INSTITUTO OSWALDO CRUZ
Pós-Graduação em Biologia Celular e Molecular

Imunidade de *Anopheles aquasalis* contra *Plasmodium vivax*

Ana Cristina Bahia Nascimento

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ANA CRISTINA BAHIA NASCIMENTO

Imunidade de *Anopheles aquasalis* contra *Plasmodium vivax*

Tese apresentada ao Instituto Oswaldo Cruz como parte dos requisitos para obtenção do título de Doutor em Biologia Celular e Molecular

Orientador (es): Prof[a]. Dra. Yara Maria Traub Csekö
Prof. Dr. Paulo Filemon Paolucci Pimenta

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Ao Fernando, Leo, Ana e João

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LISTA DE ABREVIATURAS

AF: *After feeding* ou após alimentação
AI: *After infection* ou após infecção
AMG: *Anterior midgut* ou Intestino médio anterior
AMPs: *Antimicrobial peptides* ou Peptídeos antimicrobianos
AprA: Protease que degrada AMPs
BRP: *Bacteria responsive protein* ou Proteína responsiva a bactéria
CAT: Catalase
cDNA: Ácido desoxiribonucleíco complementar
CLIPs: Serino proteases com domínios clip
CTLs: *C-type lectins* ou lectinas do tipo C
DD: *Death domain* ou Domínio de morte
Dif: *Dorsal immune factor* ou Fator imune homólogo à dorsal
dsRNA: *Double strand RNA* ou RNA dupla fita
DUOX: Proteínas dual oxidases
FBN: Fibrinogênio
FREPs: *Fibrinogen-related proteins* ou Proteínas do tipo fibrinogênio
FTs: Fafores de transcrição
GC: Gametócitos
GNBPs: *Gram-negative bacterial-binding proteins* ou Proteínas que se ligam a bactérias Gram negativas
H_2O_2: Peróxido de hidrogênio
HO: heme oxigenase
IAP2: *Inhibitor of apoptosis 2* ou Inibidor de apoptose 2
IkB: Inibidor do NF-kB
IKK: Quinase do inibidor de NF-kB
IMD: *Immune deficiency* ou deficiência imunológica
JAK: Janus quinase
LPS: Lipopolisacarídeos
LRRs: *Leucine rich repeats* ou repetições ricas em Leucina
RNAm: Ácido ribonucleíco mensageiro
NF-kB: *Nuclear factor kappa B* ou Fator nuclear kappa 5
NO: *Nitric oxide* ou Óxido nítrico

NOS: *Nitric oxide sinthase* ou Óxido nítrico sintase

OC: Oocisto

$OH^{\bullet}$: radical hidroxila

OK: *Ookinete* ou Oocineto

PAMPS: *Pathogens associated molecular patterns* ou padrões moleculares associados à patógenos

PCR: Reação em cadeia da polimerase

PER: Peroxidase

PGN: peptideoglicano

PGRPs: *Peptidoglycan recognition proteins* ou proteína de reconhecimento de peptideogicanos

PGRPLC: *Peptidoglycan recognition protein long chain* ou proteína reconhecedora de peptidioglicana de cadeia longa

PIAS: *Protein Inhibithor of activated STAT* ou Proteína inibitória de STAT ativado

PMG: *Posterior midgut* ou Intestino médio posterior

PRRs: *Pattern recognition receptor* ou Receptors de reconhecimento de padrões

$R^{\bullet}$: Radical alquil

RACE: *Rapid Amplification of cDNA Ends* ou Amplificação rápida das porções finais dos cDNAs

RISC: *RNA-Inducing Silencing Complex* ou Complexo inductor de silenciamento por RNA

RNA: *Ribonucleic acid* ou Ácido ribonucleíco

RNAi: *RNA Interference* ou Interferência por RNA

$RO^{\bullet}$: Radical alcoxil

$ROO^{\bullet}$: Radical peroxil

ROOH: Hidroperóxido orgânico

ROS: *Reactive oxygen species* ou espécies reativas de oxigênio

RTPCR: *Real Time PCR* ou PCR em Tempo Real

S: *Sporozoites* ou Esporozoítos

siRNA: *Small interferring RNA* ou pequeno RNA de interferência

SF: *Sugar-fed* ou alimentados com açúcar

SG: *Salivary gland* ou glândula salivar

SOCS: *Suppressor of cytokine signaling* ou Supressor da sinalização por citocinas

SOD: Superóxido dismutase

SRP: Serpina

STAT: *Signal Transducers and Activators of Transcription* ou Transdutor de sinal e ativador de transcrição

TAB: *TAK1 binding protein* ou Proteína ligadora de TAK1

TAK1: *TGF-β-activated kinase 1* ou Kinase 1 ativada por TGF-β

TEP: *Thioester-containing protein* ou Proteína rica em thio-ester

TIR: *Toll/interleukin-1 receptor* ou receptor Toll/Interleucina 1

TLRs: *Toll like receptors* ou receptores do tipo Toll

TOR: *Target of Rapamycin* ou alvo de rapamicina

UpD - Ligante *Unpaired*

2 ou 24hF-I: Biblioteca de *A. aquasalis* 2 ou 24 horas após alimentação sanguínea menos 2 ou 24 horas após infecção com *P. vivax*

2 ou 24hI-F: Biblioteca de *A. aquasalis* 2 ou 24 horas infecção com *P. vivax* menos 2 ou 24 horas após alimentação sanguínea

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Imunidade de *Anopheles aquasalis* contra *Plasmodium vivax*

RESUMO

Anualmente, a malária afeta cerca de 300 milhões de pessoas no mundo (causando cerca de um milhão de mortes). No Brasil 450.000 casos são notificados anualmente. Recentemente, muitos trabalhos têm procurado identificar moléculas mediadoras da interação mosquito/plasmódio. *Anopheles aquasalis* é o vetor de malária mais importante nas regiões litorâneas do Brasil e *Plasmodium vivax* o agente etiológico causador da maior parte dos casos da doença. Este estudo visa examinar moléculas que participam da interação entre estes dois organismos. Para tal, duas estratégias foram utilizadas: subtração de cDNAs e PCR com iniciadores degenerados. As bibliotecas subtrativas foram produzidas a partir de amostras de *A. aquasalis* 2 e 24 horas após alimentação sanguínea ou infecção por *P. vivax.* Após a análise dos cDNAs obtidos foram observadas diferenças na expressão de genes entre *A. aquasalis* alimentados com sangue e com sangue infectado em ambos os intervalos de tempo investigados. Os resultados das subtrações de cDNA para os genes serino protease, fibrinogênio, proteína responsiva a bactéria e carboxipeptidase foram corroborados utilizando PCR em tempo real. Por exemplo, uma cecropina teve a sua expressão diminuída após a infecção com *P. vivax* e uma serpina apresentou um resultado oposto. Análise de um fator de transcrição GATA revelou um aumento de RNAm 36 horas após infecção com *P. vivax* assim como um aumento da infecção após silenciamento deste gene, demonstrando a importância deste fator de transcrição para a resposta imune do inseto contra *P. vivax.* Em paralelo, cDNAs de *A. aquasalis* específicos para genes relacionados com a via JAK-STAT [fator de transcrição STAT, proteína inibitória de STAT ativado (PIAS) e óxido nítrico sintase (NOS)] e com o sistema de detoxificação celular (catalase e duas superóxido dismutases) foram amplificados utilizando iniciadores degenerados. Resultados de expressão destes genes revelaram que STAT, PIAS e NOS são induzidos pela infecção com *P. vivax* e experimentos de genética reversa comprovaram que a via de sinalização JAK/STAT é importante na resposta imune do *A. aquasalis* contra este parasito. Com relação às enzimas de detoxificação, foi observado um aumento da expressão 36 horas após infecção e uma diminuição da atividade das enzimas SOD e catalase 24 horas após infecção. Este aumento de RNAm 36 horas após infecção pode estar relacionado à tentativa das células de diminuírem a quantidade intracelular de espécies reativas de oxigênio mantida pela diminuição da atividade destas enzimas 24 horas pós infecção. Surpreendentemente, o silenciamento da catalase exacerbou a infecção do *P. vivax.* Este resultado pode ser correlacionado com a produção de uma rede de ditirosina que protegeria os parasitos da resposta imune do inseto. Nossos resultados apontam mecanismos imunes adotados pelo *A. aquasalis* para combater o *P. vivax,* fornecendo informações importantes sobre moléculas envolvidas no processo de interação e imunidade deste vetor de malária no Brasil com seu parasito.

INSTITUTO OSWALDO CRUZ

Anopheles aquasalis immunity against *Plasmodium vivax*

ABSTRACT

Each year, malaria affects 300 million people worldwide, causing 1 million deaths. In Brazil, 450,000 cases are reported annually. Recently, many studies have attempted to identify molecules that mediate the interaction mosquito-parasite. *Anopheles aquasalis* is the most important vector of malaria in the coastal regions of Brazil and the etiological agent *Plasmodium vivax* causes most cases of the disease. This study aims examining molecules that participate in the interaction between these two organisms. For this, two strategies were used: cDNA subtraction and PCR using degenerate primers. The subtractive libraries were produced from samples of *A. aquasalis* 2 and 24 hours after blood feeding or infection by *P. vivax*. After analyzes of cDNAs, differences were observed in gene expression between *A. aquasalis* fed with blood or infected blood at both times investigated. The expression of some candidates obtained through subtraction cDNA (serine protease, fibrinogen, carboxypeptidase and bacteria responsive protein genes) were confirmed using real time PCR. For example, the expression of a cecropin decreased after *P. vivax* infection while a serpin presented increased expression. Analysis of a transcription factor, GATA, revealed an increase in the mRNA levels 36 hours after infection. Silencing of this gene produced increased infection, demonstrating the importance of this transcription factor for the insect's immune response against *P. vivax*. In parallel, *A. aquasalis* cDNA sequences for the JAK-STAT pathway [transcription factor STAT, protein inhibitor of activated STAT (PIAS) and nitric oxide synthase (NOS)] and for genes related to cellular detoxification (catalase and two superoxide dismutases) were obtained using degenerate primers. Results of expression of these genes revealed that STAT, PIAS and NOS are induced by infection with *P. vivax* and reverse genetics experiments have shown that this pathway is important in the immune response of *A. aquasalis* against *P. vivax*. Regarding the detoxification enzymes, an increase in mRNA expression was observed 36 hours after infection and a decrease in activity 24 hours after infection. This increase in mRNA 36 hours after infection may be related to the attempt of cells to decrease the amount of intracellular ROS due to the diminished activity of these enzymes 24 hours after infection. Surprisingly, the silencing of catalase exacerbated the infection of *P. vivax*. This result may be correlated with the production of a dytirosine network that protects the parasites from the insect's immune response. Our results indicate immune mechanisms adopted by *A. aquasalis* to combat *P. vivax*, providing important information about molecules involved in the process of interaction and immunity of this vector with its Brazilian malaria parasite.

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1. Introdução

1. Introdução

1.1 Mosquitos como vetores de doenças

Em 1878, Patrick Manson, médico escocês, descobriu que os insetos poderiam veicular parasitos ao observar o desenvolvimento de filárias da espécie *Wuchereria bancrofti* no interior dos mosquitos *Culex pipiens quinquefasciatus*. Posteriormente, em 1881, Finlay identificou o mosquito *Aedes egypti* como vetor responsável pela transmissão da febre amarela. Vinte anos depois, a idéia de que os insetos transmitiam microorganismos foi confirmada através de experimentos realizados pelo médico Walter Ready em prisioneiros de guerra. A transmissão da malária por mosquitos foi comprovada em 1898, por Ronaldo Ross, após estudos de malária em aves. Atualmente, já é bem estabelecido que os insetos são transmissores de uma gama de patógenos como protozoários, vírus, bactérias e helmintos. Mosquitos do gênero *Anopheles* são insetos de grande importância epidemiológica por serem vetores de doenças ao homem, como a filariose e a malária (Lozovei 2001).

1.2 Os anofelinos

Os anofelinos são mosquitos pertencentes à ordem Diptera e à família Culicidae. Cerca de 400 espécies fazem parte do gênero *Anopheles*, porém apenas poucas espécies são importantes como vetores dos parasitos da malária (Figura 1). Cerca de 40 espécies de anofelinos são consideradas vetores importantes e outras como vetores secundários. A principal espécie transmissora de malária é *A. gambiae.* Cinco espécies são consideradas como vetores importantes no Brasil, *A. darlingi*, *A. aquasalis, A. albitarsis s.l.*, *A. cruzi s.l.* e *A. bellator* (Ministério da Saúde 2007); a distribuição das duas primeiras está mostrada nos mapas das figuras 1 e 2). As características diagnósticas deste grupo de mosquitos são: palpos de comprimento semelhante ao da probóscide, margem posterior do escutelo arredondada (exceto no gênero Chagasia) e primeiro tergito abdominal sem escamas. Em repouso, estes insetos apresentam uma posição oblíqua em relação ao substrato. Devido a este hábito, são, no Brasil, popularmente chamados de

“mosquito-prego”. São também conhecidos como “carapanã”, “muriçoca”, “sovela”, etc. Estes insetos em geral possuem hábitos crepusculares ou noturnos.

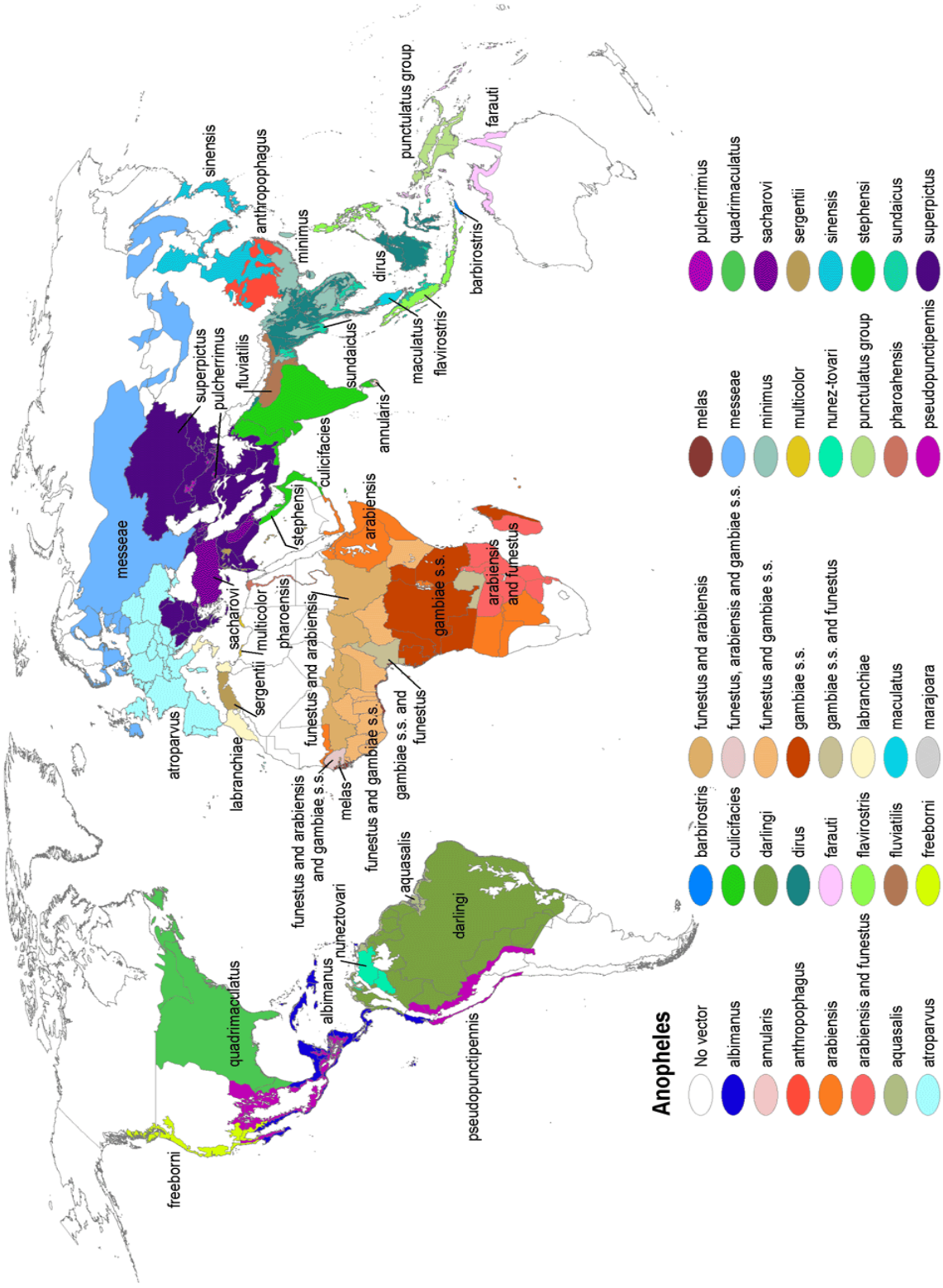

Figura 1: Distribuição de anofelinos pelo mundo (De: en.academic.ru/dic.nsf/enwiki/296541 – agosto/2010).

Figura 2: Distribuição de anofelinos vetores nas Américas (modificado de http://www.itg.be/itg/DistanceLearning/LectureNotesVandenEndenE/02_Malariap8.htm#T4 – agosto/2010).

Como todos os mosquitos, os anofelinos são holometábolos, isto é, possuem quatro estádios de desenvolvimento: ovo, larva (1° - 4°), pupa e adulto (Figura 3). Somente as fêmeas adultas são hematófagas, necessitando de sangue como fonte de nutrientes para a produção dos seus ovos (Pennington e Wells 2004). Em climas tropicais, cada fêmea adulta põe de 50 a 200 ovos no segundo e terceiro dias após o repasto sanguíneo. Os três primeiros estágios são aquáticos e duram cerca de cinco a 14 dias, dependendo da espécie e das condições ambientais (principalmente temperatura e umidade). A última fase do ciclo de vida, que dura cerca de um mês, é a do adulto alado. Os sítios de oviposição preferenciais para a maioria de espécies de anofelinos são águas claras, calmas e não poluídas. Larvas de anofelinos já foram encontradas em ambientes de águas doce, salobra e salgada, como pântanos, mangues, campos de arroz, valas relvadas, margens de córregos e rios, e em pequenas piscinas de chuva temporária (Rezende e cols. 2010).

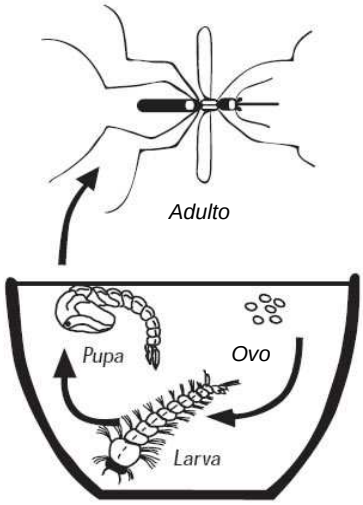

Figura 3: Ciclo de vida dos anofelinos. Desenho mostra os quatro estádios de desenvolvimento: ovo, larva (1º - 4º estágios larvares), pupa e inseto adulto (modificado de ag.arizona.edu/pubs/insects/az1221/ - agosto/2010).

A. aquasalis é um dos vetores mais importantes de malária, além de vetor secundário da filariose bancroftiana no Brasil. Sua distribuição geográfica é limitada pela salinidade, favorável para o desenvolvimento de suas larvas. Portanto, sua distribuição vai de São Paulo, Brasil, até a Costa Rica, no litoral Atlântico; e da Costa Rica até o Equador, no Pacífico, além de nas Antilhas Menores e em Trinidad e Tobago (Consoli e Lourenço-de-Oliveira 1994; Figura 2). A espécie pode também ser encontrada mais para o interior em locais onde haja solos ricos em cloreto. Embora preferencialmente zoofílico, eventualmente pica o homem durante o crepúsculo.

1.3 A malária

A malária é uma das doenças parasitárias mais importantes do mundo, em termos de saúde pública, devido ao seu grande impacto e custo associado. Segundo a Organização Mundial de Saúde (OMS; 2009a), cerca de 3,3 bilhões de pessoas estão sob o risco de contraí-la, em aproximadamente 109 países. Acomete cerca de 250 milhões de pessoas por ano em áreas tropicais e subtropicais, causando a morte de um milhão, principalmente crianças, OMS 2009a). A maioria dos casos (e

mortes) por malária ocorre na África subsaariana. Porém, esta doença também afeta populações humanas na Ásia, América latina, Oriente Médio e partes da Europa. Apesar do sucesso da erradicação da doença em diversos países, 40% da população mundial ainda habita áreas de risco (Figura 4).

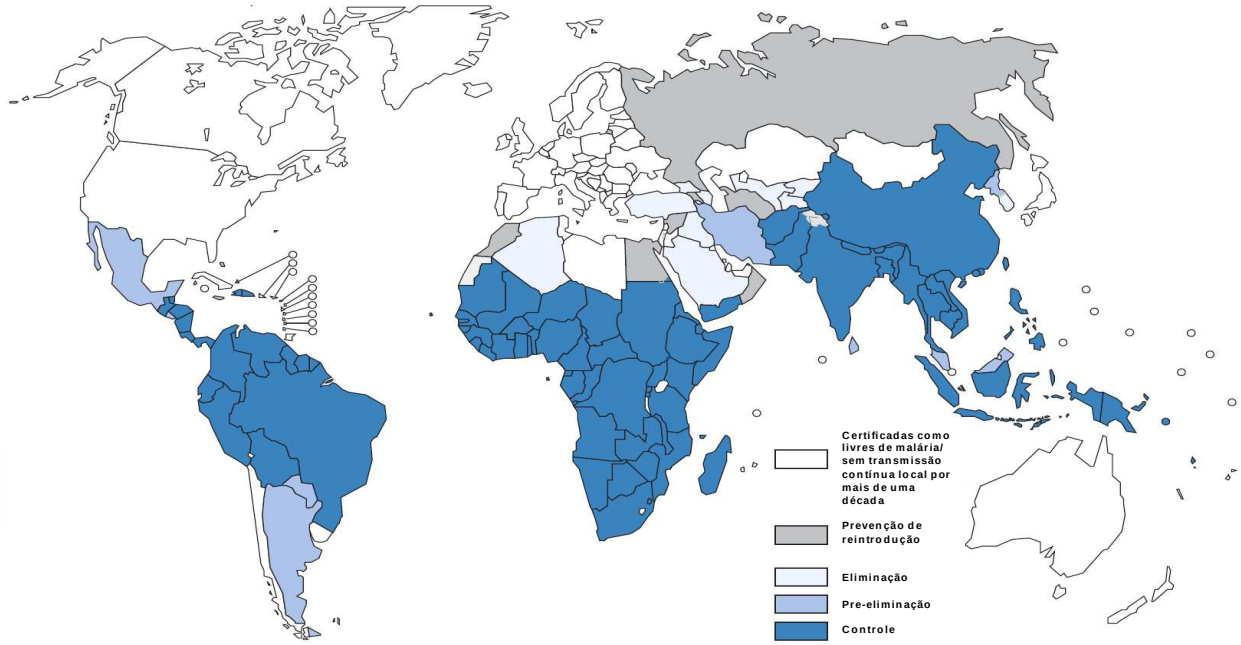

Figura 4: Mapa mundi mostrando regiões sob controle; com status de pré-eliminação, de eliminação, prevenção de reintrodução, e livres de malária (modificado de OMS 2008).

A malária é causada por parasitos do filo Apicomplexa, Classe Aconoidasida, Ordem Haemosporida, gênero *Plasmodium*. Seu ciclo de vida é heteroxênico, pois depende de dois hospedeiros, o vertebrado, onde ocorre o ciclo esquizogônico e o invertebrado onde ocorre a reprodução sexuada (Figura 5). Os invertebrados que hospedam e disseminam estes parasitos entre os hospedeiros vertebrados são mosquitos da subfamília Anophelinae, gênero *Anopheles*, sendo *A. gambiae* o principal vetor. A única exceção é a malaria aviária que é transmitida por mosquitos da subfamília Culicinae. Existem cinco espécies de plasmódio que infectam o homem: *P. falciparum*, *P. vivax*, *P. malariae, P. ovale* e *P. knowlesi*. O *P. falciparum*

é o parasito responsável pelos casos mais severos da doença em humanos, devido à sua capacidade de aderir ao epitélio dos capilares sanguíneos, podendo causar falência renal aguda, malária cerebral e edema pulmonar. Se não tratada a tempo, a malária causada por *P. falciparum* pode ser fatal. O *P. ovale* e *P. malariae* causam quadros menos severos e raramente mortais. Infecções pelo *P. vivax*, antes consideradas benignas, são vistas hoje como possíveis causadoras de quadros graves e eventualmente letais (Anstey e cols. 2009, Oliveira-Ferreira e cols. 2010). *P. knowlesi,* parasito que normalmente acomete macacos, ocasionalmente infecta e causa malária em seres humanos, com manifestações clínicas variando entre moderadas a severas (Singh e cols. 2004).

Epidemias grandes e devastadoras podem ocorrer quando o parasito da malária é introduzido em áreas onde as pessoas nunca tiveram contato prévio com o plasmódio ou possuem pouca ou nenhuma imunidade contra ele (OMS 2009a). A eficiência na transmissão da malária dependerá de diversos fatores como: pluviosidade, proximidade dos criadouros às propriedades humanas, e espécies de mosquitos presentes.

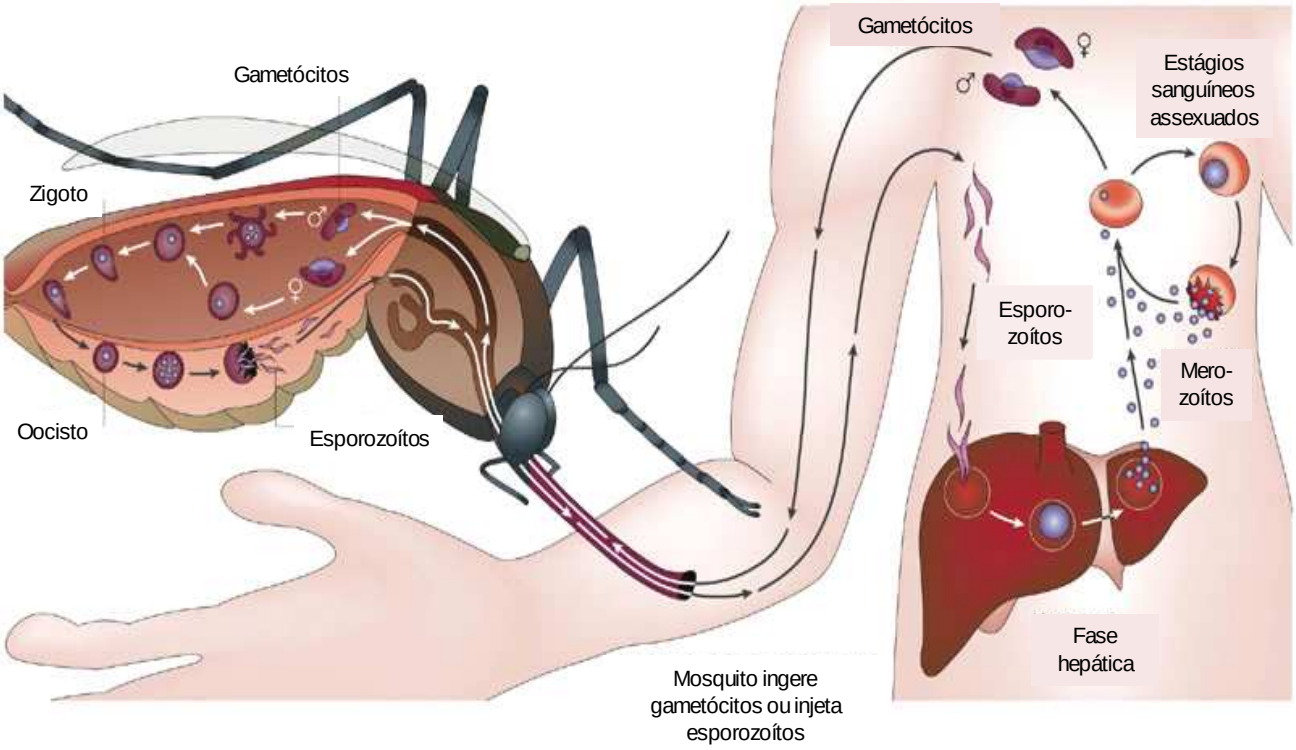

Figura 5: Ciclo de vida do *Plasmodium* nos hospedeiros invertebrados e vertebrados (Modificado de Su e cols. 2007).

O Brasil concentra mais da metade dos casos de malária nas Américas. O número de casos relatados elevou-se de 388.303 em 2001 para 606.067 em 2005, voltando a diminuir para 315.642 em 2008 (OMS 2009a). A Amazônia é a região brasileira de maior endemicidade para a malária. Em 2008, aproximadamente 97% dos casos se concentraram em seis estados da região amazônica: Acre, Amapá, Amazonas, Pará, Rondônia e Roraima. Os outros três estados amazônicos, Maranhão, Mato Grosso e Tocantins foram responsáveis por apenas 3% dos casos (Oliveira-Ferreira e cols. 2010; Figura 6). A maioria dos casos ocorre em áreas rurais, mas há registros que mostram que cerca de 15% ocorre em áreas urbanas. No Brasil circulam atualmente três espécies de plasmódios, *P. vivax*, *P. falciparum* e *P. malariae*, sendo que o *P. vivax* é o predominante sendo responsável por mais de 80% dos casos (Oliveira-Ferreira e cols. 2010).

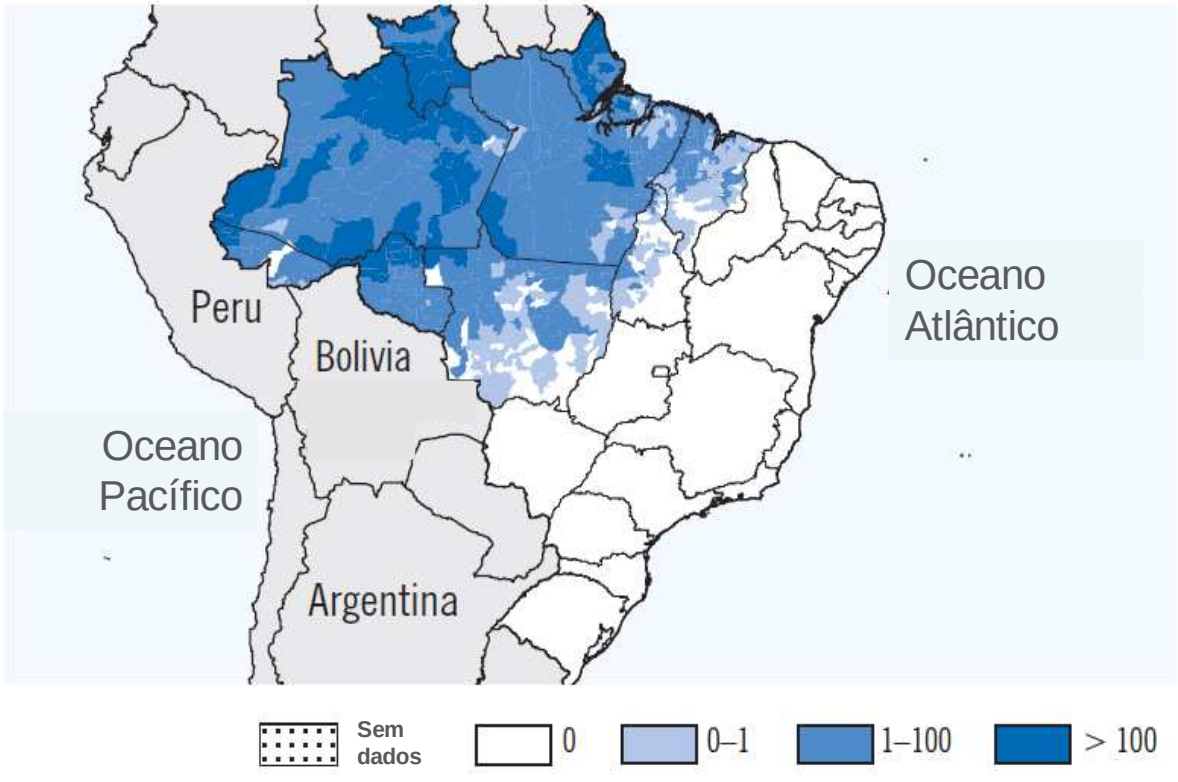

Figura 6: Estratificação dos casos de malária no Brasil (casos reportados por 1000; modificado de OMS 2009b).

