) *R. sanguineus* (Rs). The titers from immunized animals are shown on the right. Arrows indicate immunization days. Star indicates the first day of infestation. Asterisks (\*) show significant differences between immunized and control groups ( $p < .005$ ).

maintaining stable antibody levels until the end of vaccination experiment. At the time of tick infestation, sera titer to rGST-HI ranged from 128,000 to 2,048,000 (Fig. 3), showing that rGST-HI is immunogenic in rabbits.

To evaluate whether the observed humoral response against rGST-HI was protective, rabbits were infested with *R. sanguineus* or *R. appendiculatus* nymphs and adults. Tick physiological parameters evaluated were: number and weight of replete ticks recovered, fecundity, and molting success of nymph (Table 1: *R. appendiculatus* and Table 2: *R. sanguineus*). Vaccinated rabbits challenged with *R. appendiculatus* showed a statistically significant ( $p < .05$ ) reduction in the number of detaching replete ticks (11.5%), and the engorgement weight (22.7%) in females. Moreover, a decrease of 35% in the amount of eggs, and 32.3% in egg hatching rate was observed. The overall protection achieved with rGST-HI against adult *R. appendiculatus* infestation was 62.7% (based on number of engorged ticks) or 67.1% (based on the weight of engorged ticks). Fig. 4 shows the differences in phenotype and egg production between engorged adult females fed on vaccinated versus control groups, showing that host immunization with rGST-HI partially disrupted normal blood feeding and egg laying by adult *R. appendiculatus*. Interestingly, no protection against *R. appendiculatus* nymphs was observed in any of the analyzed parameters.

Despite the anti-rGST-HI antibody response produced in immunized rabbits, and the cross-reactivity between heterologous GSTs demonstrated by western blotting, no significant differences in biological parameters were observed for *R. sanguineus* fed on immunized or control rabbits (Table 2).

### 3.3. Histological analysis

To investigate the effects of rGST-HI antibodies on histophysiological aspects of *R. appendiculatus* ticks fed on immunized rabbits, tissue specimens were examined. Salivary glands from the control group did not show pathological alterations (Fig. 5a), with cells from salivary gland acini types I, II and III showing normal morphology as described previously [30]. The acini retained their normal rounded shape while the lumen diameter of glandular ducts was equally preserved. In contrast, salivary glands from ticks fed on host immunized with rGST-HI exhibited severe morphological damage characterized mainly by irregular-shaped acini (Fig. 5b). In addition, extensive cytoplasmic vacuolation of acinar cells, loss of cell limits, and nuclear damage were evident. Besides losing its typically rounded morphology, nuclei exhibited pyknosis. The granular acini also presented disruption in secretory activity,



**Table 1**  
Biological parameters of *Rhipicephalus appendiculatus* fed in rGST-HI immunized and control rabbits.

Group	Nymph					
	Engorged <sup>a</sup>				Weight <sup>b</sup>	
Control	95.26% ± 7.31				10.40 ± 0.66	
Vaccine	97.72% ± 1.99				9.54 ± 0.52	
Reduction	–3.16%				8.27%	
Adult female						
	Engorged females <sup>a</sup>	Female weight <sup>b</sup>	Egg laying <sup>c</sup>	Egg fertility <sup>d</sup>	Vaccine efficacy	
					Number <sup>e</sup>	Weight <sup>f</sup>
Control	97.78% ± 3.85	477 ± 16.68	0.53 ± 0.04	0.29 ± 0.04		
Vaccine	86.67% ± 3.33	373 ± 66.31	0.34 ± 0.08	0.20 ± 0.04		
Reduction	11.36% <sup>*</sup>	21.68% <sup>*</sup>	35.03% <sup>*</sup>	32.67% <sup>*</sup>	62.75%	67.13%

<sup>a</sup> Percentage of engorged ticks recovered on rabbits (average values ± S.D.).

<sup>b</sup> Average weight (mg) of engorged ticks (average values ± S.D.).

<sup>c</sup> Total weight of laid eggs per total weight of females.

<sup>d</sup> Total weight of larvae per total weight of laid eggs.

<sup>e</sup> Vaccine efficacy =  $100 \times [1 - (NFE \times WE \times WL)]$ , where NFE, WE and WL are the number of fully engorged females, egg laying capacity and egg fertility from vaccinated cattle/control cattle, respectively.

<sup>f</sup> Vaccine efficacy =  $100 \times [1 - (WFE \times WE \times WL)]$ , where WFE, WE and WL are the weight of fully engorged females, egg laying capacity and egg fertility from vaccinated cattle/control cattle, respectively.

<sup>\*</sup>  $p < .05$  (Student's *t*-test).

**Table 2**  
Biological parameters of *Rhipicephalus sanguineus* fed in rGST-HI immunized and control group rabbits.

Group	Nymph		
	Engorged <sup>a</sup>	Weight <sup>b</sup>	Moulting <sup>e</sup>
Control	43.12% ± 34.47	3.72 ± 0.01	65.55% ± 17.28
Vaccine	51.25% ± 10.61	3.78 ± 0.13	86.50% ± 11.00
Reduction	–18.60%	–1.61%	–31.96%
Adult female			
	Engorged females <sup>a</sup>	Female weight <sup>b</sup>	Egg laying <sup>c</sup>
Control	95.00% ± 7.07	127 ± 0.98	0.44 ± 0.01
Vaccine	95.00% ± 7.07	126 ± 3.00	0.46 ± 0.02
Reduction	0.00%	0.79%	–5.54%
			Egg fertility <sup>d</sup>
Control			0.52 ± 0.03
Vaccine			0.50 ± 0.07
Reduction			3.85%

<sup>a</sup> Percentage of engorged ticks recovered on rabbits (average values ± S.D.).

<sup>b</sup> Average weight (mg) of engorged ticks (average values ± S.D.).

<sup>c</sup> Total weight of laid eggs per total weight of females.

<sup>d</sup> Total weight of larvae per total weight of laid eggs.

<sup>e</sup> Rate of molting success.

displaying irregularities in distribution and constitution, which were deduced from cytoplasmic granules strongly stained by eosin.

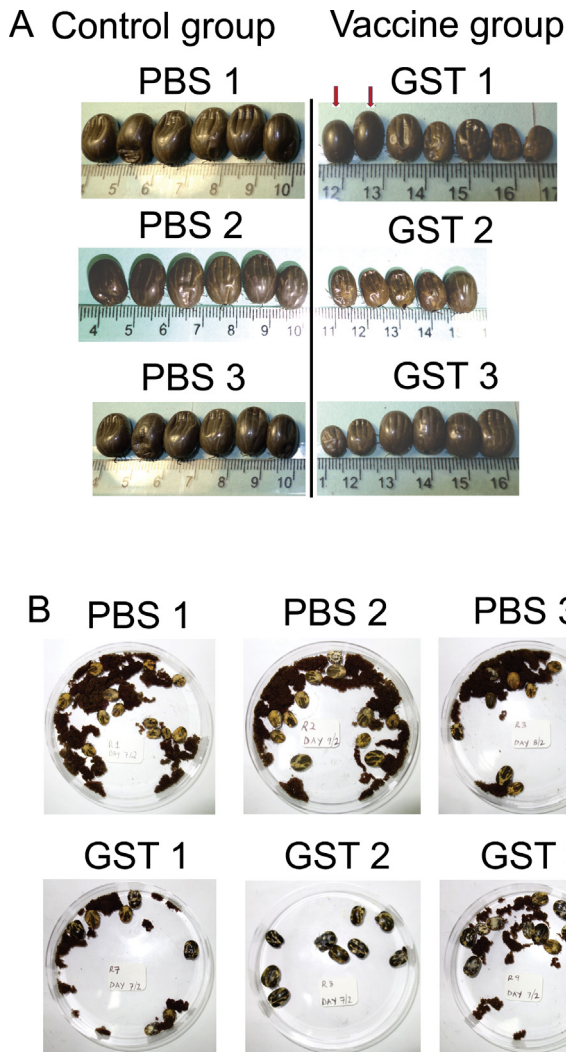
The oocytes at different stages of development (I–V oocytes) from control group all showed regular morphological characteristics as described previously [31] (Fig. 5c). For ticks fed on rGST-HI immunized rabbits, alterations in oocytes at early stages of development (types I, II and III) were found, with loss of the characteristic rounded shape and the structure of germinal vesicle (Fig. 5d). Surrounding the entire extent of oocytes IV and V periphery, small vacuoles below the cytoplasmic membrane were observed; they were mostly evident in the area adjacent to the pedicel, which means that this structure allows the absorption of hemolymph components into the oocytes. On the other hand, yolk granulation was preserved in shape and color in oocytes of all stages.

#### 4. Discussion

A number of proteins have been proposed for inclusion as antigens in vaccines against ticks [32]. Some studies went further and have evaluated tick antigens that potentially protect against heterologous tick infestation [8–10], aiming to reduce the number of antigens required in a vaccine to control multiple tick species.

Along these lines, the development of a cross-protective vaccine against *Rhipicephalus* spp. could control *R. decoloratus* and *R. appendiculatus* cattle infestation in east Africa, where both species are prevalent [33]. An important step in the selection of antigens with high level of cross-species reactivity is the degree of sequence identity among target species. The presence of conserved antigenic regions is desirable to elicit broad-spectrum protective immune responses [10,34,35]. Based on these premises, we have searched for conserved epitopes between *H. longicornis* GST and its homologues in *R. appendiculatus* and *R. sanguineus*. We found conserved GST sequences in surface-exposed regions, indicating that rGST-HI and native GSTs from *R. appendiculatus* and *R. sanguineus* contain epitopes able to be bind anti-rGST-HI antibodies. *In silico* deduced cross-binding was corroborated by western blot analysis showing cross-reactivity between anti-rGST-HI rabbit sera and native GSTs from *R. appendiculatus* and *R. sanguineus* tissues. Previously, Parizi et al. [17] demonstrated through *in silico* and *in vitro* analysis that antibodies against rGST-HI are able to bind *R. microplus* GSTs; furthermore, rGST-HI experimental vaccination cross-protected cattle against *R. microplus* infestation [17,18]. Here, *R. appendiculatus* feeding on rabbits immunized with rGST-HI had deleterious effects on the ticks blood ingestion, oviposition and egg hatching. Further histological analysis on these ticks showed unusual lesions in the





**Fig. 4.** Phenotypes and egg laying rates in *R. appendiculatus* females affected by rGST-HI vaccination. Representative examples of female phenotypes (A) and egg mass recovery (B) of engorged adult *R. appendiculatus* from control (PBS) and vaccinated groups (GST). Red arrows indicate dead ticks. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

ovaries and salivary glands. These effects could result from anti-GST antibodies inhibition of GSTs functions, such as repairing molecules damaged by reactive oxygen species (ROS). The presence of high levels of ROS induces oxidative damage at protein and DNA level, which may lead to cellular apoptosis [36].

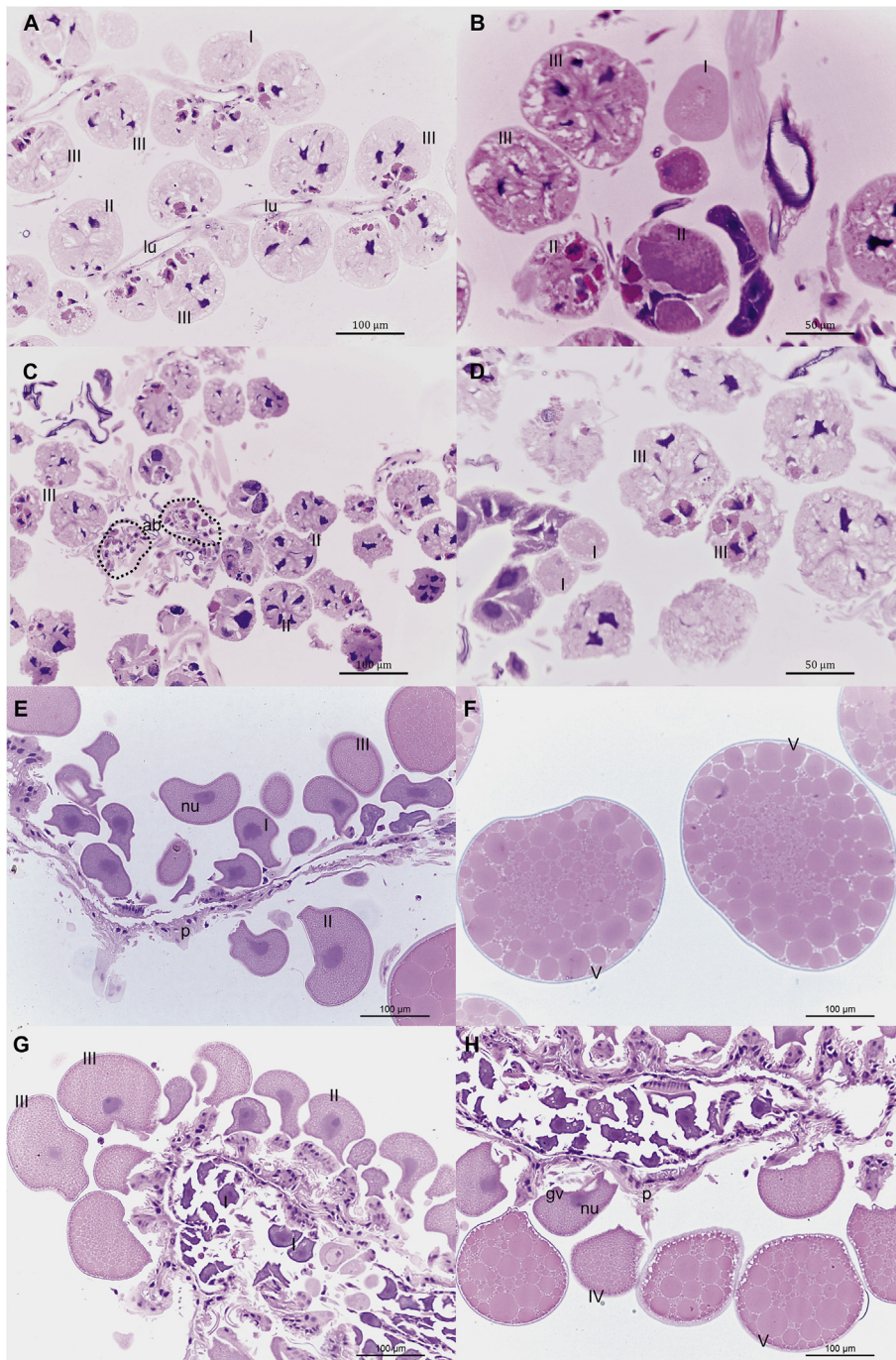
Recombinant GST-HI was found to be immunogenic in rabbits, with individual variation in serum titers ranging from 128,000 to 2,024,000. The immunogenicity of rGST-HI reported here in rabbits was also observed in cattle during two previous experiments [17,18]. For *R. appendiculatus*, the vaccination caused reduction of all parameters evaluated in adult female ticks: number, weight, egg laying, and fertility, but no significant difference in number and weight of nymphs. Adult female ticks feed larger amounts of blood compared with nymphs, leading to larger amounts of heme being processed and demanding detoxification in this phase, which could explain the differential vaccine effect. GSTs play an essential role in heme detoxification, therefore anti-GST antibodies could be blocking enzyme activity, causing cell and tissue injury and leading to the observed phenotypical damage. In addition, a number of studies have shown that tick immature stages (larvae and nymph) have lower host specificity compared with adult stages [37–39]. It

is possible that the loss of phenotypical plasticity in adults impairs the tick responses against antibodies, exacerbating vaccination effects in this phase. The total protection was assessed using a formula that considers the number of fully engorged females [28], as well as an adapted formula, which uses the female weight. This parameter was considered because the average weight was different between ticks in the vaccinated and control groups, thus allowing a more comprehensive assessment of the protection rate achieved. It is important to observe that the adult phase is the major stage involved in pathogen transmission, therefore vaccination with rGST-HI could potentially affect the tick-host-pathogen interface [40]. Interestingly, rGST-HI cattle vaccination showed similar final protection against *R. microplus* [17,18], another tick species adapted to cattle. In cattle trials, *R. microplus* was affected during larva, nymph, and/or initial fed adult stages, decreasing the total number of fully engorged females recovered. For *R. appendiculatus*, in contrast, rGST-HI vaccination was effective during female engorgement and egg development, indicating a distinct protection mechanism for this tick. The diversity of GST isoforms observed in genomes and transcriptomes of arthropods [41–43], and GSTs transcripts processed through alternative splicing, result in a potentially large number of GST isoforms, which could explain different susceptibilities along *R. appendiculatus* and *R. microplus* life cycle.