1.4 O aparelho digestivo dos mosquitos e a alimentação com sangue

O intestino dos mosquitos, além seu papel na digestão e absorção dos nutrientes, serve de local de entrada de patógenos. É composto por uma monocamada de células epiteliais e pode ser dividido em intestino anterior, médio e posterior (Figura 7). O intestino anterior está envolvido principalmente com a ingestão, condução e armazenamento do alimento. O intestino médio é o local onde o sangue fica estocado e onde ocorre todo o processo digestivo. O intestino posterior é responsável por uma parte da absorção dos nutrientes e pela excreção das dejeções da alimentação (Pennington e Wells 2004).

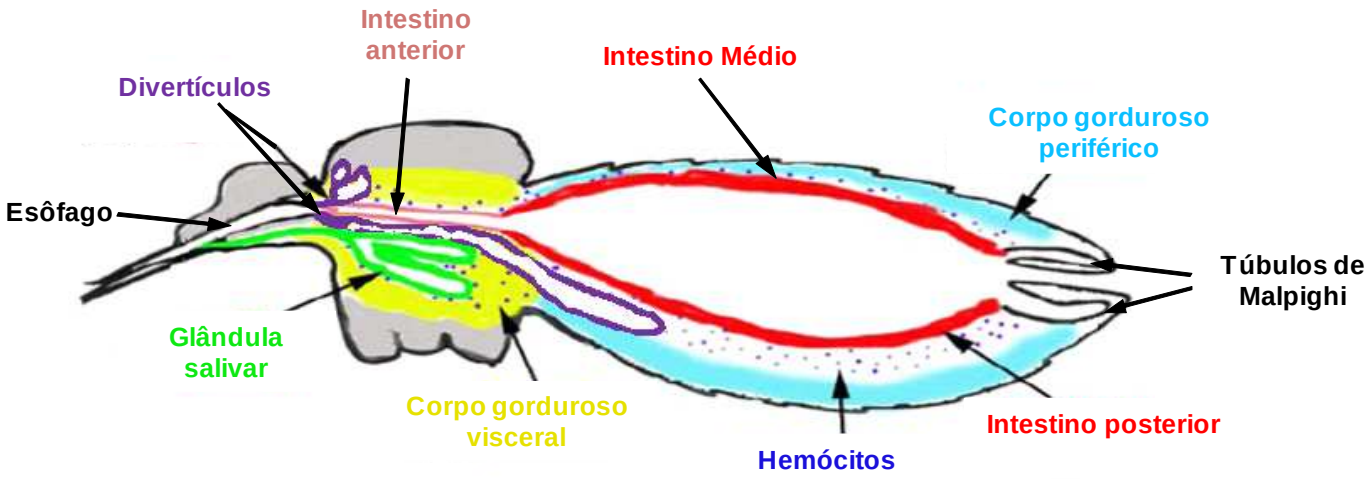

Figura 7: Anatomia interna dos mosquitos. A figura destaca os principais órgãos e tecidos destes organismos, como esôfago; divertículos; glândula salivar; intestinos anterior, médio e posterior; tubúlos de malpighi; corpo gorduroso; e hemócitos (Modificado de http://www.dimopoulosgroup.org/ presentations.html – agosto/2010).

O intestino médio é o local de maior vulnerabilidade dos vetores, pois é onde o sangue fica estocado, além de ser a única parte desprovida de quitina devido à sua origem endodérmica. Uma forma que os insetos encontraram para minimizar os prováveis problemas desta falta de quitina foi produzir, após a alimentação sanguínea, uma matriz acelular constituída de quitina e proteínas. Esta matriz, denominada de matriz peritrófica, envolve o bolo alimentar e o separa do epitélio do tubo digestivo (Devenport e Jacobs-Lorena 2004).

Na natureza, os mosquitos, assim como outros dípteros, se alimentam de néctar de plantas para manter o seu metabolismo basal e as atividades de vôo (Clements 1992). Contudo, as fêmeas de mosquitos necessitam de uma dieta mais rica para a produção de ovos, que é obtida através da hematofagia. O sangue é um alimento nutricionalmente rico, pois possui cerca de 20% de proteínas além de carboidratos e lipídeos (Pennington e Wells 2004). Órgãos distintos são responsáveis pelo armazenamento das diferentes substâncias ingeridas pelos mosquitos. O alimento açucarado é armazenado no divertículo, enquanto o alimento sanguíneo é armazenado no intestino.

Os anofelinos ingerem, em média, três vezes o seu peso em sangue em cada repasto sanguíneo. A ingestão desta quantidade grande de sangue foi a estratégia encontrada por estes insetos para solucionar seu problema energético, porém cria inúmeros desafios para a manutenção da homeostase. Inicialmente, os insetos eliminam uma grande quantidade de líquido para concentrar a parte mais nutritiva, que são as hemácias. A concentração das hemácias gera um acúmulo de toxinas e resíduos nitrogenados na forma de ácido úrico e ânions orgânicos resultantes do catabolismo das proteínas (O´Donnel e cols. 2009). Além disso, a digestão da hemoglobina, proteína mais abundante do sangue, resulta na liberação de grandes quantidades de heme no intestino do inseto (Graça-Souza e cols. 2006). A molécula de heme livre é extremamente tóxica, pois pode: a) se intercalar às membranas biológicas modificando suas funções estruturais (Schmitt e cols. 1993); b) gerar reações de oxi-redução, funcionando como um agente pró-oxidante capaz de potencializar a geração de radicais livres nas células aeróbicas (Kumar e Bandyopadhyay, 2005; Figura 8); c) induzir o desenovelamento e fragmentação do DNA e a oxidação e degradação de proteínas (Aft e Mueller 1983); e d) causar peroxidação lipídica (Tappel 1955). Os insetos desenvolveram diversas estratégias para diminuir os danos causados pela presença do heme livre. Os mosquitos, por exemplo, agregam o heme junto à matriz peritrófica (Devenport e cols. 2006) e diminuem a quantidade de radicais livres no lúmem do intestino (Oliveira 2007).

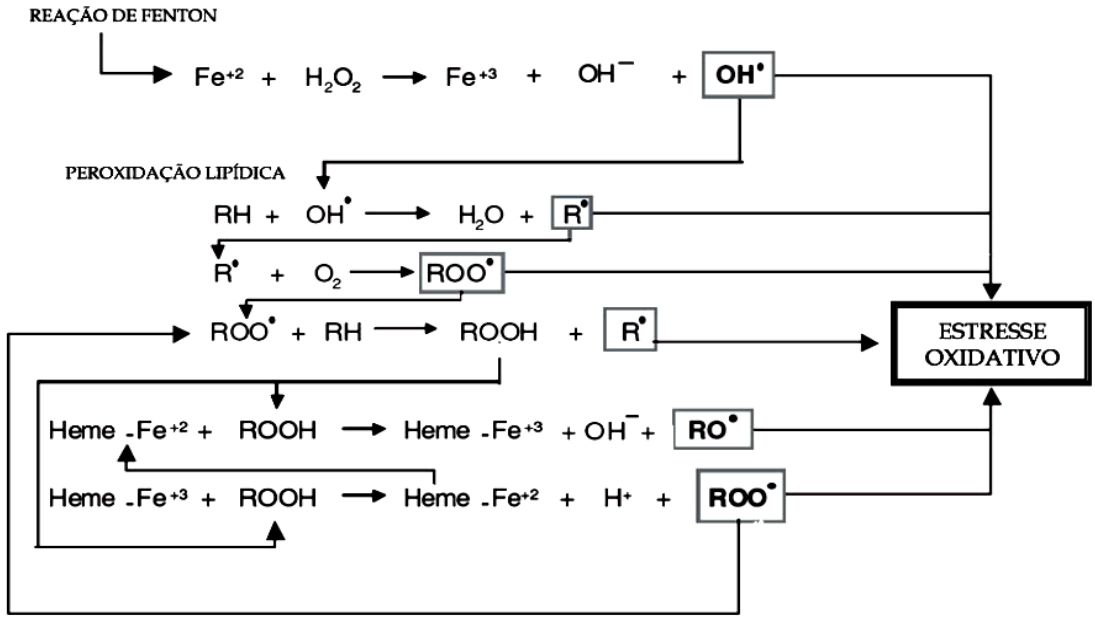

Figura 8: Molécula heme e estresse oxidativo. Ferro e heme promovem a peroxidação lipídica por mecanismos diferentes. O ferro pode gerar radicais hidroxil ($OH^{\bullet}$), através da reação de Fenton. Estes radicais são capazes de desencadear várias reações de peroxidação lipídica removendo elétrons de outras moléculas como ácidos graxos insaturados (RH) gerando radicais alquil ($R^{\bullet}$). Em contrapartida, o heme induz a conversão de hidroperóxidos orgânicos de baixa reatividade (ROOH) em radicais alcoxil ($RO^{\bullet}$) e peroxil ($ROO^{\bullet}$) extremamente reativos (Modificado de Graça-Souza e cols. 2006).

A digestão do sangue é realizada em quatro etapas: (1) lise dos eritrócitos e (2) digestão do alimento ingerido, (3) absorção dos nutrientes e (4) excreção. Em mosquitos a lise das células vermelhas do sangue acontece mecanicamente com o auxílio da armadura cibarial (Pennington e Wells 2004). A digestão das proteínas do sangue é realizada por enzimas digestivas secretadas pelo epitélio intestinal. A produção dessas enzimas digestivas é regulada transcricionalmente. Recentemente, foi demonstrado que uma via de sinalização chamada TOR (*Target of rapamycin*) é importante na "percepção" dos nutrientes pelo inseto e no desencadeamento do processo de digestão e embriogênese (Hansen e cols. 2004 e 2005, Park e cols. 2006, Brandon e cols. 2008).

A ingestão do sangue pela fêmea do mosquito induz a produção de enzimas

digestivas como glicosidases, tripsinas, quimiotripsinas, carboxipeptidades e aminopeptidades, sendo as tripsinas as principais enzimas responsáveis pela digestão sanguínea em anofelinos (Billingsly e Hecker 1991). Após o repasto sanguíneo, o tubo digestivo dos insetos transforma-se em um ambiente hostil e difícil para o estabelecimento e desenvolvimento de patógenos. Visto que os insetos são são vetores de microorganismos e que estes conseguem se desenvolver no inóspito ambiente intestinal, pode-se supor que tenham desenvolvido, ao longo da sua co-evolução com os seus vetores, adaptações que os ajudassem a evitar a ação das enzimas digestivas, como por exemplo: regulação da atividade destas enzimas (e.g. Jahan e cols. 1999, Somboon e cols. 2002); produção de moléculas como a quitinase, que facilitam o escape para o espaço entre a matriz peritrófica e o epitélio intestinal (e.g. Huber e cols. 1991, Schlein e cols. 1991, Shahabuddin e cols. 1995, Pimenta e cols. 1997); e transformação em formas menos susceptíveis à lise (Sacks e cols. 2001, Secundino e cols. 2010).

1.5 Os anofelinos e suas relações com os parasitos da malária

Dois hospedeiros, um vertebrado e outro invertebrado, são necessários para o desenvolvimento dos parasitos da malária. O ciclo no hospedeiro vertebrado se inicia quando esporozoítos são inoculados pelo mosquito (Figura 5). Estes esporozoítos são transportados para o fígado, onde invadem os hepatócitos e se multiplicam. Fases haplóides do parasito, chamados de merozoítos, caem na corrente sanguínea e sofrem nova fase de multiplicação dentro dos eritrócitos, onde podem passar por rodadas repetitivas de invasão, crescimento, divisão e rompimento da célula hospedeira. No decorrer deste parasitismo na corrente sanguínea, que pode durar meses se a doença não for tratada, alguns plasmódios em deixam as rodadas de multiplicação assexuada, se desenvolvem e amadurecem, ao longo de um período de cerca de duas semanas, em gametócitos masculino e feminino. O ciclo de vida do parasito da malária no inseto vetor é longo e possui diversas etapas (Figura 5 e 11). Após o repasto sanguíneo do inseto em um hospedeiro infectado, formas do plasmódio são ingeridas e chegam ao intestino médio do vetor. Dentre todas as formas sanguíneas de plasmódio encontradas no

hospedeiro vertebrado infectado, os gametócitos são as únicas aptas a infectar o mosquito. Os gametócitos são capazes de reconhecer mudanças no ambiente (pH, temperatura e a presença de algumas moléculas como o ácido xanturênico) logo após entrada no intestino do inseto, e se diferenciam em macro e microgametócitos (Billker e cols. 1997, 1998). O oocineto, ou ovo móvel, forma-se da fecundação do macrogameta pelo microgameta. Aproximadamente um dia após a ingestão do alimento infectado, o oocineto atravessa a matriz peritrófica e o epitélio intestinal e se fixa na lâmina basal do intestino médio. Neste momento, estes parasitos se transformam em oocistos e sofrem inúmeras divisões mitóticas para formar milhares de novas formas chamadas de esporozoítos. Nove a quinze dias após a alimentação, os oocistos se rompem e, como consequência, esporozoítos são liberados na hemocele. Ao encontrar a glândula salivar, a invadem e migram para seu lúmen. Uma vez no lúmen, os esporozoítos passam por um curto estágio de maturação até serem inoculados em um hospedeiro vertebrado durante um novo repasto sanguíneo do vetor. Alguns pontos do ciclo do plasmódio no mosquito são críticos e as interações que ocorrem entre os parasitos e os epitélios do inseto ainda são pouco esclarecidos. Segundo Huber e cols. (1991), para atravessar a matriz peritrófica, o parasito tem que produzir e secretar uma quitinase. Acredita-se, porém, que uma quitinase produzida pelo metabolismo do próprio inseto também auxilie nesta etapa (Shen e Jacobs-Lorena 1997). De acordo com Barillas-Mury e cols. (2000) e Ghosh e cols. (2001), o parasito se adere ao epitélio do intestino médio e da glândula salivar e só então penetra as células epiteliais. Entretanto, as moléculas responsáveis por esta interação ainda não foram descritas. Han e cols. (2000) e Kumar e cols. (2005) observaram que a passagem do *P. berghei* através das células epiteliais do intestino médio do *A. stephensi* causava uma série de danos, levando a apoptose destas células e posterior reconstrução do epitélio. Foi também demonstrado que as células do epitélio do intestino médio e da glândula salivar são ativadas imunologicamente após a invasão pelos parasitos da malária (e.g. Ribeiro e Francischetti 2003, Vlachou e cols. 2005, Kumar e cols. 2004).

Após ser ingerido pelo mosquito durante a alimentação sanguínea, o plasmódio necessita cruzar diversas barreiras de forma a realizar uma infecção bem sucedida. Durante todo o ciclo de desenvolvimento do plasmódio dentro do inseto são observadas perdas importantes no número de parasitos (Christophides 2004;

Figura 9). A primeira barreira encontrada pelo plasmódio é a barreira de infecção do aparelho digestivo. Uma perda importante no número de parasitos ocorre neste órgão devido à ação de enzimas digestivas e fatores imunes. Após passar pela primeira, o plasmódio encontra a segunda barreira, a barreira de escape do tubo digestivo. Nesta etapa (passagem através das células epiteliais do intestino médio do mosquito e transformação de oocineto em oocisto), ocorre uma redução drástica no número de parasitos, devido à ação do sistema imune inato com produção de proteínas imunes e óxido nítrico (NO) (Han e cols. 2000, Kumar e cols. 2004, Alavi e cols. 2003, Blandin e Levashina 2004). A última barreira é a chamada barreira de transmissão, na qual o plasmódio necessita infectar a glândula salivar e escapar para o seu lúmen. Perdas menores são observadas nas glândulas salivares, devido à magnitude de amplificação de cerca de dois a oito mil vezes na transformação de oocisto para esporozoíto.

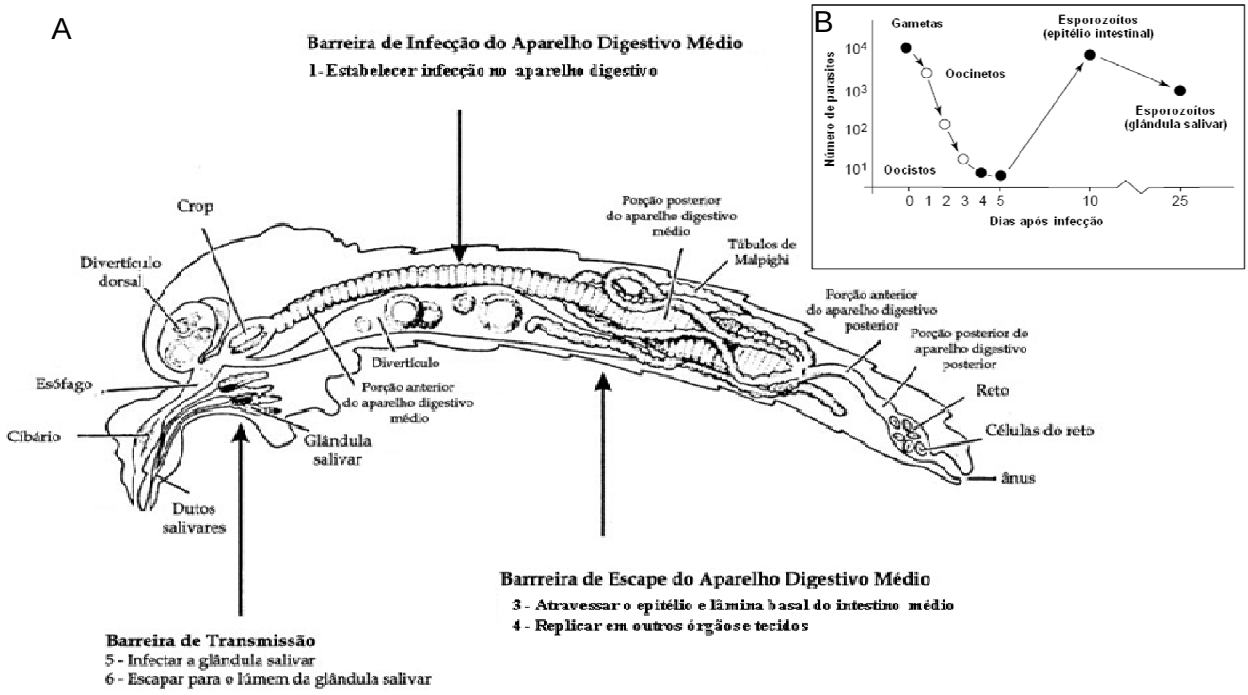

Figura 9: Desenho esquemático das barreiras de transmissão de patógenos e regulação do número de *Plasmodium* em mosquitos. A: Figura ressalta as principais barreiras de transmissão de patógenos em mosquitos e a sua morfologia interna. B: Gráfico mostrando as perdas de plasmódio e a sua amplificação dias após infecção do mosquito (modificado de Black e cols. 2002, Blandin e Levashina 2004).

1.6 O sistema imune dos insetos

Organismos multicelulares, vertebrados ou invertebrados, exploram diversos ambientes aquáticos e terrestres, onde se expõem a uma grande diversidade de vírus e parasitos (fungos, bactéria, protozoários e helmintos). Para tanto, estes organismos continuamente desenvolvem mecanismos de defesa celular e humoral contra estes invasores. O sistema imune dos insetos, embora menos complexo que o dos vertebrados é muito eficiente em combater uma vasta gama de microorganismos invasores. Apesar de não possuir células de memória, anticorpos e linfócitos iguais aos dos vertebrados, estes organismos apresentam mecanismos de memória imunológica inata (Rodrigues e cols. 2010), moléculas semelhantes a anticorpos (Dong e cols. 2006) e células similares aos linfócitos chamadas de hemócitos. Uma das razões para este grande sucesso evolutivo é a capacidade do seu sistema imune em lidar com uma grande diversidade de patógenos. Populações naturais de insetos apresentam diferentes graus de susceptibilidade a organismos invasores, indo de “muito susceptíveis” a “muito resistentes” (e.g. Collins e cols. 2002, Hume e cols. 2007). Em experimentos de laboratório, Collins e cols. (1986) e Kumar e cols. (2003) foram capazes de selecionar populações de *A. gambiae* refratárias ao plasmódio. A resistência à infecção exibida por populações naturais ou de laboratório, pode ser atribuída a existência de um sistema imune capaz de destruir os parasitos ou impedir o desenvolvimento dos mesmos dentro do inseto. Portanto, o estudo do sistema de defesa dos insetos é fundamental para o desenvolvimento de novas estratégias de controle vetorial.

As principais células e órgãos que participam da resposta imune dos insetos são o corpo gorduroso (principal órgão imune do inseto), as células epiteliais e os hemócitos (Figuras 10 e 12). Os hemócitos são células sanguíneas circulantes que possuem papel determinante no combate sistêmico a infecções (Hillyer e cols. 2009). A hematopoiese, que ocorre nas glândulas linfáticas, é responsável por produzir os progenitores dos hemócitos, chamados de prohemócitos. Em *Drosophila, o*s prohemócitos podem se diferenciar em três diferentes tipos de hemócitos que possuem funções distintas: as células cristalinas que possuem papel na melanização, os plasmatócitos que atuam na fagocitose e os lamelócitos que são importantes no encapsulamento (Lemaitre e Hoffmann 2007; Figura 10).

A primeira linha de defesa dos insetos é constituída por barreiras estruturais (exoesqueleto rígido de quitina e o revestimento quitinoso das traquéias) que dificultam o contato do patógeno com o organismo. Quando o microorganismo consegue transpor estas barreiras e entra em contato com o ambiente interno do organismo, uma série de respostas imunes é ativada, culminando em respostas celulares e humorais. Outras barreiras utilizadas na tentativa de evitar que o patógeno se espalhe para outros tecidos, são os epitélios e a matriz peritrófica (Figuras 9 e 11). Organismos invasores são reconhecidos pelos insetos através de receptores de reconhecimento de padrões (*Pattern recognition receptors* - PRR) que reconhecem padrões moleculares associados à patógenos (*Pathogen-associated molecular patterns* - PAMPs). Após o reconhecimento dos patógenos, algumas reações do sistema imune podem ser são desencadeadas: (1) resposta humoral com produção de peptídeos antimicrobianos (AMPs) e moléculas efetoras pequenas (espécies reativas de oxigênio e de nitrogênio); (2) resposta celular que resulta na fagocitose, encapsulamento dos invasores e indução de apoptose; e (3) reação de profenoloxidases que depositam melanina em volta dos microorganismos (Hultmark 2003, Lemaitre e Hoffman 2007, Muller e cols. 2008; Figura 10).

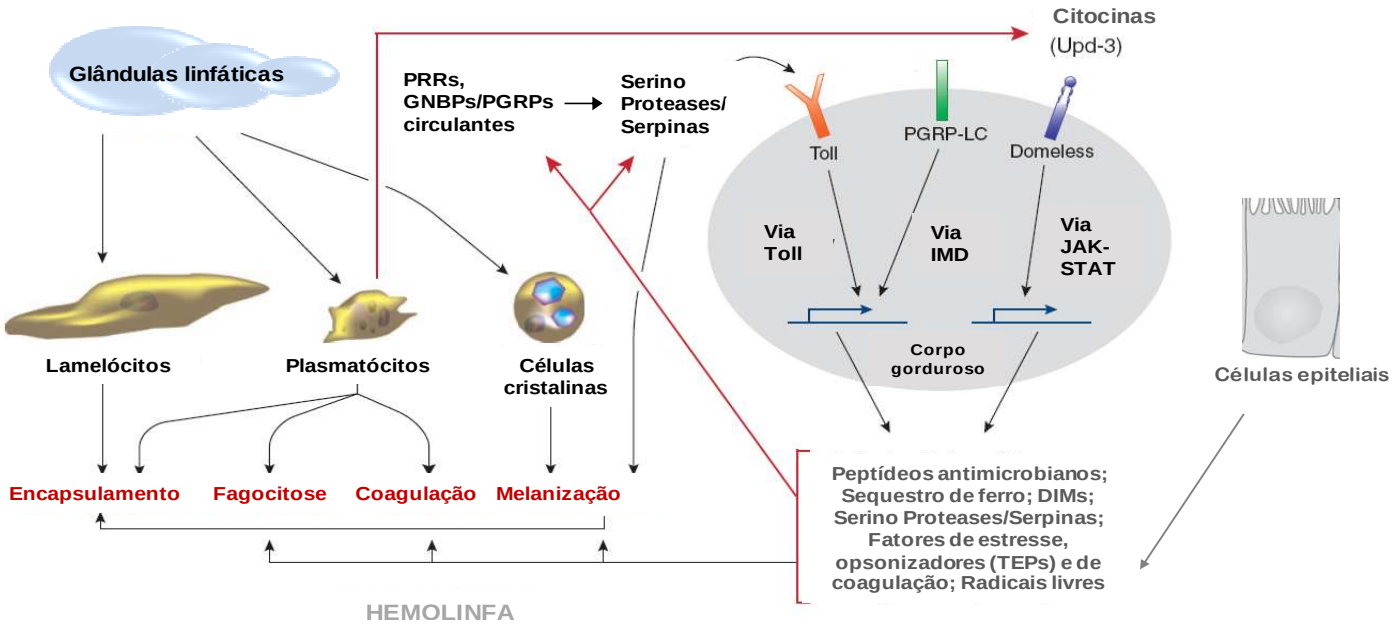

Figura 10: Resumo esquemático do sistema de defesa dos insetos. A detecção do patógeno leva à produção de um grande espectro de moléculas de defesa em tecidos que respondem imunologicamente (modificado de Lemaitre e Hoffmann 2007).

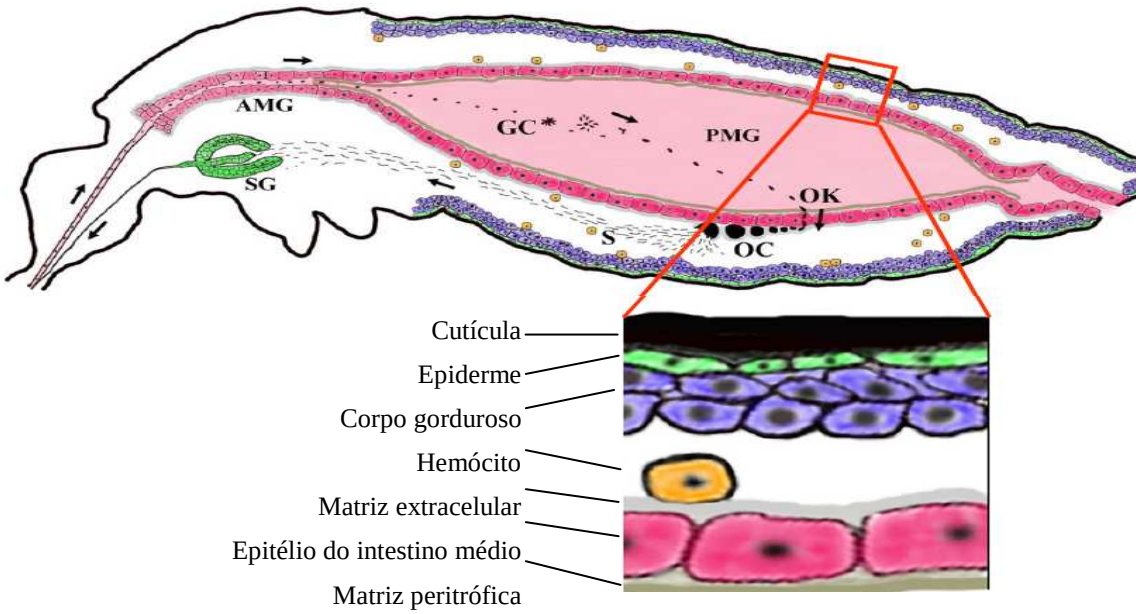

Figura 11: Órgãos e tipos celulares envolvidos na interação *Plasmodium*-mosquito. GC – Gametócitos, OK – oocineto, OC – oocisto, S – esporozoítos, AMG – intestino anterior, PMG – intestino posterior, SG – glândula salivar (modificado de Dimopoulos e cols. 2003).

1.6.1 Mecanismos de defesa humoral

A resposta imune humoral dos insetos ocorre em quatro etapas principais: (1) reconhecimento dos PAMPs pelos PRRs, (2) amplificação e distribuição do sinal de reconhecimento; (3) produção de um conjunto de moléculas efetoras e ativação de cascatas de coagulação; e (4) aumento de moléculas de imunidade através da ativação de vias de transdução de sinal como Toll, *Immune Deficiency* (IMD) e *Janus Kinase-Signal Transducer and Activator of Transcription* (JAK-STAT) (Michel e Kafatos 2005, Christophides e cols. 2002; Figura 10).

Embora ausentes na maioria dos animais, a utilização de PAMPs é recorrente em grupos distintos de microorganismos, provavelmente em função de sua importância na manutenção de aspectos básicos de sua fisiologia. São exemplos de PAMPs os componentes das paredes e membranas celulares dos patógenos, como peptidoglicanos (PGN), lipopolissacarídeos (LPS) e β-1,3 glucanos. Alguns PRRs estudados em insetos são as proteínas que reconhecem peptidoglicanos

(*Peptidoglycan recognition proteins* - PGRPs) e proteínas que se ligam a bactérias (*Gram-negative bacterial-binding proteins* – GNBPs; Osta e cols. 2004). O reconhecimento destas moléculas exclusivas de patógenos é importante na montagem de uma resposta imune eficiente e direcionada ao invasor.

A ligação entre PAMPs e PRRs, responsável pelo reconhecimento de moléculas não-próprias, ativa cascatas proteolíticas de serino proteases que amplificam o sinal e acionam a resposta efetora. Serino proteases com domínios clip (CLIPs) são componentes essenciais dessas cascatas, pois ativam vias de sinalização que levam à síntese de AMPs, aglutinação da hemolinfa e melanização (Osta e cols. 2004, Michael e Kafatos 2005; Figura 10). Estas cascatas são finamente reguladas por serpinas, moléculas que inibem as serino proteases através de uma ligação covalente com o centro ativo da enzima (Osta e cols. 2004, Reichhart e cols. 2005). As serpinas fazem parte de uma grande família de inibidores encontrada em animais, plantas, bactérias e vírus (Law e cols. 2006). Em humanos, podem atuar na coagulação sanguínea, ativação do complemento, fibrinólise, fertilização, inflamação, remodelamento tecidual, apoptose, transporte hormonal e regulação da pressão sanguínea (Janciauskiene 2001, Charron e cols. 2008). Em artrópodos, atuam na regulação das vias Toll e das profenoloxidases, na coagulação da hemolinfa e na proteção contra proteinases de microorganismos. Em decorrência de sua importância na modulação da resposta imune, o papel das serpinas no desenvolvimento de *Plasmodium* em *A. gambiae* também vem sendo objeto de estudo (Michel e cols. 2005, Abraham e cols. 2005, Danielli e cols. 2005).

Várias moléculas efetoras são produzidas após desafio dos insetos com microorganismos, como por exemplo: AMPs, espécies reativas de oxigênio, proteínas com domínio do tipo fibrinogênio (FBN) (*fibrinogen-related proteins* - FREPs), proteínas responsivas a bactérias (*Bacteria responsive proteins* - BRP), proteínas contendo domínios ricos em leucina (*Leucine rich-repeat* (LRR) *domain - containing proteins*) e lectinas do tipo C (*C-type lectins* – CTLs). As FREPs são proteínas altamente conservadas (Gokudan e cols. 1999, Wang e cols. 2005) que têm sido implicadas na resposta imune de mosquitos contra plasmódios e bactérias (Dimopoulos e cols. 2002, Dong e cols. 2006). Estudos funcionais revelaram que essas proteínas possuem funções complementares e sinérgicas e que podem formar homodímeros (Dong e cols. 2009). Especula-se atualmente que diferentes FBN

podem se associar para formar proteínas multiméricas com o intuito de aumentar o repertório de PRRs e, consequentemente, o reconhecimento de patógenos. As BRPs fazem parte de um grupo de proteínas da família 18 das glicosil hidrolases. Estas proteínas têm sido implicadas em funções imunes como: proliferação e migração celular, e agregação (Shi e Paskewitz 2004). As LRR são proteínas envolvidas em respostas imunes de melanização e morte de plasmódio, agindo como parte do sistema complemento (Osta e cols. 2004, Warr e cols. 2006). As LRR podem se localizar no citoplasma, ligadas às membranas, ou serem secretadas. As CTLs fazem parte de uma família bastante diversa de lectinas que possui a característica de se ligar a carboidratos e mediar processos como adesão celular, interações célula-célula, reposição de glicoproteínas e reconhecimento de patógenos (Cirimotich e cols. 2010). Foi demonstrado por Osta e cols. (2004) que duas lectinas (CTLA4 e CTLAM2) protegiam os oocinetos contra a resposta de melanização.

1.6.1.1 AMPs

Os AMPs são as proteínas efetoras mais bem caracterizadas. São moléculas catiônicas pequenas, de até 10 kDa (com exceção da atacina de 25 kDa), importantes na imunidade contra bactérias e fungos (Lemaitre e Hoffman 2007). São principalmente produzidas pelo corpo gorduroso, hemócitos e por estruturas que representam barreiras físicas como intestino médio, túbulos de malpighi, traquéia e glândula salivar (Levashina 2004; Figuras 10 e 12). Os AMPs atuam na membrana do patógeno e são muito estáveis, podendo permanecer na hemolinfa do inseto semanas após o desafio. Os 20 AMPs já descobertos podem ser divididos em sete classes: defensinas, cecropinas, drosomicina, metchnikowina, atacina, diptericina e drosocina. As cecropinas fazem parte de uma grande família de peptídeos tóxicos que atuam formando canais de poros nas membranas dos patógenos. São proteínas pequenas, de 35 aminoácidos em média, que atuam quase sempre em sinergia realizando defesas rápidas e não específicas tanto contra bactérias Gram-negativas quanto Gram-positivas (Tamang e Saier 2006). Em *A. gambiae* infectados com bactérias e plasmódios ocorre um aumento na expressão do gene para cecropina

(Vizioli e cols. 2000). *A. gambiae* transgênicos superexpressando cecropina apresentaram uma redução de 60% na infecção por *P. berghei* (Kim e cols. 2004).

1.6.1.2 Espécies reativas de oxigênio e nitrogênio

O papel dos radicais livres como moléculas efetoras do sistema imune de insetos foi demonstrado primeiramente em hemócitos de *D. melanogaster* infectados por bactérias gram-positivas e negativas (Nappi e cols. 1995, Nappi e Vass 1998). Em 1998, Luckhart e colaboradores demonstraram a indução da enzima óxido nítrico sintase (NOS) e a produção do radical antimicrobiano "NO" em *A. gambiae* e *A. stephensi* desafiados com *P. berghei*. Em 2003, Kumar e colaboradores estudando duas linhagens de mosquitos *A. gambiae,* uma refratária e outra susceptível ao parasito da malária, observaram grandes diferenças na transcrição de genes ligados ao metabolismo redox. Estes pesquisadores notaram que, após a infecção, a inhagem refratária se encontrava em um processo crônico de estresse oxidativo que provavelmente era responsável pela resistência ao *Plasmodium*. Logo após, Kumar e colaboradores (2004) demonstraram que a invasão do epitélio intestinal pelo plasmódio, além de estimular a produção do radical NO, aumentava a expressão de uma peroxidase (PER) intestinal. A PER utiliza o nitrito (sub-produto do radical "NO") juntamente com peróxido de hidrogênio para formar espécies altamente oxidantes, como dióxido de nitrogênio, que são responsáveis pela nitração de proteínas e morte tanto da célula invadida como do oocineto (Kumar e cols. 2003 e 2004, Gupta e cols. 2005, Kumar e Barillas-Mury 2005; Figura 13).

Uma enzima DUOX, produtora de ROS, foi descrita no epitélio intestinal de *A. gambiae* (Kumar e cols. 2004) e *D. melanogaster* (Ha e cols. 2005a) e está relacionada ao controle de infecções. Esta enzima funciona em associação à catalase, que é responsável por detoxificar o ambiente e manter a homeostase local (Ha e cols. 2005b; Figura 12).

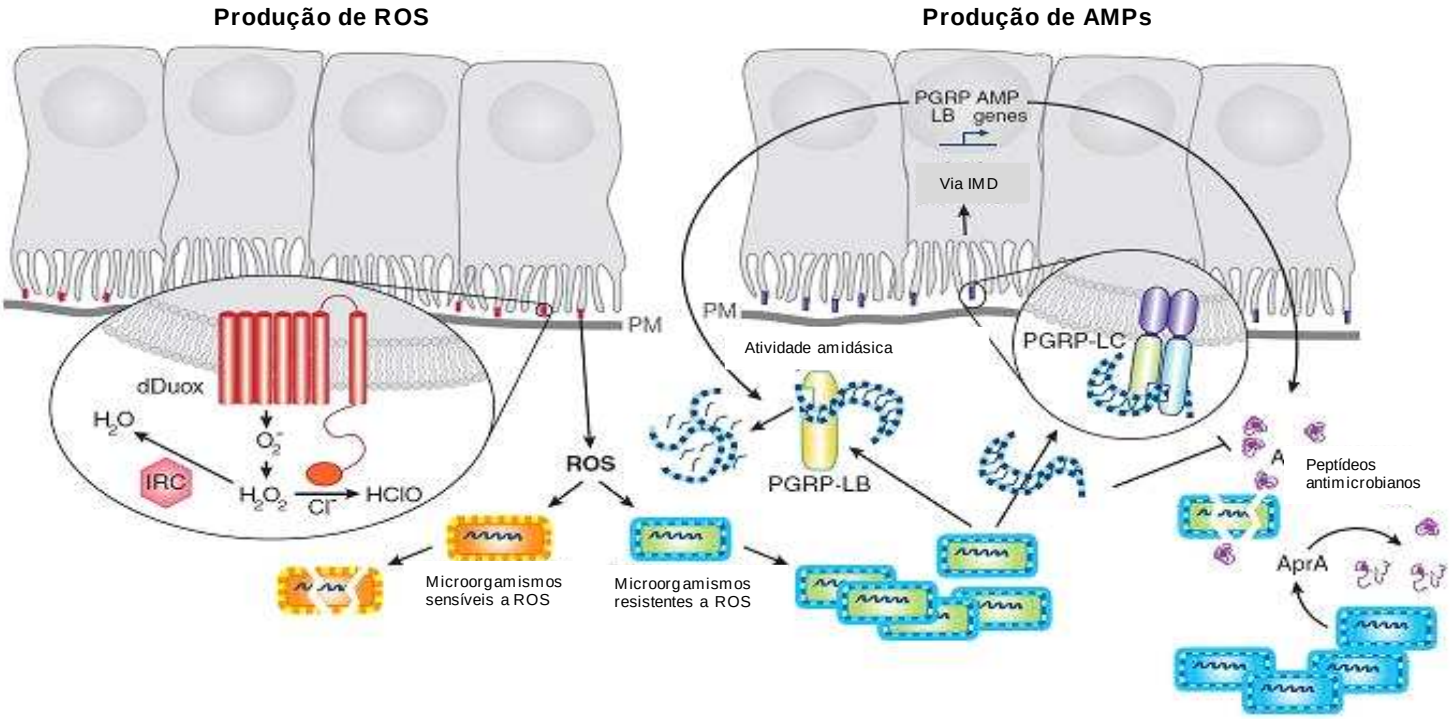

Figura 12: Esquema ilustrando a resposta imune do intestino de *D. melanogaster* com produção de ROS e peptídeos antimicrobianos (AMPs). ROS são produzidos pela proteína DUOX e detoxificados pela catalase (induzida imunologicamente). AMPs são produzidos pelas células epiteliais sob o controle da via IMD após o reconhecimento de PGN (peptideoglicanos) liberados por bactérias Gram-negativas (modificado de Lemaitre e Hoffman 2007).

Os radicais livres são produzidos constantemente pelo organismo através da respiração celular e, como descrito acima, em desafios imunológicos. Apesar do seu efeito benéfico na cura de infecções, estas moléculas também podem ser altamente danosas para o hospedeiro que as produz. Para que os organismos produtores não sejam muito prejudicados com a produção de radicais livres em excesso, estes desenvolveram mecanismos antioxidantes capazes de reduzir a quantidade destas moléculas. Os processos utilizados pelos antioxidantes são vários, como por exemplo: (1) remoção catalítica das espécies reativas por enzimas como catalase, superóxido dismutase e glutationa peroxidase; (2) diminuição da disponibilidade de moléculas pró-oxidantes, como a ferritina que estoca ferro e a hemopexina que liga heme; (3) conversão de moléculas oxidantes, como a heme oxigenase (HO) que catalisa a degradação do heme; e (4) reação e conversão para espécies menos

reativas de radicais livres, como as vitaminas E e C, bilirrubina e o ácido úrico (Halliwell e Gutteridge 1999).

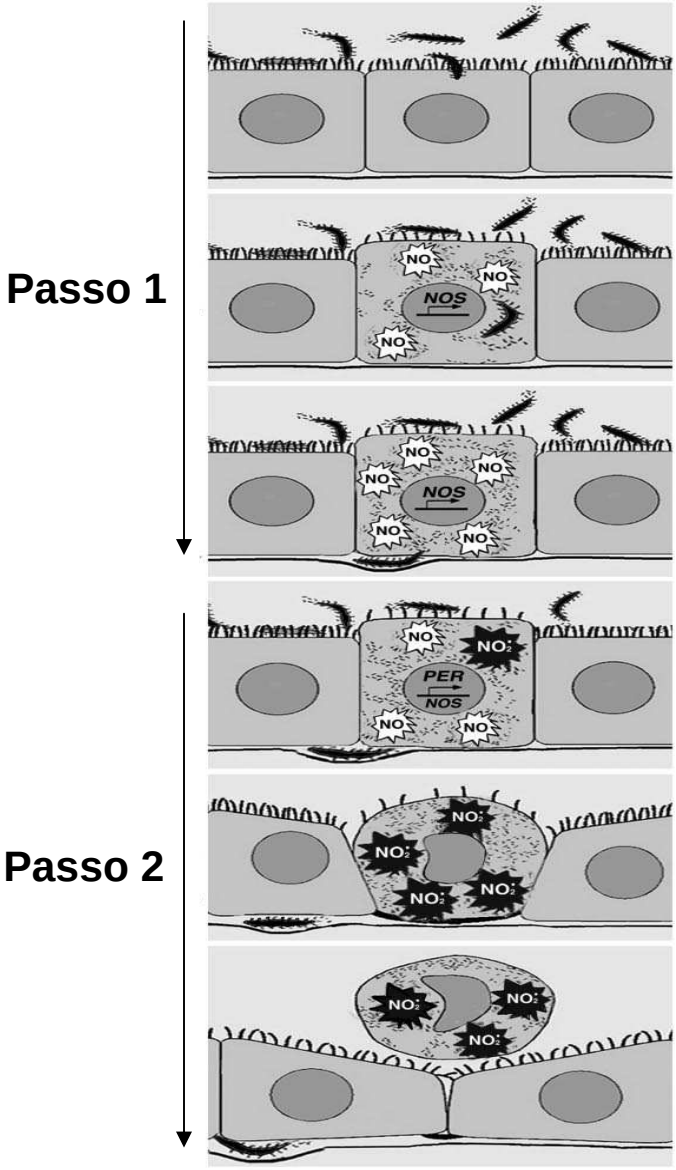

Figura 13: Representação esquemática da resposta das células epiteliais à invasão dos oocinetos. A invasão das células pelos oocinetos induz a expressão da enzima óxido nítrico sintase (NOS) que catalisa a produção do óxido nítrico (NO). Além disso, o ápice da célula se projeta para o lúmen e as microvilosidades são perdidas. O NO é bastante instável e rapidamente se converte em nitrito. O nitrito e o peróxido de hidrogênio servem de substrato para a peroxidase (PER) produzir NO_2 e mediar a nitração. Consequentemente, as células exibem degeneração nuclear e são liberadas no lúmen do intestino (modificado de Kumar e cols. 2004).

Todas as proteínas efetoras produzidas após um desafio são reguladas primariamente ao nível transcricional, através de moléculas (fatores de transcrição - FTs) que se ligam a regiões regulatórias dos genes efetores e promovem o aumento ou a diminuição da sua transcrição. Muitos genes efetores como os AMPs possuem em suas regiões regulatórias sítios de ligação a FTs da família NF-κB. Estudos recentes têm demonstrado o papel destas moléculas na regulação dos AMPs através de duas vias de sinalização distintas, Toll e IMD (Lemaitre e Hoffman 2007). A via Toll é principalmente ativada por bactérias Gram-positivas, fungos e vírus, enquanto a IMD por bactérias Gram-negativas (Tzou e cols. 2002, Hoffman e Reichhart 2002, Xi e cols. 2008). Outro FT, o STAT e a via de sinalização da qual faz parte, a JAK-STAT, têm papel importante na ativação da resposta imune dos insetos contra uma variedade de patógenos (Barillas-Mury e cols. 1999, Dostert e cols. 2005, Molina-Cruz e cols. 2008, Souza-Netto e cols. 2009). Outro grupo de FTs vinculado a aspectos imunológicos é o GATA. Estes FTs possuem este nome, por se ligarem a sequências de DNA (A/T) GATA (A/G). São encontrados em uma diversidade de grupos de organismos como, por exemplo, fungos, plantas e animais. Atuam em diversas funções como: desenvolvimento, diferenciação e proliferação. Mutações ou modulações que comprometam a expressão destes genes podem causar doenças ao homem (Patient e McGhee 2002). Fatores de transcrição do tipo GATA apresentam papel na imunidade contra diferentes patógenos em *D. melanogaster* e *Caenorhabditis elegans* (Tingvall e cols. 2001, Kerry e cols. 2006, Senger e cols. 2006, Shapira e cols. 2006).

1.6.2 Regulação do sistema imune

Pesquisadores têm procurado correlacionar a ativação das vias de sinalização imunológicas com a eficácia da resposta imune de mosquitos contra patógenos (e.g. Molina-Cruz e cols. 2008, Gupta e cols. 2009, Garver e cols. 2009, Souza-Netto e cols. 2009). Dada sua relevância nos processos imunológicos, todas as vias de sinalização descritas acima são altamente conservadas evolutivamente (Figuras 14 e 17).

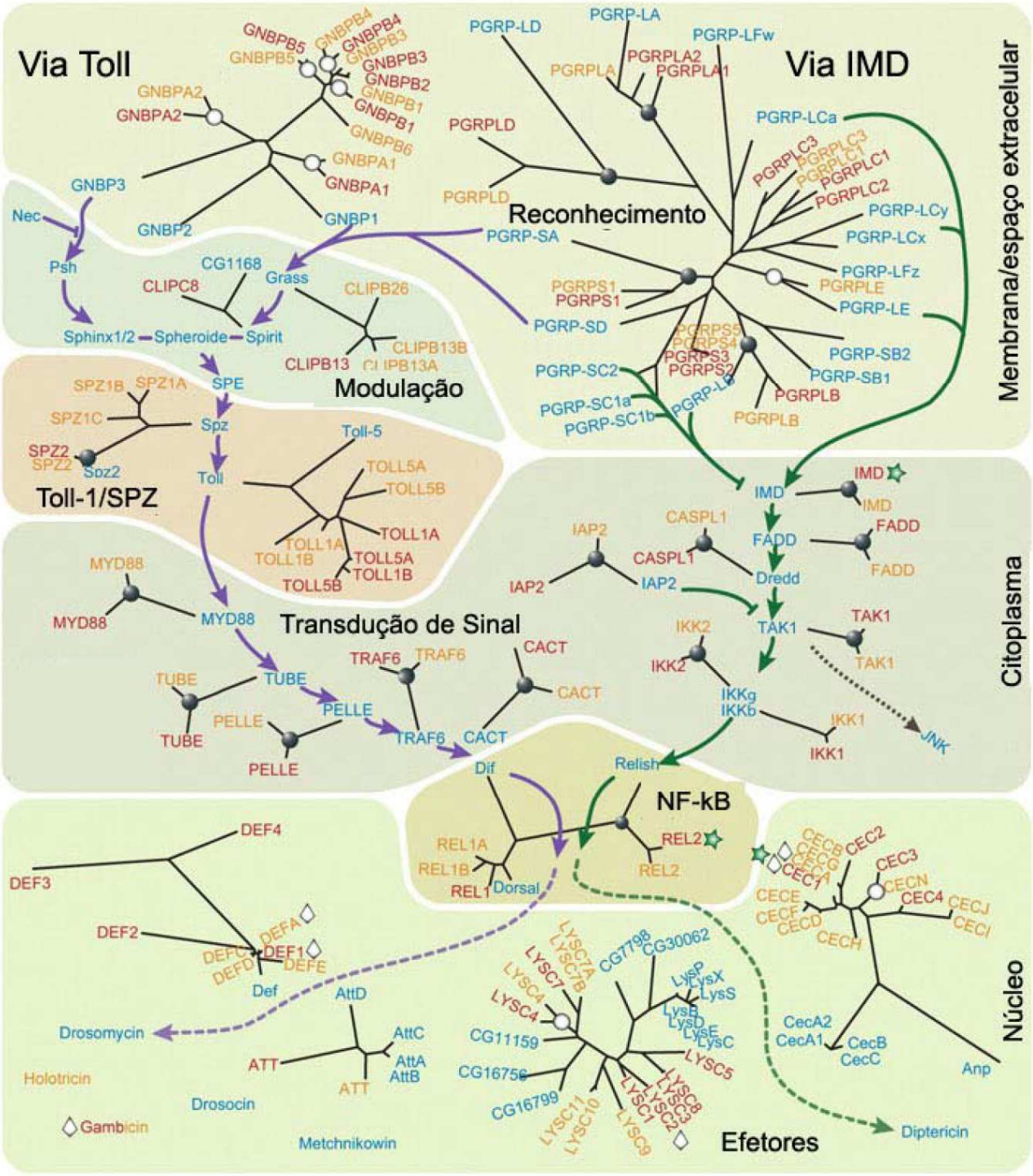

Figura 14: Genes e famílias gênicas envolvidas nas vias Toll (verde) e IMD (roxo). Os genes de *D. melanogaster* encontram-se indicados em azul, de *A. gambiae* em vermelho e de *A. aegypti* em verde (modificado de Waterhouse e cols. 2007).

1.6.2.1 Via Toll

Receptores Toll foram descritos pela primeira vez em *D. melanogaster.* Insetos e mamíferos possuem 10 cópias de genes desta família, os receptores do tipo Toll (*Toll like receptors* - TLRs), em seus genomas, porém, enquanto todos TLRs de humanos possuem funções relacionadas à imunidade, somente alguns TLRs de inseto possui função imune (Imler e Zheng 2004). Em *D. melanogaster*, esta via, além de funções imunes como a produção de peptídeos antimicrobianos e antifúngicos, desempenha funções relacionadas à ontogenia (Belvin e Anderson 1996, Lemaitre e cols. 1996). A via Toll em *D. melanogaster* é muito similar à cascata ativada pela citocina IL-1 e TLRs de vertebrados, o que sugere uma origem comum para estas duas vias. A via de sinalização Toll é desencadeada pela ligação de PAMPs de microorganismos como bactérias ou fungos a PRRs (PGRPs ou GNBPs). Essa ligação ativa uma cascata de serino proteases que cliva a proteína Spatzle. A ligação da Spatzle (clivada) ao receptor Toll (que contem repetições de leucina, LRRs - *leucine-rich repeats*) faz com que o receptor sofra alterações conformacionais que resultam no recrutamento de pelo menos três proteínas com domínios *Death* (MyD88, Tube e Pelle). MyD88 e Tube compartilham o domínio TIR semelhante à Toll e possivelmente interagem diretamente. Pelle contem um domínio adicional de serina-treonina quinase e por isso pode estar envolvido na via de degradação proteolítica de Cactus, proteína homóloga a IkB. A degradação do repressor negativo da via permite que as proteínas homólogas ao NF-κB, Dorsal (maioria dos insetos) e Diff (*Drosophila*), se transloquem para o núcleo e promovam a transcrição de genes efetores, como por exemplo, o AMP drosomicina (Figura 15; Lemaitre e Hoffmann 2007). Recentemente, a via Toll foi apontada como sendo importante na imunidade de insetos vetores contra *Plasmodium* (Garver e cols. 2009).

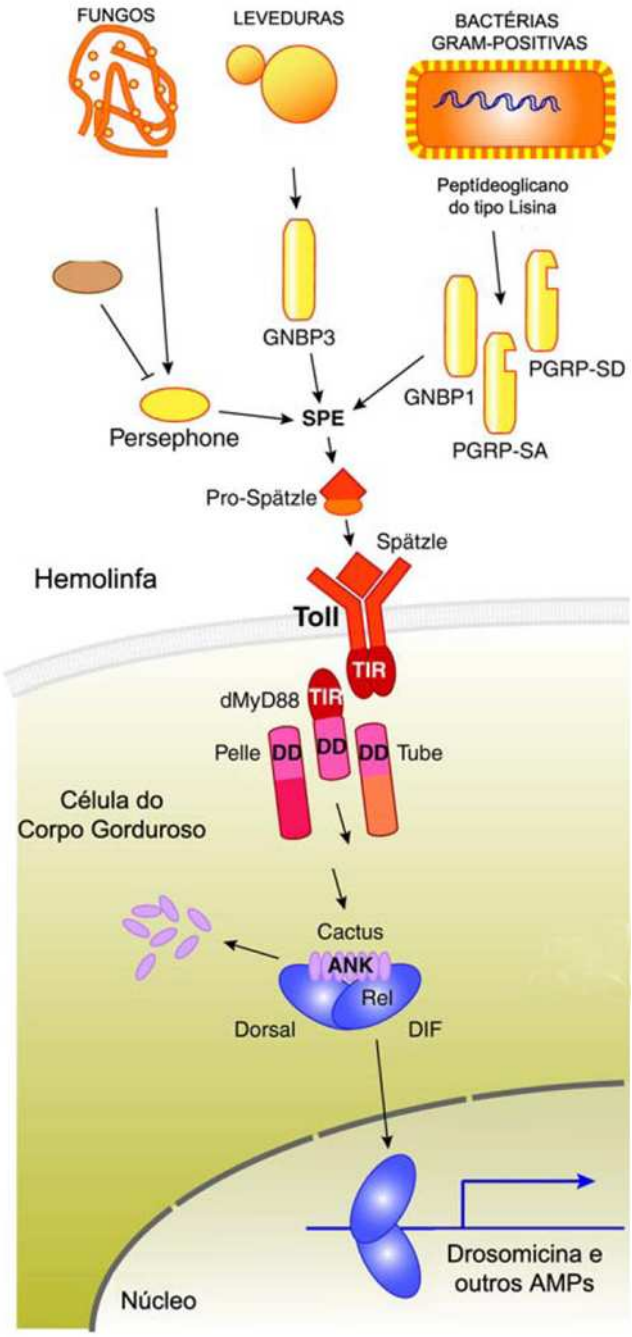

Figura 15: Desenho esquemático da via Toll de *D. melanogaster* (modificado de Lemaitre e Hoffmann 2007).

1.6.2.2 Via IMD

A via IMD foi descoberta através da observação de um mutante de *D. melanogaster* susceptível a bactérias gram-negativas, mas resistente a bactérias gram-positivas e fungos (Lemaitre e cols. 1995). Este variante foi chamado de mutante com deficiência imunológica (*Immune deficiency* - IMD) e a proteína mutada recebeu o nome de IMD e veio a dar nome à via. Nenhuma outra função além da ligada à imunidade do inseto foi descrita para esta via. Esta via apresenta similaridades com a via TNF-R de mamíferos. A via IMD é ativada de modo parecido à via Toll. O receptor transmembranar da via de IMD parece ser uma proteína reconhecedora de peptidioglicana de cadeia longa (*Peptidoglycan recognition protein long chain* - PGRPLC). Apesar deste receptor não possuir domínios de interação proteína-proteína em sua porção citoplasmática, acredita-se que, ao ser ativado, se ligue a algum co-fator que possui domínios de interação com proteínas que serão responsáveis pela passagem do sinal para a via IMD. Portanto, a ativação do receptor pelos PAMPs é capaz de recrutar as proteínas adaptadoras IMD, FADD e a caspase DREDD. Uma vez em proximidade, DREDD cliva IMD, deixando um o resíduo N-terminal A31 exposto. Esse resíduo exposto permite a ligação da IMD a DIAP2. Em conjunto com as enzimas E2 conjugadoras de ubiquitina (*E2-ubiquitin-conjugating enzymes*), UEV1a, Bendless (Ubc13) e Effete (Ubc5), IMD (e um pouco DIAP2) são, então, poliubiquitiniladas (Paquette e cols. 2010). Essas cadeias de poliubiquitinas induzem a ativação de quinases [os complexos formado por dTAK1 (*TGF-β-activated kinase 1*) e TAB (*TAK1 binding protein*)] e IKKb-IKKg que darão continuidade ao sinal (Kleino e cols. 2005, Paquette e cols. 2010). A proteína dFadd liga-se então a DREED, que, por sua vez, se associa e cliva a proteína Relish fosforilada. A fosforilação de Relish é realizada pelo complexo kinase iKB que é ativado pela MAPKKK TAK1. Uma vez clivada, Relish libera sua porção C-terminal (inibidora de sua translocação para o núcleo), da porção C-terminal (Figura 16). Sua entrada no núcleo promove a transcrição de genes efetores como os AMPs. Recentemente, foi demonstrado que a via IMD controla a resistência de anofelinos a *P. falciparum* (Garver e cols. 2009).

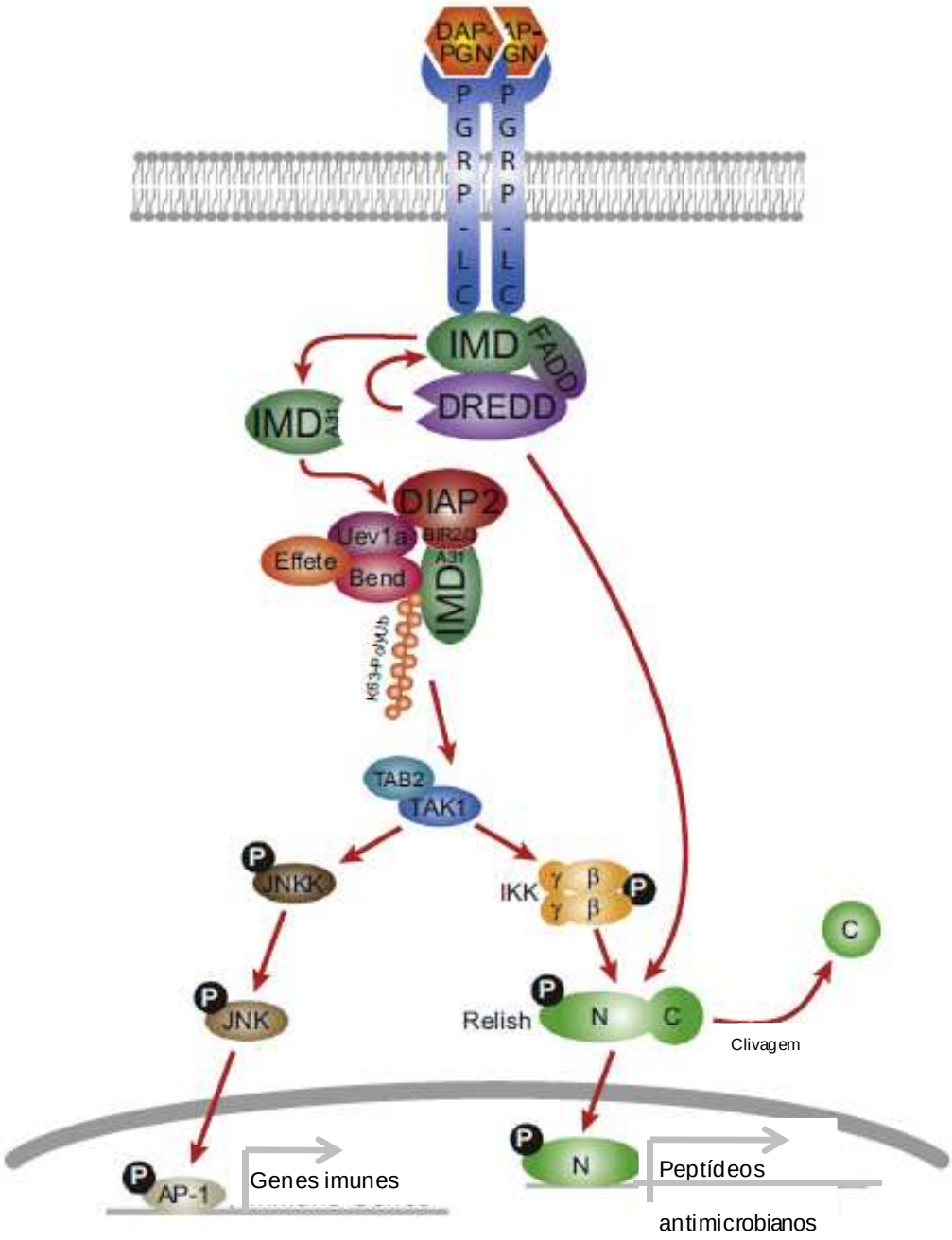

Figura 16: Desenho esquemático da via IMD de *D. melanogaster* (modificado de Paquette e cols. 2010).