Interestingly, vaccination with rGST-HI against *R. sanguineus* failed to induce significant differences in all tick physiologic parameters analyzed. Despite both *R. appendiculatus* and *R. sanguineus* having a three-host life cycle, *R. appendiculatus* is more adapted to feed in bovidae, while *R. sanguineus* can parasite a higher number and diversity of host species [44]. Generalist ticks like *R. sanguineus* are better adapted to environmental variations [45], being able to more efficiently change salivary protein profile to evade distinct host responses against tick feeding [46,47]. This phenomenon could account for a lower vaccine effectiveness against *R. sanguineus*. Also, the presence of different GST isoforms, which have functional redundancies in different sub-cellular compartments, cell conditions or requirements, can be a reason for the reduced effectivity of rGST-HI vaccine. Moreover, the efficacy of the host natural immune response developed against ticks depends on the host species and immune background, affecting tick protein expression [48]. For example, *R. sanguineus* repeated infestations trigger low immune protection levels for dogs, its natural host [49–51]. In contrast, high immune protection is developed after repeated infestations in experimental hosts, such as guinea pig and rabbits [49,51,52]. A direct comparison between these apparently discrepant observations is not possible, however, since experimental infestation are performed in naive animals inoculated with one or a few antigens, as opposed to the repeated exposure to several saliva antigens in successive natural infestations. Despite the distinct levels of host susceptibility, there are reports of cross-protective antigens reducing *R. sanguineus* fitness in dogs (Bm86 antigen) [53], guinea pigs (64TRPs antigen) [34], and rabbits (ATAQ antigen) [10]. Taken together, these results show that even susceptible hosts can be protected by vaccination against *R. sanguineus* infestation, although in the current protocol rGST-HI was not able to develop protective immunity.

## 5. Conclusions

The results presented here demonstrate rGST-HI as a potential vaccine antigen against different tick species. Vaccinating animals with this antigen raised protective immunity against *R. appendiculatus* in rabbits, similarly to what was previously observed for *R. microplus* in cattle [17,18]. *R. appendiculatus* parasitism on rGST-HI immunized rabbits led to salivary glands and ovary damage in the ticks, decreasing adult female fitness and resulting in a protec-



**Fig. 5.** Histologic sections of *R. appendiculatus* tissues stained with hematoxylin and eosin (HE). (A and B) Salivary glands of females fed on host from control group. (C and D) Salivary glands from females fed on host immunized with rGST-HI. I, II and III: acini types; ab: apoptotic body; lu: lumen. (E and F) Ovaries of females fed on host from control group. (G and H) Ovaries from females fed on host immunized with rGST-HI. I, II, III, IV and V: ovaries types; p: pedicel; nu: nucleus; gv: germinal vesicle.

tion level of about 65%. Despite the high similarity among tick GSTs, *R. sanguineus* life cycle was not affected by vaccination, showing the need to investigate protection levels developed for rGST-HI against specific target tick species. Field vaccination trials against *R. appendiculatus* and *R. microplus* infestations performed in regions where these two ticks occur could lead to a new generation of anti-vector vaccines for these notorious ectoparasites.

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#### Conflict of interest

The authors certify that they have no affiliations with, or involvement in any organization or entity with any financial interest in the subject matter or materials discussed in this manuscript.



## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.vaccine.2017.10.026>.

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### **3.2. Capítulo II**

#### **An insight into the functional role of antioxidant and detoxification enzymes in**

#### ***Rhipicephalus microplus* tick.**

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**Insight into the functional role of antioxidant and detoxification enzymes in  
*Rhipicephalus microplus* females ticks**

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### **Highlights**

- *R. microplus* blood feeding alter the transcriptional level and activity of antioxidant and detoxification enzymes
- *R. microplus* blood feeding alter GSH levels in salivary glands, ovaries, midgut and fat body.
- *R. microplus* blood feeding did not interfere in NADPH levels in salivary glands, ovaries, midgut and fat body.
- Catalase and thioredoxin reductase gene silencing do not impair tick blood feeding, digestion and reproduction.

## Abstract

Ticks have developed physiological adaptations to transport, store, metabolize and secrete toxic components from the diet and environment. Different classes of enzymes are involved in these processes, however, the role of several of these enzymes is not yet characterized in *Rhipicephalus microplus*. In this context, this work reveal the action of antioxidant and detoxification enzymes, as well as the levels of essential cellular reductants in partially (PEF) and fully engorged (FEF) *R. microplus* females. Results demonstrate that transcriptional levels and enzymatic activity from ovary and fat body are predominantly higher in PEF than in FEF. With exception of GPx activity in ovary, which was the only enzyme that showed highest enzymatic activity in the FEF stage. These results indicate a higher requirement of the antioxidant potential at the initial feeding moments than in the laying phase in these organs. In midgut, however, there is a greater variation on the transcriptional levels and enzymatic activity between the PEF and FEF phases, possibly due to a coordinated system between ROS control and maintenance of the intestinal microbiota. According to the great ability of the tick to maintain redox homeostasis without causing oxidative stress, NADPH levels were unchanged between the PEF and FEF phases. However, GSH levels showed to be variable between the PEF and FEF phases according to what was observed at the transcriptional gene level and the enzymatic activity of most of the enzymes analyzed, possibly to support a higher requirement of the reducing potential in FEF phase. The silencing of CAT and TRx reductase were not able to impair the females feeding and laying capacity, and larvae hatching, indicating the high capacity of remaining enzyme activity, or the activation of compensatory mechanisms. Altogether, results demonstrate the complexity of the antioxidant enzyme system and detoxification in the tick and its relation with several physiological mechanisms.

## 1. Introduction

Hematophagous animals have evolved innumerable mechanisms to adapt the blood feeding and digestion to nutrient acquisition and energy production (Mans et al., 2002; Ribeiro, 1995). Among this animals, ticks have a special relevance as hematophagous arthropod, since it can ingest between 50-200 times their initial weight in blood (Mehlhorn, 2016). Hemoglobin comprises one of the most abundant proteins in vertebrate blood (Sojka et al., 2016) and derived from hemoglobin digestion, free heme and Fe (Horn et al., 2009; Lara et al., 2003) are toxic to biological systems, damaging cell membranes (Schmitt et al., 1993) and unleashing peroxidation reactions through the Fenton reaction (Kehrer, 2000), leading to oxidative stress disturbance and cell death. For this reason, ticks evolved physiological adaptations to transport, store, metabolize and secrete possible toxic components from its diet (Graca-Souza et al., 2006; Lara et al., 2003). Oxidative stress is defined as an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage (Sies and Jones, 2007). In this context, antioxidant enzymes have an important role in cellular protection against damage caused by reactive oxygen species (Aruoma et al., 2006).

Scientific community have been studying hematophagous animals to understand how it can ingest high amounts of blood, maintaining a physiological homeostasis without causing oxidative stress (Maya-Monteiro et al., 2000; Oliveira et al., 1995; Pereira et al., 2007). As expected, antioxidant enzymes are often involved in this mechanisms: specifically, it was demonstrated the role of catalase in the control of H<sub>2</sub>O<sub>2</sub> levels in adult females of *R. microplus* during blood meal and digestion. By inhibiting catalase activity, it was observed an increase in H<sub>2</sub>O<sub>2</sub> concentration in digestive cell and impaired heme detoxification. *In vivo*, these effects reduced survival and diminished the rate of oviposition (Citelli et al., 2007). Also, the transcriptional up-regulation of several enzyme genes is induced by the blood acquisition. This effect was described with catalase in *A. aegypti*. (Oliveira et al., 2017), sulfotransferase in *Ixodes ricinus* (Perner et al., 2016a), superoxide dismutase and catalase in *R. prolixus* (Paes et al., 2001) and glutathione S-transferase (GST) in *Dermacentor variabilis* (Dreher-Lesnack et al., 2006). Other studies demonstrated the biological damages of catalase silencing in hematophagous, such *Anopheles gambiae* (DeJong et al., 2007; Magalhaes et al., 2008), *Aedes aegypti* (Bottino-Rojas et al., 2015) and *Lutzomyia longipalpis* (Diaz-Albiter et al., 2011), especially related to insect reproductive capacity. In contrast, thioredoxin reductase silencing in *Amblyomma maculatum* showed



that depletion of thioredoxin reductase activity did not interfere in tick haematophagy, lethality and oviposition, but did reduce the viability of the microbiome within the tick organs (Budachetri and Karim, 2015). This results showed a multifunctional role of antioxidant enzymes in hematophagous arthropods and their relation with blood feeding. In addition, studies related to identification and characterization of the functional role of antioxidant proteins in tick physiology could contribute to development of new strategies to parasite control.

In the present work, it was showed an alternated transcriptional pattern in 13 gene and activity levels of four enzymes related with antioxidant and detoxification systems in different organs of partial and fully engorged *R. microplus* female ticks. The GSH levels in the same organs showed also differences between fully and partially engorged females, while NADPH remained unchanged. Furthermore catalase or thioredoxin reductase gene silencing by RNAi did not interfere in tick feeding and reproduction, indicating the capacity of remaining enzyme function, or the activation of compensatory mechanisms.

## **2. Methods:**

### **2.2. Animals and ticks**

*Rhipicephalus microplus* (Porto Alegre strain), were reared on Hereford calves (*Bos taurus taurus*) obtained from a naturally tick-free area (Santa Vitória do Palmar, RS, Brazil; 33°32'2" S, 53°20'59" W) maintained in individual sheds at the Faculdade de Veterinária of Universidade Federal do Rio Grande do Sul (UFRGS). Calves were infested with 15-day-old tick larvae. After 21 days, partially and fully engorged adult female ticks were collected for organs dissection. Partially engorged females (PEF) were collected directly from the host, while fully engorged females (FEF) were collected after spontaneous detachment. During the off-host period of the life cycle, ticks were kept at 27°C and 85% relative humidity. Experiments were approved and conducted following the guidelines of the Ethics Committee on Animal Experimentation of UFRGS.

### **2.3. Organ dissection, protein and RNA extraction**

Salivary glands, ovaries, midguts and fat bodies were dissected from PEF ranging from 25-70 mg and FEF collected after spontaneous detachment. For activity assays, tissues were kept in phosphate buffer saline (PBS) with a protease inhibitor cocktail (Pepstatin A 1 µM; E-64 10 µM; TPKC 50 µM; EDTA 5 µM). Organs were disrupted by sonication

(Qsonica sonicator Q125) on ice at 40 MHz in 5 pulses of 30 s at 30 s intervals. The homogenate was centrifuged at  $16,000 \times g$  for 15 min at 4 °C. Soluble fractions were divided into 20  $\mu$ l aliquots and stored at -20 °C until the use.

Total RNA was extracted using TRIzol reagent (Invitrogen) following the manufacturer's recommendations. The concentration and quality of the RNA were determined by spectrophotometry (Ultrospec 1000, Amersham Biosciences, Pharmacia Biotech) assessing A 260 $\lambda$  and the A 280 $\lambda$ /260 $\lambda$  ratio, respectively.

For cDNA synthesis, total RNA was diluted in diethylpyrocarbonate (DEPC)-treated water and treated with DNase I (Invitrogen). A PCR of RNA samples was performed to ensure no DNA contamination. Total RNA was reverse-transcribed using oligo (dT) p12–18 primer and Superscript<sup>®</sup>III (Invitrogen), following the manufacturer's protocol. The resulting cDNA was stored at -70 °C until use.

#### **2.4. Selection of qPCR target**

Sequences encoding *R. microplus* antioxidant enzymes were identified in a *R. microplus* transcriptome database (RmINCT-EM; transcripts of seven organs of *R. microplus*) created by our research group, sequenced by Illumina technology following the database assembly (unpublished). To validate the accuracy of sequence encoding enzymes, sequences were blasted with BLAST (Basic Local Alignment and Search Tool) with the BLASTN algorithm against NCBI GenBank nucleotide and genome databases. When more than one sequence of an enzyme were identified, one sequence was selected based in the reads per kilobase million (RPKM) values in tick organs, so sequence with best e-value and coverage was selected to qPCR analyses.

#### **2.5. mRNA transcriptional level of antioxidant enzymes (Real-time quantitative PCR)**

For analysis of mRNA transcriptional levels using quantitative RT-PCR (qRT-PCR), ORF specific primers were designed (table S1) to amplify approximately 200-bp fragment of enzyme genes: *phospholipid-hydroperoxide glutathione peroxidase* (PHGPx; accession number: MK262714); *glutathione S-transferase 2* (SG2; accession number: MK262715); *glutathione S-transferase 3* (GST3; accession number: MK262716); *glutathione S-transferase 4* (GST4; accession number: MK262717); *glutathione synthetase* (GS;

accession number: MK262718); *catalase* (*CAT*; accession number: KY953208.1); *CuZn superoxide dismutase* (*CuZnSOD*; accession number: MK262723); *sulfotransferase* (*RmSult*; accession number: MK262724); *peroxiredoxin* (*Prx*; accession number: MK262719); *thioredoxin reductase* (*TrxR*; accession number: MK262720); *thioredoxin peroxidase* (*TrxP*; accession number: MK262721); *thioredoxin* (*Trx*; accession number: MK262722); *dual oxidase A* (*DuoxA*; accession number: MK262713). Actin (accession number: AY255624.1) was used as reference gene (details in table S1).

Relative quantification was carried out with 200 ng of cDNA prepared from the salivary glands, ovaries, midgut, and fat body of partially and fully engorged females. Additionally, cDNA from ovaries dissected during RNAi silencing experiments, were analyzed by this protocol. Cycling parameters were 5 min at 95 °C, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. After amplification was complete, a melting curve analysis was performed using the default parameters of the instrument (Rotor Gene<sup>®</sup> Q, Qiagen). All samples were analyzed in duplicate and each qPCR reaction was repeated twice. The relative expression ratio of each gene was calculated according to the mathematical model described by (Pfaffl et al., 2001), and used in the Relative Expression Software Tool (REST –MCS©, version 2; (Pfaffli et al., 2002). PCR efficiency was calculated from the log-linear portion of the standard curves. Data were corrected for efficiencies (above 90%) for each primer assay individually, followed by normalization of expression of target genes to the expression of reference gene. The qPCR experiment was performed in accordance with the Minimum Information Required for Publication of Quantitative Real-time PCR Experiments (MIQE) Guidelines (Bustin et al., 2010).

## **2.6. Enzyme activity levels in tick organs**

### **2.6.1. Catalase (CAT)**

Catalase activity was kinetically evaluated by measuring the rate of hydrogen peroxide consumption in a 96-well plate in a VersaMAX microplate spectrophotometer (Molecular Devices, USA) at 25 °C, according to the protocol described by (Aebi, 1984) with slight modifications. Briefly, extracts were added to assay buffer (10 mM Tris–HCl pH 8.0) and then H<sub>2</sub>O<sub>2</sub> was added to reaction. CAT activity was determined spectrophotometrically by monitoring the disappearance of H<sub>2</sub>O<sub>2</sub> at 240 nm, using the

extinction coefficient of  $43.6 \text{ M}^{-1} \text{ cm}^{-1}$ . This same protocol to measure CAT activity in tick tissues was previous utilized in other study of our research group (Freitas et al., 2007a).

### **2.6.2. Superoxide dismutase (SOD)**

Total SOD activity was kinetically measured using the RanSOD<sup>®</sup> kit (Randox, UK), with absorbance measured at 505 nm in a 96-well plate in a VersaMAX microplate spectrophotometer (Molecular Devices, USA) at 25 °C, according to the manufacturer's protocol. The assay employs xanthine and xanthine oxidase to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) to form a red formazan dye.

### **2.6.3. Glutathione S-transferase (GST)**

The GST activity of the extracts was kinetically measured as described by (Habig et al., 1974) using 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma) as substrate. Reaction mixture (50 mM CDNB in methanol, 5 mM glutathione in 100 mM Tris-HCl pH 7.5) and organ extract were tested in a 96-well plate, The absorbance at 340nm was measured each 15 s in a VersaMAX microplate spectrophotometer (Molecular Devices, USA) at 25 °C. The concentration of the product formed was calculated using the millimolar extinction coefficient of 9.6, corrected for the 96-well microplate light path. This same protocol to measure GST activity in tick tissues was previous utilized in other studies of our research group (de Freitas et al., 2008; Freitas et al., 2007a).