1.6.2.3 Via JAK-STAT

A via JAK-STAT foi descrita primeiramente em mamíferos como tendo um papel importante em respostas antivirais (Dupius e cols. 2003, Karst e cols. 2003, Ho e cols. 2005). Em *D. melanogaster*, esta via possui funções descritas tanto na imunidade como no desenvolvimento. Regula diversos processos relacionados com a embriogênese, como o desenvolvimento dos olhos, a segmentação embrionária e a manutenção de células-tronco (Arbouzova e Zeidler 2006). Esta via é também ativada após infecção dos insetos com vírus, bactérias e plasmódios (Barillas-Mury e cols. 1999, Dostert e cols. 2005, Molina-Cruz e cols. 2008, Souza-Netto e cols. 2009). Além disso, sua participação na resposta imune celular, através da regulação da proliferação de hemócitos e na diferenciação de prohemócitos, já foi demonstrada (Hanratty e Dearolf 1993, Harrison e cols. 1995, Krzemièn e cols. 2007).

A via JAK-STAT em *D. melanogaster* é ativada após a união de moléculas do tipo citocinas da família *unpaired* (UpD) ao receptor transmembrana Domless, homólogo do receptor de citocinas tipo I de mamíferos. Essa ligação causa uma alteração conformacional do receptor que leva à fosforilação de proteínas JAK, associadas a estes receptores. As proteínas JAK ativadas fosforilam Dome, resultando na formação de sítios de ligação para as proteínas citoplasmáticas STAT. O recrutamento das STATs pelo complexo JAK-Dome induzem sua fosforilação e dimerização de STAT. Os dímeros formados migram para o núcleo e promovem a transcrição de genes efetores. Essa via é finamente regulada por dois reguladores negativos, a proteína inibitória de STAT ativado (*Protein inhibitor of activated STAT* - PIAS) e o supressor de sinalização por citocinas (*Suppressor of cytokine signaling* – SOCs) (Arbouzova e Zeidler 2006; Figura 17).

Alguns dos produtos finais desta via já são conhecidos. Em *D. melanogaster* foi mostrado que esta via ativa a transcrição do gene TEP2 e de genes de estresse do tipo Turandot. Enquanto a função de Turandot permanece desconhecida, é sabido que TEP2 possui homologia com as proteínas do sistema complemento de mamíferos e é importante na opsonização de patógenos (Lagueux e cols. 2000, Boutros e cols. 2002, Agaisse e cols. 2003). Em *A. gambiae,* esta via induz a transcrição e expressão da enzima óxido nítrico sintase, responsável pela produção do radical antimicrobiano óxido nítrico após infecções com *P. berghei* (Gupta e cols.

2009). Já em *A. aegypti*, esta via é importante em respostas contra o vírus dengue (Souza-Netto e cols. 2009).

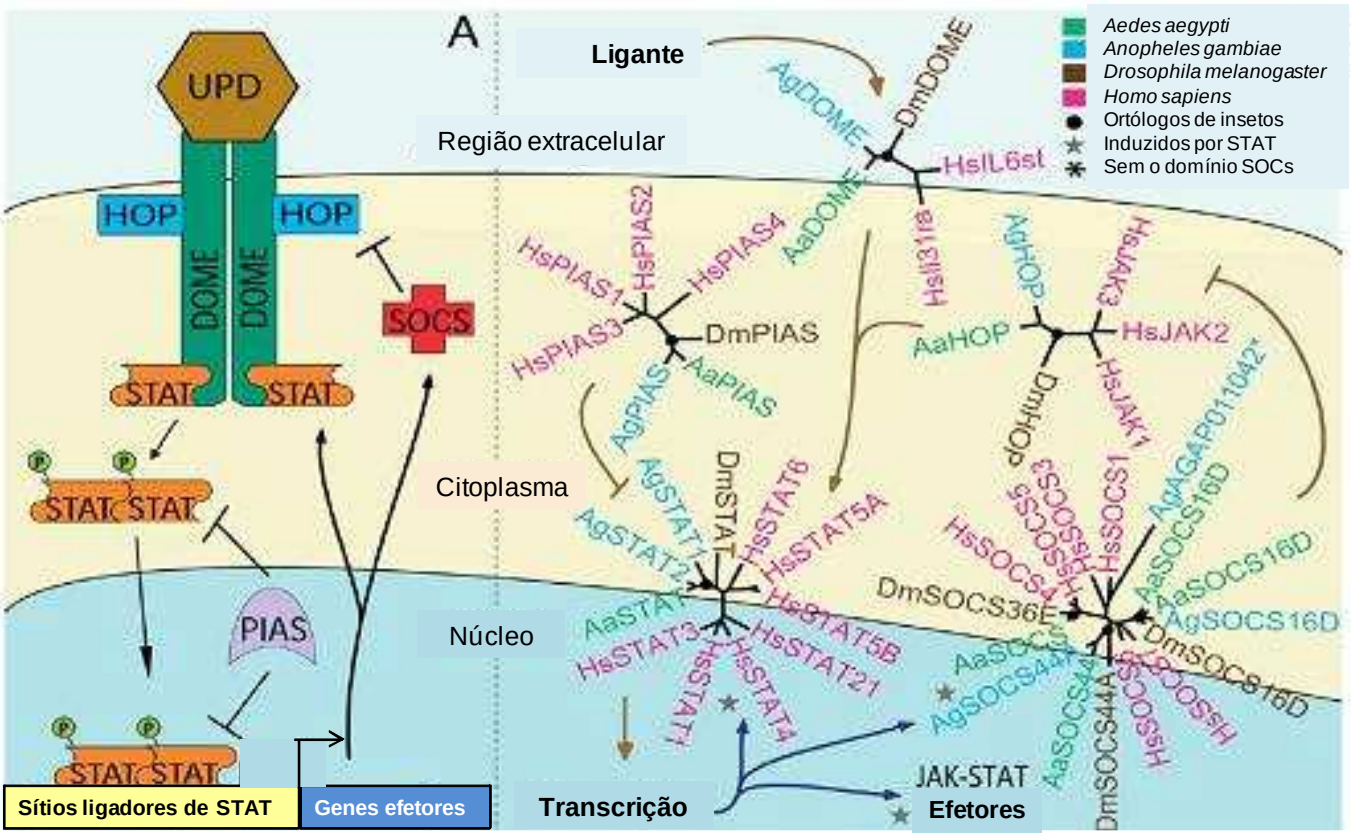

Figura 17: Esquema da via JAK-STAT em insetos. A) Desenho esquemático da via de sinalização JAK-STAT de *D. melanogaster* mostrando todos os componentes conhecidos e suas possíveis funções. B) Conservação evolutiva entre componentes da via JAK-STAT de insetos [*D. melanogaster* (marrom), *A. aegypti* (verde), *A. gambiae* (azul)] e *Homo sapiens* (rosa) (modificado de Souza-Netto e cols. 2009).

1.6.3 Mecanismos de defesa celular

1.6.3.1 Melanização e coagulação

A melanização é uma reação imune, somente encontrada em artrópodos, que envolve a síntese de melanina e sua deposição em volta do patógeno. Neste grupo de organismos, é o tipo mais rápido de resposta imune, desencadeada poucos minutos após o desafio. A produção de melanina é promovida após o reconhecimento de patógenos, ativando de cascatas de serino proteases que clivam a forma inativa da pro-fenoloxidase para a forma ativa fenoloxidase. As fenoloxidases catalizam a conversão de dopamina em melanina. As quinonas e os ROS gerados durante a melanização são tóxicos para os parasitos (Christensen e cols. 2005). A reação de melanização interage com outras repostas imunes, como coagulação sanguínea, fagocitose, produção de AMP e cicatrização (Cerenius e cols. 2008). A reação de melanização precisa ser finamente regulada, caso contrário, produtos intermediários tóxicos são produzidos. As serpinas desempenham papel importante na regulação da reação de melanização através da inibição de serino proteases. A melanização foi observada em vários insetos desafiados com plasmódios, bactérias e helmintos (Christensen e cols. 2005).

O processo de coagulação envolve a ativação de proteínas via cascata de serino proteases. Dois genes relacionados com fatores de coagulação, um fibrinogênio e uma anexina, são altamente expressos após lesão séptica em *Drosophila* (De Gregorio e cols. 2001).

1.6.3.2 Fagocitose

A fagocitose (em insetos) é um processo de defesa celular baseado no reconhecimento, engolfamento e destruição intracelular do patógeno pelos hemócitos. É mediada por PRRs que se ligam à PAMPs e desencadeiam uma cascata de sinalização que leva à internalização do invasor através de um mecanismo dependente de actina. Uma PGRP de *D. melanogaster* tem sido implicada na fagocitose de bactérias Gram-negativas por hemócitos (Ramet e cols. 2002). Em mosquitos, uma proteína similar à do complemento humano C3 chamada

TEP1 (*Thioester-containing protein 1*) participa da fagocitose de *Escherichia coli* e *Staphylococcus aureus*. Além desta, proteínas com domínio do tipo imunoglobulina chamadas de DsCam, formadas a partir do *splicing* alternativo de um único gene, são importantes no reconhecimento e opsonização de invasores (Moita e cols. 2005, Dong e cols. 2006).

1.6.4 RNAi e imunidade

O mecanismo de defesa antiviral pelo processo de interferência por RNA (*RNA interference* – RNAi) se baseia no silenciamento da expressão gênica através da degradação sequência-específica de RNAs mensageiros (RNAm). Tem sido apontada como mecanismo fundamental no controle da expressão gênica e na imunidade contra vírus de RNA. A via de RNAi é iniciada após o reconhecimento de dupla-fitas de RNA (*Double-strand RNA* – dsRNA) pela enzima Dicer, da família das RNAs polimerases III, que cliva dsRNA viral recém-sintetizadas gerando pequenos RNAs de interferência (*Small interfering RNAs* – siRNA) de 21 a 25 pares de bases. Os siRNAs servem como molde para a proteína argonauta, componente catalítico do complexo RISC (*RNA-Inducing silencing complex*), reconhecer e degradar o RNA viral. Uma forma de amplificação deste mecanismo acontece quando a porção senso do siRNA é submetida à amplificação por uma RNA polimerase, gerando assim novos siRNAs do gene em questão (Dykxhoorn e cols. 2003; Figura 18). Mutações nos genes argonauta2 e Dicer2 aumentam a susceptibilidade de *Drosophila* a diversos vírus (e.g. Galiana-Arnoux e cols. 2006, van Rij e cols. 2006).

Desde 1998, a via de RNAi tem sido utilizada como instrumento para reprimir genes específicos através da introdução de dsRNA sintética no organismo de interesse (Fire e cols. 1998). Desta forma, esta via se tornou uma excelente ferramenta para a identificação de componentes necessários para um processo celular particular.

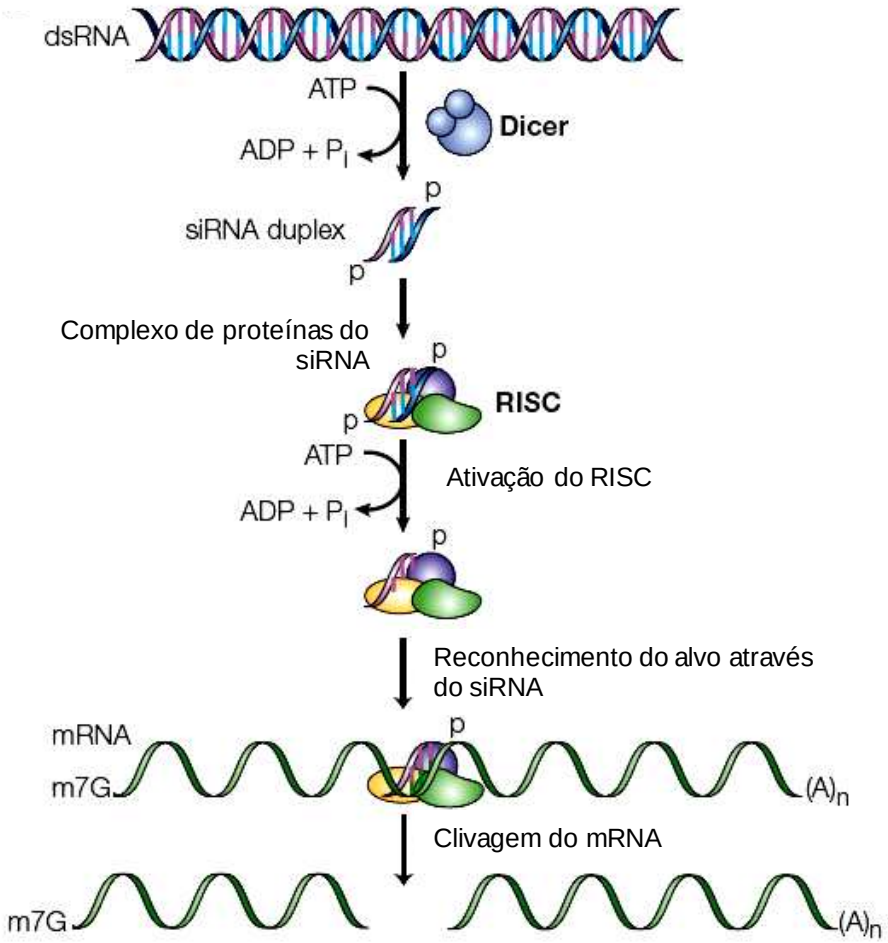

Figura 18: A via de siRNA (modificado de Dykxhoorn e cols. 2003).

1.7 Justificativa

Após mais de um século de desenvolvimento e implementação de estratégias de controle, doenças transmitidas por insetos ainda representam um desafio para a saúde pública mundial. A malária, considerada passível de controle há cinco décadas, continua a afetar 250 milhões de pessoas, causando um milhão de mortes por ano (OMS 2009). Mesmo considerando que os níveis de mortalidade em adultos sejam baixos, a malária é a doença parasitária que resulta na maior perda econômica, de acordo com o Banco Mundial. No Brasil, como em outros países tropicais, esta doença é um problema sério de saúde pública e representa 10% de

todos os casos reportados fora da África. *A. aquasalis* é o vetor de malária mais importante nas regiões litorâneas do Brasil e o *P. vivax* o agente etiológico causador da maior parte dos casos da doença.

Apesar da relevância epidemiológica de *P. vivax* (que contribui com aproximadamente 50% dos casos reportados fora da África), pesquisadores têm dedicado pouco tempo (e dinheiro) a estudos direcionados a este parasito específico. Há duas explicações possíveis para esta observação: (1) a dificuldade de se cultivar o parasito (diferentemente do que ocorre com *P. falciparum*) e (2) a crença de que este parasito não causa sintomatologias graves (de Lacerda e cols. 2007, Oliveira-Ferreira e cols. 2010, Udomsangpetch e cols. 2008). Apesar de ter sido considerada por muito tempo uma infecção benigna, a malária causada por *P. vivax* é agora vista como grave e letal (Anstey e cols. 2009). Portanto, novos esforços devem ser realizados para que se melhor compreenda a biologia deste importante parasito.

O aumento recente de casos de malária, o aumento de resistência dos parasitos a drogas, e o surgimento de populações de mosquitos anofelinos resistentes a inseticidas, têm chamado a atenção para a necessidade de desenvolvimento de novas estratégias de controle e do incremento das pesquisas em vacinas e fármacos. Embora investigações recentes venham contribuindo para o avanço do conhecimento da interação vetor-parasito, estudos que investiguem os eventos moleculares que ocorrem durante a alimentação sanguínea e após a infecção com plasmódio abrem novas perspectivas para o controle desta doença.

Os aspectos gerais do ciclo de vida do parasito dentro do inseto vetor já foram elucidados, porém pouco se sabe sobre os eventos que determinam o desenvolvimento do plasmódio e as interações entre estes dois organismos.

Aspectos como (1) a mudança de estágios do parasito; (2) os eventos que determinam a invasão da matriz peritrófica, do intestino médio e das glândulas salivares; e (3) que moléculas do inseto são eficazes no combate dos parasitos; necessitam ser melhor compreendidos.

O conhecimento das moléculas de interação vetor-patógeno e das suas vias de regulação poderá ser utilizado no desenvolvimento de estratégias para o combate à transmissão da malária, como, por exemplo, na produção de insetos modificados geneticamente que sejam potencialmente mais resistentes a patógenos (Marrelli e cols. 2007).

Esta proposta poderá gerar novas informações sobre a interação do vetor de malária, *A. aquasalis*, com o *P. vivax*, através de descrição de moléculas mediadoras deste processo.

2. Objetivos

2. Objetivos

Objetivo geral

Estudar mecanismos de interação entre o inseto vetor *A. aquasalis* e o parasito *P. vivax.*

Objetivos específicos

a) Gerar sequências de *A. aquasalis* envolvidas na interação vetor-parasito através da produção de bibliotecas subtrativas;

b) Anotar estas sequências e confirmar a modulação de alguns genes por RTPCR;

c) Estudar a via de sinalização JAK-STAT em *A. aquasalis* e seu possível papel na resposta imune deste inseto ao *P. vivax*;

d) Estudar o papel de radicais livres na resposta imune do *A. aquasalis* ao *P. vivax*;

e) Estudar o papel do fator de transcrição GATA na imunidade de *A. aquasalis* contra *P. vivax.*

3. Resultados

3. Resultados

Este trabalho teve como objetivo central estudar moléculas de interação entre *A. aquasalis* e *P. vivax*, principalmente aquelas relacionadas com o sistema de defesa do inseto. Antes do começo do presente estudo, apesar da importância do *A. aquasalis* como vetor de malária no Brasil, poucos trabalhos haviam sido realizados com este inseto. Além disso, poucos genes deste mosquito eram conhecidos. A maioria das sequências de *A. aquasalis* depositada nos bancos de dados públicos foi gerada para estudos de genética de populações e não serviam para o nosso propósito. Deste modo, duas estratégias foram adotadas para a identificação de genes de *A. aquasalis* potencialmente envolvidos na interação com *P. vivax*: a) construção de bibliotecas de subtração de cDNA e b) PCR usando iniciadores degenerados. Estas técnicas proporcionaram a identificação de diversos genes importantes na interação do *A. aquasalis* com seu parasito *P. vivax*.

Capítulo 1

Anopheles aquasalis **infectado pelo** ***Plasmodium vivax*** **apresenta perfis únicos de expressão de genes quando comparado com outros vetores de malária e plasmódios**

Publicado: PLoS One. 2010 Mar 22;5(3):e9795.

Justificativa:

Apesar da importância do *A. aquasalis* como vetor de malária na América do Sul poucos estudos foram realizados com este vetor. Nenhum gene de *A. aquasalis* relacionado com moléculas situadas na interface mosquito-plasmódio, como por exemplo, as moléculas de imunidades, havia sido caracterizado ou se encontrava depositados nos bancos de dados públicos. Portanto, bibliotecas subtrativas foram preparadas a partir de amostras deste inseto alimentado com sangue ou infectado com o *P. vivax* com o intuito de revelar genes importantes na interface vetor-parasito que pudessem vir a ser utilizados em estratégias de controle desta enfermidade.

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PLoS one

Anopheles aquasalis Infected by *Plasmodium vivax* Displays Unique Gene Expression Profiles when Compared to Other Malaria Vectors and Plasmodia

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Abstract

Malaria affects 300 million people worldwide every year and is endemic in 22 countries in the Americas where transmission occurs mainly in the Amazon Region. Most malaria cases in the Americas are caused by *Plasmodium vivax*, a parasite that is almost impossible to cultivate *in vitro*, and *Anopheles aquasalis* is an important malaria vector. Understanding the interactions between this vector and its parasite will provide important information for development of disease control strategies. To this end, we performed mRNA subtraction experiments using *A. aquasalis* 2 and 24 hours after feeding on blood and blood from malaria patients infected with *P. vivax* to identify changes in the mosquito vector gene induction that could be important during the initial steps of infection. A total of 2,138 clones of differentially expressed genes were sequenced and 496 high quality unique sequences were obtained. Annotation revealed 36% of sequences unrelated to genes in any database, suggesting that they were specific to *A. aquasalis*. A high number of sequences (59%) with no matches in any databases were found 24 h after infection. Genes related to embryogenesis were down-regulated in insects infected by *P. vivax*. Only a handful of genes related to immune responses were detected in our subtraction experiment. This apparent weak immune response of *A. aquasalis* to *P. vivax* infection could be related to the susceptibility of this vector to this important human malaria parasite. Analysis of some genes by real time PCR corroborated and expanded the subtraction results. Taken together, these data provide important new information about this poorly studied American malaria vector by revealing differences between the responses of *A. aquasalis* to *P. vivax* infection, in relation to better studied mosquito-*Plasmodium* pairs. These differences may be important for the development of malaria transmission-blocking strategies in the Americas.

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These authors contributed equally to this work.

Introduction

Malaria affects 300 million people worldwide every year. Among the endemic countries 22 are in the Americas, where transmission occurs mainly in the Amazon region. Brazil had an estimated 1.4 million malaria cases in 2006, over half of the total for the Americas, while Colombia had an estimated 408,000 cases ("WHO/HTM/GMP/2008.1"). The malaria parasites that circulate in this region are *Plasmodium falciparum*, *Plasmodium vivax* and *Plasmodium malariae* while the main vectors are *Anopheles darlingi*, *Anopheles aquasalis* and some species of the *Anopheles albitarsis* complex [1]. The ineffectiveness of vaccines, the resistance of *Plasmodium* to drugs and of insects to insecticides indicates the need for discovering new strategies to combat this disease. In some A*nopheline* species studies aiming at blocking malaria transmission have been successful [2].

The parasite responsible for the majority of malaria cases in the Americas is *P. vivax*. The mosquito *A. aquasalis* is an important American malaria vector that breeds in brackish coastal marsh habitats from Nicaragua to Southern Brazil [3–5]. The *Plasmodium sp.* life cycle in the insect vector is very complex. During 14 to18 days the parasite passes through various stages of development, and interacts with different tissues of the insect. The study of the reproduction and development of these parasites in their vector is essential for the development of new malaria control strategies. However, almost all previous studies are based on African and Asian anopheline species such as *Anopheles gambiae* infected with *P. falciparum* or *Plasmodium berghei* and *Anopheles stephensi* infected with *P. berghei* [6–7].

The main goal of this study is to analyze the effect of *P. vivax* infection on *A. aquasalis* gene expression. Due to the lack of a practical continuous cultivation system for *P. vivax* [8] insects used in these studies were fed on blood from *P. vivax* malaria patients.

Different subtraction mRNA libraries were constructed and analyses of these libraries revealed numerous mosquito genes that are up and down–regulated by infection. The analysis of these

Table 1. Data of subtraction libraries.

Library	Average length of inserts	Number of Sequences	Sequences with high quality	Unique Sequences
2hF-I	335 bp	606	285	180
2hI-F	236 bp	521	358	179
24hF-I	245 bp	376	234	73
24hI-F	365 bp	635	170	64
Total/Average	295 bp	2138	1047	496

F-I: libraries of cDNA after feeding minus after infection and I-F: libraries of cDNA after infection minus after feeding. h–Hours of feeding or infection.
bp: base pair.
doi:10.1371/journal.pone.0009795.t001

parasite induced *A. aquasalis* genes should lead to a better understanding of this vector-parasite relationship and to the identification of targets for blocking malaria transmission.

Results and Discussion

Subtraction experiments

Based on the precedent from anopheline studies [6–7] that *Plasmodium* infection can induce genes responsible for immunity, as well as more general aspects of stress, we constructed mRNA subtraction libraries with the aim of identifying genes regulated by this challenge. *A. aquasalis* were infected with *P. vivax* through artificial feedings with blood of malaria patients. All infected insect samples utilized in this study were tested for *P. vivax* infection by PCR and all of them amplified the expected 84 bp band (Figure S1).

Subtraction cDNA libraries were constructed for these studies from RNAs obtained from insects at 2 and 24 hours post-feeding on uninfected blood (F) or on infected blood (I). Four libraries were constructed using cDNAs from 2 and 24 hours infected minus non-infected insects (named 2hI-F and 24hI-F, respectively) and 2 and 24 hours non-infected minus infected insects (2hAF-I and 24hAF-I, respectively). The 2 and 24 hour timepoints were chosen for library generation since they represent the first stages of infection prior to parasite differentiation into oocysts, and thus should provide data most relevant to development of transmission blocking strategies. At 2 hours after infection (AI) the gametocytes differentiate and fertilization and zygote formation occur, and at approximately 24 hours AI the ookinetes pass through the midgut epithelium, a crucial and traumatic step in infection.

The amplicons obtained from these libraries (Figure S2) were cloned and 2,138 cDNA fragments were sequenced. A total of 1,047 high quality sequences were clusterized generating 496 unique fragments (Table 1). For protein function prediction, the sequences were compared to insects and plasmodia sequences, to curated databases and databases of conserved domains and orthologs. Of the total sequences, an elevated percentage had best matches with insect (98%) databases. A low number of sequences presented hit with *Plasmodium* database, and since these were related to very conserved sequences, they could be of mosquito origin. This absence of *Plasmodium* sequences can be

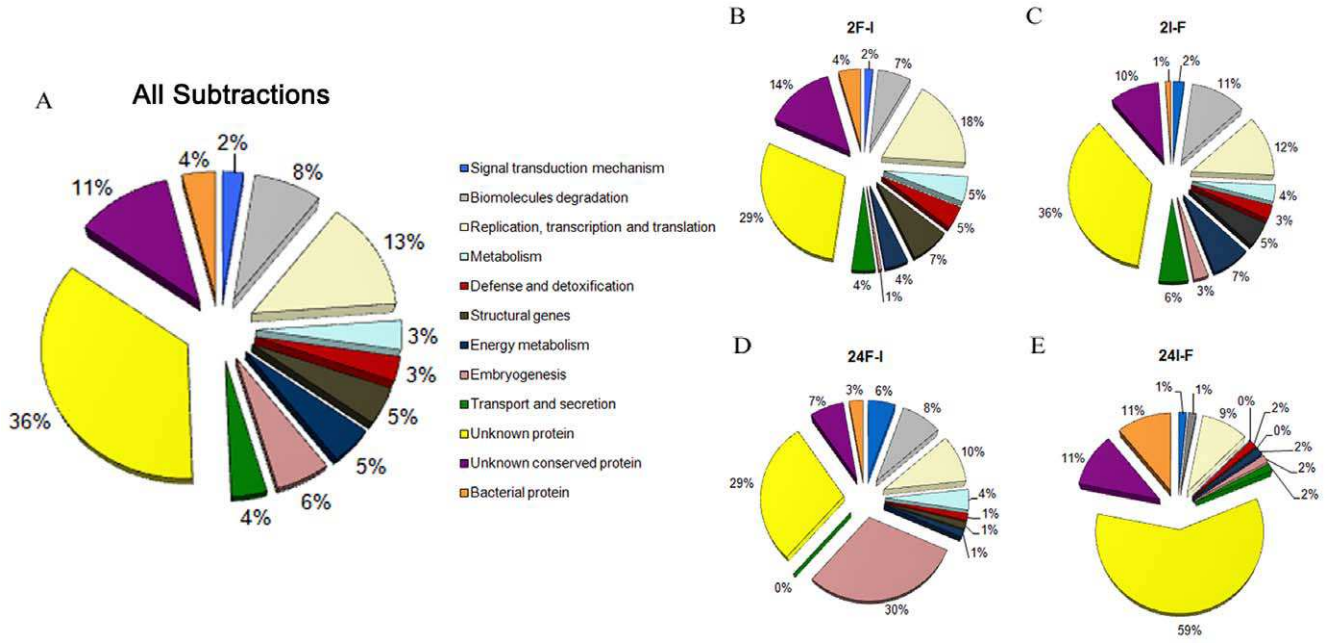

Figure 1. Overview of the functional breakdown of subtraction library ESTs based on blast similarities. Pie charts indicating the relative proportions of gene groups in all libraries (A), 2hF-I (B), 2hI-F (C), 24hF-I (D) and 24hI-F (E) libraries. Deduced proteins were grouped by similarity in predicted biological functions. The functional categories of genes and their corresponding colors are indicated.
doi:10.1371/journal.pone.0009795.g001

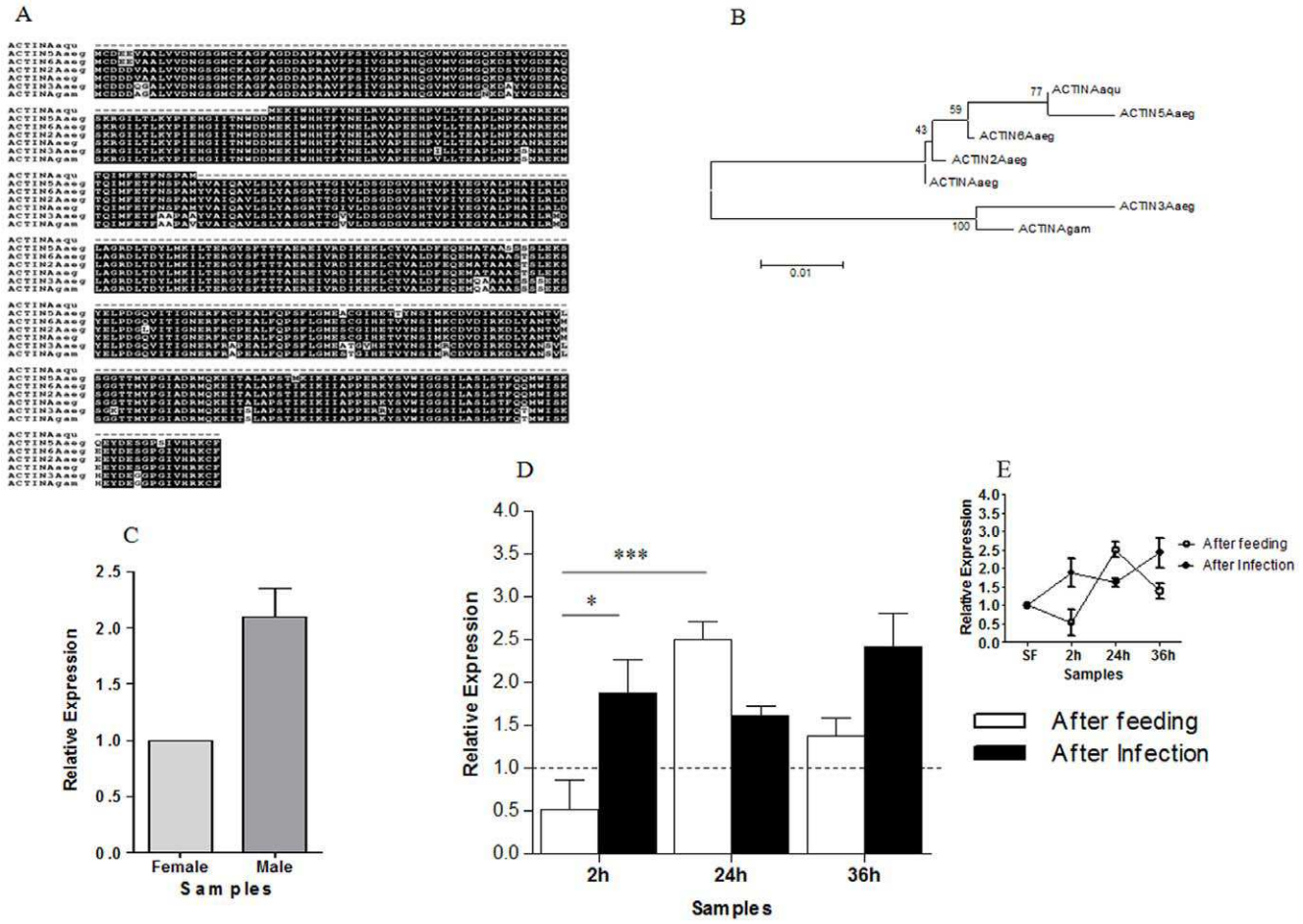

Figure 2. Characterization of actin induction in mosquitoes fed on sugar, blood, or infected blood. A: Multiple alignment of mosquito actin sequences. B: The phylogenetic tree was constructed using neighbor-joining. C–E: Expression levels of actin determined by RTPCR following various feeding regimens. C: Sugar-fed males and females, D and E: sugar-fed (dotted line and SF), blood-fed (AF) and *P. vivax* infected (AI) females. h–hours. Accession numbers of actin sequences from: *A. aquasalis* (Aaqu) (GR486917); *A. aegypti* (Aaeg) (ACTIN - EMBLEAT47188.1, ACTIN2 - EMBLAAQ24506.1, ACTIN3 - EMBLAAQ24507.1, ACTIN5 - EMBLAAY81972.1, and ACTIN6 - EMBLAAZ31061.1); and *A. gambiae* (Agam) (ACTIN - EMBLCAJ14142.1). +−: s.e.m.; * $p<0.05$, ** $0.03>p>0.01$, *** $p<0.01$.
doi:10.1371/journal.pone.0009795.g002

explained by the low parasite load observed in this natural vector-parasite pair or the early times of infection, before sporozoites amplification.