### **2.6.4. Glutathione peroxidase (GPx)**

Enzymatic kinetics of GPx was assessed by the Ransel<sup>®</sup> Kit (Randox, UK) in a 96-well plate in a VersaMAX microplate spectrophotometer (Molecular Devices, USA) at 25 °C, according the manufacture's protocol. The activities were expressed as U/mg of protein. Briefly, oxidation of NADPH was measured in the presence of organs extract, reduced glutathione (GSH), glutathione reductase, and tert-butyl hydroperoxide at 340 nm over 3 min (Pinto and Bartley, 1969).

## **2.7. Total glutathione content in tick organs**

Total glutathione (tGSH) was assayed in a 96-well plate in a VersaMAX microplate spectrophotometer (Molecular Devices, USA) by measuring the formation of TNB

quantitated at 412 nm. GSH reacts with DTNB [5,5'-dithio-bis (2-nitrobenzoic acid)] to form TNB (5-thio-2-nitrobenzoic acid) and GS-TNB (oxidized glutathione–TNB). GS-TNB is then reduced to GSH and TNB by GR in the presence of NADPH (Rahman et al., 2006). Because GR reduces also GSSG into 2GSH, the amount of glutathione measured represents the sum of reduced and oxidized glutathione in the sample. The rate of formation of TNB is proportional to the concentration of glutathione in the sample. The glutathione levels were expressed as  $\mu\text{mol}$  of glutathione/mg of protein.

## **2.8. NADPH content in tick organs**

The NADPH was measured by a commercial NADPH quantification kit (#MAK038; Sigma-Aldrich) in a 96-well plate in a VersaMAX microplate spectrophotometer (Molecular Devices, USA). Briefly, organs were extracted with extraction buffer, homogenized, and centrifuged. An aliquot of the supernatant was heated at 60 °C for 30 min to remove the precipitate. Sample was reacted with NADP<sup>+</sup> cycling buffer and enzyme mix (containing G6PDH). The solutions were then incubated with NADPH developer and the absorbance measured at 450 nm. The amount of NADPH heated sample was quantified from an NADPH standard curve.

## **2.9. RNA interference**

### **2.9.1. Synthesis of dsRNA**

The CAT and TrxR gene sequence were amplified by PCR from the *R. microplus* cDNA with gene-specific primers containing the T7 promoter recognition site at the 5' ends. dsRNA produced from an unrelated gene, MSP1 from *Plasmodium falciparum* (accession number: AF061132), was used as control. This gene was obtained from a plasmid kindly provided by Dr. Gerhard Wunderlich (Departamento de Parasitologia, USP, Brazil). dsMSP1 was previously used as dsRNA non-related control in *R. microplus* RNA interference experiments (Kalil et al., 2017; Leal et al., 2018; Seixas et al., 2018).

The PCR product was purified using a Glassmilk DNA purification kit (BIO101 Systems) and used to synthesize dsRNA with a T7 Ribo Max Express RNA Kit (Promega, USA) according to the manufacturer's protocol. The dsRNA synthesis was evaluated by 2% agarose gel electrophoresis, and the concentration was determined spectrophotometrically at 260 nm (Ultrospec 1000, Amersham Biosciences). The *R. microplus* genome is not

available, for this reason, *Drosophila melanogaster* genome sequences (accession number AC092233) were used to ensure dsRNA specificity and to exclude potential nonspecific thought dsCheck program (Naito et al., 2005). In addition, dsRNA sequences were searched in BLAST algorithm to identify possible off-target sequences using sequences deposited in Genbank (Sabirzhanov et al., 2011).

### **2.9.2. Artificial capillary feeding**

*R. microplus* larvae infestation were performed as described in section 2.1. On day 21 after infestation, partially engorged females (PEF; weighing between 25 mg and 70 mg) were collected from the bovine. Groups of 23-19 females homogeneously distributed were immobilized on a glass plate covered with double-sided adhesive tape. dsRNA solutions were injected into the tick (1  $\mu$ L, 8  $\mu$ g/tick) using syringe (Hamilton). Immobilized ticks were artificially fed using microhematocrit capillary tubes (75  $\times$  1.0  $\times$  1.5mm) filled with bovine blood, collected with the addition with sodium citrate (Alzugaray et al., 2017; Gonsioroski et al., 2012). Females were allowed to feed for approximately 24 h, and then kept in separate tubes at 27–28 °C and 80-90% relative humidity for egg laying.

Before and after the feeding process, the females were weighed to determine blood ingestion. After 24 h of dsRNA injection three ticks from each group were dissected and the ovaries were collected for RNA and protein extraction. cDNA was synthesized from extracted RNA to verify the mRNA silencing by qPCR, according section 2.3. After that, females were placed in Petri dishes and maintained at 27 °C and 85% humidity. Biological parameters analyzed were: weight gain, reproductive efficiency index and hatching rate (Bennett, 1974; Fabres et al., 2010). One experiment was performed with dsTrxR and three independent experiments were performed with dsCAT.

### **2.10. Data normalization**

Enzyme activities, NADPH and GSH quantification were expressed as units, ng and nmol per milligram of protein, respectively. To normalize the results, the total protein content of the cell extracts was quantified based on the Bradford method, using bovine serum albumin as standard (Bradford, 1976). Real time experiments used Actin (accession number: AY255624.1) as reference gene.

### 2.11. Statistical analysis

One-way ANOVA and Tukey multiple comparison post hoc test ( $p < 0.05$ ) were used for qRT-PCR analysis, and enzymatic assays comparing the activity in tick organs. For RNAi biological parameters (weight gain, reproductive efficiency index and hatching rate) one-way ANOVA followed by Dunnett's multiple comparisons test was used. All analyses were performed using Graph Pad Prism version 7.00 for Windows (Graph Pad Software, Inc.).

## 3. RESULTS AND DISCUSSION

### 3.2. Antioxidant and detoxification enzymes have different activity and transcriptional levels

The antioxidant enzyme machinery is related with the control of reactive oxygen species generated during tick blood feeding and with their physiological redox homeostasis (Galay et al., 2015; Graca-Souza et al., 2006). In order to determine the relationship between tick blood feeding and antioxidant enzymes in organs of fully engorged females (FEF) and partially engorged females (PEF), the transcriptional profiles of 13 genes related to oxidative stress and detoxification were determined. Also, enzymatic activity of GST, GPx, CAT and SOD were kinetically measured.

Results showed that *RmSult* and *GST4* are transcribed only in midgut (fig. 1 D) and fat body (fig. 1 C), respectively. The transcription observed only in these organs suggests a connected physiological role of these enzymes with the organ function. Both SULTs and GSTs act mainly in phase II detoxification processes, conjugating a sulphate group or a reduced glutathione (GSH), respectively, to an array of physiological substrates or xenobiotics, making them more available for clearance (James and Ambadapadi, 2013; Tew and Townsend, 2012). *Ixodes scapularis* genome showed 25 and 55 genes annotated as *GSTs* and *Sults*, respectively (Gulia-Nuss et al., 2016). Its high number of isoforms compared with most of antioxidant enzymes is related with the broad range of GST and sulfotransferase substrates, as endogenous compounds, dietary constituents and drugs (Negishi et al., 2001). The organ-specific and microenvironments localization, as well as their transcriptional regulation along tick development could be a support to characterization of their physiologic roles. For example, in *Ixodes ricinus*, a *sulfotransferase* transcript was up regulated in midgut by blood feeding in FEF stage (Perner et al., 2016a). In the present work, however,

the analyzed *RmSult* was up-regulated in PEF ticks. Besides that, considering its specific transcription in midgut under physiological conditions, is possible to suggest that this *RmSult* may have a role in heme detoxification process. Moreover, the sulfate conjugation with insect molting hormones was suggested to have important physiological implications in the formation of biologically inactive conjugates (prehormones) in order to storage and transport (Yang and Wilkinson, 1973), which could be controlled by *RmSult* in *R. microplus* midgut. Alternatively, putative substrates to *RmSult* could be hormones and cholesterol from host blood, which first gets on to tick midgut (Cherry, 1976). It was previously described the presence of heparan sulfate, an anticoagulant glycosaminoglycan, in *R. microplus* midgut and ovary (Onofre et al., 2003), probably with important functions related with embryogenesis and the tick feeding. An *in vivo* biosynthesis of heparan sulfate requires a sulfotransferase activity (Liu and Pedersen, 2007; Sterner et al., 2014), in this context, this sulfotransferase also may be acting on the biosynthesis of heparan sulfate in midgut of *R. microplus*.

Elevated levels of GST activity has been related with tick acaricide resistance (Ghosh et al., 2017; Nandi et al., 2015; Ziapour et al., 2017), that is why most studies are focused on GST role in drug detoxification and less is known about their endogenous substrates. However, at least seven distinct GSTs of *Drosophila melanogaster* were able to conjugate 4-hydroxynonenal (4-HNE), a product of lipid peroxidation (Hernandez et al., 2018; Sawicki et al., 2003; Singh et al., 2001), although toxic at supraphysiological concentrations it is an essential component of signaling functions as cell proliferation, differentiation and apoptosis. In addition, a positive regulation was observed in both transcription and expression of GSTs triggered by blood feeding in the *H. longicornis* tick (Hernandez et al., 2018). In the present work, the specific transcription of *GST4* in the fat body could be related with the clearance of potential lipid hydroperoxides and its derivatives, since this organ can store a great amount of lipid reserves in the PEF phase, and *GST4* transcripts were uniquely observed in PEF. Moreover, peroxidase activity of GSTs has been shown in insect, using the model substrate cumene hydroperoxide (Ortelli et al., 2003; Singh et al., 2001; Vontas et al., 2001), demonstrating the potential functional roles of GSTs also in the clearance of ROS and maintenance of redox cell homeostasis under physiological conditions. The *SG2* relative expression showed to be 34 times higher in salivary glands than in midgut of partially engorged females (fig. 1A). Also, the *R. microplus* transcriptome showed a higher RPKM



of *SG2* in salivary glands (data not shown). The presence of transcripts in tick salivary glands generally is related with its secretion as proteins to the host throughout the saliva. GST was found in a *R. microplus* salivary proteome (Tirloni et al., 2014), besides that, a recent work showed *R. microplus* infestation trigger antibody response against GST (Garcia et al., 2017), showing its secretion through tick saliva and suggesting GST function also in a tick-host interface. However, no GST activity was detected in and saliva of *R. microplus* females (Rosa de Lima et al., 2002). *GST3*, in contrast, was transcribed in all tested organs (fig. 1 B). This ubiquitous transcription can suggest that *GST3* have a substrate present in all tested organs, probably related to a basal cell function. For example, in *D. melanogaster*, GSTs are also involved in biosynthesis of hormones and protection against oxidative stress (Enya et al., 2017; Townsend et al., 2009). Besides that, GSTs have functions in signaling pathways as the S-glutathionylation a post-translational modification of proteins, well characterized in Jun N-terminal kinase (JNK), a member of the family of stress-activated kinases (Townsend et al., 2009).

Considering the relative high number of *GST* isoforms compared with other antioxidant enzymes found in tick genomes (Cramaro et al., 2015; Gulia-Nuss et al., 2016) and transcriptomes (Cramaro et al., 2015; Oleaga et al., 2018), it is expected that these *GST* transcriptional levels analyzed here are not able to show the total GST activity present in tick organs. Here, the GST activity assay showed its ubiquitous expression in all tested organs of PEF and FEF. A previous study demonstrated the presence of GST in all analyzed organs from *Rhipicephalus sanguineus* (Sabadin et al., 2017), reflecting its fundamental function for the maintenance of cell homeostasis. It was previous demonstrated the GST activity is triggered by *Dermacentor variabilis* blood meal in partially engorged female stage (Dreher-Lesnick et al., 2006), and in *A. aegypti* GST transcription were up regulated by ingestion of blood and heme but not by sugar (Bottino-Rojas et al., 2015), supporting the hypothesis that the modulation of GST gene expression is likely altered by heme released during blood feeding. In the present work, salivary glands of both stages demonstrated to have the same GST activity levels, while midgut, ovaries and fat body of PEF had more GST activity than FEF (fig. 2 A). So, it is possible that blood meal induces GST activity during early feeding in these organs, and it is not maintained during tick engorgement, may having another mechanism triggered for detoxification of heme and its possible ROS products during FEF phase. Similar results were observed in *H. longicornis*, showing a positive

regulation in the expression of GSTs in PEF and negative regulation in the FEF in fatty body, ovary and salivary glands (Hernandez et al., 2018). Additionally, here, other three enzymes were quantified regarding its activity levels, and GPx had the only higher activity in the FEF compared to PEF stage in ovary (fig. 2 B).

GPx is a cytosolic enzyme that catalyzes the reduction of hydrogen peroxide to water as well as lipid peroxide to lipid alcohols, thereby preventing membrane lipoperoxidation (Frank and Sosenko, 1987). GSH is an essential co-factor to GPx activity, for this reason, glutathione synthetase also comprises fundamental enzymes to the maintenance of antioxidant glutathione system. Transcription profile of *GS* and of *PHGPx* showed similar patterns for both enzyme genes (fig. 1 A and E): no difference between both stages were observed in salivary glands and midguts, but showed higher transcriptional level in the PEF stage in ovary and fat body. These similar transcriptional pattern suggests that these enzymes have similar transcriptional control and could be acting as a couple in antioxidant system. The enzymatic assays demonstrated, in contrast, that there is a major GPx activity in ovary of FEF than in PEF (fig. 2 B). Considering there are two GPx isoforms, is possible that the second GPx could be acting to reduce lipid hydroperoxides in FEF ovary. The activity of GPx in ovary and fat body in PEF and FEF phases, respectively, follows the tick lipid concentration in this organs along tick life cycle: in periods of food abundance (PEF) lipids are stored in fat body, while during the maturation of tick oocytes (FEF), their lipid content increase several fold in a short period. The vast majority of the lipid accumulated in oocytes originates in the fat body (Arrese and Soulages, 2010). This high lipid concentration and traffic within ovaries could increase the lipid peroxidation rates, which could be cleared by GPx, since any other enzyme tested here demonstrated to have higher activity in ovary of FEF phase. In addition, GPx is a knowing enzyme that has specialized capability of reducing membrane-bound ester lipid hydroperoxides (Kuhn and Borchert, 2002). Together with glutathione, the thioredoxin system make up the major biological thiol-dependent antioxidant mechanisms, providing electrons to peroxidases to remove ROS. Besides that, thioredoxin (Trx) are also involved in DNA replication, by reducing ribonucleotide reductase, and regulates the activity of many redox sensitive enzymes and transcriptional factors (Lu and Holmgren, 2014). Relative transcription of the electron donor *Trx* (fig. 1 I) showed an interesting pattern since its exclusively expressed in ovary and fat body in PEF phase and absent in FEF stage, and it shows a reverse patten in midgut, showing a direct

effect of tick blood digestion on regulation of thioredoxin transcriptional level and different organ dependence of this electron source. A number of works related thioredoxin/glutaredoxin as interconvertible systems acting as functional backup to support intracellular redox potential in evolutionarily diverse organisms (plant (Marty et al., 2009), vertebrate (Du et al., 2012), and fungi (Wang et al., 2015)). It is possible a main role of glutathione system when thioredoxin is absent in *R. microplus*. In the same context, there were reports of the absence of glutathione reductase in diverse organisms as *D. melanogaster* (Kanzok et al., 2001), *Schistosoma mansoni* (Alger and Williams, 2002), and other parasitic Platyhelminthes (Salinas et al., 2004), which was substituted by thioredoxin reductase, that supports GSSG reduction. In ticks, its phenomenon is not described, however, a *glutathione reductase* is not found in *I. scapularis* genome (Gulia-Nuss et al., 2016), neither in *I. ricinus* (Cramaro et al., 2015) and *Ornithodoros erraticus* (Oleaga et al., 2018) midgut transcriptomes, which supports the hypothesis that ticks also link its glutathione-thioredoxin systems. The RmINCT-EM *R. microplus* transcriptome used here (data not shown) and a *R. microplus* ovary proteome (Xavier et al., 2018) also lacks *glutathione reductase*, showing only *thioredoxin reductases*, which could be acting along with glutathione system. As expected, *TrxR* is transcribed in all tested organs of both PEF and FEF phases. In salivary glands, the transcription remains constant across both phases, while midgut undergo a positive transcriptional regulation after tick blood feeding. This same salivary glands and midguts transcriptional profile along the tick engorgement phases was previously described in *A. maculatum* (Budachetri and Karim, 2015), in agreement with the results shown here, and demonstrating the increase in thioredoxin reductase requirement in midgut after complete blood meal. Transcriptional levels of *TrxP* and *Prx* were also evaluated here, both are transcribed in all tested organs, and were mostly down regulated after blood meal, except in midgut, when one *TrxP* markedly increase, possibly following the observed increased transcriptional level of *TrxR* and *Trx* in midgut in FEF stage.

In the present work, *CAT* was transcribed in all analyzed organs in both stages (fig. 1 K). After complete blood feeding, it was down regulated in midguts and fat body. It was previously reported that catalase transcriptional level were up regulated by blood meal in *Aedes aegypti* midgut, but also decrease after 48 h (Budachetri and Karim, 2015). In *R. prolixus*, catalase activity markedly decreases in midgut four days after blood ingestion (Gandara et al., 2016). These data suggests a first requirement of catalase as an adaptive

mechanism triggered against oxidative stress caused by an initial blood meal that is not maintained after the blood uptake. It was not possible to observe the down regulation of CAT activity in FEF midgut in enzymatic assay (Fig. 2 D), maybe due the slight regulation in *R. microplus* transcriptional levels that do not affect CAT activity and only could be detected by qPCR. In contrast, in fat body, where CAT activity was higher than in other organs, its down regulation after blood meal observed in qPCR was corroborated in the activity levels. It was previous observed in *R. microplus* egg and larvae the relation with increased O<sub>2</sub> consumption and catalase activity (Freitas et al., 2007a), that could be possibly related with the high energetic demand, which could also occur in fat body in PEF stage.

Superoxide dismutase exists in different isoforms in order to scavenge superoxide anion from mitochondria (MnSOD), cytosol (CuZnSOD), nucleus, membranes and extracellular fluids (Miao and St Clair, 2009). The *I. scapularis* genome showed 10 *SOD* genes (Gulia-Nuss et al., 2016). In the present work, *R. microplus* transcriptional levels of cytosolic *CuZnSOD* gene were markedly up regulated in midgut in FEF tick (fig. 1 L). Beside its main action against cellular stress responses such as those triggered by blood feeding, CuZnSOD act as heme binding protein (Pacello et al., 2001), being possible that in addition to its antioxidant function in *R. microplus* it could play a role in heme trafficking, which would be important in the intracellular tick blood meal digestion. *CuZnSOD* transcription also demonstrated to be about 20 times higher in ovaries than in salivary glands and twice than midgut, which can be related with the essential heme traffic in ovaries during vitellogenesis (Perner et al., 2016b). SOD activity assay showed to decrease in ovaries and fat body in FEF stage (fig. 2 C). A transcriptomic study revealed that in *R. microplus* FEF phase ovaries are almost fully mature, with protein synthesis reduced (Xavier et al., 2018) possibly reflecting in decrease of general metabolic activity and less mitochondrial ROS production and a lower SOD requirement.

Besides the deleterious effect of ROS, their fundamental role in cell signaling pathways is known (Schieber and Chandel, 2014). Moreover it is revealed the role of Dual oxidase (Duox), a NADPH oxidase that provide H<sub>2</sub>O<sub>2</sub> essential to supports dityrosine-mediated protein cross-linking and eggshell hardening in *Rhodnius prolixus* blood sucking (Dias et al., 2013). However *R. microplus* the transcriptional levels of *DuoxA* gene in ovaries, showed no differences between tick phases analyzed (fig. 1 M). Interestingly, it was elevated in midgut of FEF, that could be protecting epithelial cells from potential pathogens and

regulation of intestinal microbiota, as previously described in *A. gambiae* and fruit fly *Bactrocera dorsalis* (Kumar et al., 2010; Yao et al., 2016). However, conflicting results with *A. aegypti* revealed heme ingestion decreases ROS levels and allow intestinal microbiota proliferation (Oliveira et al., 2011). It suggests that different control mechanisms can be acting since, in insects digest blood meals is extracellular and in contrast, tick blood digestion occurs intracellularly (Lara et al., 2005; Walker and Fletcher, 1987). Moreover, considering the biological difference, it is possible that they are involved in midgut microbiota control. In FEF salivary glands an increase of *DuoxA* transcriptional levels was also observed (fig. 1 M). It was previously revealed numerous degeneration signaling in tick salivary glands related to apoptosis starting after tick drop of the host (Freitas et al., 2007b). NADPH oxidase-mediated ROS formation and activates JNK (Reinehr et al., 2005), which could be an initial step to ROS signaling pathway, triggering apoptosis in tick salivary glands. Furthermore, an overall analysis showed that salivary glands transcriptional levels of antioxidant enzymes is mainly invariable between PEF and FEF phases, which is corroborated with enzymatic assays.

The overview of transcriptional levels and activity of antioxidant enzymes showed that in ovary and fat body most of transcripts and all enzymatic activities are higher in the PEF phase than in FEF phase (Fig. 2), suggesting a higher requirement of reducing power in initial feeding moments than in laying phase. In midguts, however, it was a balance in transcriptional levels and activities of tested enzymes. We hypothesize that enzymes are working in a coordinate system to control both redox homeostasis and tick microbiota.

### **3.3. The glutathione levels is higher in partially engorged organs but not in salivary glands**

The role of GSH in maintaining cell homeostasis against ROS is well known. Reduced levels of intracellular glutathione indicate a missing of redox potential to reduce reactive species and prevent cell damage (Matsumaru et al., 2003). The intracellular glutathione concentration is a result of GSH generation by de novo synthesis and recycling from GSSG by glutathione reductase, and the excretion of the resulting GSSG, under normal conditions, GSSG amounts to <1% of GSH (Circu and Aw, 2012). When supraphysiological levels of oxidized glutathione are formed by an oxidized environment, it is exported to the extracellular matrix (Hirrlinger et al., 2001). Therefore, the total glutathione levels in tick

organs were measured, comparing the partially and fully engorgement phases (Fig. 3). In agreement with previous results showed here, revealing higher enzymatic antioxidant activity and transcriptional levels in organs of PEF, total glutathione levels in ovary and fat body was higher in PEF than in FEF, possibly in order to provide reduced potential to antioxidant enzymes. Indeed, as previously observed with other arthropod blood suckers, start to blood feeding almost immediately induce enzymatic antioxidant responses, but this systems not always keeps active after feeding process (Gandara et al., 2016; Oliveira et al., 2017). Reduced levels of glutathione observed in FEF stage could be possibly due to GSSG exportation after PEF stage. Salivary glands, however, showed the glutathione level increased during the fully engorged phase. Several works revealed that a depletion of cellular glutathione levels constitutes an apoptotic signal (Boggs et al., 1998; Cazanave et al., 2007; Circu and Aw, 2012; Matsumaru et al., 2003). It was previously showed that *R. microplus* salivary glands starts to demonstrate numerous apoptotic signaling after tick drop of the host, however, highest damages could be observed after 72 hours (Freitas et al., 2007b), indicating that a first signaling induce apoptosis, but real damages and decrease in glutathione levels could occurs not immediately, but is late effect.

#### **3.4. NADPH levels in female organs**

The NADP reduced form, NADPH, is the donor of reductive potential to oxidized glutathione (GSSG) and thioredoxin, which are used by glutathione and thioredoxin peroxidases to neutralize  $H_2O_2$ . Thus, NADPH serves as the ultimate donor of reductive power for the large majority of ROS-detoxifying enzymes, recycling essential reductant co-factors. NADPH is also used in anabolic pathways, such as lipid and cholesterol synthesis and fatty acid chain elongation (Wamelink et al., 2008). Heme exposure of *A. aegypti* cells up-regulates genes encoding enzymes involved in the pentose phosphate pathway, the major NADPH production pathway, which may account for the need of NADPH as a provider of reducing equivalents for the thiol-based antioxidant defenses (Bottino-Rojas et al., 2015). In fact, an important correlation exists between the activity of the PPP and the triggering of an antioxidant response (Kruger et al., 2011). We propose that, in *R. microplus*, the high amount of ingested blood could increase NADPH levels, which may help prevent an oxidative burst. However, results showed a constant NADPH levels between PEF and FEF in the organs analyzed (Fig. 4), indicating that blood feeding does not interfere in tick redox homeostasis related with this electron donor, possibly by action of enzymes related with NADPH

production. Therefore, ticks could have multiple mechanisms to maintain blood digestion homeostasis to prevent the formation of toxic radicals (Graca-Souza et al., 2006; Sanders et al., 2003). It is important to note that the changes in tick physiology during blood feeding do not cause significant changes in intracellular ratio of NADPH to its oxidized form  $\text{NADP}^+$ . Results showed that despite the variance observed in transcriptional gene, enzyme activity and GSH, NADPH levels was constant between fully and partially engorged females. In fact, large changes in this parameter are usually related with oxidative stress causing toxicity rather than signaling associated with a physiological redox biology (Murphy, 2012).

### **3.5. Antioxidant gene silencing did not interfere in tick feeding and reproductive capacity**

The role of antioxidant enzymes and redox potential could be increasing the ROS antioxidant control by tick. Several works showed the biological implications in *catalase* silence of blood feeding arthropods, specially related to reproductive ability of insects as *Anopheles gambiae* (DeJong et al., 2007), *Aedes aegypti* (Oliveira et al., 2017) and *Lutzomyia longipalpis* (Diaz-Albiter et al., 2011). In *R. microplus*, it was demonstrated that FEF ticks injected with 3-amino-1,2,4-triazole (AT), a CAT inhibitor, diminished life span and egg-laying rates (Citelli et al., 2007). In *Amblyomma maculatum* oviposition efficiency was significantly reduced in ds*CAT* plus AT injected females (Budachetri and Karim, 2015). However, ds*CAT* knockdown alone did not interfere with *A. maculatum* hematophagy or phenotype. In the RmINCT-EM *R. microplus* transcriptome only one *thioredoxin reductase* contig were found. Considering also the lack of *glutathione reductase* in transcriptome and in a *R. microplus* ovary proteome (Xavier et al., 2018), and the previous described potential of thioredoxin reductase to reduce GSSG (Kanzok et al., 2001), we hypothesize the gene silencing of this enzyme could impact in tick biological parameters. Both enzymes showed to be transcribed in all tested organs (Fig. 1 H and K), for these reasons, catalase and thioredoxin reductase was chosen to compose RNAi experiment.

Partially engorged females were submitted to gene silencing through ds*CAT* or ds*TrxR* inoculation. Then, ticks were artificially blood fed by a capillary-tube during approximately 24 hours until female increase the weight and are able to lay eggs. Subsequently, biological parameters as tick fed capacity, egg laying and hatching were

evaluated (Alzugaray et al., 2017). The successful gene silencing of *CAT* and *TrxR* were confirmed through real time qPCR 24 h after dsRNA injection. The reduction in transcriptional level achieved 99% for *CAT* (Fig. 5A) and 85% for *TrxR* (Fig. 5C) compared with the transcriptional level from ds*MSP* (a non-related gene) injected ticks. The enzyme activity of catalase was also decreased in ovary from silenced ticks, showing a reduction of 72% when compared with the ds*MSP* injected ticks (Fig 5B). Biological parameters analyzed however, showed no difference when compared with de control groups (ds*MSP*- or PBS-injected ticks) for *CAT* (Fig. 6A, B and C) and *TrxR* (Fig. 6D, E and F). These results suggest that even a remaining catalase activity is enough to stabilize tick engorgement, fecundity and survival. Since several other works showing a biological implication by CAT absence in arthropods, probably there is not a *R. microplus* compensatory mechanism, but an effective catalase activity even in lower concentrations. A previous work showed depletion of TrxR activity through RNAi did not interfere in haematophagy or phenotype in the tick *A. maculatum* (Budachetri and Karim, 2015). It could be related with no complete absence of TrxR activity, but also with the trigger of some compensatory mechanism, since many reductase enzymes that uses NADPH as cofactor could act to reduce thioredoxin or GSH. Although the gene silencing was confirmed after 24 hours, the RNAi effect on gene silencing could be smaller or absent after this period, turning over the enzyme expression and function in tick physiology.

In summary, it was demonstrated a variable transcriptional and activity modulation of a range of antioxidant and detoxification enzymes by *R. microplus* blood feeding. Most enzymes have a major transcriptional level in fat body and ovary of PEF, which was corroborated by enzyme assays. GSH levels is also higher in PEF phase in fat body and ovary. These results reveal a major requirement of antioxidant potential during tick blood acquisition than egg laying phase in this organs. In midgut no pattern of transcription and activity modulation of antioxidant and detoxification enzymes was observed. Such data reinforced the complexity of ROS and microbiota control in tick midgut by this systems. GSH levels showed to be also modulated by blood feeding in the organs analyzed, demonstrating its higher levels during PEF phases in midgut, ovary and fat body. In contrast, NADPH levels is constant between PEF and FEF in the organs analyzed, showing an efficient tick control of oxidative stress during blood acquisition. CAT and TrxR gene



silencing were not able to interfere in the tick biological parameters analyzed, indicating the activation of potential compensatory mechanisms, or an efficient remaining enzyme activity.

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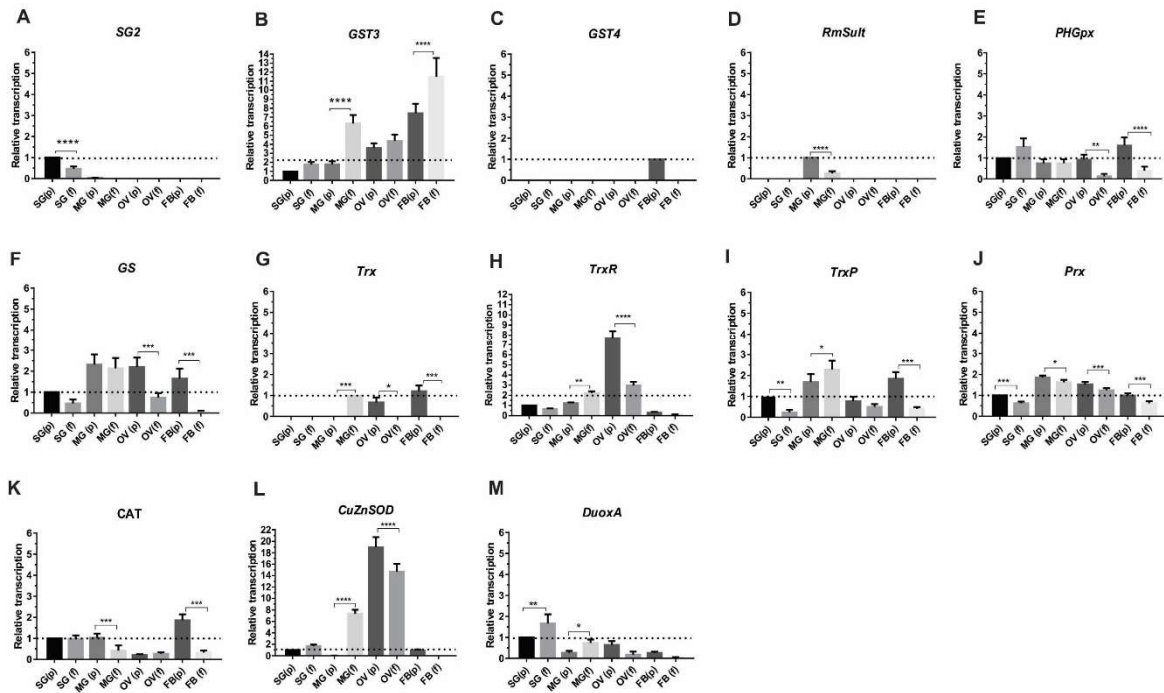
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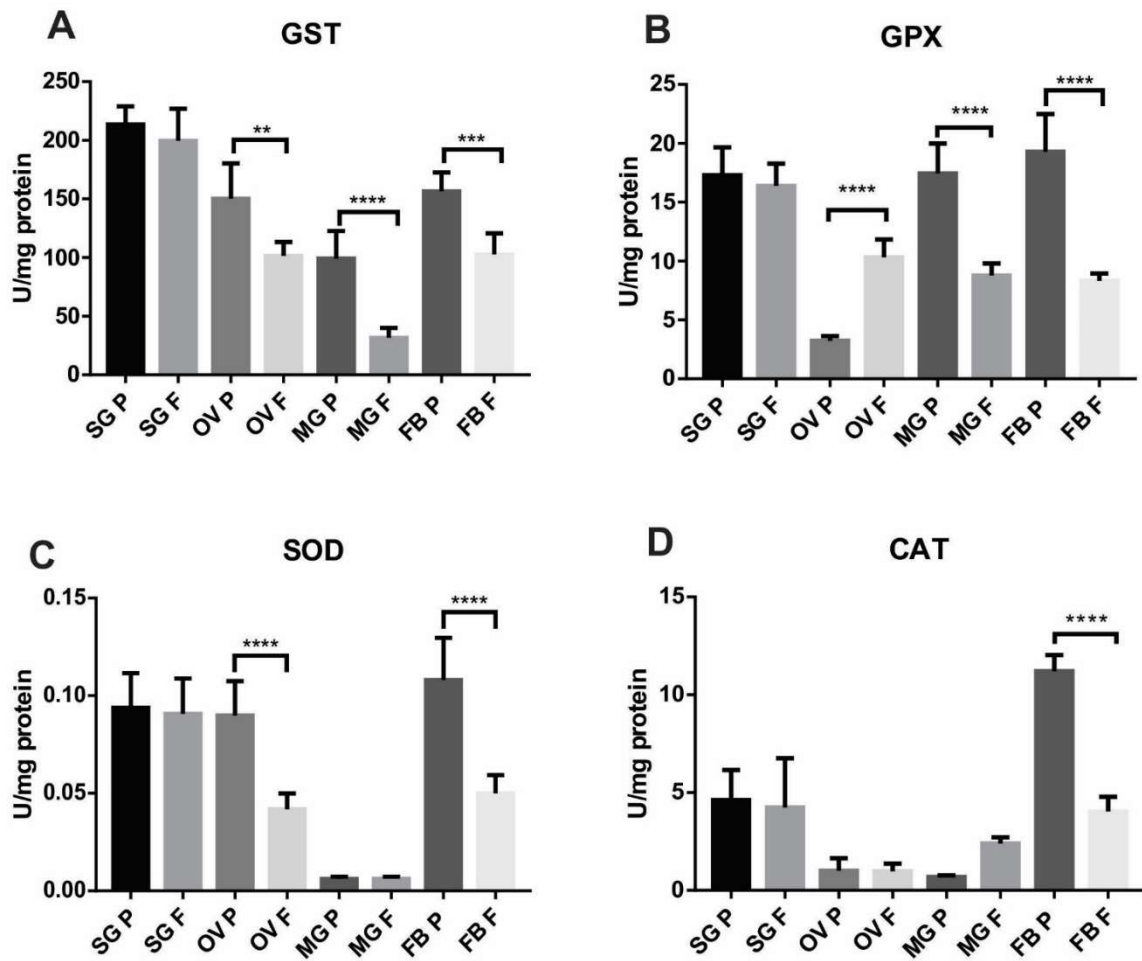