After annotation, sequences were divided into 13 different categories based on their biological functions (Figure 1A, Tables S1, S2, S3, S4). Altogether, 36% of the sequences did not present hits in any database. These could be specific for *A. aquasalis* or represent untranslated regions of genes poorly conserved among species. Also, 11% of the sequences coded for unknown conserved proteins, normally related with other mosquito sequences which might represent conserved genes useful in ample spectrum control strategies.

One major category of genes (59%) identified in the 24hI-F library codes for unknown proteins (Figure 1E). These genes may play as-of-yet unknown roles in the interaction of the mosquito with the parasite. Another important difference between the 24hI-F and the 24AF-I libraries was the number of embryogenesis-related genes, almost entirely composed by vitellogenin ESTs, which were down-regulated by the insect infection. While 30% of the sequences from the 24hF-I library were embryogenesis related, only 1.6% were found in the 24hI-F library (Figures 1D and E). Interestingly, some authors have described interference of *Plasmodium* infection on the mosquito reproductive fitness [9], which is consistent with these results. This may be due to the cost of building an immune response that leads to the functional tradeoff between mating and immunity [10]. Another possible explanation is a metabolic deficiency generated by the down modulation of genes related to energy metabolism. As an example, proline oxidase transcripts were only observed in the 2hF-I library (Table S1) [11]. Alternatively, parasite nutrient acquisition or expression alteration of some digestive enzymes, as observed for the chymotrypsin-like serine protease discussed below, could inhibit blood digestion and absorption of nutrients leading to decreased number of embryogenesis related genes expression.

Unexpectedly, only 3% of all ESTs (15 sequences) were related to immunity (Figure 1A), and no differences were observed in numbers of immune related genes among the libraries (Figures 1B-E). A feeble response to the parasite, as we report here, was seen in *A. stephensi* infected with *P. berghei* [6]. In contrast, a strong immune response was seen in the natural vector-parasite pair *A. gambiae-P. falciparum* [7]. The lack of a strong immune response of *A. aquasalis* in the early steps of *P. vivax* infection could be responsible for the success of colonization and transmission of this malaria parasite. These differences in the biology of Old and New World parasite-

vector interactions could well be due to genetic/evolutionary factors since, albeit belonging to the same genus, these species have evolved in distant parts of the world, submitted to very different environmental pressures. Still, we cannot exclude completely the possibility of the subtraction approach missing some immune genes. Future analyses using more sensitive mRNA abundance assays will be used to address this issue when genome sequences become available.

Validation of cDNA subtractions and analyzes of gene expression

To validate differential expression results a subset of the genes identified in the subtraction libraries were selected for analysis by real time PCR (RTPCR). In some cases, the expression of some of these genes was investigated in relation to blood-feeding, development, and comparing males and females. The majority of RTPCR experiments corroborated the subtraction library results. The few inconsistencies observed are related to the nature of the subtraction screening methodology. Nevertheless, these did not invalidate this approach, but rather confirmed its utility as a technique to identify differentially expressed sequences and rare transcripts [12].

Cellular structural genes

Among the genes related to cellular structure, we chose to characterize the expression of actin. Four actin ESTs were identified. Phylogenetic approaches showed that one of these (GR486917) was more closely related to actin5 of *Aedes aegypti* (Figures 2A and B). The expression of this gene was higher in males than sugar-fed (SF) females (Figure 2C). In females, expression increased after blood ingestion at 24 hours after feeding (AF) and returned to almost basal levels at 36 hours AF (Figures 2D and E), which may be due to the distention of the insect abdomen caused by ingestion of almost three parts of its weight in blood. Also, actin expression was higher at 2 h AI when compared with 2 h AF. This result is in agreement with the subtraction result, where all actin transcripts were found in 2hI-F library (Table S2). Most importantly, infection of mosquitoes with *P. vivax* increased slightly the expression of this gene between 2 and 36 hours AI (Figures 2D and E). This coincides with the passage of the *Plasmodium* ookinete between epithelial cells and establishment in the basal lamina of the epithelium. This parasite route has been already shown to disrupt and reorganize actin filaments, with expulsion of the infected cells to the midgut lumen, in other mosquito-*Plasmodium* models [13,14]. We suggest that this phenomenon can be the cause for the increase in actin mRNA levels in *A. aquasalis* after *P. vivax* infection. The actin gene, which has been widely used as a constitutive control in quantitative expression experiments [15], in *A. aquasalis* varied with feeding and infection, showing that this gene is not useful to normalize RTPCR, as had already been shown for *A. gambiae* [16].

Digestive enzymes

Based on the importance of digestive enzymes for insect reproduction and in premises that these enzymes may affect positively or negatively the parasite development and insect vector competence, the expression of two digestive enzymes, chymotrypsin-like serine protease and carboxypeptidase were evaluated.

Twelve serine proteases sequences were obtained through subtractions. Seven of these were found in the 2hI-F library, three in the 24hF-I library, one in the 24hI-F library and one in the 2hF-I library (Tables S1, S2, S3, S4). One chymotrypsin-like molecule (Figures 3A and B) from the 2hI-F library (GR486809) was evaluated by RTPCR. This gene was not expressed in immature stages and males. Phylogenetic analysis showed this gene to be related to digestive serine proteases, which was corroborated by up-regulated expression at 24 hours AF (Figures 3C and D). Interestingly, *P. vivax* infection decreased the expression of this gene (Figures 3C and D). In contrast, some previous work in other mosquitoes showed differences in digestive enzymes activity but not in mRNA levels after pathogen challenge [17,18]. The modulation of chymotrypsin-like serine protease transcription by the parasite could increase its survival and development in the insect, since early insect forms of *Plasmodium* are susceptible to protease digestion [19]. Parasite interference in the signaling pathways leading to transcription could explain this observation.

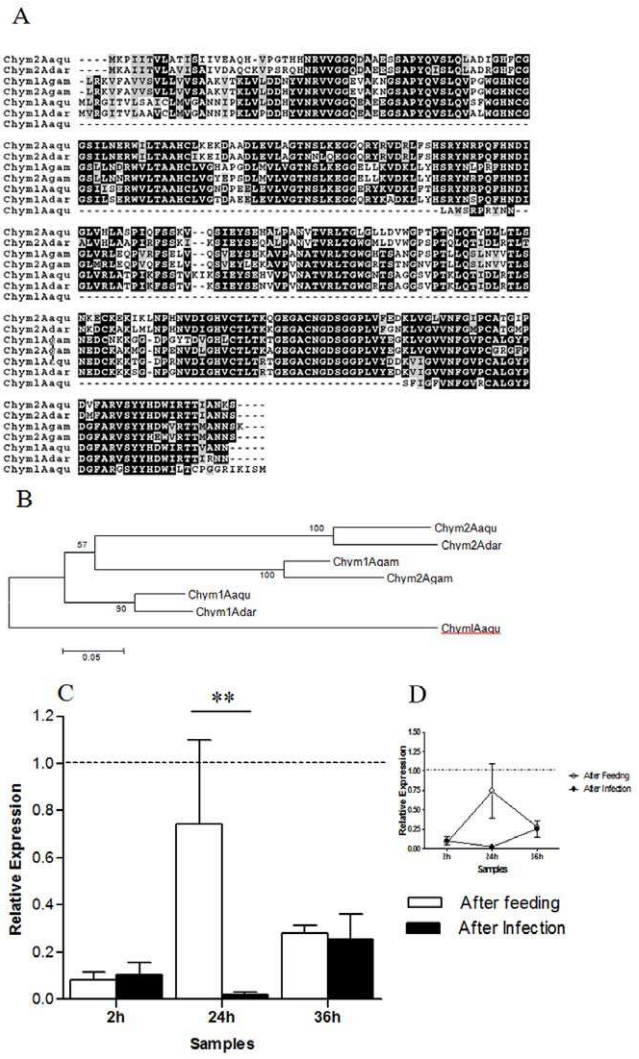

Figure 3. Characterization of chymotrypsin-like protease (Chymlp) induction in mosquitoes fed on sugar, blood, or infected blood. A: Multiple aminoacid sequence alignment of mosquito Chymlp sequences. B: Phylogenetic tree constructed using neighbor-joining method. C–D: Expression levels of Chymlp determined by RTPCR n sugar-fed (dotted line), blood-fed and *P. vivax* infected females. Chym–chymotrypsin, h–hours. Accession numbers of serine proteases from: *A. aquasalis* (Aaqu) (Chyml - GR486809, Chym1 - O97097, Chym2 - O97098); *A. darlingi* (Adar) (Chym1- O97099 and Chym2 - O97100); and *A. gambiae* (Agam) (Chym1 - ENSANGP00000024897, Chym2 - ENSANGP00000026162. +−: s.e.m.; * $p<0.05$, ** $0.03>p>0.01$, *** $p<0.01$.
doi:10.1371/journal.pone.0009795.g003

Recently, Brandon *et al.* [20] described that the target of rapamycin (TOR) kinase, which has been implicated in nutrient sensing, was involved in early trypsin transcription and synthesis in the mosquito *A. aegypti* midgut in response to feeding. Also, three sequences related to mosquitoes GATA transcription factor, final downstream step in the TOR pathway, were found in the 2hF-I library. In *A. gambiae* GATA has been associated with upstream sequences of the trypsin genes [21]. This pathway may also be related to the reduction of the infected insects' embryogenesis, since association between TOR pathway and vitellogenin production was described in *A. aegypti* [22].

Two carboxypeptidase clones representing non-overlapping regions of the same cDNA (GR486815 and GR486794) were found only in the 2hI-F library. This gene was more closely related to carboxypeptidase A than B based on phylogenetic analyses (Figures 4A and B). Expression was low in males in contrast with SF females (Figure 4C). No significant differences in mRNA levels were observed for this digestive enzyme in *A. aquasalis* AF and AI (Figures 4D and E). This is in contrast with results observed with carboxypeptidase B1 and 2 of *A. gambiae* that are up-regulated by *P. falciparum* [23], and with observations of blood meal induction of different classes of carboxypeptidase A and B of *A. aegypti* [24]. Lavazec *et al.* [2] demonstrated that antibodies against carboxypeptidase B reduced *P. falciparum* development in *A. gambiae*.

Immunity-related genes

Although we found few immunity related genes in our subtractions, those detected should be good candidates for the development of strategies to interrupt malaria transmission and were therefore further characterized. Three fibrinogen related sequences were identified in libraries 2hF-I (techylectin-like) and 2hI-F (ficolin and fibronectin-like). The expression of the first one (GR486377) related to mosquito and horse-crab techylectins (Figures 5A and B), a molecule important in mosquito immune activation [25], was evaluated. Fibrinogen was expressed in immature stages of *A. aquasalis*, mainly in first instar larvae (L1)

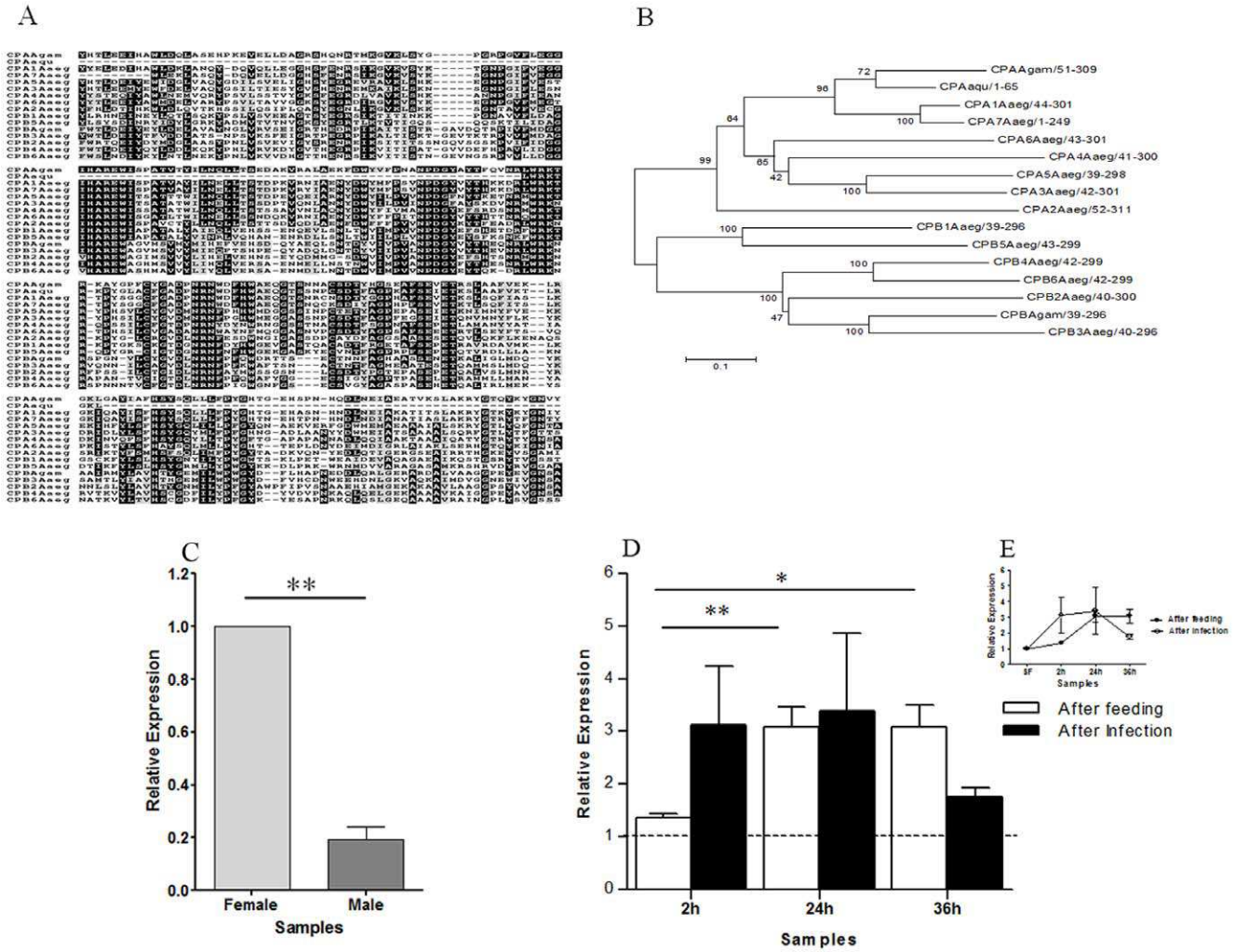

Figure 4. Characterization of carboxypeptidase induction in mosquitoes fed on sugar, blood, or infected blood. A: Multiple aminoacid sequence alignment of *A. aquasalis* and *A. gambiae* carboxypeptidases. B: Phylogenetic tree constructed using neighbor-joining method. C–E: Expression levels of carboxypeptidase determined by RTPCR following various feeding regimens. C: Sugar-fed males and females, D and E: sugar-fed (dotted line and SF), blood-fed (AF) and *P. vivax* infected (AI) females. h–hours. Accession numbers of carboxypeptidase sequences from: *A. aquasalis* (Aaqu) (CP–consensus of GR486815 and GR486794); *A. gambiae* (Agam) (CPA - AGAP009593 and CPB-CAF28572.1); and *A. aegypti* (CPA1-AAD47827.1, CPA2-AAT36726.1, CPA3-AAT36727.1, CPA4-AAT36728.1, CPA5-AAT36729.1, CPA6-AAT36730.1, CPA7-AAT36731.1, CPB1-AAT36735.1, CPB2-AAT36733.1, CPB3-AAT36734.1, CPB4-ABO21075.1, CPB5-ABO21076.1, and CPPB6-ABO21077.1). +−: s.e.m.; * $p<0.05$, ** $0.03>p>0.01$, *** $p<0.01$.
doi:10.1371/journal.pone.0009795.g004

and pupae (Figure 5C) and the expression in males was higher than in SF females (Figure 5D). No significant expression of this techylectin related protein was seen 2, 24 and 36 hours AF and AI in females (Figures 5E and F). Nevertheless, a modest two fold increase of mRNA expression was seen 36 hours AI. Similar results were seen in other mosquito species, where levels of proteins with fibrinogen domains were increased in *A. gambiae* immediately after challenge with Gram-negative plus Gram-positive bacteria, and 24 hours after *P. berghei* infection [26]. These levels were also increased 48 hours after infection of *A. stephensi* with *P. berghei* [6]. The increase of this gene expression 36h AI can be important for early oocysts recognition and insect immune activation.

A sequence with 94% similarity to bacteria responsive protein (BRP) (GR486800) was identified in the 2hI-F library. Phylogenetic analysis showed the deduced peptide to be more related with BRP2 than BRP1 of *A. gambiae* (Figures 6A and B). BRP proteins are modulated by bacterial and peptidoglycan challenges in *A. gambiae* [27]. BRP2 presented higher expression in males than SF females (Figure 6C) and was induced by *P. vivax* infection. The induction of BRP2 by *P. vivax* 24 and 36 hours AI (Figures 6D and E) indicates that the parasite passage and persistence in the basal lamina of the midgut could be stimulating the immune system. According to Shi and Paskewitz [27], BRP proteins may have a function in cell proliferation or regulation of immune cell migration and aggregation. Then, increased levels of BRP2 production in *A. aquasalis* may promote these secondary immune functions in haemolymph, in response to *P. vivax* presence.

Cecropin is a potent AMP with a wide range of antimicrobial targets. Two overlapping ESTs (GR486610 and GR486612) with high similarity (96%) to cecropin were found in the 2hF-I library. These two ESTs generated the complete open reading frame for an *A. aquasalis* cecropin protein (Fig. 7A). Phylogenetic analyses showed that this cecropin was closely related with cecropin 3 (or B) of *A. gambiae* (Figures 7A and B). Males presented more mRNA for cecropin than SF females (Figure 7C). In females, cecropin was up-regulated 2 hours after ingestion of both infected blood or uninfected blood and decreased at 24 and 36 hrs post blood feeding (Figures 7D and E). This induction may be a strategy to control the commensal bacterial burst induced by blood nutrients [28] due the negative regulation of reactive oxygen species production in response to heme toxicity [29]. Still, in *A. aquasalis* this gene was down-regulated 24 hours AI (Figures 7D and E), at a time when *Plasmodium* are passing through the mosquito midgut, strongly activating the immune system and producing AMPs in other mosquito species [30]. This cecropin down-regulation in *A. aquasalis* upon *P. vivax* infection is in contrast with findings in *A. aegypti* and *A. gambiae* infected with bacteria, filamentous fungi, yeast and *Plasmodium*, which cause an increase in AMP production [31-32]. *A. gambiae* transgenic mosquitoes overexpressing cecropin reduced *P. berghei* infection by 60% [33]. These observations suggest that the infection with *P. vivax* could be regulating the bacteria load in the insect or suppressing some signaling pathway, as NF-κB pathway, that leads to the increase of this AMP. The down-regulation of cecropin in *A. aquasalis* infected by *P. vivax* is probably important for parasite survival and development in the vector.

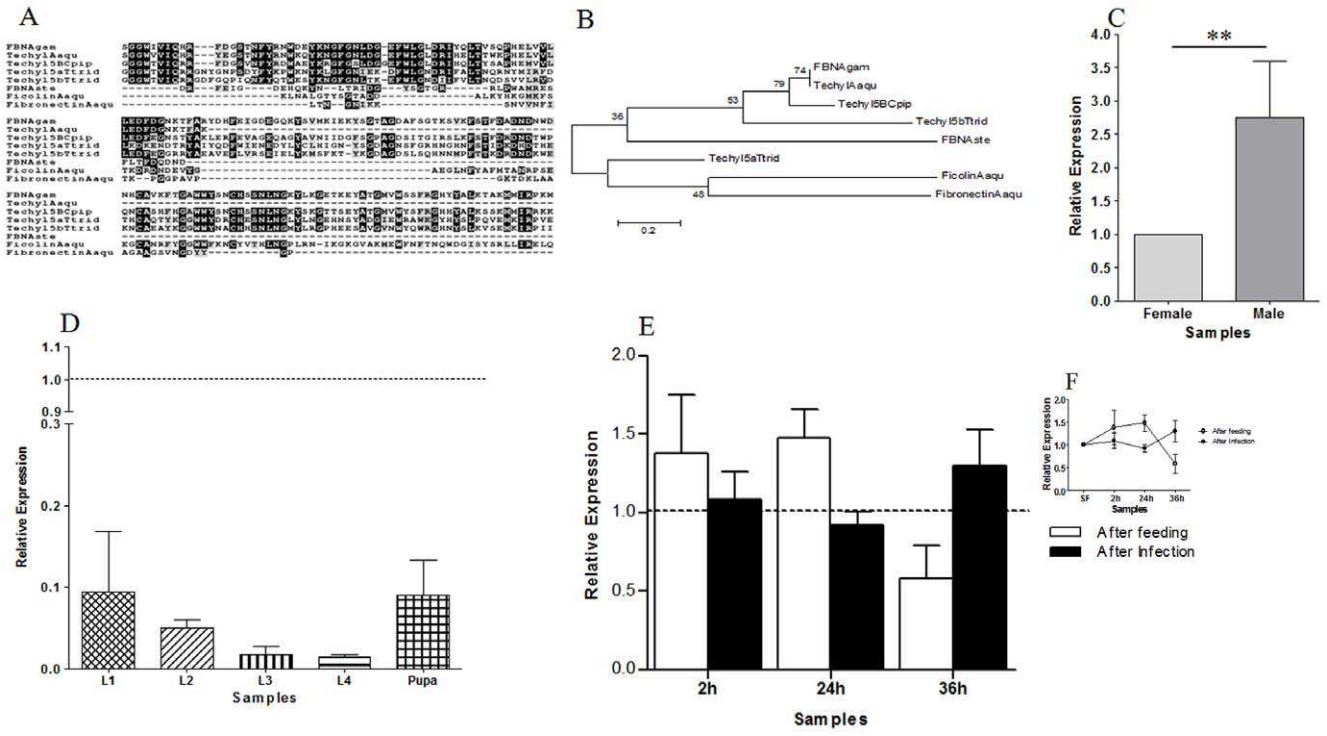

Figure 5. Characterization of techylectin related protein induction in mosquitoes fed on sugar, blood, or infected blood. A: Multiple aminoacid sequence alignment of mosquito fibrinogen related proteins. B: Phylogenetic tree for fibrinogen constructed based on the neighbor-joining method. C–F: Expression levels of techylectin related protein in *A. aquasalis* following different feeding regimens. C: sugar-fed males and females, D: immature stages, E and F: sugar-fed females (dotted line), and females after feeding and after *P. vivax* infection . h–hours, L1–first instar larva, L2–second instar larva, L3–third instar larva and L4–fourth instar larva. Accession numbers of fibrinogen sequences from: *A. aquasalis* (Aaqu) (Techyl - GR486377, Fibronectin - GR486898 and Ficolin - GR487133); *A. gambiae* (Agam) (FBN-AGAP004917-PA); *A. stephensi* (Aste) (FBN–CB602443), *C. pipiens* (Cpip) (Techyl5B - CPIJ000938); and *Tachypleus tridentatus* (Ttrid) (Techyl5a - AB024737 and Techyl5b - AB024738). +−: s.e.m.; * $p<0.05$, ** $0.03>p>0.01$, *** $p<0.01$.

doi:10.1371/journal.pone.0009795.g005

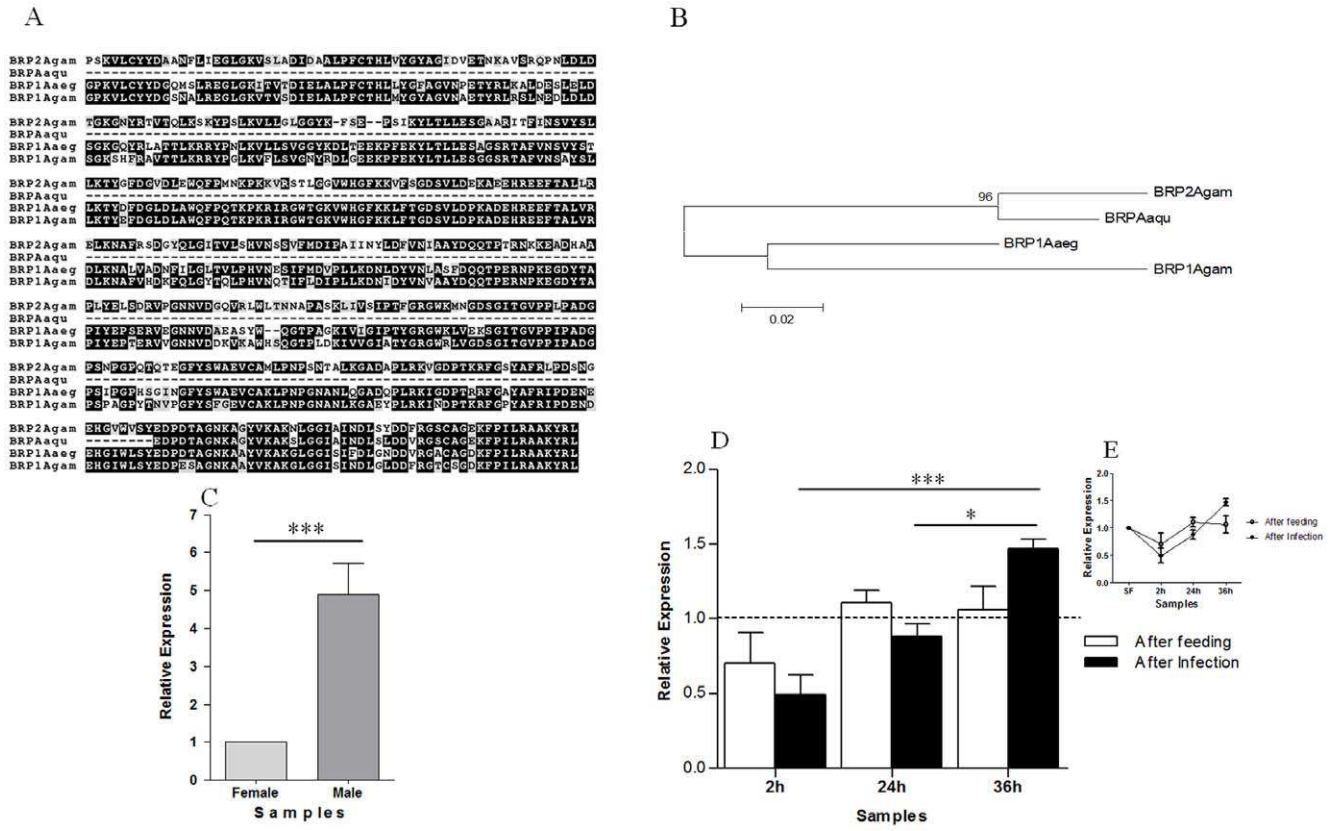

Figure 6. Characterization of BRP related protein induction in mosquitoes fed on sugar, blood, or infected blood. A: Multiple aminoacid sequence alignment of mosquito BRPs. B: Phylogenetic tree for BRP sequences based on the neighbor-joining method. C–E: Expression levels of BRP determined by RTPCR following various feeding regimens. C: Sugar-fed males and females, D and E: sugar-fed (dotted line and SF), blood-fed (AF) and *P. vivax* infected (AI) females. h–hours. Accession numbers of BRP sequences from: *A. aquasalis* (Aaqu) (GR486800); *A. gambiae* (Agam) (BRP1 - AGAP008061 and BRP2 - AGAP008060) and *A. aegypti* (Aaeg) (BRP1 - AAEL001965). +−: s.e.m.; * $p<0.05$, ** $0.03>p>0.01$, *** $p<0.01$.
doi:10.1371/journal.pone.0009795.g006

Serpins play a critical role in the regulation of invertebrate innate immune responses [34] and some serpins were shown to be important for the successful development of *Plasmodium* in the insect [e.g. 35]. A unique EST related to serpin was found in the 2hF-I library (GR486572). Blast results and phylogenetic approaches showed the *A. aquasalis* serpin to group with inhibitory proteins more related to the *A. gambiae* serpin 4 (Figures 8A and B), which, in this mosquito, was induced by *Staphylococcus aureus* and *Escherichia coli*, but not by *Plasmodium* infection [36]. In contrast, in *A. aquasalis*, the expression of this gene was higher 36 hours after *P. vivax* infection (Figures 8D and E). Expression was also higher in SF females than in males (Figure 8C). Our results indicate that increased expression of this serpin in *A. aquasalis* may be triggered by parasite passage through the midgut epithelium and the parasite exposure in the haemolymph, and that this increase can be responsible for infection success of *P. vivax* by the suppression of the mosquito immune response such as melanization. Functional studies need to be done to prove if this serpin acts as a negative immunomodulator. If *A. aquasalis* serpin indeed carries out a protective function for the parasite, this protein would be an ideal target for an anti-malaria strategy.

General comments and conclusions

RTPCR experiments revealed high expression of mRNAs for some digestion and immune genes in sugar-fed female and male *A. aquasalis*. Males presented more elevated levels of mRNA for immunity genes (BRP and cecropin) and lower levels of the negative immune regulator serpin than females, indicating that these insects are immunologically ready for the ingestion of contaminated sugar or other fluids. On the other hand, prior to blood feeding, females presented higher levels of mRNA for the digestive enzymes chymotrypsin-like serine protease and carboxypeptidase, possibly involved with digestion of blood necessary for egg maturation, than males.

Although blood was obtained from many different donors, and RNA was extracted from pools of insects, we cannot totally exclude the possibility, albeit improbable, that some of the gene expression differences observed were due to heterogeneity of the blood ingested by the insects or to anemic state commonly found in malaria patients [37].