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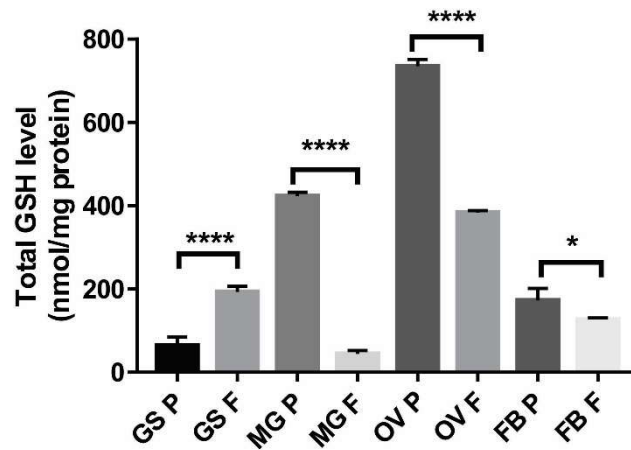
## Legends



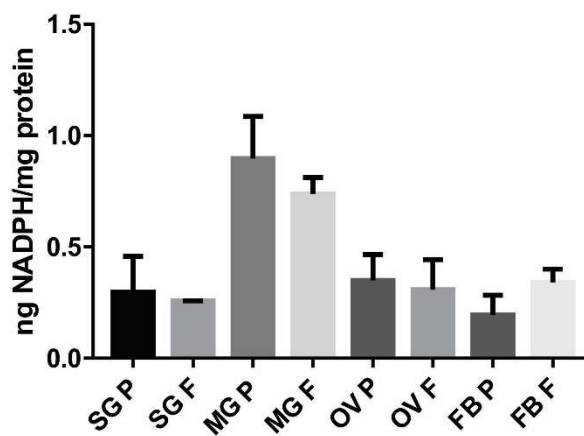
**Figure 1:** Relative transcription of antioxidant and detoxification genes in *R. microplus* organs of fully (f) and partially (p) engorged females. SG: salivary glands; MG: midgut; OV: ovary FB: fat body. (A) *glutathione S-transferase 2* (*SG2*; accession number: MK262715); (B) *glutathione S-transferase 3* (*GST3*; accession number: MK262716); (C) *glutathione S-transferase 4* (*GST4*; accession number: MK262717); (D) *sulfotransferase* (*RmSult*; accession number: MK262724); (E) *phospholipid-hydroperoxide glutathione peroxidase* (*PHGpx*; accession number: MK262714); (F) *glutathione synthetase* (*GS*; accession number: MK262718); (G) *thioredoxin* (*Trx*; accession number: MK262722); (H) *thioredoxin reductase* (*TrxR*; accession number: MK262720); (I) *thioredoxin peroxidase* (*TrxP*; accession number: MK262721); (J) *peroxiredoxin* (*Prx*; accession number: MK262719); (K) *Catalase* (*CAT*; accession number: KY953208); (L) *CuZn superoxide dismutase* (*CuZnSOD*; accession number: MK262723); (M) *dual oxidase A* (*DuoxA*; accession number: MK262713). Experiments were performed in duplicate with two experimental replicate. Results are expressed as the mean  $\pm$  S.D. The ANOVA was used to determine significant differences between groups when data were normally distributed and an *a posteriori* Tukey's test for pair-wise comparisons. Significance was set at \*  $p \leq 0.05$ ; \*\*  $p \leq 0.01$ ; \*\*\*  $p \leq 0.001$ ; \*\*\*\*  $p \leq 0.0001$ .



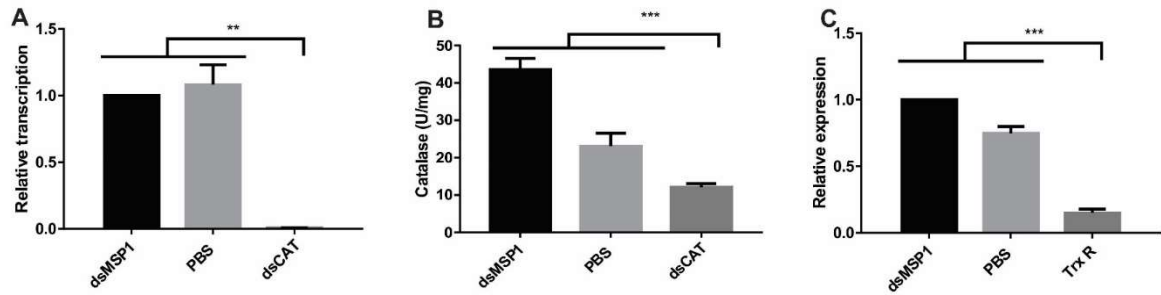
**Figure 2:** Enzyme activity levels in organs of fully (F) and partially (P) engorged *R. microplus* females. SG: salivary glands; MG: midgut; OV: ovary FB: fat body. Catalase (CAT); Superoxide dismutase (SOD); Glutathione S-transferase (GST); Glutathione Peroxidase (GPx). CAT and GPX assays were performed in duplicate with four experimental replicates. SOD assays were performed in triplicate with four experimental replicates. GST assays were performed in duplicate with six experimental replicates. Results are expressed as the mean  $\pm$  S.D. The ANOVA was used to determine significant differences between groups when data were normally distributed and an *a posteriori* Tukey's test for pair-wise comparisons. Significance was set at \*\*  $p \leq 0.01$ ; \*\*\*  $p \leq 0.001$ ; \*\*\*\*  $p \leq 0.0001$ .



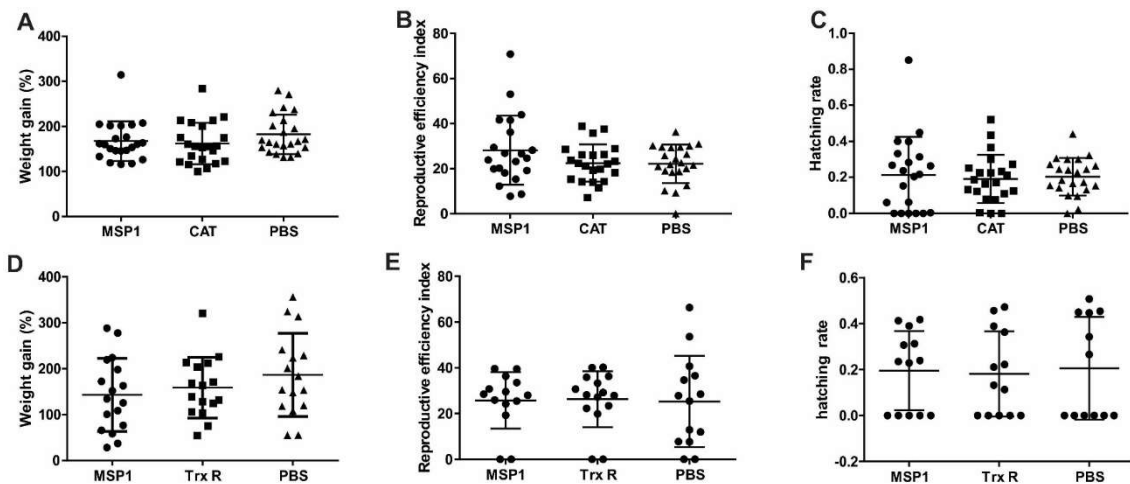
**Figure 3:** Total glutathione levels in *R. microplus* organs of fully (F) and partially (P) engorged females. SG: salivary glands; MG: midgut; OV: ovary FB: fat body. Experiments were performed in triplicate. Results are expressed as the mean  $\pm$  S.D. The ANOVA was used to determine significant differences between groups when data were normally distributed and an *a posteriori* Tukey's test for pair-wise comparisons. Significance was set at \*  $p \leq 0.05$ ; \*\*\*\*  $p \leq 0.0001$ .



**Figure 4:** Total NADPH levels in *R. microplus* organs of fully (F) and partially (P) engorged females. SG: salivary glands; MG: midgut; OV: ovary FB: fat body. Experiments were performed in duplicate with two experimental replicate. Results are expressed as the mean  $\pm$  S.D. The ANOVA was used to determine significant differences between groups when data were normally distributed and an *a posteriori* Tukey's test for pair-wise comparisons.



**Figure 5:** Relative expression of *CAT* (A) and *TrxR* (C) genes and Catalase activity (B) in *R. microplus* ovary of indicated dsRNA (dsMSP1, dsCAT or dsTrx R) or PBS injected ticks. Real times were performed in duplicate with two experimental replicate. Catalase enzyme assay was performed in duplicate with four experimental replicates. Results are expressed as the mean  $\pm$  S.D. The ANOVA was used to determine significant differences between groups when data were normally distributed and an *a posteriori* Dunnetts's test for pair-wise comparisons. Significance was set at \*\*  $p \leq 0.01$ ; \*\*\*\*  $p \leq 0.0001$ .



**Figure 6:** Tick blood feeding capacity (A and D), reproductive index (egg weight/tick weight; C and E) and hatching rate (larval weight/egg weight laid by each tick; D and F) of indicated dsRNA (MSP1, CAT or Trx R) or PBS injected ticks. RNAi experiments with dsCAT and dsTrxR were performed in tree and one biological replicates, respectively. Results are expressed as the mean  $\pm$  S.D. The ANOVA was used to determine significant differences between groups when data were normally distributed and an *a posteriori* Dunnetts's test for pair-wise comparisons. Significance was set at \*\*  $p \leq 0.01$ ; \*\*\*\*  $p \leq 0.0001$ .

**Supplementary table 1:** Accession numbers, oligonucleotide sequences used as primers and their efficiency for q-PCR amplification, primer melting temperature (T<sub>m</sub>; °C) and PCR product size of 13 *R. microplus* genes and one reference gene.

Target gene	Accession number	Primer sequence (5'-3')	T <sub>m</sub> (°C)	PCR product size	Primer efficiency
<i>Actin</i>	AY255624.1	F: GTACATGGTGGTGCCGCCG	59.1	184	2.00
		R: TCAGGTCATCACCATCGGCAAC	59.3		
<i>PHGPx</i>	MK262714	F: CGTCAACGGGGACAAAGCG	58.6	206	2.07
		R: CGTGCCAGCTGTTGACAGCTC	58.2		
<i>SG2</i>	MK262715	F: GGTGAGCGTGTTGCCGAGG	58.7	181	1.96
		R: GCGGAACTGTGGCTCATGGA	58.2		
<i>GST3</i>	MK262716	F: GCCGACAACATCGACAACGTG	58.5	199	2.02
		R: GGTTGGGCAGTTCCTCGAACC	59.4		
<i>GST4</i>	MK262717	F: GGCAGAGGTCAGCAAGCGTG	58.1	193	2.02
		R: GTGCTTGCAGCAGCCAATGG	58.9		
<i>GS</i>	MK262718	F: TTGCACACCCTCGCAACTACGT	59.2	191	2.06
		R: GCTGGCCTCCTCAGTTGATCGT	59.1		
<i>CAT</i>	KY953208	F: TCATGTGCCTGTAGCCATCGG	58.1	186	2.01

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		R: ATCTTCTTCATCCGGGACCCG	58.8		
<i>CuZn-SOD</i>	MK262723	F: GGAATAGTCCCGACTTGCGCC	59.1	183	1.99
		R: TTGCGAATGAACACGGACGC	59.1		
<i>RmSult</i>	MK262724	F: CACGGAGCACGAGTGCGCT	59.6	235	2.01
		R: TCGTCGGCGATTGGCAGAA	59.7		
<i>Prx</i>	MK262719	F: GAAAGGCCCATCAGGCAGCT	57.9	191	1.96
		R: GCATCCTATTCTCGCACCCAGC	59.6		
<i>TrxR</i>	MK262720	F: CGCTTGAGTGTGCGGGCTTT	59.8	146	2.26
		R: TCAATCAGTTCCAGCTTCGTGGG	59.9		
<i>TrxP</i>	MK262721	F: ACAAGGTGGCCTCGGACCTATC	58.0	188	2.06
		R: TCTCGTCCACAGATCGACCCAC	58.5		
<i>Trx</i>	MK262722	F: GGAGCCAGCCTTGTCATAGTGGA	59.1	205	1.81
		R: TTAGCTTCGCCTTGGCCCG	59.8		
<i>DuoxA</i>	MK262713	F: CTGCCTGCGAAACGGTCAAC	57.9	192	2.05
		R: GGGTGCATCAGACGAGCCG	58.6		

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\*Primer Melting Temperature (T<sub>m</sub>) Forward (F); reverse (R); *phospholipid-hydroperoxide glutathione peroxidase (PHGPx)*; *glutathione S-transferase 2 (SG2)*; *glutathione S-transferase 3 (GST3)*; *glutathione S-transferase 4 (GST4)*; *glutathione synthetase (GS)*; *Catalase (CAT)*; *CuZn superoxide dismutase (CuZnSOD)*; *sulfotransferase (RmSult)*; *peroxiredoxin (Prx)*; *thioredoxin reductase (TrxR)*; *thioredoxin peroxidase (TrxP)*; *thioredoxin (Trx)*; *dual oxidase A (DuoxA)*.

**Supplementary table 2:** Accession number, oligonucleotide sequences used as primers to dsRNA template amplification, primer melting temperature (T<sub>m</sub>; °C) and PCR product size of two genes used for RNA interference experiment in *R. microplus*.

Target gene	Accession number	Primer sequence (5'-3')	T <sub>m</sub> (°C)	PCR product size
<i>CAT</i>	KY953208	F:GGATCCTAATACGACTCACTATAG GATCGAGCCAAGTCCAGACAAGA	55.3	481
		R:GGATCCTAATACGACTCACTATAG GTGCACTCTGCACGAGCTTCTC	55.6	
<i>TrxR</i>	MK262720	F:GGATCCTAATACGACTCACTATAGGC ACTGCTAATGCAAATGGCACA	56.1	592
		R:GGATCCTAATACGACTCACTATAGGG ATGGTGGCATCCAAGTCTGC	56.6	



\*Primer Melting Temperature (T<sub>m</sub>) Forward (F); reverse (R); *Catalase (CAT)*; *thioredoxin reductase (TrxR)*.