Our present studies revealed new aspects of interactions between the important American vector *A. aquasalis* and its natural parasite *P. vivax*. Subtraction experiments and RTPCR showed that the early steps of *P. vivax* infection did not activate the *A. aquasalis* immune system as powerfully as observed in other mosquito-*Plasmodium* pairs. In the case of *A. aquasalis*, the presence of the parasite in insect haemolymph 36 hours after infection, rather than its presence in the midgut or during passage through its epithelium 24 hours after infection, appeared to correlate with the induction of a potent anti-microbial immune response. This information could contribute for the development of new strategies intended to interrupt the *P. vivax* cycle within its vector. Differences detected in relation to others malaria vectors might help direct new malaria transmission-blocking strategies specific

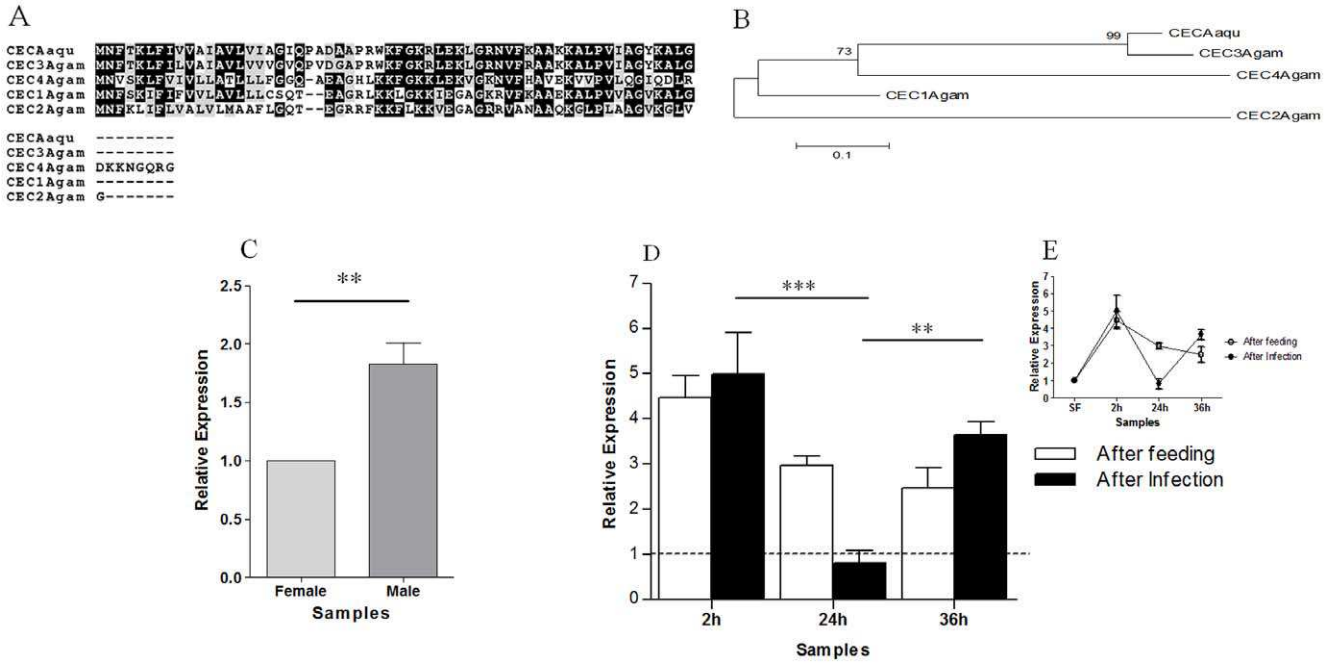

Figure 7. Characterization of cecropin induction in mosquitoes fed on sugar, blood, or infected blood. A: Multiple aminoacid sequence alignment of *A. aquasalis* and *A. gambiae* cecropin sequences. B: Phylogenetic tree constructed using neighbor-joining method. C–E: Expression levels of cecropin determined by RTPCR following various feeding regimens. C: Sugar-fed males and females, D and E: sugar-fed (dotted line and SF), blood-fed (AF) and *P. vivax* infected (AI) females. h–hours. Acession numbers of cecropin (cec) sequences from: *A. aquasalis* (Aaqu) (consensus of GR486610 and GR486612), and from *A. gambiae* (Agam) (Cec1 - AGAP000693-PA, Cec2 - AGAP000692-PA, Cec3–AGAP000694-PA and Cec4 - AGAP006722-PA). +−: s.e.m.; * $p<0.05$, ** $0.03>p>0.01$, *** $p<0.01$.
doi:10.1371/journal.pone.0009795.g007

for *A. aquasalis*. Garver *et al.* [38] showed differences of immune response of *A. gambiae* to *P. falciparum* and *P. berghei*, and also revealed some common aspects of the immune response of three anopheline species, *A. gambiae*, *A. stephensi* and *Anopheles albimanus* to a single *Plasmodium* species, *P. falciparum*. These observations demonstrate the importance of obtaining specific information on the various malaria parasite and vector pairs, since it apparently is impossible to predict their responses and outcome of infection.

Materials and Methods

Ethics statement

For the acquisition of *P. vivax* infected human blood, eight patients were selected among the people visiting the Health Center (Posto Estadual de Saúde da Vigilância em Saúde do Município de Iranduba, Distrito de Cacau Pirêra, Amazonas, Brazil) looking for malaria diagnosis and treatment during outbreaks. Diagnosis was performed by Giemsa stained blood smear. After positive diagnosis and visualization of gametocytes, patients were interviewed and inquired about the possibility of volunteer donation of a small amount of blood for research purposes. After verbal agreement, a term of consent was first read to the potential volunteers, with detailed verbal explanation, and, after final consent, signed by the patient. After this, one 200 µl sample of venous blood was drawn from each patient and placed in heparinized tubes. Blood samples were kept under refrigeration in an ice box (at approximately 15°C) for about 15 minutes, taken to the laboratory and used to feed *A. aquasalis*. Patient selection criteria were: to be *P. vivax* positive, to have about 4–8% of circulating gametocytes, determined by the National Institutes of Health international protocols, and to consent to be part of the research (consent form was approved by the Brazilian Ministry of Health, National Council of Health, National Committee of Ethics in Research (CONEP), written approval number 3726).

Insects and infection

A. aquasalis were reared at 27°C and 80% humidity. Insect infections were performed in an endemic area of Manaus, Amazonas state. Malaria patients were diagnosed by microscopic examination of Giemsa-stained blood smears. Infected or control blood was offered to the insects by artificial feeding at 37°C constant temperature, maintained using a water circulation system, to prevent exflagellation of microgametocytes. After the experimental feeding, the mosquitoes were kept in cages and given 20% sucrose *ad libitum*. Whole mosquitoes were separated in pools of 25 insects for subtraction experiments at 2 and 24 hours AF and AI, and at 2, 24 and 36 hours AF and AI were separated in pools of 5 insects for RTPCR. Infection was evaluated by PCR using a specific *Plasmodium* 18 s rRNA gene [39].

Subtraction experiments

RNA from *A. aquasalis* fed on human blood infected or not with *P. vivax* was extracted with TRIzol (Invitrogen), messenger RNA (mRNA) was purified using the NucleoTrap® mRNA Mini Purification Kit (Clontech) and cDNAs used for subtractions were synthesized using the PCR-Select™ cDNA Subtraction Kit (Clontech). Equal amounts of cDNA from each sample were pooled to construct the four libraries using cDNAs from 2 and 24 hours infected minus non-infected insects and 2 and 24 hours non-infected minus infected insects. After two PCR rounds, the amplicons were cloned into pGEM®-T Easy Vector (Promega) and utilized to transform high efficiency DH5-α *E. coli*. Sequencing of 2,138 selected clones was performed using an

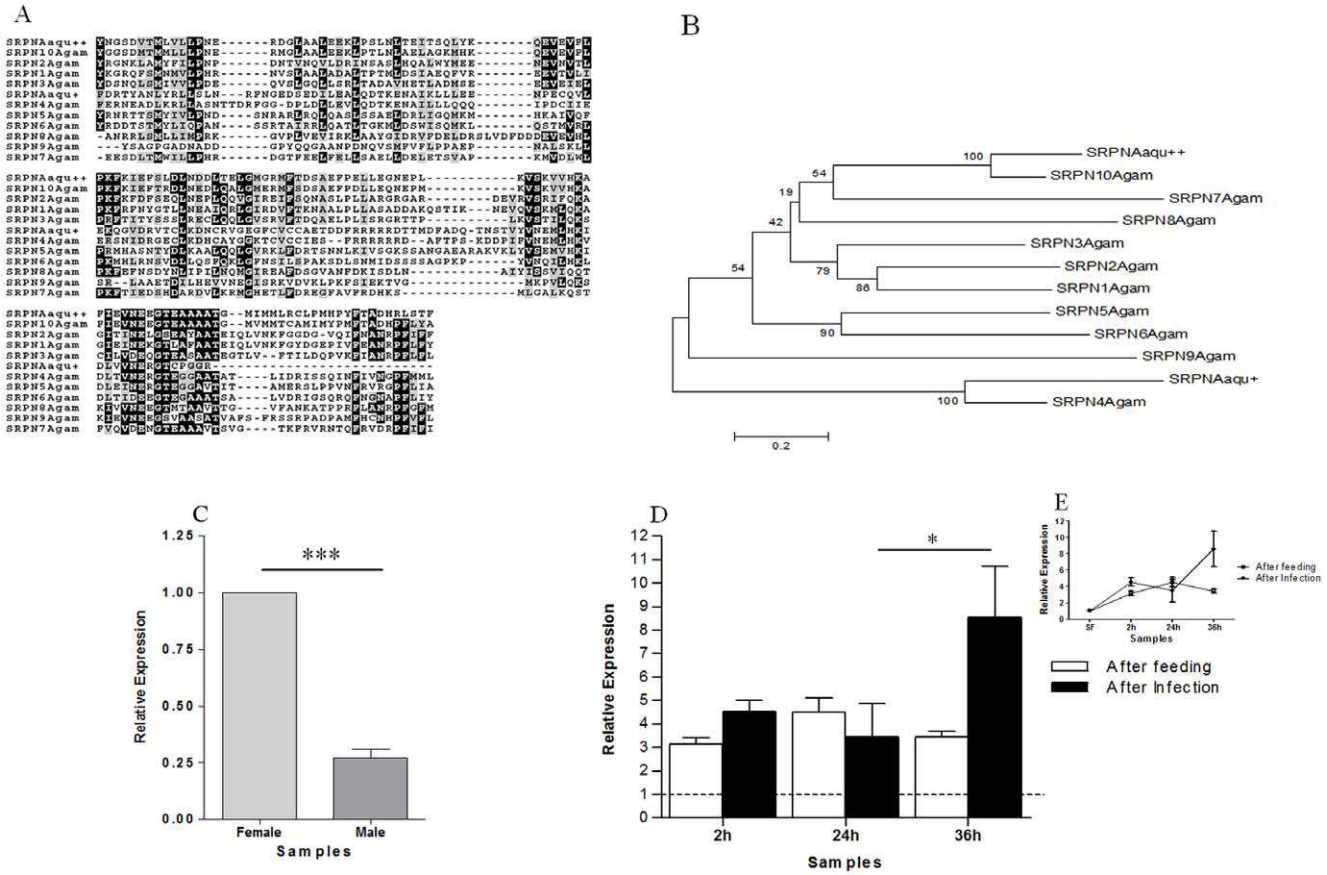

Figure 8. Characterization of serpin induction in mosquitoes fed on sugar, blood, or infected blood. A: Multiple aminoacid sequence alignment of *A. aquasalis* and *A. gambiae* serpin sequences. B: Phylogenetic tree constructed using neighbor-joining method. C–E: Expression levels of serpin determined by RTPCR following various feeding regimens. C: Sugar-fed males and females, D and E: sugar-fed (dotted line and SF), blood-fed (AF) and *P. vivax* infected (AI) females. A: Multiple aminoacid sequence alignment of *A. aquasalis* and *A. gambiae* serpin sequences. B: Phylogenetic tree was performed using neighbor-joining method. C - E: Relative expression of serpin in sugar-fed males and females (C); sugar-fed (dotted line), blood-fed and *P. vivax* infected females (D - E). h–hours. Accession numbers of serpin (SRPN) sequences from: *A. gambiae* (Agam) (SRPN1–AGAP006909-PA, SRPN2–AGAP006911-PA, SRPN3 - AGAP006910-PA, SRPN4–AGA009679-PA, SRPN5–AGAP009221-PA, SRPN6–AGA009212-PA, SRPN7AGAP007693-PA, SRPN8–AGAP003194-PA, SRPN9–AGAP003139-PA, and SRPN10–AJ420785). + Sequence of serpin found in the 2hAF-I library (GR486572). ++ *A. aquasalis* serpin sequence from GenBank, accession number EX809758. +−: s.e.m.; * $p<0.05$, ** $0.03>p>0.01$, *** $p<0.01$.
doi:10.1371/journal.pone.0009795.g008

ABI 3700 sequencer (Applied Biosystems) in the PDTIS/FIOCRUZ Sequencing Platform.

Sequences annotation

The sequences obtained were submitted to the STINGRAY platform (System for Integrated Genomic Resources and Analyses) (http://stingray.biowebdb.org/). Vector and primer sequences were trimmed and quality evaluated by Phred, Phrap and Consed programs. The sequences were clustered using the CAP3 program and clusters were searched for similarity using the BLASTN and BLASTX algorithm with sequences of insects, plasmodia and different databases (RefSeq, UniRef, UniProt, InterProt, KOG, COG, Prk, Smart and CDD). The cutoff e-values utilized were $\geq 10^{-5}$ for tBLASTX similarity and 10^{-10} for BLASTN. Sequences were annotated and grouped in functions. Nucleotide sequences have been submitted to Genbank and their respective accession numbers are indicated in Tables S1, S2, S3, S4.

RTPCR

RNA was extracted from whole insects submitted to different experimental conditions. The extracted RNA was treated with RQ1 DNAse free-RNAse (Promega) and utilized for cDNA synthesis. RTPCR reactions were performed using the SyberGreen fluorescent probe in an ABI 7000 machine (Applied Biosystems). The PCR cycles used were 50°C 2 min, 95°C 10 min, 95°C 15 sec and 63°C 1 min for 35 times for all reactions. Primer sequences and amplicon lengths are listed in Table S5. The relative expression of the selected genes was based on gene expression CT difference formula [40]. Quantifications were normalized in relation to the housekeeping gene rp49 [41]. All the experiments were performed using four to six biological replicates. The statistic method used in the analysis was ANOVA test with multiple comparisons of Tukey or Games-Howell. When the parametric model (ANOVA) was not adequate, we utilized the Kruskal-Wallis test with multiple comparisons of Mann-Whitney. For the male versus female analyses the t-student or the Wilcoxon tests were utilized. All tests were performed with reliable level of 95% ($\alpha = 0.05$). The statistical analyses were accomplished using the *Graph pad Prism5®*, R, software.

Sequence analyses

The sequences were aligned using the ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/) and presented with BOXSHADE

(http://bioweb.pasteur.fr/seqanal/interfaces/boxshade.html) programs. For phylogenetic analyses, the sequence alignments were examined with the Mega program (Molecular Evolutionary Genetics analysis, version 4) [42]. Relationships between the sequences were assessed by neighbor-joining method with amino acid distances with 1,000 replications in the bootstrap test.

Supporting Information

Figure S1 PCR to confirm A. aquasalis experimental infection with P. vivax. MW - molecular weight marker, Pv18s - P. vivax 18 s rRNA gene, I - infected insects, C- - negative control, C+ - blood of humans infected with P. vivax, I (25) - pool of 25 P. vivax infected insects and I (1) - one P. vivax infected insect.
Found at: doi:10.1371/journal.pone.0009795.s001 (0.69 MB TIF)

Figure S2 Differentially expressed products amplified after different subtractions (2hF-I 2hI-F, 24hF-I and 24hI-F). W - molecular weight marker, F-I - cDNA after feeding minus after infection and I-F - cDNA after infection minus after feeding. h - Hours of feeding or infection. bp - base pairs.
Found at: doi:10.1371/journal.pone.0009795.s002 (0.82 MB TIF)

Table S1 List of sequences from the 2 hours non-infected minus infected insects library. Sequences with significant similarity on BLASTN or BLASTX were grouped based on the function of the homologous protein.
Found at: doi:10.1371/journal.pone.0009795.s003 (0.29 MB DOC)

Table S2 List of sequences from the 2 hours infected minus non-infected insects library. Sequences with significant similarity on BLASTN or BLASTX were grouped based on the function of the homologous protein.
Found at: doi:10.1371/journal.pone.0009795.s004 (0.28 MB DOC)

Table S3 List of sequences from the 24 hours non-infected minus infected insects library. Sequences with significant similarity on BLASTN or BLASTX were grouped based on the function of the homologous protein.
Found at: doi:10.1371/journal.pone.0009795.s005 (0.13 MB DOC)

Table S4 List of sequences from the 24 hours infected minus non-infected insects library. Sequences with significant similarity on BLASTN or BLASTX were grouped based on the function of the homologous protein.Sequences with significant similarity on BLASTN or BLASTX were grouped based on the function of the homologous protein.
Found at: doi:10.1371/journal.pone.0009795.s006 (0.12 MB DOC)

Table S5 Primers used for quantitative PCR.
Found at: doi:10.1371/journal.pone.0009795.s007 (0.03 MB DOC)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: ACB NFS YMMTC PFP. Performed the experiments: ACB MSK. Analyzed the data: ACB AJT YMMTC PFP. Contributed reagents/materials/analysis tools: WDP WPT YMMTC PFP. Wrote the paper: ACB YMMTC PFP.

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Capítulo 2

A via de sinalização JAK-STAT controla a carga parasitária do *Plasmodium vivax* em etapas iniciais da infecção do *Anopheles aquasalis*

Submetido ao periódico PLoS Pathogens

Justificativa:

Os insetos possuem um sistema imune poderoso especializado em combater infecções. O sistema imune dos insetos é controlado por três grandes vias, Toll, IMD e JAK-STAT. A via JAK-STAT é muito conservada evolutivamente. Em humanos, existe evidência de que a desregulação desta via pode causar vários tipos de doenças. Em insetos, ela está associada a diversas funções, como desenvolvimento embrionário, manutenção da homeostase, regeneração e resposta imune. Trabalhos recentes com insetos têm descrito a ativação desta via após infecções por bactérias, protozoários e vírus. Devido à sua importância na imunidade de insetos, seu papel na imunidade de *A. aquasalis* contra *P. vivax* foi avaliado.

The JAK-STAT pathway controls *Plasmodium vivax* load in early stages of *Anopheles aquasalis* infection

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Abstract

Malaria affects 300 million people worldwide every year and 450.000 only in Brazil. In the Brazilian coast the main malaria vector is *Anopheles aquasalis,* and *Plasmodium vivax* is responsible for the majority of malaria cases in the Americas. Insects possess a powerful immune system to combat infections. Three pathways control the insect immune response: Toll, IMD and JAK-STAT. The main goal of this study is to analyze the immune role of the *A. aquasalis* JAK-STAT pathway after *P. vivax* infection. Three genes belonging to this pathway, the transcription factor Signal Transducers and Activators of Transcription (STAT), the regulatory Protein Inhibitors

of Activated STAT (PIAS) and the nitric oxide synthase enzyme (NOS) were characterized. Expression of STAT and PIAS was higher in males when compared to females and in eggs and first instar larvae when compared to larvae and pupae. RNA levels for STAT and PIAS increased 24 and 36 hours after *P. vivax* challenge. NOS transcription increased 36h after infection while the enzyme was already detected in some midgut epithelial cells 24 hours after infection. Imunocytochemistry experiments using specific antibodies showed that in non-infected insects STAT and PIAS were found mostly in the fat body, while in infected mosquitoes the proteins were dispersed along all body. The knock-down of *STAT* by RNAi increased the number of oocysts in the midgut of *A. aquasalis*. This is the first clear evidence for the involvement of a specific immune pathway in the interaction of the Brazilian malaria vector *A. aquasalis* with *P. vivax,* delineating a potential target for the future development of disease controlling strategies.

Keywords: *Anopheles aquasalis*, *Plasmodium vivax*, JAK-STAT pathway, STAT, PIAS, NOS, RNAi

Author Summary

Malaria is endemic in 22 countries in the Americas where *Anopheles aquasalis* is an important vector and *Plasmodium vivax* is responsible for most malaria cases. This natural vector-parasite pair is difficult to study due to the lack of a continuous cultivating system for *P. vivax*, and of genome data for *A. aquasalis*. Moreover, almost all previous studies are based on African and Asian anopheline species. Understanding the interaction mechanisms between mosquito vectors and plasmodia is important for the development of malaria control strategies. Our results showed that the JAK-STAT immune pathway is activated in *A. aquasalis* after *P. vivax* challenge and is important to maintain the low levels of *P. vivax* load observed in this vector. Our results add to the understanding of the *A. aquasalis* interaction with *P. vivax* and lead to possible explanations for this vector competence in *P. vivax* transmission. All information generated here may be used to direct the development of new or specific strategies to block malaria transmission by *A. aquasalis* in some parts of the Americas.

Introduction

Malaria is one of the most important parasitic diseases, affecting 300 million people worldwide every year and 22 countries in America. Brazil presents over half of the total estimated cases with numbers varying from 300 to 600 thousands over the past years [1]. The lack of effective vaccines, the development of drug resistance in *Plasmodium* parasites and of insecticide resistance in mosquitoes, have prevented the successful control of human malaria in many tropical regions. Understanding the biology of the interactions between mosquito vectors and *Plasmodium* is important to identify potential targets for the development of novel malaria control strategies to disrupt the development of *Plasmodium* in the insect vectors and prevent disease transmission to humans. The mosquito immune system limits parasite development and over-activation of some immune pathways has been shown to decrease *Plasmodium* infection [2, 3].

The insect immune system is very efficient in defending against a diversity of pathogens through multiple innate immune responses, which are also present in higher organisms [4]. Genetic studies in *Drosophila* identified four major signaling pathways that regulate expression of immune effector genes: TOLL, Immune deficiency (IMD), Janus kinase (JAK) and Signal Transducer and Activator of Transcription (STAT) pathways [5].

The JAK-STAT pathway was first described as a cytokine induced intracellular signaling pathway [6, 7]. This pathway is regulated very tightly by a series of activators and suppressors and in humans over-activation of this pathway has been associated with neoplastic transformation [8]. In *Drosophila*, the JAK-STAT pathway has been implicated in several cellular processes such as regeneration, homeostasis, eye development, embryonic segmentation, and participates in some cellular immune responses as differentiation of prohemocytes and hemocyte proliferation, as well as antibacterial responses [9-12]. Recent studies showed that the JAK-STAT pathway mediates *Anopheles gambiae* immune response to *Plasmodium berghei* and *Plasmodium falciparum* [13] and *Aedes aegypti* response to dengue virus II [14].

In *Drosophila melanogaster,* activation of the STAT pathway is initiated when the peptide ligand Unpaired (Upd) binds to the transmembrane receptor Domeless. This activates the JAK kinase Hopscotch to phosphorylate the transcription factor

Stat92E. The phosphorylated STAT protein forms a dimer, translocates to the nucleus and activates transcription of target genes [10].

This pathway is tightly regulated by various proteins, such as suppressor of cytokine signaling (SOCS) and protein inhibitor of activated STAT (PIAS). The SOCS gene is transcriptionally activated by the STAT pathway as part of a negative feedback loop that modulates STAT signaling by preventing STAT phosphorylation, while PIAS inhibits signaling by directly binding to STAT proteins and targeting them for degradation [15].

Anopheles aquasalis is an important malaria vector in the Brazilian coast. Although *P. vivax* infections accounts for more than 50 percent of malaria cases outside Africa and causes high morbidity in endemic areas, research on the biology and transmission of *P. vivax* has been neglected for several decades. This is mostly due to the lack of an efficient continuous cultivation system and to the belief that this parasite does not cause severe malaria [16, 17]. Although it has long been considered a benign infection, it is now accepted that *P. vivax* can cause severe and even lethal malaria [18].

We cloned and characterized three genes from the JAK-STAT pathway: the transcription factor *STAT*, the *PIAS* regulatory proteins and the enzyme *NOS*. The main goal of this study is to determine whether the JAK-STAT pathway is activated in *A. aquasalis* mosquitoes in response to *P. vivax* infection and, if so, whether this response limits *Plasmodium* infection.

Results

Identification and characterization of *Anopheles aquasalis* STAT and PIAS

Two genes of the JAK-STAT pathway of *A. aquasalis*, the transcription factor *STAT* (AqSTAT) and its regulatory protein *PIAS* (AqPIAS) were amplified by PCR, using degenerate primers and genomic DNA as template. The 1150bp (STAT) and 891bp (PIAS) PCR fragments were cloned and sequenced. After *in silico* predictions of exons and introns, 836bp and 549bp coding sequences were obtained for STAT and PIAS, respectively. These sequences were used to design perfect-matching primers and the SMART RACE technique was used to obtain the complete cDNA sequences of these two genes using a mixture of cDNAs from males and infected

and non-infected females as template. A full-length AqSTAT cDNA sequence of 1599bp was obtained, consisting of a 1491bp open reading frame (ORF) coding for a 497 amino acid residues protein, plus a 108bp 3' untranslated region (UTR) (Figs. S1 and 1A). The full-length AqPIAS cDNA consists of 2407bp including a 1953bp ORF, which encodes a protein of 651 amino acid residues, as well as a 211bp 5´ UTR and 243bp 3´ UTR (Figs. S2 and 2A). These two sequences were deposited in GenBank with accession numbers HM851178 and HM851177, respectively.

The schematic organization and the deduced amino acid residues of AqSTAT are shown in Fig. 1A. Sequence analyses and comparison with other mosquitoes STAT showed that AqSTAT presents the SH2 domain, the STAT binding domain and a portion of the alpha domain, but lacks the STAT interaction domain (Fig. 1A). Phylogenetic approaches showed that AqSTAT grouped with STATs from other mosquitoes and was more closely related to *A. gambiae* STAT-A (the ancestral gene) than to STAT-B (a gene duplication that probably resulted from a retro-transposition even) (Figs. 2B and C). AqPIAS presents two very conserved domains, the SAP domain and the MIZ/SP-RING zinc finger domain (Fig. 2A). The deduced AqPIAS protein has higher homology to putative ortholog genes from other mosquitoes that to those of other insects, such as *D. melanogaster* and *Appis mellifera.*

Gene expression of the AqSTAT and AqPIAS investigated by RTPCR revealed that these genes are expressed in all mosquito developmental stages and in both genders in adult insects. STAT is highly expressed in eggs (Fig. 3A), while PIAS has high levels of expression in both eggs and first instar larvae (Fig. 4A). In adult stages, both STAT and PIAS were expressed at higher levels in males than in females (Figs. 3A and 4A). We then investigated the effect of *P. vivax* infection on expression of these two genes. To circumvent the inability to culture *P. vivax*, all mosquitoes used in these studies were fed on blood from human donors infected with *P. vivax* malaria. Both STAT and PIAS genes were transcriptionally activated by *P. vivax* infection by 24 and 36 hours post-infection (hpi). This induction was transient and was no longer observed by 48 hpi (Figs. 3B and 4B). Furthermore, PIAS protein expression was also induced in protein homogenates obtained from infected females 24 and 36 hpi (Fig. 4C).

Identification and characterization of the effector gene nitric oxide synthase

A 702 bp cDNA fragment of *A. aquasalis* NOS (AqNOS) was cloned using degenerate primers and sequenced. This fragment is part of the nitric oxide synthase domain of NOS proteins (Fig. 5A). The *A. aquasalis* NOS is closely related to mosquitoes NOS (Fig. 5B-C). This sequence was deposited in GenBank with accession number HM851179. NOS mRNA expression is higher in sugar-fed males than in females (Fig. 6A) and is induced by *P. vivax* infection 36 hpi (Fig. 6B). Immunocytochemistry of *A. aquasalis* midguts infected with *P. vivax* 24 hpi revealed high levels of NOS expression in the cytoplasm of some epithelial cells when compared to the sugar-fed insects (Fig. 6C and D).

Immunocytochemistry location of STAT and PIAS

To reveal the tissues responsible for the expression of STAT and PIAS, immunocytochemistry experiments were carried out. Antibodies against STAT and PIAS were incubated with tissue sections of *A. aquasalis* submitted to different conditions. While males presented an elevated expression of these proteins in all body parts, with stronger expression in the fat body (Fig. 7), expression was lower in sugar-fed females. In blood-fed females, as in males, the expression of both proteins was mainly in the fat body, although some expression was also observed in eggs (Figs. 8-10). When the insects were infected with *P. vivax* the levels of these proteins increased and were located in dispersed cells distributed through almost all insect parts and organs (Figs. 8-10). This corroborated our mRNA and protein expression results.

Silencing of STAT

To test whether activation of the JAK-STAT pathway limits *P. vivax* infection in *A. aquasalis*, the effect of silencing the transcription factor AqSTAT by systemic injection of double strand RNA (dsRNA) was evaluated. As a control, females were injected with ß-galactosidade dsRNA (dsß-gal), a gene not present in the mosquito genome. STAT expression was greatly reduced (70%) in mosquitoes injected with STAT dsRNA (dsSTAT), relative to those injected with dsß-gal (Figs.11A and B). This effect was already observed 1 day post-injection and was still present 5 days post-injection. Mosquitoes were infected with *P. vivax* two to three days after dsRNA

injection. Three to five days after infection, the guts were dissected and the oocysts were counted. These experiments revealed that reducing expression of the STAT gene increased the proportion of infected *A. aquasalis* females as well as oocysts density (Figs. 11C, D and E).

Discussion

The JAK-STAT pathway is very conserved among species all the way from insects to humans. This pathway is important in insect immune response against some pathogens as bacteria [19-23], virus [14] and *Plasmodium* [13]. A single STAT gene (STAT92E) was found in *Drosophila* as well as several other components of this signaling pathway such as: two homologous receptor ligands (*Upd2* and *Upd3*), a membrane receptor (*Domeless*) and a JAK-kinase homologue (*Hopscotch*) [10]. Some JAK-STAT repressors have also been characterized in *D. melanogaster*, as for example SOCS (*SOCS36E*) [24] and PIAS (*dPIAS*) [25]. Bioinformatic analysis of the *A. aegypti* and *A. gambiae* genome sequences revealed the existence of Domeless, Hopscotch, STAT, PIAS and SOCS orthologs in these two mosquito species [14, 26]. All dipteran insects examined so far have a single STAT gene, except for *A. gambiae*, in which two functional genes (AgSTAT-A and AgSTAT-B) have been characterized [13]. The AgSTAT-A gene is ancestral and is the putative ortholog of STAT genes from other insects; while AgSTAT-B is an intronless gene that is evolving fast and appears to be the result of a retro-transposition event in which an AgSTAT-A cDNA was re-inserted back into the genome. Interestingly, AgSTAT-B regulates transcription of AgSTAT-A in adult stages and is the only STAT gene expressed in pupae [13].

In this work, three genes of the JAK-STAT pathway of *A. aquasalis*, the transcription factor STAT, its regulatory protein PIAS, and NOS were cloned, sequenced and characterized. The domain organization of the PIAS protein is very similar to that of the *A. gambiae* and *A. aegypti* orthologs. The deduced *A. aquasalis* STAT, on the other hand, lacks some of the N-terminal conserved domains present in *A. gambiae, A. aegypti* and *Drosophila* STATs. It is probably the product of alternative splicing, as a similar cDNA (ΔN-STAT92), giving rise to a protein that lacks 113 aa at the N–terminus, has been characterized in *Drosophila* [27].