	*	20	*	40
R._microplus_DuoxA	:	GLKPGEKRKLEEVGNDVIMVMRTSLSKQEFADALGMKPDS	:	40
R._microplus_PHGPx	:	MATADDSWKDASSTYDFSVAVDIDGNEVSLDKYKGHVALIV	:	40
R._microplus_GST	:	MAPTPVVGYYTTARGLAQSI RNLLVYKGVHFEDKRYEFGPA	:	40
R._microplus_GST3	:	MAPIVGYWDV RALAQFIRNLLVYKGVQFEDKRYKFGPAPD	:	40
R._microplus_GST4	:	MPVLLYNLEGS PHCSFIRCLAKEIGVQLSIKEINCAKGEH	:	40
R._microplus_GS	:	KCEHGGIASVIARCSFSQAGVCPNLKASSCNVAENALVSE	:	40
R._microplus_Prx	:	MPPLNLGDPFPNFTCETTIGTIDFHEWLGD SWGILFSHPA	:	40
R._microplus_TrxR	:	SGGLAASKEAAELGKKVAVCDFVKPTPKGTTWGLGGTCVN	:	40
R._microplus_TrxP	:	MASCWGWF LVALTIGPQLITCAEEACRSYDGGQVYPQENT	:	40
R._microplus_Trx	:	MPVKQVTDDARFQAELS NSGASLVIVDFTASWCGPCQMA	:	40
R._microplus_CuZnSOD	:	LLVLLCVLHVTRTVERRAVCYAPGSVFMQLFFVQESVEHS	:	40
R._microplus_RmSult	:	PYSKEAKYIYVARNPYDCCVSYFYHTRNIPDYKQDGTDFD	:	40

	*	60	*	80
R._microplus_DuoxA	:	LFVKQMFNCV DDKDKGQISFQEF LDTVVLFTRGKSEDKLR	:	80
R._microplus_PHGPx	:	NVASKUGKTNKNYTQLVELHKKYAESKGLRILAFPCNQFG	:	80
R._microplus_GST	:	PTYEKLGWAA DSASLGFTFPNLPYYIDGDVRLTQSLAILR	:	80
R._microplus_GST3	:	FDRSDWLKEKFTLGLKFPNLPYYIDGNVKITQSLAILRYL	:	80
R._microplus_GST4	:	RSAEFIKKNPFHKVPTIDDGGLIVYESNAIAYLLRKYAP	:	80
R._microplus_GS	:	LGTPIRPQSITMERLQSCMAALQDE D VRKRAVQDAKEYAI	:	80
R._microplus_Prx	:	DYTPVCTTE LARAAQLAHVFEKKGVKI IALS CDSVESHHG	:	80
R._microplus_TrxR	:	VGCI PKKLMHNAALLGQGIKDSTSFGWEMQDVKFKWETMR	:	80
R._microplus_TrxP	:	KSSAHNLHWSKTQISK P APYFSGTAVVNGEFKELKLS DYK	:	80
R._microplus_Trx	:	PVFESLSNKYTQAVFLKVDIDQCQDIAAAHGVS AVPTFIF	:	80
R._microplus_CuZnSOD	:	VVITGEITGLQPGAHLHVHAYGDLTNGCNSTKGFHNP MH	:	80
R._microplus_RmSult	:	QFFEMFLEGKVD FGDYFDHLLSWYEH CDDPNVFFVTYEQL	:	80

	*	100	*	120
R._microplus_DuoxA	:	IIFDMCDNHGDGMIQKQELTRMLRSLVDIAKTNSLSEYET	:	120
R._microplus_PHGPx	:	GQEPGTEADIKKFVEKYNVKFDMFSKVN VNGDKAHLWKY	:	120
R._microplus_GST	:	YLGKKHGLDARS DQEAELWLMEQQANDLLWALVVTAMNP	:	120
R._microplus_GST3	:	GRKHDLAARNEDE TLELDVLEQQARDLTTRLIFATAPNPN	:	120
R._microplus_GST4	:	ESQLYPNCIKTRARIDQV LAAANGNISPI LGTYLAARCLK	:	120
R._microplus_GS	:	SHGLCMKSKSQEPDSYTFVFPCLLPSPYPREAFQQVKALQ	:	120
R._microplus_Prx	:	WIKDIEAYGELPDGPFYPYPIIADEKREIAVKLGMLDPVEK	:	120
R._microplus_TrxR	:	GNIRDYIASLNWKYRVSLREAGVDYMNAYAHFVDPHKLKL	:	120
R._microplus_TrxP	:	GKYL VFFFYPLDFTFVCPT EIIAFSDRVQEFKALNAEVVA	:	120
R._microplus_Trx	:	FRAKAKLTTMRGADPNTLEAKIQELLSSEPPEGAATEKVQ	:	120
R._microplus_CuZnSOD	:	KDHGGPEDRERHVGD LGNIKAEADGKARVYITDSMISLIG	:	120
R._microplus_RmSult	:	KKDAKTSVLKIAEFIGE QYQGQKLRNDKSRFESVLTNITVE	:	120

	*	140	*	160
R._microplus_DuoxA	:	SDIIRSMYVSAGLDNSDTLSYEDFKMMKEHRGDVIAIGL	:	160
R._microplus_PHGPx	:	LKQKQSGFLTD AIKWNFTK FVVDKEGQPVHRYAPT DPLD	:	160

R.\_microplus\_GST : NATEARKSQEKRLADSLPRWQELLKKRRWALGNTLTLYVDF : 160  
R.\_microplus\_GST3 : YEETIKAYADNIDNVLPWEEHLANREWALGDRLTYVDFL : 160  
R.\_microplus\_GST4 : RTKPSADEVKECEEKVLKCLEHLLGESKFAAGDKITLADL : 160  
R.\_microplus\_GS : EDINLLIHRVAHDYSFLRDCLQSTIETDEFTASIFNIYQK : 160  
R.\_microplus\_PrX : DKEGLPLTCRAVFIIGPDKKMKLSMLYPATTGRNFDEVLR : 160  
R.\_microplus\_TrxR : T----- : 121  
R.\_microplus\_TrxP : CSVDSPFTHLAWINTPRKQGGLGPIKIPLLSDLTHQISKD : 160  
R.\_microplus\_Trx : GHVDMSTFFDRNAQECLNESDDHPLSGCLSSGDGYLESDC : 160  
R.\_microplus\_CuZnSOD : HHNIIGRAMVVHANPDDLKGGTNDSTSGSAGPRLACCV : 160  
R.\_microplus\_RmSult : SMKESVNESMQTSMRTLAAALGGKVPKWIVLMKGTAGAES : 160

\* 180 \* 200

R.\_microplus\_DuoxA : DCKGANQNFLDTSSNVARMTSFQINQPAEDPPSWTVLKWN : 200  
R.\_microplus\_PHGPx : IEPDLLKLF\*AVNSWHAEVSANVDCFLGKRPYDQSTNRN : 199  
R.\_microplus\_GST : LLYEALDWNRQFAPDAFANRPELLDYLRREFQLPNLKEYF : 200  
R.\_microplus\_GST3 : LYEGFDTHREFKGDVQRYPRIVAYLKRFEELPNLKEYFA : 200  
R.\_microplus\_GST4 : CLIGHVAVYVELPCVNRKYPKLAAYYDRVKGALPYFNEV : 200  
R.\_microplus\_GS : VWEAEFPQPISLGLLRSDYMLDLKSGDTKELHEARPKQVE : 200  
R.\_microplus\_PrX : ATDSLIVTETTRKVATPAGWKKGTPCMVLPVSVTDEEIPQLF : 200  
R.\_microplus\_TrxR : ----- : -  
R.\_microplus\_TrxP : YGVYLEDLGHTRLGLFIIDDKGNLRQITMNDMPVGRSVDE : 200  
R.\_microplus\_Trx : DEQLIISLGFQTPVKLHSIKFQAPSSQ----- : 187  
R.\_microplus\_CuZnSOD : IGFVSGSAWNSPDLR----- : 175  
R.\_microplus\_RmSult : CEKPMMSGDFVRKGIWGDWQNHFSSEEQVKRLQKKIHEKTRG : 200

\* 220 \* 240

R.\_microplus\_DuoxA : QVTTFLEENRQHVFYLFVYVFTIVLFIERFIYYCYMSEH : 240  
R.\_microplus\_PHGPx : LRLACPDVFPVLSRERRRLCRNFGKPCF----- : 227  
R.\_microplus\_GST : ASDKYVKWPIMAPYMFWGKH----- : 220  
R.\_microplus\_GST3 : SDKYHKYPILGPHRKGWFKK----- : 220  
R.\_microplus\_GST4 : FGPAIAQAKKLWDMVH----- : 216  
R.\_microplus\_GS : VNTIASSFGGITPPLYKLQKYTMSNLGVVATEVEMPKCKT : 240  
R.\_microplus\_PrX : PTGIKQYDVPSGKKYLRTTMD----- : 221  
R.\_microplus\_TrxR : ----- : -  
R.\_microplus\_TrxP : TLRLVQAFQYTDKHGEVCPAGWKPGGDTIIPTPSEKLYF : 240  
R.\_microplus\_Trx : ----- : -  
R.\_microplus\_CuZnSOD : ----- : -  
R.\_microplus\_RmSult : SDVMKLWKDTDIPH----- : 214

\* 260 \* 280

R.\_microplus\_DuoxA : MDLRHVMGVGIAITRGSAAASLSFCYSLLLLLTMCRNLIKI : 280  
R.\_microplus\_PHGPx : ----- : -  
R.\_microplus\_GST : ----- : -

```

R._microplus_GST3      : ----- : -
R._microplus_GST4      : ----- : -
R._microplus_GS        : DKLLGKGLLAGWRAYKNDKAAILFVVEEETYNVCDQRNLE : 280
R._microplus_PrxC      : ----- : -
R._microplus_TrxC      : ----- : -
R._microplus_TrxC      : SKVVDPE----- : 247
R._microplus_TrxC      : ----- : -
R._microplus_CuZnSOD   : ----- : -
R._microplus_RmSult    : ----- : -

```

\*                    300                    \*                    320

```

R._microplus_DuoxA     : RELPLQQYIPLDSHVQFHKIVALTALFFSLVHTVGHYINF : 320
R._microplus_PHGPx     : ----- : -
R._microplus_GST       : ----- : -
R._microplus_GST3      : ----- : -
R._microplus_GST4      : ----- : -
R._microplus_GS        : YAIEMHESVKVMRRKFSELHNCTIQEGLNLIVDGVEIAVV : 320
R._microplus_PrxC      : ----- : -
R._microplus_TrxC      : ----- : -
R._microplus_TrxC      : ----- : -
R._microplus_TrxC      : ----- : -
R._microplus_CuZnSOD   : ----- : -
R._microplus_RmSult    : ----- : -

```

\*                    340                    \*                    360

```

R._microplus_DuoxA     : YHVSTQPAEHLRCMTKEMHFDSDFKSTITFWVFQTVTGIT : 360
R._microplus_PHGPx     : ----- : -
R._microplus_GST       : ----- : -
R._microplus_GST3      : ----- : -
R._microplus_GST4      : ----- : -
R._microplus_GS        : YYRTAYAPQQMDAKCWDTRLRIELSRAIKCPSVQYHLAGT : 360
R._microplus_PrxC      : ----- : -
R._microplus_TrxC      : ----- : -
R._microplus_TrxC      : ----- : -
R._microplus_TrxC      : ----- : -
R._microplus_CuZnSOD   : ----- : -
R._microplus_RmSult    : ----- : -

```

\*                    380                    \*                    400

```

R._microplus_DuoxA     : GVLLFIVMCIIFIFAHPKIRQKAYSIFWMTHSLYILLYIL : 400
R._microplus_PHGPx     : ----- : -
R._microplus_GST       : ----- : -
R._microplus_GST3      : ----- : -
R._microplus_GST4      : ----- : -

```

```

R._microplus_GS      : KKVQQVLAQPDVLERFFTNKVIANSIRATFTGLYSLDLTP : 400
R._microplus_Prx    : ----- : -
R._microplus_TrxR   : ----- : -
R._microplus_TrxP   : ----- : -
R._microplus_Trx    : ----- : -
R._microplus_CuZnSOD : ----- : -
R._microplus_RmSult : ----- : -

```

\*            420            \*            440

```

R._microplus_DuoxA  : CLLHGQAKLTGSPRFWMFFIGPAIVFTLTKVVSLSLQTKYME : 440
R._microplus_PHGPx  : ----- : -
R._microplus_GST    : ----- : -
R._microplus_GST3   : ----- : -
R._microplus_GST4   : ----- : -
R._microplus_GS     : QGHKAAEEAIAHPRNYVLKPPQREGGNNVYGDDVRNKLQA : 440
R._microplus_Prx    : ----- : -
R._microplus_TrxR   : ----- : -
R._microplus_TrxP   : ----- : -
R._microplus_Trx    : ----- : -
R._microplus_CuZnSOD : ----- : -
R._microplus_RmSult : ----- : -

```

\*            460            \*            480

```

R._microplus_DuoxA  : LDILDTELLPSDVTRVKFTRPPNFKYLSGQWVRLSCTGFR : 480
R._microplus_PHGPx  : ----- : -
R._microplus_GST    : ----- : -
R._microplus_GST3   : ----- : -
R._microplus_GST4   : ----- : -
R._microplus_GS     : LGRTKEREYILMDLIQPPITTNFYIVRSTEEASKCNVSE : 480
R._microplus_Prx    : ----- : -
R._microplus_TrxR   : ----- : -
R._microplus_TrxP   : ----- : -
R._microplus_Trx    : ----- : -
R._microplus_CuZnSOD : ----- : -
R._microplus_RmSult : ----- : -

```

\*            500            \*            520

```

R._microplus_DuoxA  : ATEYHSLTLTSAPHENYLSVHVKAQGPWTWKLARNYFDPSN : 520
R._microplus_PHGPx  : ----- : -
R._microplus_GST    : ----- : -
R._microplus_GST3   : ----- : -
R._microplus_GST4   : ----- : -
R._microplus_GS     : LGIFGVILGDQYELIENYEAGHIVRTKMTGTSEGGICAGF : 520
R._microplus_Prx    : ----- : -

```

```

R._microplus_TrxR      : ----- : -
R._microplus_TrxP     : ----- : -
R._microplus_Trx      : ----- : -
R._microplus_CuZnSOD : ----- : -
R._microplus_RmSult   : ----- : -

```

```

                *           540           *           560
R._microplus_DuoxA   : LHESMLPKVRLEGPFGGGNQDWYKFEIAVMVGGGIGVTPY : 560
R._microplus_PHGPx   : ----- : -
R._microplus_GST     : ----- : -
R._microplus_GST3    : ----- : -
R._microplus_GST4    : ----- : -
R._microplus_GS      : ASLDSVFLV----- : 529
R._microplus_Prx     : ----- : -
R._microplus_TrxR    : ----- : -
R._microplus_TrxP    : ----- : -
R._microplus_Trx     : ----- : -
R._microplus_CuZnSOD : ----- : -
R._microplus_RmSult  : ----- : -

```

```

                *           580           *           600
R._microplus_DuoxA   : ASILNDLVFGTSTNRYSGVACKKVYFMWICPSHRHFEWFI : 600
R._microplus_PHGPx   : ----- : -
R._microplus_GST     : ----- : -
R._microplus_GST3    : ----- : -
R._microplus_GST4    : ----- : -
R._microplus_GS      : ----- : -
R._microplus_Prx     : ----- : -
R._microplus_TrxR    : ----- : -
R._microplus_TrxP    : ----- : -
R._microplus_Trx     : ----- : -
R._microplus_CuZnSOD : ----- : -
R._microplus_RmSult  : ----- : -

```

```

                *           620           *           640
R._microplus_DuoxA   : DVLRDVERKDVTNVLEIHIFITQFFHKFDLRTTMLYICEN : 640
R._microplus_PHGPx   : ----- : -
R._microplus_GST     : ----- : -
R._microplus_GST3    : ----- : -
R._microplus_GST4    : ----- : -
R._microplus_GS      : ----- : -
R._microplus_Prx     : ----- : -
R._microplus_TrxR    : ----- : -
R._microplus_TrxP    : ----- : -