AqSTAT and AqPIAS are expressed in all insect stages and both in males and females. The high expression in eggs and first instar larvae may be indicating that, as in *D. melanogaster* [28, 29], the JAK-STAT pathway in *A. aquasalis* may also participate in embryogenesis. The expression pattern of AqSTAT in adult stages is very similar to *A. gambiae* STAT-A [13], as in both anophelines males express higher STAT mRNA levels than sugar-fed females. In *A. gambiae*, AgSTAT-A expression remained unchanged 24 hours after infection with *P. berghei* [13]. In contrast, AqSTAT expression was activated transiently by *P. vivax* infection at 24 and 36 hpi. AqPIAS presented similar mRNA expression pattern as AqSTAT and the induction of these two genes suggests that the JAK-STAT pathway is activated in response to *P. vivax* infection. The induction of PIAS protein expression corroborated the transcriptional results and provided direct evidence that the JAK-STAT pathway is also carefully regulated in *A. aquasalis.* Silencing AgSTAT-A in *A. gambiae* females infected with *P. berghei* reduced the number of early oocysts present 2 days post-infection, nevertheless enhancing the overall infection by increasing oocyst survival [13]. AqSTAT silencing also increased the number of oocysts present, but its effect on very early stages of infection remains to be established. The peak transcriptional activation of the JAK-STAT pathway 36 hours after infection was similar to what has been observed for other immune genes such as serpins, bacterial responsive protein and fibrinogen [30], indicating that the immune system is activated at the time when *Plasmodium* parasites have invaded the midgut and come in contact with the mosquito haemolymph.

In vertebrates, STAT1 regulates nitric oxide synthase (NOS) expression [31]. DNA sequences capable of binding to STAT and NF-κB have been described in the regulatory regions of the NOS gene in *Anopheles stephensi* [32]. In *A. gambiae*, AgSTAT-A participates in the transcriptional activation of NOS in response to bacterial and plasmodial infections, NOS expression being activated by *P. berghei* 24 hpi [13]. In *A. aquasalis*, we observed high levels of NOS expression at a later time (36 hpi) in response to *P. vivax*. Luckhart *et al.* [33, 34] detected an increase in *A. stephensi* midgut NOS mRNA at several times (6, 24, 48 and 72h) after *P. berghei* infection. In *A. gambiae* infected with *P. falciparum* induction of NOS mRNA was also observed [35]. High expression of NOS protein was also seen in the cytoplasm of some midgut cells of *A. aquasalis* 24 hpi. These observations suggest that activation

of the JAK-STAT pathway may be regulating NOS expression and that NO may be an important mediator of the antiplasmodial response.

In some models of vector-parasite interaction, as *A. stephensi-P. berghei*, insect midgut cells suffer damages after parasite invasion. Among these are protrusions toward the lumen, loss of microvilli, induction of NOS and production of NO, which is converted into nitrite and then into NO_2, and causes protein nitration that leads to cell death [36, 37]. This epithelial immune response is important to control the parasite number and in some cases can be decisive for clearance of infection. Nevertheless, this mechanism is not universal, as induction of NOS and peroxidase activities were not observed in other vector-parasite combinations such as *A. aegypti–Plasmodium gallinaceum* and *A. stephensi–P. gallinaceum* [38].

Immunocytochemistry revealed that *A. aquasalis* STAT and PIAS not only had concomitant expression but also were expressed in the same tissues. The expression of these proteins in sugar-fed males and females was mostly observed in the fat body, but males presented stronger labeling than females. This corroborated the role of the fat body as the main immune organ of the insects. The high expression in males is in agreement with our previous results for other *A. aquasalis* immune genes such as fibrinogen, bacteria responsive protein and cecropin [30]. This seems to indicate that male mosquitoes are more prepared for eventual challenges, as opposed to what was observed in vertebrates and some invertebrate species where females are more immunocompetent than males [39]. The expression of this protein also presented differences between non-infected and infected insects. The non-infected insects were immunologically marked mainly in the fat body while the infected ones were marked in dispersed cells along all body and in the ingested blood. This pattern of expression of proteins from the JAK-STAT pathway demonstrated that *A. aquasalis* is producing a systemic immune response against *P. vivax.*

Our results showed that the *A. aquasalis* JAK-STAT pathway is activated in response to *P. vivax* challenge. Furthermore, preventing activation of the JAK-STAT pathway by silencing the AqSTAT transcription factor increased the infection, as well as the number of *P. vivax* oocysts in *A. aquasalis* mosquitoes. These results confirm the role of the JAK-STAT in limiting *P. vivax* infection of *A. aquasalis*. Enhancing

these responses by using a transgenic approach may be effective in preventing *P. vivax* malaria transmission to humans by *A. aquasalis* mosquitoes.

Material and Methods

Ethics Statement

For the acquisition of *P. vivax* infected human blood, patients were selected among people visiting the Health Center (Posto Estadual de Saúde da Vigilância em Saúde do Município de Iranduba, Distrito de Cacau Pirêra, Amazonas, Brazil) looking for malaria diagnosis and treatment during outbreaks. Diagnosis was performed by Giemsa stained blood smear. After positive diagnosis and visualization of gametocytes, patients were interviewed and inquired about the possibility of volunteer donation of a small amount of blood for research purposes. After verbal agreement, a term of consent was first read to the potential volunteers, with detailed verbal explanation, and, after final consent, signed by all patients involved in the study. After this, 200 microliters of venous blood was drawn from each patient and placed in heparinized tubes. Blood samples were kept under refrigeration in an ice box (at approximately 15^{o}C) for about 15 minutes, taken to the laboratory and used to feed *A. aquasalis*. Patient selection criteria were: to be *P. vivax* positive, to have about 4-8% of circulating gametocytes, determined by the National Institutes of Health international protocols, and to consent to be part of the research. The study, including its consent form, was approved by the Brazilian Ministry of Health, National Council of Health, National Committee of Ethics in Research (CONEP), written approval number 3726).

Insect infection

A. aquasalis were reared at 27º C and 80% humidity [40]. Insect infections were performed in an endemic area of Manaus, Amazonas state, as described in Bahia *et al.* [30]. Infected or control blood were offered to the insects by artificial feeding at 37º C constant temperature, maintained using a water circulation system, to prevent exflagellation of microgametocytes. After the experimental feeding, the mosquitoes were kept in cages and given 20% sucrose *ad libitum*.

PCR using degenerate primers

PCR reactions were performed as described using degenerate primers designed on conserved regions of *STAT* and *PIAS*, based in sequences of *A. gambiae*, *A. stephensi*, *A. aegypti* and *D. melanogaster* [19]. The PCR cycles used were: two cycles (1 min steps at 95º C, 55º C and 72º C, and 95º C, 42º C and 72º C) followed by 30 cycles at moderate stringency (1 min steps at 95º C, 52º C and 72º C) and a final 7 min extension at 72º C. All amplicon generated were cloned into pGEM®-T Easy Vector (Promega) and utilized to transform high efficiency DH5-α *Escherichia coli*. Sequencing of the selected clones was performed using an ABI 3700 sequencer (Applied Biosystems) in the PDTIS/FIOCRUZ Sequencing Platform.

RACE

The SMART cDNA RACE amplification kit (Becton Dickinson Clontech) was used to obtain the 5' and 3' ends of the PIAS and STAT cDNAs. All amplicons generated were cloned and sequenced as described above. After sequencing, the cDNAs of STAT and PIAS were assembled using the CAP3 Sequence Assembly Program (http://pbil.univ-lyon1.fr/cap3.php) and aligned with other insect sequences with the Clustal W Program (http://www.ebi.ac.uk/Tools/clustalw2/).

Real Time PCR (RTPCR)

RNA was extracted from whole insects submitted to different experimental conditions [immature stages – egg, first to fourth instar larvae and pupa; sugar-fed males and females; females fed on blood and blood from *P. vivax* malaria patients]. The extracted RNA was treated with RQ1 RNAse-free DNAse (Promega) and utilized for cDNA synthesis. RTPCR reactions were performed using the SyberGreen fluorescent probe employing an ABI 7000 machine (Applied Biosystems). The PCR cycles used were 50º C 2 min, 95º C 10min, 95º C 15 sec and 63º C 1 min for 35 times for all reactions. The primer sequences were: STATFwd 5' CTGGCGGAGGCGTTGAGTATGAAAT 3' and STATRev 5' CGGATAAGGAAGGC TCGTTTTGAAT 3', PIASFwd 5' TAGCAGCTCACAGTATAGCCTCGAT 3' and PIASRev 5' TCCCATTCCAACCAACAAACCA 3', and NOSFwd 5' AGGATCTGGCC CTCAAGGAAGCCGA 3' and NOSRev 5' ATCGTCACATCGCCGCACACGTACA 3'. The relative expression of the selected genes was based on gene expression CT

difference formula [41]. Quantifications were normalized in relation to the housekeeping gene rp49 [42]. All the experiments were performed using four to six biological replicates and three experimental replicates. The statistics method used in the analyses was ANOVA test with multiple comparisons of Tukey or Games-Howell. When this parametric model was not adequate, we utilized the Kruskal-Wallis test with multiple comparisons of Mann-Whitney. Bonferroni correction was used when necessary. All tests were performed with reliable level of 95% (a= 0.05). The statistical analyses were accomplished using the GraphPad Prism5® and R 2.9.0.

Immunocytochemistry

Sugar-fed male and female *A. aquasalis* submitted to different treatments (sugar-feeding, infected and non-infected blood-feeding) were collected, had their heads, legs and wings removed and were fixed overnight at 25º C in 4% paraformaldehyde in PBS. The insects were dehydrated in 30% to 100% ethanol, and then infiltrated with hystoresin kit (Leica) at room temperature for 5-7 days. Hystoresin-embedded mosquitoes were sectioned using a rotary microtome to obtain 3 µm sections that were adhered to slides. Slides were dried, blocked for 20 minutes in PBS BSA 1% and 20 minutes in RPMI medium. Sections were then incubated overnight with 1:250 anti-rabbit STAT or PIAS antibodies diluted in PBS/BSA 1%. After that, the tissue sections were washed 5-8 times with PBS/BSA 1% and then incubated with rabbit secondary antibody conjugated to FITC (Molecular Probes), diluted 1:250 in blocking solution. After two washes in PBS, the slides were mounted using Mowiol anti-photobleaching Mounting Media (Sigma Aldrich). Immunostaining was analyzed with a confocal laser microscope (LMS 510). Photos are representative of at least five mosquitoes for each treatment.

Alternatively, guts of females 24 hours after infection were dissected and fixed for 20 minutes in 4% paraformaldehyde in PBS at 4^0 C. After this, the insect guts were blocked for 20 minutes in PBS BSA 1% followed by 20 minutes of a new block in RPMI medium. Then, the guts were incubated with commercial anti-NOS antibody (Sigma Aldrich SAB4300426) diluted 1:250 in PBS BSA 1%. Five washes were performed and the guts were incubated with anti-rabbit conjugated to Alexa 594 also diluted 1:250 in PBS BSA 1%. Five more washes with PBS were performed before the mounting of the guts in slides with Mowiol. The same steps were performed in the

control samples, except for the incubation with the primary antibody. The material was analyzed by confocal laser microscopy.

Western Blot

Proteins of whole insects submitted to different feeding regimens (sugar-fed males and females, and females after different times of blood-feeding and infection) were extracted by Trizol Reagent (Invitrogen) following the manufacturer's protocol. Samples corresponding to one insect were separated on 12% SDS-PAGE gels and subsequently transferred to Hybond nitrocellulose membranes. The membranes were blocked with 5% non-fat milk TBS Tween 20 0.1% (TBST) for at least one hour. The membranes were then incubated with anti-PIAS antibody at a 1:250 dilution for two hours. After three washes of 10 minutes in TBST, the membranes were incubated with anti-rabbit secondary antibody at a 1:80.000 dilution for one hour. Three more washes were performed before the incubation of the membrane with the detection system Pierce SuperSignal West Pico chemiluminescent substrate (ThermoScientific).

Gene silencing

Double stranded RNAs for *STAT* and ß-*ga*l were produced from PCR-amplified fragments using the T7 Megascript kit (Ambion). Amplicons for dsß-gal were produced using plasmid templates and for dsSTAT by reverse transcriptase PCR (RT-PCR) products, from sugar-fed female cDNA, giving rise to 544 bp and 503 bp fragments, respectively. Two rounds of PCR were performed to amplify ß-gal. The first PCR round was performed with primers containing a short adaptor sequence at the 5' end (tggcgcccctagatg). The primers used for the first round of PCR were ß-galFwd 5'tggcgcccctagatgTGATGGCACCCTGATTGA 3' and ß-galRev 5' tggcgcccctagatgTCATTGCCCAGAGACCAGA 3'. The PCR cycles utilized were 95º C for 3 min, 35 cycles of 95º C for 30 s, 57º C for 45 s and 72º C for 45 s followed by 72º C for 7 min. Two microliters of the first PCR were used in the second PCR reaction. The second round of PCR was utilized to insert the bacteriophage T7 DNA-dependent RNA polymerase promoter to the DNA templates. The second round of PCR utilized the same conditions of the first reaction. The second round PCR primer, which has the T7 (bold letters) and the adaptador sequences, was 5'

ccg**TAATACGACTCACTATAGG**tggcgcccctagatg 3'. STAT amplification was performed in one round of PCR, which also inserted the T7 sequence. The STAT primer used was STATFwd 5' **TAATACGACTCACTATAGG**GGATGATGTACCGGA CCTGCT 3' and STATRev 5' **TAATACGACTCACTATAGG**GGTGTACGATGACGA CAACCG 3'. The amplification of STAT sequence was done using the PCR cycles as follows: 95º C for 5 min and 35 cycles of 95º C for 30 s, 55º C for 45 s and 72º C for 45 s.

dsSTAT or dsß-gal (69nL of 3 µg/µL) diluted in water were introduced into the thorax of cold anesthetized 3–4 day old female mosquitoes by a nano-injector (Nanoject, Drummond) with glass capillary needles. After the injection, the insects were maintained in an air incubator and fed on sugar solution.

At two to three days after the dsRNA injections, the insects were fed with *P. vivax* infected blood. Three to five days after infection, the oocysts in the basal lamina of the gut epithelium were counted to estimate the *P. vivax* load in the infected mosquito. Each dissected mosquito gut was stained with 2% mercurochrome and observed under light microscopy. At least 30 guts were used for each experimental condition. Oocyst numbers in dsSTAT injected insects were compared to non-injected insects and insects injected with β-gal dsRNA, a control for a gene not found in the insect. The significance of gene silencing effect on oocysts loads was determined by the Mann-Whitney statistical test.

Semi-quantitative RT-PCR

Total RNA was extracted from females, either sugar-fed or one to five days after dsRNA injections. Up to 5 µg of RNA were treated with RQ1 RNAse-free DNAse (Promega) and used for first strand cDNA synthesis utilizing the ImProm-II™ Reverse Transcription System (Promega). PCR reaction conditions were the same utilized for RTPCR, as were the primers (STAT and RP49). Biological and experimental triplicates were performed. The PCR reactions were separated in a 2.5% ethidium bromide-containing agarose gel. The intensity of amplified products was measured using ImageJ 1.34s software (http://rsb.info.nih.gov/ij) and plotted for semi-quantitative analysis. The ANOVA test was used as statistics method.

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Figure legends

Figure S1: **Sequence of STAT obtained from PCR fragments produced using degenerate primers and RACE PCR.** Numbers on the left indicate nucleotide

sequence length and on the right indicate amino acid sequence length; asterisk indicates the stop codon; aminoacids in italics represent the hydrophobic binding pocket; aminoacids in bold format indicate the phosphotyrosine binding pocket; underlined aminoacids represent the alpha domain; dashed aminoacids represent the binding domain; uperlined aminoacids indicates the SH2 domain. The nucleotides in bold format indicate the poly(A) tail. AqSTAT sequence was deposited under accession HM851178.

Figure S2: **Sequence of PIAS obtained from PCR fragments produced using degenerate primers and RACE PCR**. Numbers on the left indicate nucleotide sequence length, on the right indicate amino acid sequence length and asterisk indicates the stop codon. Underlined aminoacids represent the SAP domain and dashed aminoacids the MIZ/SP-RING zinc finger domain. The nucleotides in bold format indicate the poly(A) tail. AqPIAS sequence was deposited under accession number HM851177.

Figure 1: **Characterization of transcription factor STAT gene.** A: Schematic representation of STAT proteins from *A. aquasalis* (AqSTAT-A), *A. gambiae* (AgSTAT-A and AgSTAT-B) and *A. aegypti* (AeSTAT-A) showing the STAT interaction domain (yellow), STAT alpha domain (green), STAT binding domain (blue) and SH2 domain (red). B: Phylogenetic tree for STAT using insect and human sequences, constructed based on the neighbor-joining method. C: Multiple aminoacid sequence alignment of STAT of insects. Accession numbers of STAT sequences from: *A. aquasalis* (Aq) – HM851178, *A. gambiae* (Ag) (STAT-A – ACO05014.1 and STAT-B – CAA09070.1, *A. aegypti* (Ae) – ABO72629.1, *Culex quinquefasciatus* (Cq) – XP_001866606.1, *Culex tritaeniorhynchus* (Ct) – AAQU64663.1, and *D. melanogaster* (Dm) – NP_996243.1.

Figure 2: **Characterization of *PIAS* gene.** A: Schematic representation of *A. aquasalis, A. gambiae* and *A. aegypti* PIAS proteins showing the SAP domain (blue) and the MIZ/SP-RING zinc finger domain (red). B: Phylogenetic tree for *PIAS* of insects and humans constructed based on the neighbor-joining method. C: Multiple aminoacid sequence alignment of *PIAS* from insects. Accession numbers of PIAS

sequences from: *A. aquasalis* (Aq) – HM851177, A. *gambiae* (Ag) – XP_001688469.1, *A. aegypti* (Ae) – XP_001647815.1, *Drosophila pseudobscura* (Dp) – XP_002138569, and *A. mellifera* (Am) – XP_623571.

Figure 3: **Transcription levels of *A. aquasalis* STAT determined by RTPCR .** A: immature stages (eggs, larvae (L1-L4) and pupae), sugar-fed males and females, B: sugar-fed females (dotted line), and blood-fed control and blood-fed infected females. h – hours, L1 – first instar larva, L2 – second instar larva, L3 – third instar larva and L4 – fourth instar larva. +–: s.e.m.; * 0.05>p>0.03, ** 0.03>p>0.01, *** p>0.01.

Figure 4: Expression of **PIAS in *A. aquasalis* determined by RTPCR.** A: Expression of mRNA of PIAS in immature stages (eggs, larvae (L1-L4) and pupae) sugar-fed males and females, B: Expression of mRNA of PIAS in sugar-fed females (dotted line), and females after blood-feeding and after *P. vivax* infection, C: Expression of PIAS protein in *A. aquasalis* submitted to different feeding regimens [sugar-fed male (♂) and female (♀), blood-fed (control) (BFC) and blood-fed infected (BFI) females] and human blood. h – hours, L1 – first instar larva, L2 – second instar larva, L3 – third instar larva and L4 – fourth instar larva. +–: s.e.m.; * 0.05>p>0.03, ** 0.03>p>0.01, *** p>0.01.

Figure 5: **Characterization of** *NOS* **gene.** A: Schematic representation of *A. aquasalis* NOS protein showing nitric oxide synthase (red), flavodoxin (green) and NOS oxygenase (green) domains. B: Phylogenetic tree of insects NOS constructed based on the neighbor-joining method. C: Multiple aminoacid sequence alignment of insects NOS . Accession numbers of PIAS sequences from: *A. aquasalis* (Aq) – HM851179, *A. gambiae* (Ag) – AGAP008255-PA, *A. aegypti* (Ae) – AAEL009745, *A. stephensi* (As) – O61608, and *D. melanogaster* (Dm) – CG6713.

Figure 6**: Expression of NOS in *A. aquasalis.*** A: Transcription of NOS in *A. aquasalis* following different feeding regimens determined by RTPCR. A: sugar-fed males and females, and B: sugar-fed females (dotted line), and blood-fed control and blood-fed infected females. h – hours. * 0.05>p>0.03, ** 0.03>p>0.01, *** p>0.01. D

and E: Immunofluorescence staining of blood-fed control and blood-fed infected female midguts. NOS was detected with a universal anti-NOS antibody.

Figure 7: **Expression of STAT and PIAS in different tissues of males and females insects.** The figures show the expression of the STAT and PIAS proteins in adult *A. aquasalis*. A-C – sugar-fed males and D-F – sugar-fed females. Arrowheads show the fat body expressing STAT and PIAS proteins. Ab – abdomen, SF – sugar-fed insects. CTRL – control pictures.

Figure 8: **Expression of STAT and PIAS in different tissues of the *A. aquasalis*.** A-C: 24 hours blood-fed (control) (BFC) females. D-F: 24 hours blood-fed infected (BFI) females**.** The figures are representative of the expression of STAT and PIAS in adult *A. aquasalis*. Arrowheads represent fat body tissue. Arrows represent disperse cells expressing STAT and PIAS proteins. To – torax, Ab – abdomen and Bl – blood. CTRL- control pictures.

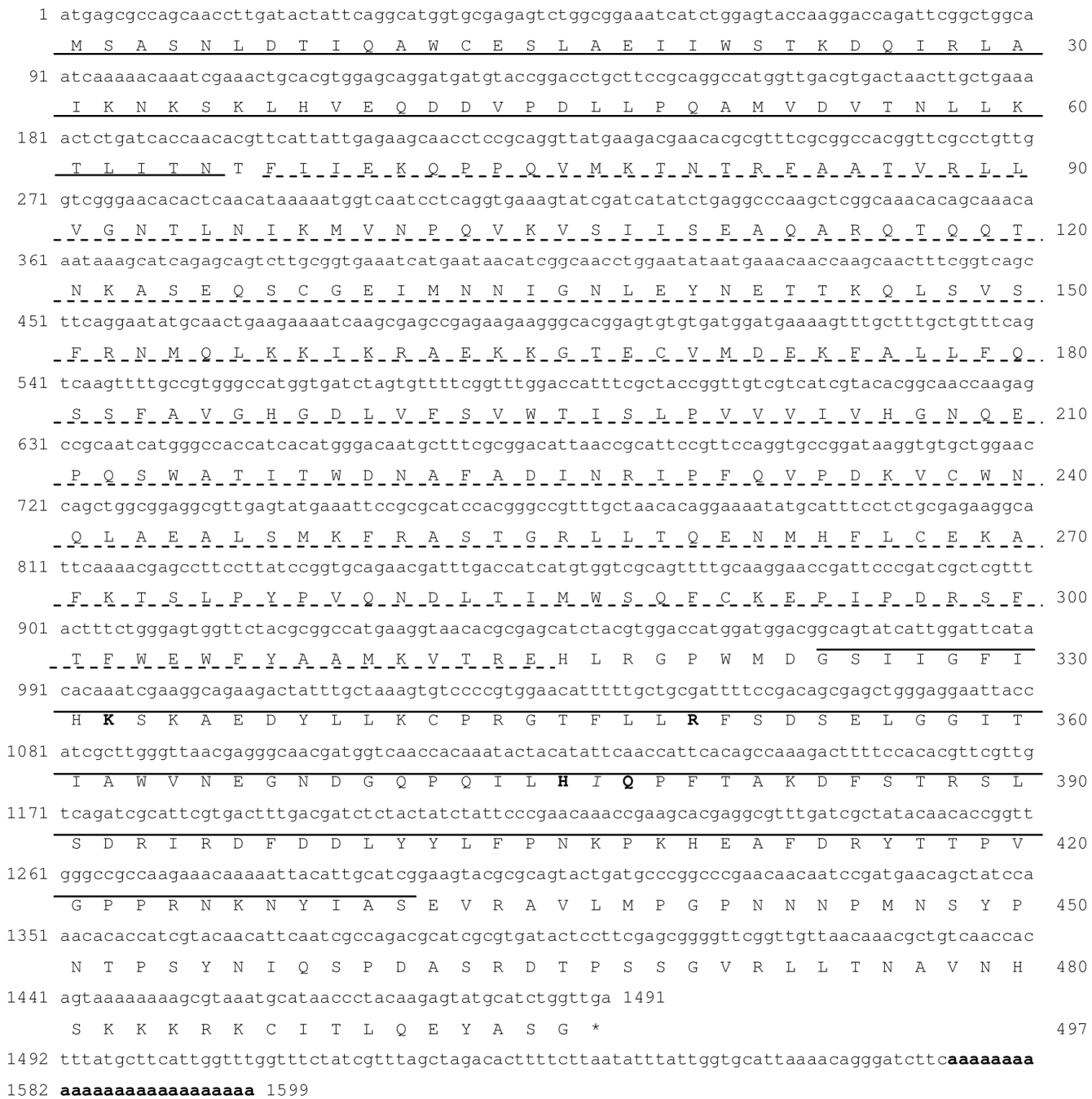
Figure 9: **Expression of STAT and PIAS in different tissues of the *A. aquasalis***. A-C: 36 hours blood-fed (control) (BFC) females. D-F: 36 hours blood-fed infected (BFI) females**.** The figures are representative of the expression of STAT and PIAS in adult *A. aquasalis*. Arrowheads show the fat body tissue, asterisks represent the eggs and setae represent disperse cells expressing STAT and PIAS proteins. To – torax, Ab – abdomen, Eg – eggs and Bl – blood. CTRL – control pictures.

Figure 10: **Expression of STAT and PIAS in different tissues of the *A. aquasalis*.** A-C: 48 hours blood-fed (control) (BFC) females. D-F: 48 hours blood-fed infected (BFI) females**.** The figures are representative of the expression of STAT and PIAS in adult *A. aquasalis*. Arrowheads show the fat body tissue, asterisks represent the eggs and setae represent disperse cells expressing STAT and PIAS proteins. To – torax, Ab – abdomen, Eg – eggs and Bl – blood. CTRL – control pictures.

Figure 11: **Effect of STAT silencing on *A. aquasalis* susceptibility to *P. vivax* infection.** A and B **-** Effect of dsRNA-mediated knockdown of *STAT* and *ß-gal* (control) on *A. aquasalis* STAT expression 1 to 5 days after dsRNA injection. Zero

day refers to *A. aquasalis* sugar-fed females. C- Number of infected insects after dsRNA injections. D and E - Oocysts numbers (D) and visualization (arrows) (E) in midguts of mosquitoes previously injected with double stranded RNA for ß-gal (D, E1 and E2) and STAT (D, E3 and E4) three to five days after *Plasmodium* infection. The significance of gene silencing on oocysts load in experimental samples, compared to water dsß-gal-treated controls, was determined by Mann-Whitney statistical test with Bonferroni correction.

FigureS1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M S A S N L D T I Q A W C E S L A E I I W S T K D Q I R L A 30
91 atcaaaaacaaatcgaaactgcacgtggagcaggatgatgtaccggacctgcttccgcaggccatggttgacgtgactaacttgctgaaa
I K N K S K L H V E Q D D V P D L L P Q A M V D V T N L L K 60
181 actctgatcaccaacacgttcattattgagaagcaacctccgcaggttatgaagacgaacacgcgtttcgcggccacggttcgcctgttg
T L I T N T F I I E K Q P P Q V M K T N T R F A A T V R L L 90
271 gtcgggaacacactcaacataaaaatggtcaatcctcaggtgaaagtatcgatcatatctgaggcccaagctcggcaaacacagcaaaca
V G N T L N I K M V N P Q V K V S I I S E A Q A R Q T Q Q T 120
361 aataaagcatcagagcagtcttgcggtgaaatcatgaataacatcggcaacctggaatataatgaaacaaccaagcaactttcggtcagc
N K A S E Q S C G E I M N N I G N L E Y N E T T K Q L S V S 150
451 ttcaggaatatgcaactgaagaaaatcaagcgagccgagaagaagggcacggagtgtgtgatggatgaaaagtttgctttgctgtttcag
F R N M Q L K K I K R A E K K G T E C V M D E K F A L L F Q 180
541 tcaagttttgccgtgggccatggtgatctagtgttttcggtttggaccatttcgctaccggttgtcgtcatcgtacacggcaaccaagag
S S F A V G H G D L V F S V W T I S L P V V V I V H G N Q E 210
631 ccgcaatcatgggccaccatcacatgggacaatgctttcgcggacattaaccgcattccgttccaggtgccggataaggtgtgctggaac
P Q S W A T I T W D N A F A D I N R I P F Q V P D K V C W N 240
721 cagctggcggaggcgttgagtatgaaattccgcgcatccacgggccgtttgctaacacaggaaaatatgcatttcctctgcgagaaggca
Q L A E A L S M K F R A S T G R L L T Q E N M H F L C E K A 270
811 ttcaaaacgagccttccttatccggtgcagaacgatttgaccatcatgtggtcgcagttttgcaaggaaccgattcccgatcgctcgttt
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S D R I R D F D D L Y Y L F P N K P K H E A F D R Y T T P V 420
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G P P R N K N Y I A S E V R A V L M P G P N N N P M N S Y P 450
1351 aacacaccatcgtacaacattcaatcgccagacgcatcgcgtgatactccttcgagcggggttcggttgttaacaaacgctgtcaaccac
N T P S Y N I Q S P D A S R D T P S S G V R L L T N A V N H 480
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S K K K R K C I T L Q E Y A S G * 497
1492 tttatgcttcattggtttggtttctatcgtttagctagacacttttcttaatatttattggtgcattaaaacagggatcttcaaaaaaaa
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FigureS2