```

```

R._microplus_Trx      : ----- : -
R._microplus_CuZnSOD : ----- : -
R._microplus_RmSult  : ----- : -

                        *           660           *           680
R._microplus_DuoxA   : HFQRVSNRSMFTGLKAINHFGRPDMSAFLKFKVQQQHSYVS : 680
R._microplus_PHGPx   : ----- : -
R._microplus_GST     : ----- : -
R._microplus_GST3    : ----- : -
R._microplus_GST4    : ----- : -
R._microplus_GS      : ----- : -
R._microplus_Prx     : ----- : -
R._microplus_TrxR    : ----- : -
R._microplus_TrxP    : ----- : -
R._microplus_Trx     : ----- : -
R._microplus_CuZnSOD : ----- : -
R._microplus_RmSult  : ----- : -

                        *           700           *
R._microplus_DuoxA   : KVGVFSCGPSALTKSVSSACETVNNNRKLPYFIHHYENF : 719
R._microplus_PHGPx   : ----- : -
R._microplus_GST     : ----- : -
R._microplus_GST3    : ----- : -
R._microplus_GST4    : ----- : -
R._microplus_GS      : ----- : -
R._microplus_Prx     : ----- : -
R._microplus_TrxR    : ----- : -
R._microplus_TrxP    : ----- : -
R._microplus_Trx     : ----- : -
R._microplus_CuZnSOD : ----- : -
R._microplus_RmSult  : ----- : -

```

Supplementary figure 1: predicted amino acid sequences of antioxidant enzymes deposited in Genebank. Glutathione S-transferase 2 (GST; accession number:MK262715); glutathione S-transferase 3 (GST3; accession number: MK262716); glutathione S-transferase 4 (GST4; accession number: MK262717); sulfotransferase (RmSult; accession number: MK262724); phospholipid-hydroperoxide glutathione peroxidase (PHGPx; accession number: MK262714); glutathione synthetase (GS; accession number: MK262718); thioredoxin (Trx; accession number: MK262722); thioredoxin reductase (TrxR; accession number: MK262720); thioredoxin peroxidase TrxP; accession number: MK262721); peroxiredoxin (Prx; accession number:MK262719); CuZn superoxidedismutase (CuZnSOD; accession number: MK262723); dual oxidase A (DuoxA; accession number: MK262713)

#### 4. DISCUSSÃO

As enzimas antioxidantes destacaram-se devido ao papel que têm na eliminação de espécies reativas de oxigênio e na diminuição da toxicidade dessas moléculas e da morte celular nos organismos (JAKOBY, 1978; LANKIN e GUREVICH, 1976; MONTGOMERY, 1977; NICKLA *et al.*, 1983). Além disso, com a caracterização de vias metabólicas, proteínas e sua regulação, a função das espécies reativas e consequentemente dessas enzimas na sinalização celular vem sendo elucidada (JIA *et al.*, 2018; MOLDOGAZIEVA *et al.*, 2018; RADY, 2018). A função dessas enzimas foi descrita na regulação da homeostase redox em uma variedade de organismos, o que aumentou o interesse da comunidade científica engajada no estudo de artrópodes hematófagos. A partir de então, diversos estudos demonstram a relação entre enzimas antioxidantes e de detoxificação com o hábito hematófago (BOTTINO-ROJAS, *et al.*, 2015; GRACA-SOUZA, *et al.*, 2006; SANDERS, *et al.*, 2003), contribuindo para o desenvolvimento de novos métodos de controle contra esses parasitas. Nesta tese composta por dois capítulos, enzimas antioxidantes e o nível de dois potenciais redutores celulares foram analisados em fêmeas do carrapato *R. microplus* parcialmente e totalmente alimentadas. Além disso, atuação de uma GST recombinante como antígeno vacinal foi observada contra as espécies de carrapato *R. appendiculatus* e *R. sanguineus*.

Os resultados obtidos a partir dos experimentos apresentados no primeiro capítulo mostraram uma alta variação no nível transcricional e na atividade das enzimas relacionadas ao estresse oxidativo e detoxificação, não apresentando um padrão estritamente definido nem em relação ao tecido, nem em relação ao estágio de alimentação. Em um trabalho similar, um perfil variado de expressão de GSTs e transcrição de duas *GSTs* também foi observado em diferentes órgãos e diferentes estágios de alimentação no carrapato *H.*



*longicornis* (HERNANDEZ *et al.*, 2018). Esses dados sugerem a atuação de um sistema de regulação complexo sobre o controle de danos e a sinalização celular gerada por radicais livres. Apesar de não haver um padrão de transcrição bem definido entre os dois estágios de alimentação e tecidos analisados, observa-se que a *RmSult* e a *GST4* são transcritas especificamente no intestino e no corpo gorduroso, respectivamente, sugerindo a atuação desses transcritos em atividades exclusivamente relacionadas ao órgão em que foram encontrados. Em *I. ricinus* foi observado um aumento nos transcritos de uma *sulfotransferase* no intestino em um primeiro momento após a alimentação com sangue (MUELLER, *et al.*, 2015), o que é compatível com a atuação dessa enzima na detoxificação do heme, ou outros componentes advindos da dieta. Sabe-se que as sulfotransferases também atuam na inativação de hormônios (MUELLER, *et al.*, 2015; SUZUKI-ANEKOJI, *et al.*, 2013), o que também poderia ser um alvo, já que os hormônios do hospedeiro também são ingeridos durante a hematofagia. Além disso, heparan sulfato, um glicosaminoglicano anticoagulante, está presente em intestino e ovário de *R. microplus* (ONOFRE *et al.*, 2003), tendo possivelmente uma função na embriogênese e alimentação do carrapato. A biossíntese *in vivo* de heparan sulfato, requer a atividade de sulfotransferases (LIU e PEDERSEN, 2007; STERNER *et al.*, 2014), nesse sentido, a *RmSult* poderia estar atuando na biossíntese de heparan sulfato no intestino do *R. microplus*.

Ainda que a maioria dos estudos apresentados na literatura relacionem a atividade de GST com a detoxificação de drogas e a resistência a acaricidas em carrapatos (GHOSH *et al.*, 2017; NANDI *et al.*, 2015; ZIAPOUR *et al.*, 2017), a *GST4* transcrita exclusivamente no corpo gorduroso da fêmea parcialmente alimentada poderia estar relacionada, também, com a degradação de 4-HNE. Esse produto é um aldeído poli-insaturado potencialmente tóxico produzido durante a peroxidação lipídica, e sua degradação por diferentes GSTs foi

descrita em *D. melanogaster* (SAWICKI *et al.*, 2003; SINGH, *et al.*, 2001). Em *D. variabilis*, foi demonstrado que a transcrição de duas GSTs é regulada positivamente depois da hematofagia em fêmeas parcialmente alimentadas (DREHER-LESNICK, *et al.*, 2006). Em *A. aegypti* a transcrição de uma GST é observada somente após a hematofagia, e não após a ingestão de açúcar (BOTTINO-ROJAS, *et al.*, 2015). Além disso, foi observada uma regulação positiva tanto na transcrição quanto na expressão de GSTs desencadeada pela alimentação com sangue no carrapato *H. longicornis* (HERNANDEZ, *et al.*, 2018), o que demonstra a direta interferência da hematofagia sobre a ativação dessas GSTs. Tendo em vista que a fêmea parcialmente alimentada já ingeriu uma considerável quantidade de sangue/heme e nessa fase há uma maior concentração de lipídeos no corpo gorduroso (ARRESE e SOULAGES, 2010), as peroxidação lipídicas provavelmente são favorecidas, o que possivelmente necessita de um controle que poderia estar sendo efetuado por essa GST e outras enzimas antioxidantes. Os dados desse trabalho mostram, ainda, que tanto a atividade enzimática de todas as enzimas testadas (CAT, SOD, GPx e GST), quanto o nível transcricional da grande maioria dos transcritos quantificados no corpo gorduroso são maiores na fase PEF. Além disso, os níveis de glutathiona no corpo gorduroso também são maiores na fase PEF, corroborando com o dado que mostra o nível transcricional de *glutathione sintetase* nessa fase, o que sugere maior requerimento desse potencial redutor para utilização por enzimas relacionadas à GSH. Corroborando com esses dados, em *H. longicornis* foi observado uma regulação positiva na expressão de GSTs na fase PEF e negativa na fase FEF tanto no corpo gorduroso, quanto no ovário e em glândula salivar (HERNANDEZ, *et al.*, 2018).

Ainda nesse contexto, é interessante observar que foi demonstrada maior atividade enzimática de GPx em ovário de FEF do que em PEF. Visto que a GPx possui uma atividade

especialmente relacionada a degradação de hidroperóxidos de lipídeos (BOTTINO-ROJAS, *et al.*, 2015; CAVAS e TARHAN, 2003; URSINI, *et al.*, 1982), tendo uma função na manutenção da integridade das membranas (COZZA, *et al.*, 2017), é possível que ela esteja atuando no controle da peroxidação lipídica no ovário durante a oogênese, uma vez que nessa fase a teleógina mobiliza uma maior quantidade de lipídeos do corpo gorduroso para o ovário como reserva energética para a formação do ovo (ARRESE e SOULAGES, 2010; MARTINS *et al.*, 2018). Além disso, a atividade das outras enzimas testadas e a grande maioria dos transcritos mostraram ser mais abundantes no ovário de PEF do que no ovário de FEF sugerindo que esse controle antioxidante pode ser exercido majoritariamente pela GPx.

O sistema tiorredoxina compreende um dos mais importantes reguladores da homeostase redox (WANG *et al.*, 2015). No presente trabalho, um dado interessante mostrou que a *Trx* é transcrita exclusivamente em ovário e corpo gorduroso na fase PEF e completamente ausente nesses tecidos na fase FEF. Já no intestino, observa-se um padrão contrário de transcrição entre as fases de alimentação, o que demonstra o efeito direto da hematofagia sobre o nível transcricional dessa enzima. Diversos estudos descrevem que os sistemas tiorredoxina-glutaredoxina atuam em conjunto, interagindo e, muitas vezes funcionando como sistemas de backup (DU *et al.*, 2012; MARTY *et al.*, 2009; WANG, *et al.*, 2015). Por isso, é possível que a própria glutathione ou o sistema glutathione esteja majoritariamente ativo quando a tiorredoxina está ausente em *R. microplus*. Ainda nesse contexto, a ausência de glutathione redutase foi descrita em diversos organismos, como *D. melanogaster* (KANZOK *et al.*, 2001), *Schistosoma mansoni* (ALGER e WILLIAMS, 2002) e outros platelmintos (SALINAS *et al.*, 2004), e foi observado que a atividade dessa enzima estava sendo substituída por tiorredoxina redutase. Em carrapatos esse fenômeno não é

descrito, no entanto, a glutathione redutase não é encontrada no genoma do carrapato *I. scapularis* (GULIA-NUSS *et al.*, 2016), nem em transcritos do intestino de *I. ricinus* (PERNER, *et al.*, 2016a) e *Ornithodoros erraticus* (OLEAGA *et al.*, 2018), nem em um proteomas de ovário de *R. microplus* (XAVIER *et al.*, 2018) sugerindo que esse mecanismo também exista em carrapatos. No transcriptoma de *R. microplus* utilizado no presente trabalho também não foram encontrados transcritos anotados como glutathione redutase, indicando que a atividade tiorredoxina redutase sobre as glutathiones oxidadas é um mecanismo atuante também nesse carrapato. O silenciamento de *TrxR* em *R. microplus* realizado no presente trabalho não levou a nenhuma deficiência em relação a alimentação, postura e eclosão das larvas, apesar de mostrar uma redução de 72% dos transcritos em relação ao controle (dsMSP1). Um resultado similar também foi observado após o silenciamento de uma *tiorredoxina redutase* em *Amblyomma maculatum* (COZZA, *et al.*, 2017), possivelmente devido à existência de um mecanismo compensatório, visto que outras redutases (isoformas ou proteínas *moonlight*) que utilizam NADPH como cofator podem estar atuando nesse sistema de redução.

A catalase é uma das enzimas antioxidantes mais estudadas em artrópodes hematófagos, destacando-se por seu papel na detoxificação do heme no intestino de *R. microplus* (CITELLI *et al.*, 2007). Os dados apresentados aqui mostram que tanto os transcritos de *CAT* quanto a atividade de CAT são presentes em todos os órgãos nos dois estágios analisados. Na fase de completo ingurgitamento, observou-se uma diminuição do nível de transcrição no intestino e no corpo gorduroso. Um padrão parecido foi observado no intestino de *Aedes aegypti*. Depois da alimentação o nível transcricional da *catalase* foi regulado positivamente, diminuindo após 48 horas (OLIVEIRA, *et al.*, 2017). Em *R. prolixus* foi observado que a atividade da catalase diminuiu significativamente no intestino

4 dias após a hematofagia (GANDARA *et al.*, 2016). Esses dados sugerem que possivelmente exista uma primeira demanda de catalase como um mecanismo adaptativo contra o potencial estresse oxidativo gerado por uma aquisição inicial de sangue mais abundante. Em contrapartida, não foi possível observar esse padrão através do ensaio enzimático, que mostrou um nível constante de atividade de catalase entre os intestinos de fêmeas parcialmente e totalmente alimentadas. Esse resultado pode estar relacionado com uma modificação sutilmente regulada em nível transcricional, que não foi rigorosamente refletida em nível proteico, ou que não foi possível constatar através de ensaios enzimáticos.

Muitos estudos demonstram as consequências biológicas do silenciamento de *catalases* em artrópodes hematófagos, especialmente relacionadas à capacidade reprodutiva de insetos como *Anopheles gambiae* (DEJONG *et al.*, 2007), *Aedes aegypti* (OLIVEIRA, *et al.*, 2017) e *Lutzomyia longipalpis* (DIAZ-ALBITER *et al.*, 2011). Em *R. microplus* já foi observado o papel da catalase na detoxificação do heme: a injeção de um inibidor específico de catalase em fêmeas de *R. microplus* totalmente alimentadas diminuiu o tempo de vida e a capacidade reprodutiva desses carrapatos (CITELLI, *et al.*, 2007). No presente trabalho, no entanto, os dados mostram que o silenciamento de *catalase* não foi capaz de alterar as atividades fisiológicas relacionadas à alimentação, postura e eclosão das larvas. Mesmo que o nível de silenciamento da enzima tenha sido de 99%, esses resultados sugerem, em conjunto, que pode haver ainda uma atividade remanescente de catalase advinda de proteínas expressas antes do silenciamento capaz de estabilizar os parâmetros biológicos analisados.

Os níveis transcricionais (RNAm) de *CuZnSOD* são quase 20 vezes mais abundantes em ovário de PEF e duas vezes mais abundantes no intestino do que em glândula salivar, o que poderia estar relacionado com o tráfego de heme do intestino para o ovário durante a vitelogênese (PERNER, *et al.*, 2016b). Os ensaios enzimáticos mostraram uma menor

atividade de SOD tanto no ovário quanto no corpo gorduroso na fase FEF. Um estudo transcriptômico mostrou que em *R. microplus* os ovários estão quase completamente maduros nessa fase, com a síntese de proteínas reduzida (XAVIER, *et al.*, 2018), possivelmente refletindo uma diminuição na atividade metabólica, uma menor atividade mitocondrial e menor produção de O<sub>2</sub> pela mitocôndria, o que poderia estar relacionado com a diminuição da atividade de SOD na fase FEF.

Apesar dos danos causados por ROS, essas moléculas também atuam na sinalização celular. Nesse contexto, foi observado que o H<sub>2</sub>O<sub>2</sub> produzido por uma NADPH oxidase (Duox) em *R. prolixus* é fundamental para a formação de estruturas de ligação entre proteínas, contribuindo para o endurecimento da casca do ovo desse hematófago (DIAS *et al.*, 2013). No presente trabalho, o ovário de PEF e FEF mostraram níveis transcricionais de *DuoxA* semelhantes. No intestino, uma quantidade mais abundante de transcritos foi detectada em FEF. Uma hipótese é de que essa enzima possa estar atuando na proteção de células epiteliais do intestino contra possíveis patógenos, ou na regulação da microbiota intestinal, como descrito em *A. gambiae* (KUMAR *et al.*, 2010) e na mosca *Bactrocera dorsalis* (YAO *et al.*, 2016). Nas glândulas salivares também foi observado um maior nível transcricional dessa enzima em FEF. A atividade dessa enzima na glândula salivar de teleógina poderia estar relacionada com o aumento de espécies reativas de oxigênio que sinalizam para vias de ativação de apoptose. A atuação de NADPH oxidases na formação de ROS para ativação de JNK já foi observada (REINEHR *et al.*, 2005), além disso, diversos sinais de degradação relacionados à apoptose nas glândulas salivares de *R. microplus* após a alimentação foram descritas (FREITAS *et al.*, 2007).

Potenciais redutores são essenciais para o metabolismo celular, uma vez que é um sistema controlado em grande parte por reações de oxi-redução. Nesse contexto, o NADPH

é a principal fonte doadora de elétrons na célula, estando envolvido em múltiplas funções, incluindo a regeneração de diversas moléculas redutoras dos sistemas antioxidantes (AGLEDAL *et al.*, 2010). No presente trabalho, a quantidade de NADPH em tecidos de PEF e FEF foi constante entre os dois estágios de alimentação, mostrando que o carrapato mantém uma homeostase redox mesmo após a ingestão de grandes quantidades de sangue, permitindo um constante equilíbrio nos níveis de NADPH. Em contrapartida, os níveis de glutathione total mostraram-se variáveis nos órgãos analisados. Em intestino, ovário e corpo gorduroso foi observado um maior nível de glutathione na fase PEF, assim como os dados anteriores de maior perfil transcricional e atividade da maioria das enzimas avaliadas. Possivelmente um maior nível de GSH seja mantido na fase PEF para suprir as necessidades redutoras das enzimas envolvidas no metabolismo da glutathione.

No segundo capítulo, os experimentos iniciais demonstraram o potencial da proteína rGST-HI (GST do carrapato *Haemaphysalis longicornis*) como um imunógeno vacinal contra *R. appendiculatus* e *R. sanguineus*, visto que foi observado seu grau de identidade com a sequência deduzida de aminoácidos de GSTs dessas duas espécies, seus sítios antigênicos e similares foram encontrados *in silico*, sua antigenicidade cruzada foi observada *in vitro* através de Western blot, e, finalmente, mostrou ser uma proteína imunogênica em coelhos. Trabalhos anteriores mostraram a eficiência da vacina baseada em rGST-HI contra *R. microplus* (PARIZI, *et al.*, 2012; PARIZI, *et al.*, 2011), neste trabalho, no entanto, destaca-se o fato de que o antígeno induziu proteção apenas contra o carrapato *R. appendiculatus* e não contra o *R. sanguineus*. Ainda não existem trabalhos que caracterizam funcionalmente GSTs de *R. appendiculatus* ou *R. sanguineus*, entretanto, sabe-se que as GSTs desempenham diferentes funções metabólicas em um mesmo organismo, atuando na detoxificação de xenobióticos (GHOSH, *et al.*, 2017; ZIAPOUR, *et al.*, 2017), endobióticos

(SAWICKI, *et al.*, 2003), e hidroperóxidos (SINGH, *et al.*, 2001; ZIEGLER e IBRAHIM, 2001), por isso é possível especular que anticorpos induzidos pela vacina podem ter sido capazes de afetar GSTs que atuam em alguma função vital em *R. appendiculatus* e não em *R. sanguineus*, ou ainda, que outras isoformas de GST não atingidas pelos anticorpos gerados pela imunização atuem nessa mesma função. Ao analisar proteomas e transcriptomas de outros carrapatos, é possível encontrar um grande número de isoformas de GSTs (OLEAGA, *et al.*, 2018; PERNER, *et al.*, 2016a), o que leva a hipótese de que as diferenças entre as GSTs em *R. sanguineus* e *R. appendiculatus*, possibilitem mecanismos compensatórios diferentes. Ou ainda, que as GSTs em *R. sanguineus* sejam mais variáveis do que em *R. appendiculatus*, aumentando o espectro de regiões das proteínas que podem não ter afinidade pelos anticorpos. Apesar de possuírem um ciclo biológico semelhante (ambos são carrapatos de três hospedeiros), uma diferença biológica importante é que o *R. appendiculatus* é um carrapato mais adaptado em parasitar bovídeos, enquanto que o *R. sanguineus* possui um maior espectro de hospedeiros (CUPP, 1991), por isso, o perfil de proteínas expresso na saliva pode ter sido selecionado para evadir dos mecanismos de defesa do hospedeiro, possibilitando o sucesso de sua alimentação (POPARA *et al.*, 2013; RODRIGUEZ-VALLE *et al.*, 2010; TIRLONI *et al.*, 2017). Essa característica também poderia ter influenciado na falta de eficiência da vacina observada contra *R. sanguineus*.

## 5. CONCLUSÕES

Uma visão geral dos dados demonstra que a hematofagia está relacionada com modificações nos níveis transcricionais e na atividade das enzimas antioxidantes e nos níveis de GSH em diferentes tecidos, no entanto, não influencia nos níveis de NADPH. Além disso, o silenciamento de genes de enzimas antioxidantes não foi capaz de alterar a fisiologia dos parâmetros analisados, sugerindo a possibilidade de existência de um mecanismo



compensatório, ou que a atividade remanescente pode ser suficiente para manter a capacidade de sobrevivência e reprodução. Os dados demonstram que as enzimas antioxidantes compreendem um sistema biológico complexo na fisiologia do *R. microplus*. Finalmente, observou-se que mesmo carrapatos evolutivamente próximos podem responder de formas diferentes frente à uma resposta imune.

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## Anexo A

### CURRICULUM VITAE

#### 1. DADOS PESSOAIS

**Nome** Gabriela Alves Sabadin

**Nascimento** 30/12/1990 - Porto Alegre/RS - Brasil

#### 2. FORMAÇÃO ACADÊMICA/TITULAÇÃO

**2015** Doutorado em Biologia Celular e Molecular.

Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil

Orientador: Itabajara da Silva Vaz Júnior

Bolsista do: Conselho Nacional de Desenvolvimento Científico e Tecnológico

**2013 - 2015** Mestrado em Biologia Celular e Molecular.

Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil

Título: PERFIL INIBITÓRIO E ANTIGENICIDADE DA CISTATINA JpIocys2a DE *Ixodes ovatus*, Ano de obtenção: 2015

Orientador: Itabajara da Silva Vaz Júnior

Bolsista do: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

**2009 - 2012** Graduação em Biotecnologia.

Universidade Federal de Pelotas, UFPEL, Pelotas, Brasil

#### 3. FORMAÇÃO COMPLEMENTAR

**2014 - 2014** Extensão universitária em Desvendando a biodiversidade: caracterização. (Carga horária: 15h).

Pontifícia Universidade Católica do Rio Grande do Sul, PUCRS, Porto Alegre, Brasil

**2014 - 2014** Curso de curta duração em Curso de Biologia de Artrópodes Vetores. (Carga horária: 90h).

Universidade Federal do Rio de Janeiro, UFRJ, Rio De Janeiro, Brasil

**2012 - 2012** Curso de curta duração em técnicas para coleta de secreções. (Carga horária: 30h).

Universidade Federal de Pelotas, UFPEL, Pelotas, Brasil

**2009 - 2009** Curso de curta duração em Biossegurança em Unidades Hemoterápicas e Laboratoriais. (Carga horária: 30h).

Universidade Federal de Pelotas, UFPEL, Pelotas, Brasil

#### **4. ATUAÇÃO PROFISSIONAL**

##### **Universidade Federal do Rio Grande do Sul - UFRGS**

###### **Vínculo institucional**

**2015 - Atual** Vínculo: Bolsista, Enquadramento funcional: Doutoranda, Carga horária: 40, Regime: Dedicção exclusiva

**2013 - 2015** Vínculo: Bolsista, Enquadramento funcional: Mestrado, Carga horária: 40, Regime: Dedicção exclusiva

##### **Universidade Federal de Pelotas - UFPEL**

###### **Vínculo institucional**

**2010 - 2012** Vínculo: Bolsista, Enquadramento funcional: Estagiária, Carga horária: 20, Regime: Parcial

##### **Revisor de periódico**

###### **Parasites & Vectors**

**2018 - Atual** Regime: Parcial

#### **5. PRODUÇÃO**

##### **Artigos completos publicados em periódicos**

1. PARIZI, L. F.; ALI, A.; TIRLONI, L.; OLDIGES, D. P.; SABADIN, G. A.; COUTINHO, M. L.; SEIXAS, A.; LOGULLO, C.; TERMIGNONI, C.; da SILVA VAZ JÚNIOR, I Peptidase inhibitors in tick physiology. MEDICAL AND VETERINARY ENTOMOLOGY, v.32, p.129 - 144, 2018.

2. SABADIN, GABRIELA ALVES; PARIZI, LUÍS FERNANDO; KIIO, IRENE; XAVIER, MARINA AMARAL; DA SILVA MATOS, RENATA; CAMARGO-

MATHIAS, MARIA IZABEL; GITHAKA, NAFTALY WANG'OMBE; NENE, VISH; DA SILVA VAZ, ITABAJARA Effect of recombinant glutathione S-transferase as vaccine antigen against *Rhipicephalus appendiculatus* and *Rhipicephalus sanguineus* infestation. VACCINE, v.35, p.6649 - 6656, 2017.

3. RANGEL, CAROLINA K.; PARIZI, LUÍS F.; **SABADIN, GABRIELA A.**; COSTA, EVENILTON P.; ROMEIRO, NELILMA C.; ISEZAKI, MASAYOSHI; GITHAKA, NAFTALY W.; SEIXAS, ADRIANA; LOGULLO, CARLOS; KONNAI, SATORU; OHASHI, KAZUHIKO; DA SILVA VAZ, ITABAJARA Molecular and structural characterization of novel cystatins from the taiga tick *Ixodes persulcatus*. Ticks and Tick-borne Diseases, v.8, p.432 - 441, 2017.

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## 6. CAPÍTULOS DE LIVROS PUBLICADOS

1. **SABADIN, G. A.**; PHILIPPSEN, F. R. RDC Anvisa nº. 234/05 Importação de produtos biológicos. In: Política e Regulação de Vacinas no Brasil: Guia Resumido da Legislação Vigente. 1 ed. Pelotas: Universitária, 2012, v.1, p. 81-.

## 7. TRABALHOS PUBLICADOS EM ANAIS DE EVENTOS (RESUMO)

1. FREITAS, M. B.; **SABADIN, GABRIELA**; DA SILVA VAZ, I. In silico Characterization and Cloning of a Histamine-Binding Protein (HBP) ORF from *Rhipicephalus microplus* Tick In: 47a. Reunião anual da Sociedade brasileira de bioquímica e biologia molecular, 2018, Joinville.

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2. **SABADIN, GABRIELA**; SALOMON, T.; BENFATO, M.; OLIVEIRA, P.; DA SILVA VAZ, I. TRANSCRIPTIONAL GENE LEVEL, ACTIVITY OF DETOXIFICATION ENZYMES AND AND CATALASE GENESILENCE IN *RHIPICEPHALUS MICROPLUS* TICK In: 47a. Reunião da Sociedade Brasileira de Bioquímica e Biologia Molecular, 2018, Joinville.

**47a. Reunião anual da Sbbq, 2018.**

3. FREITAS, M. B.; SABADIN, G. A.; Luis F. Parizi; VAZ JR, I. S. CROSS ANTIGENICITY AND IMMUNOGENICITY ANALYSIS OF *HAEMAPHYSALIS LONGICORNIS* AND *RHIPICEPHALUS SANGUINEUS* GLUTATHIONE S-TRANSFERASES In: 45a. Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular, 2016, Natal.

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6. Luis F. Parizi; Kiiro, I; RANGEL, C. K.; SABADIN, G. A.; GITHAKA, N. W.; NENE, V.; VAZ JR, I. S. Vaccine Potential of GST-HI and BrBmcys2c Antigens in a Heterologous Challenge Against the Tick *Rhipicephalus appendiculatus* In: 45a. Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular

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7. RANGEL, C. K.; SABADIN, G. A.; GITHAKA, N. W.; Luis F. Parizi; SEIXAS, A.; LOGULLO, C.; KONNAI, S.; OHASHI, K.; VAZ JR, I. S. Inhibition profile of a *Rhipicephalus appendiculatus* cystatin In: Abstracts of 44th Annual Meeting of the Brazilian Society for Biochemistry and Molecular Biology, 2015, Foz do Iguaçu.

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8. SABADIN, G. A.; Luis F. Parizi; GITHAKA, N. W.; SEIXAS, A.; LOGULLO, C.; KONNAI, SATORU; OHASHI, KAZUHIKO; VAZ JR, I. S. INHIBITORY PROFILE OF JpIocys2a, AN *Ixodes ovatus* CYSTATIN, AND CROSS-ANTIGENICITY WITH *Rhipicephalus microplus* CYSTATINS. In: 44th Annual Meeting of the Brazilian Society for Biochemistry and Molecular Biology, 2015, Foz do Iguaçu.

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9. Luis F. Parizi; **SABADIN, G. A.**; ALZUGARAY, M. F.; SEIXAS, A.; LOGULLO, C.; KONNAI, S.; OHASHI, K.; DA SILVA VAZ JR, ITABAJARA. The putative role of *Rhipicephalus microplus* and *Ixodes ovatus* cystatins in tick physiology. In: Abstracts of 25th International Conference of the World Association for the Advancement of Veterinary Parasitology, 2015, Liverpool.

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11. **SABADIN, G. A.**; Luis F. Parizi; SEIXAS, A.; OLIVEIRA, C. J. L.; GITHAKA, N. W.; OHASHI, KAZUHIKO; KONNAI, SATORU; VAZ JR, I. S. Sequence analysis, antigenicity and production of *Ixodes persulcatus*, *Ixodes ovatus*, *Rhipicephalus appendiculatus* cystatins In: XVIII CONGRESSO BRASILEIRO DE PARASITOLOGIA VETERINÁRIA, 2014, Gramado.

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12. ALZUGARAY, M. F.; **SABADIN, G. A.**; Luis F. Parizi; TIRLONI, L.; SEIXAS, A.; BERASAIN, P.; VAZ JR, I. S. Sequence analysis, transcriptional profile and vaccine potential of the *Rhipicephalus microplus* protein Bm05 In: XVIII Congresso Brasileiro de Parasitologia Veterinária, Gramado.

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13. HAUBERT, Louise; FRANÇA, Rodrigo; **SABADIN, G. A.**; CONCEIÇÃO, Fabrício; MOREIRA, A. N. Avaliação da inocuidade de *Pichia pastoris* para potencial utilização como probiótico. In: 26º CONGRESSO BRASILEIRO DE MICROBIOLOGIA, 2011, Foz do Iguaçu.

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14. FRANÇA, Rodrigo; **SABADIN, G. A.**; HAUBERT, Louise; SILVA, Wladimir Padilha da; BAMPI, Suely Ribeiro; MAGALHÃES, Carolina; SILVEIRA, Marcelle Moura; CASTELLI, Regina; CONCEIÇÃO, Fabrício. Inibição do crescimento in vitro de *Salmonella typhimurium* por *Saccharomyces boulardii* e *Bacillus cereus* var. Toyoi” In: 20º Congresso de Iniciação Científica, 10ª Mostra de Pós-Graduação e 3º Congresso de Extensão, 2011, Pelotas/RS/Brasil.

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15. HAUBERT, Louise; FRANÇA, Rodrigo; SABADIN, G. A.; CONCEIÇÃO, Fabrício; MOREIRA, A. N. Resistência da levedura *Pichia pastoris* ao armazenamento em suspensão aquosa sob refrigeração e na forma liofilizada. In: 26º CONGRESSO BRASILEIRO DE MICROBIOLOGIA, 2011, Foz do Iguaçu.

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#### **8. TRABALHOS PUBLICADOS EM ANAIS DE EVENTOS (RESUMO EXPANDIDO)**

1. SABADIN, G. A.; Luis F. Parizi; GITHAKA, N. W.; SEIXAS, A.; OLIVEIRA, C. J. L.; KONNAI, S.; OHASHI, K.; VAZ JR, I. S. Analysis of Cystatin Sequences and Prediction of Cross-Antigenic Regions for a Tick Vaccine In: XLII Reunião anual da Sociedade Brasileira de Bioquímica (SBBq), 2013, Foz do Iguaçu.

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2. FRANÇA, Rodrigo; HAUBERT, Louise; SABADIN, G. A.; CONCEIÇÃO, Fabrício; SILVA, PADILHA, Wladimir. Resistência da levedura *Pichia pastoris* ao armazenamento em solução sob refrigeração e ao trato gastrointestinal de camundongos In: Congresso de Iniciação Científica, 2010, Pelotas.

**XIX CIC Resumos, 2010.**

#### **9. ORIENTAÇÕES E SUPERVISÕES CONCLUÍDAS**

##### **Iniciação científica**

1. Carolina Rangel. **Caracterização de uma cistatina de *Rhipicephalus appendiculatus*.** 2015. Iniciação científica (Farmácia) - Universidade Federal do Rio Grande do Sul

2. Michele Freitas. **Clonagem da ORF, expressão e purificação de uma proteína de ligação à histamina (HBP) do carrapato *Rhipicephalus microplus*, 2016.** Iniciação científica (Farmácia) - Universidade Federal do Rio Grande do Sul