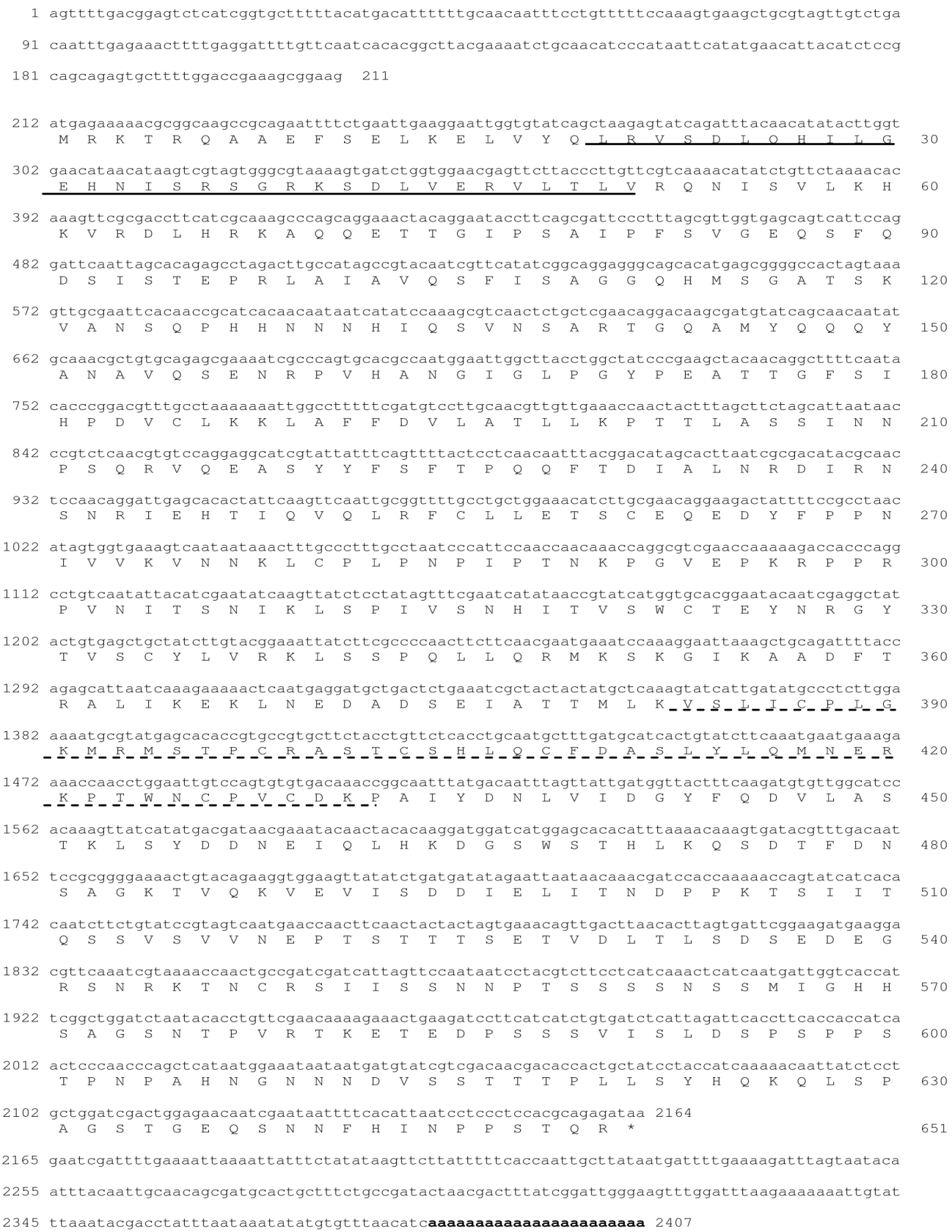
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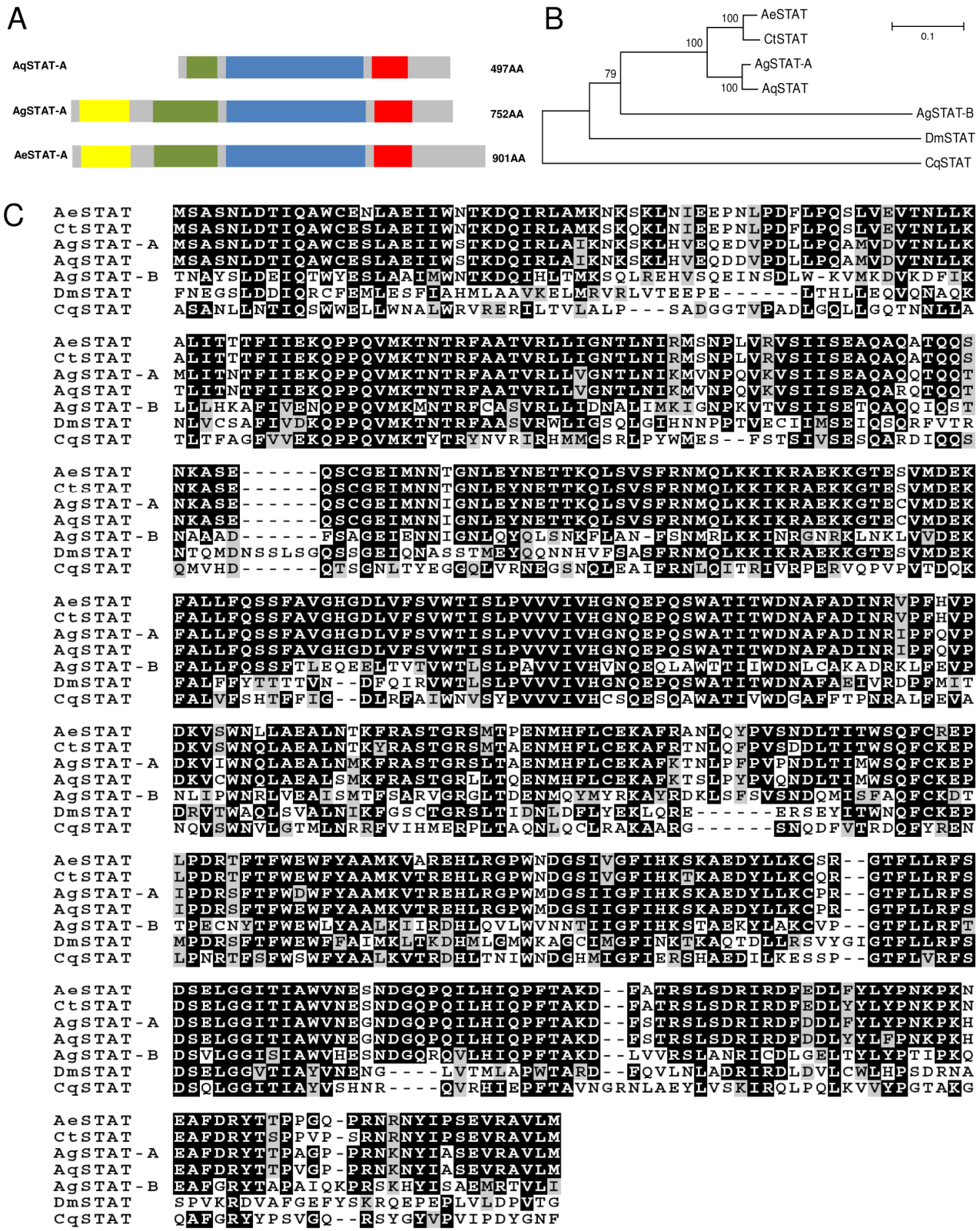
Figure 1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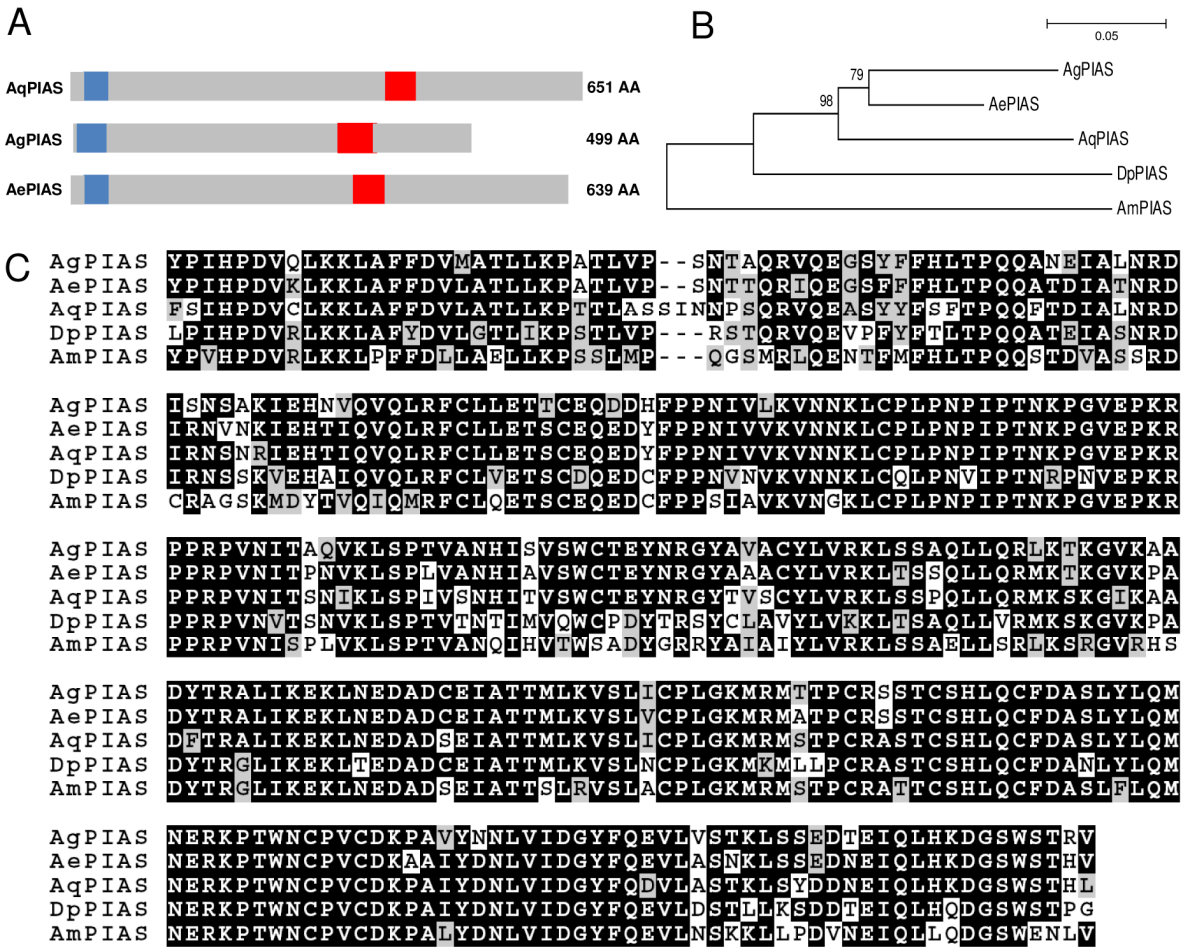
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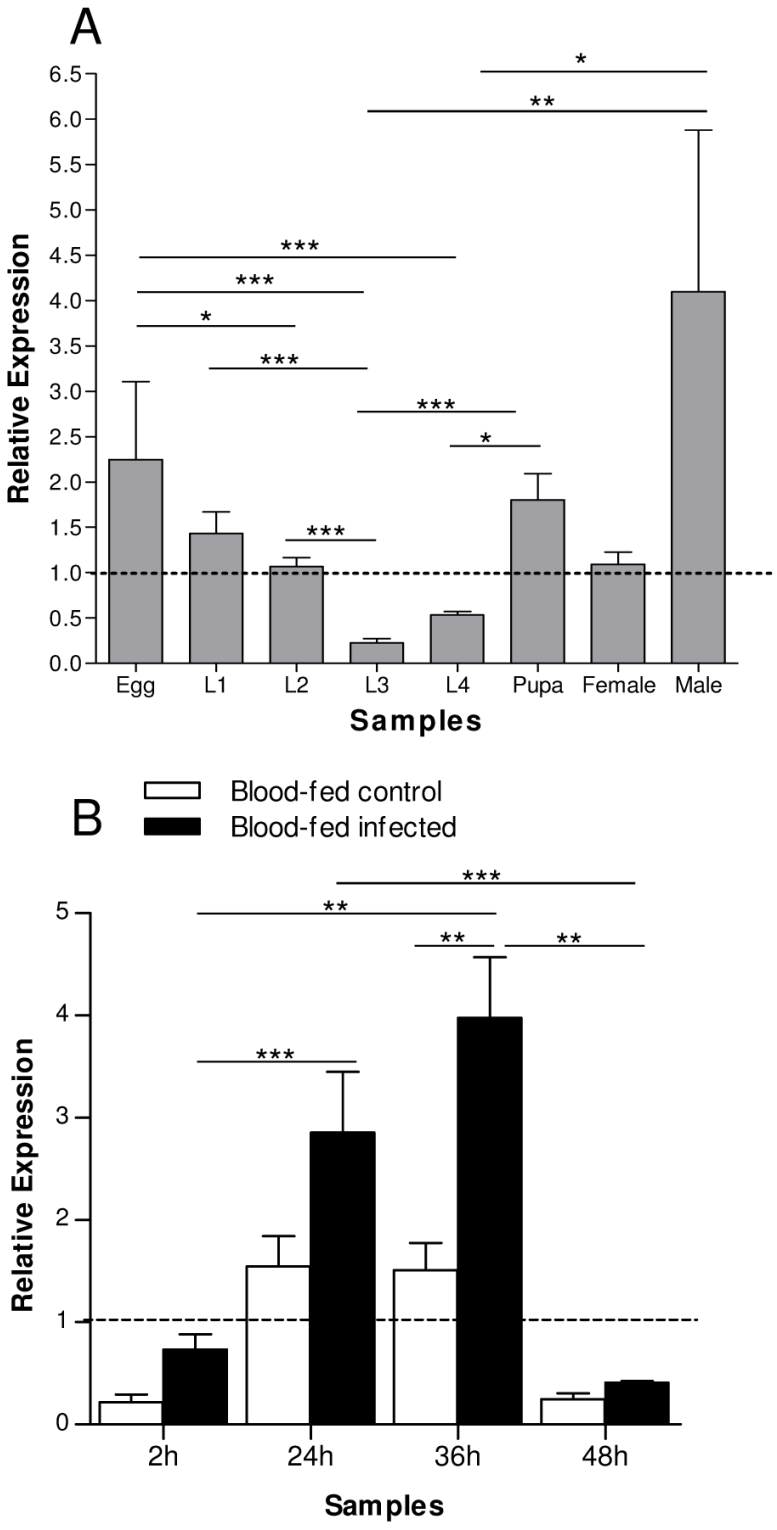
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L3
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Male
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Blood-fed control
Blood-fed infected
Relative Expression
Samples
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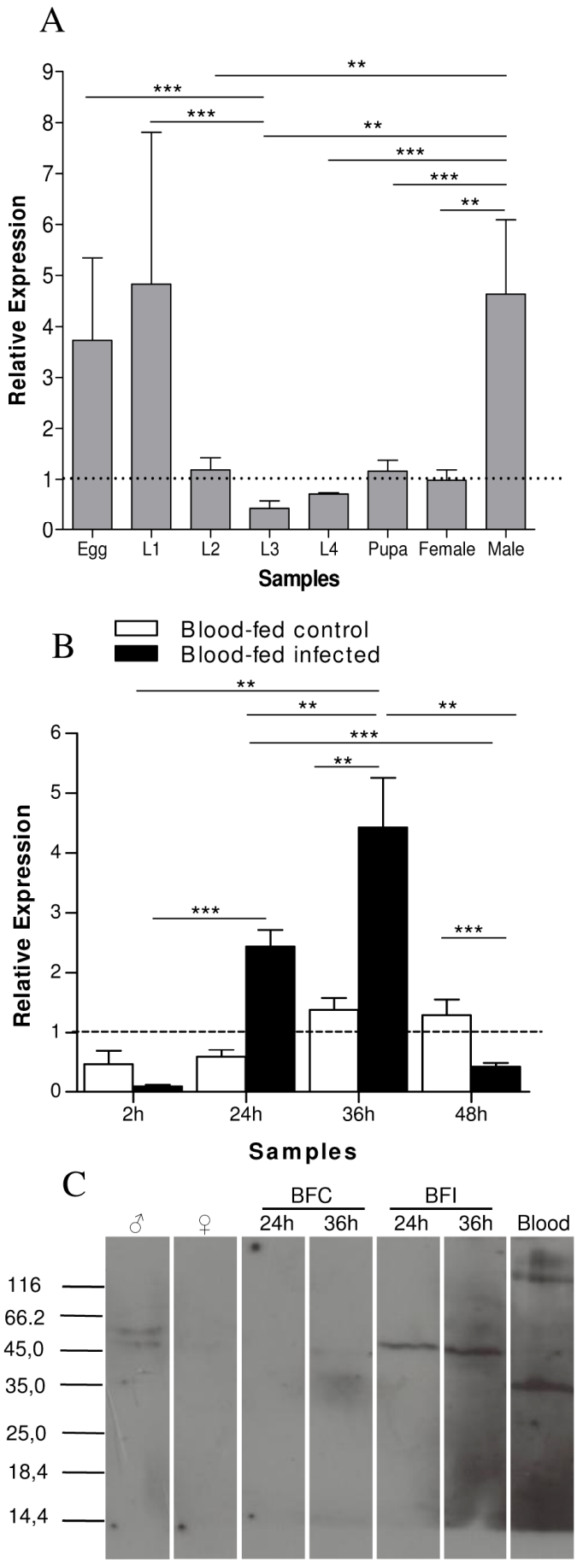
24h
36h
48h

Figure 4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24h
36h
48h
Samples
C
♂
♀
BFC
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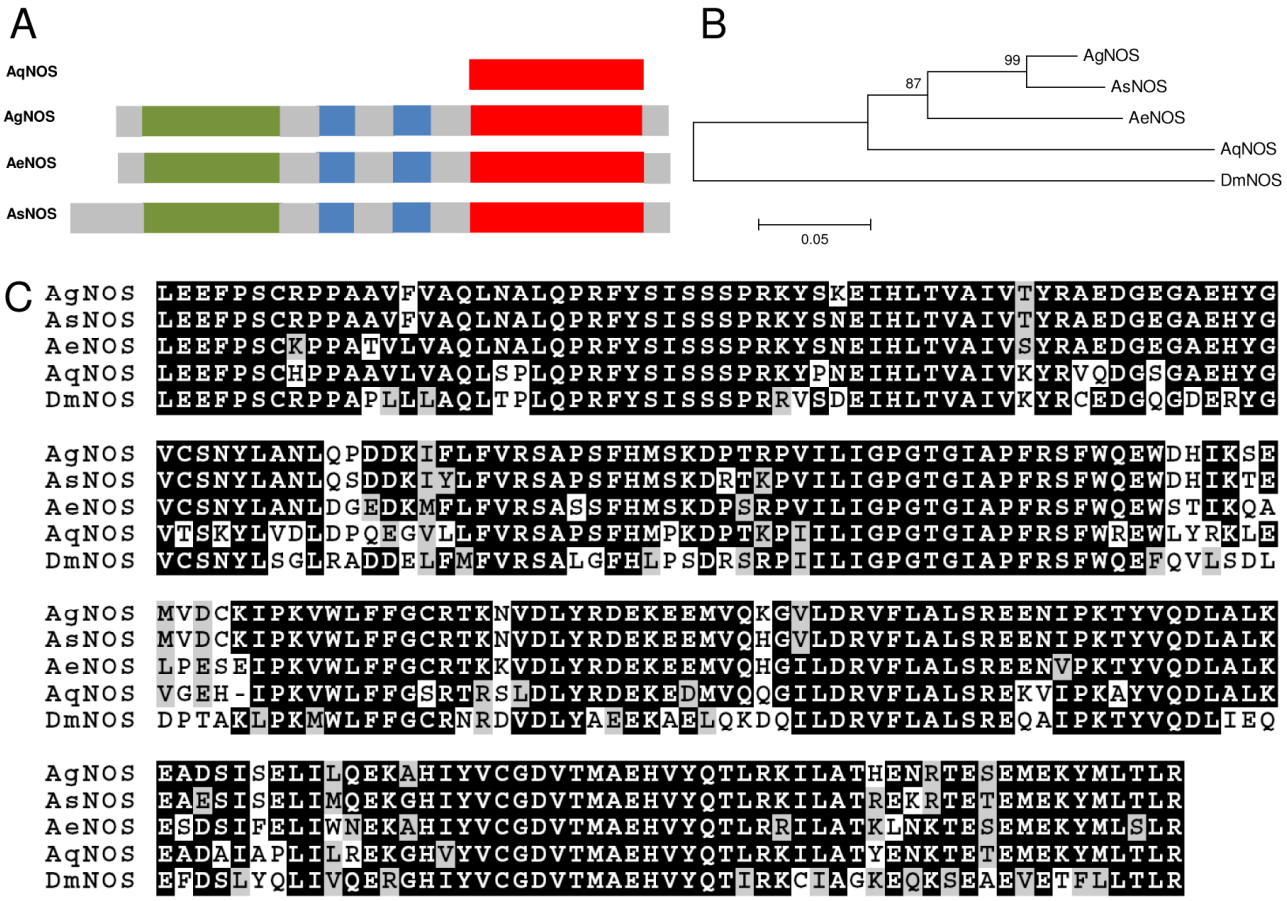
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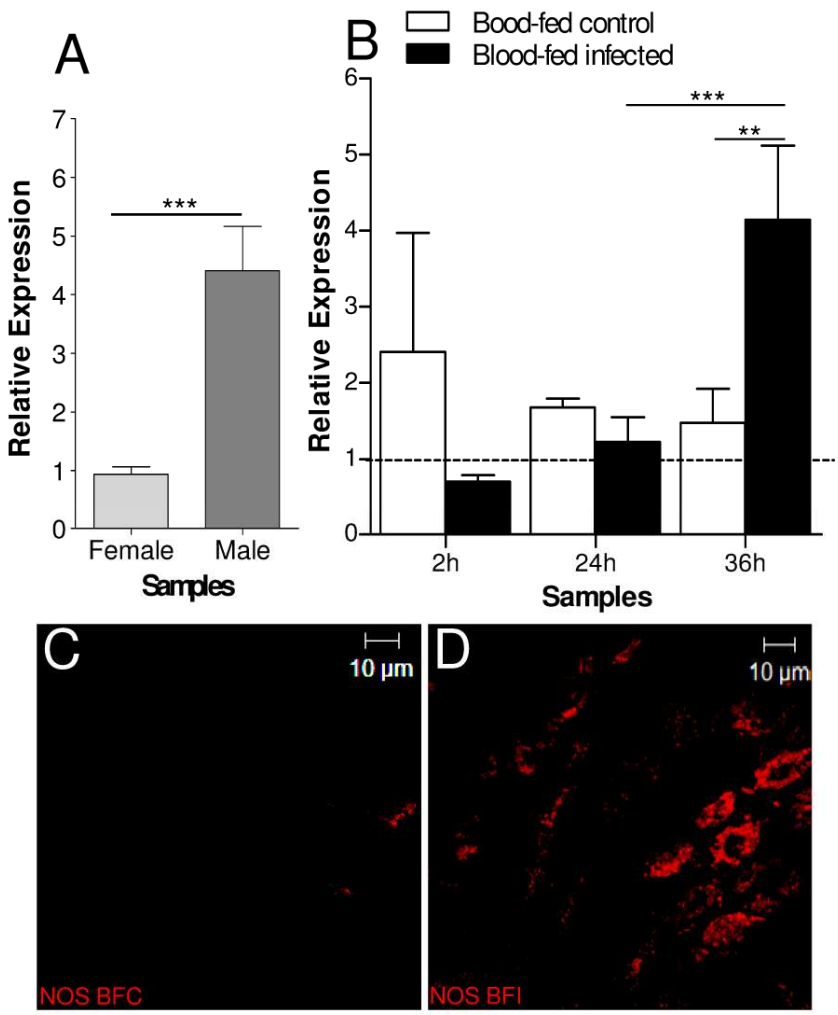
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24h
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Samples
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D
10 µm
NOS BFI

Figure 7

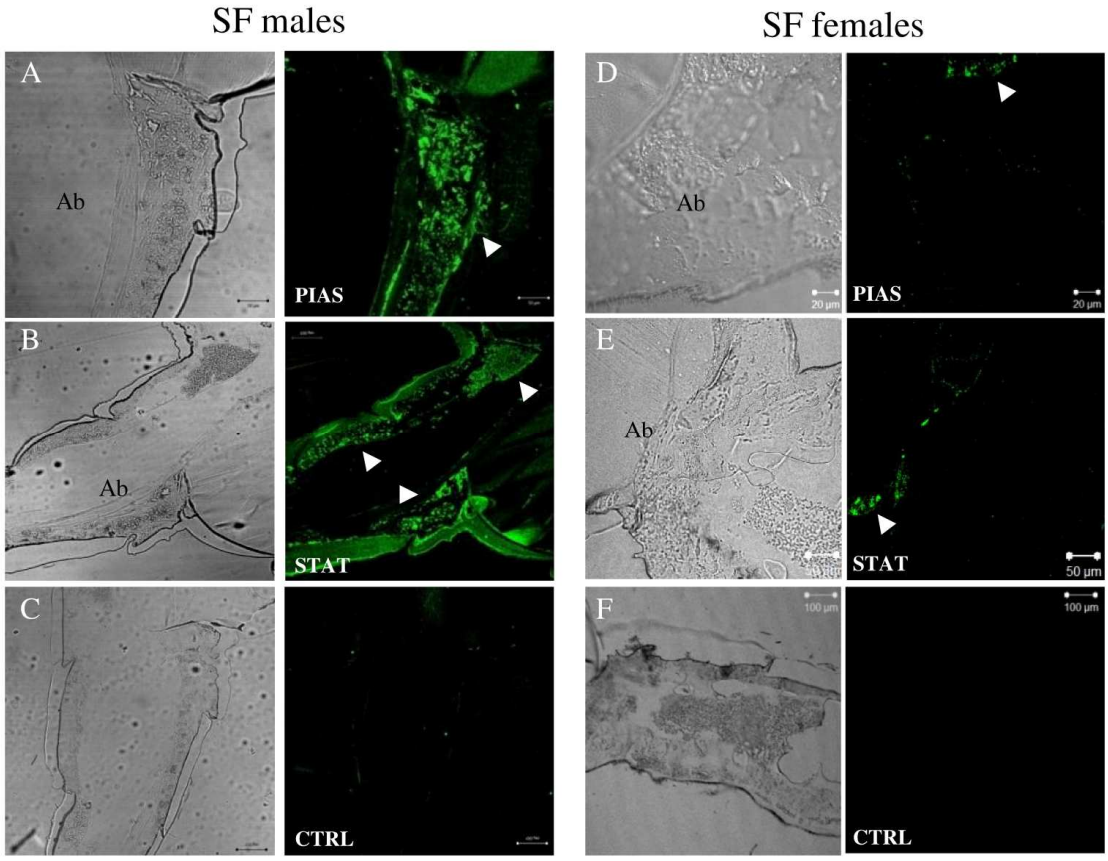
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Figure 8

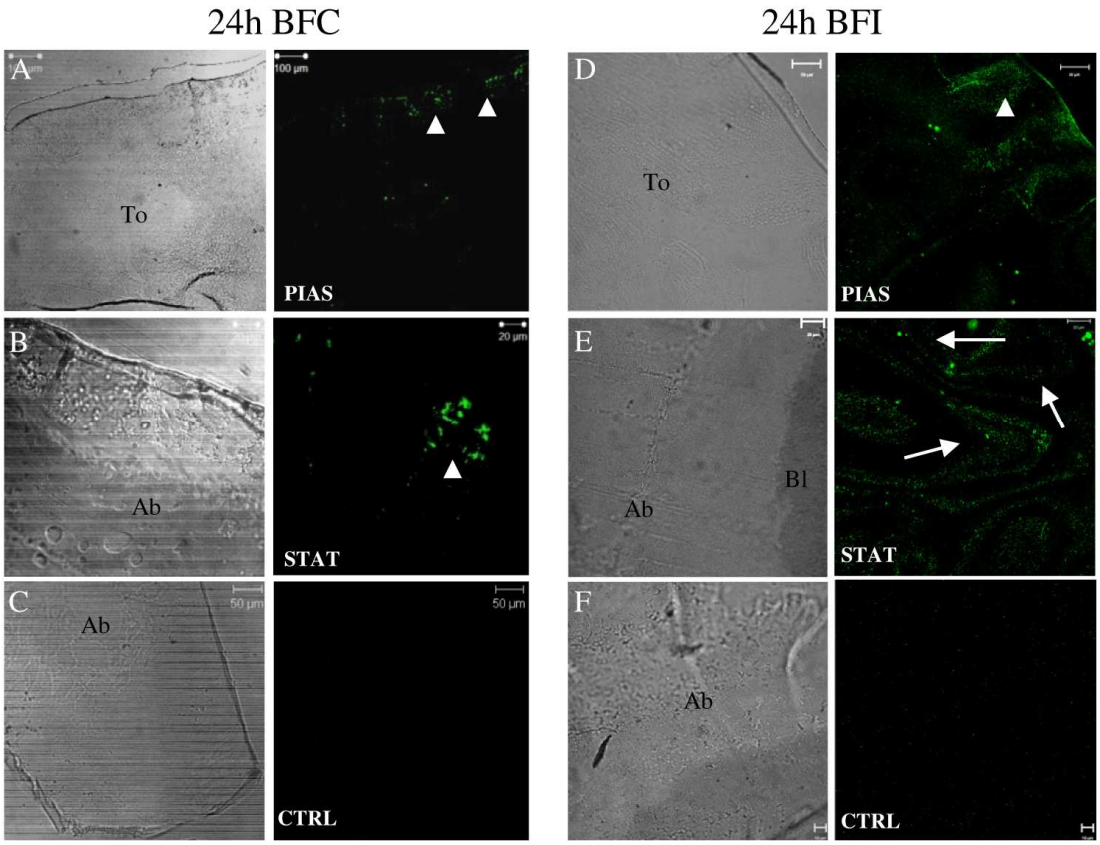
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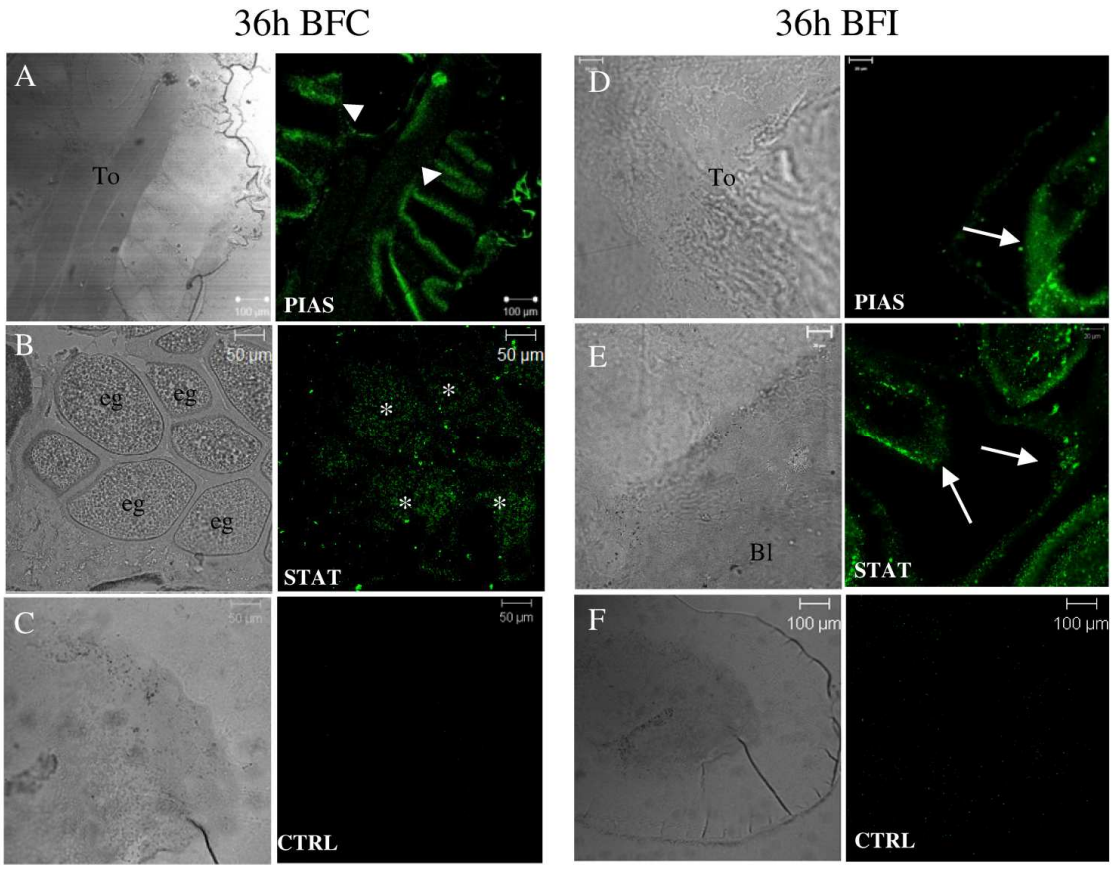
Figure 9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B
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eg
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Bl
STAT
C
50 µm
50 µm
CTRL
F
100 µm
100 µm
CTRL

Figure 10

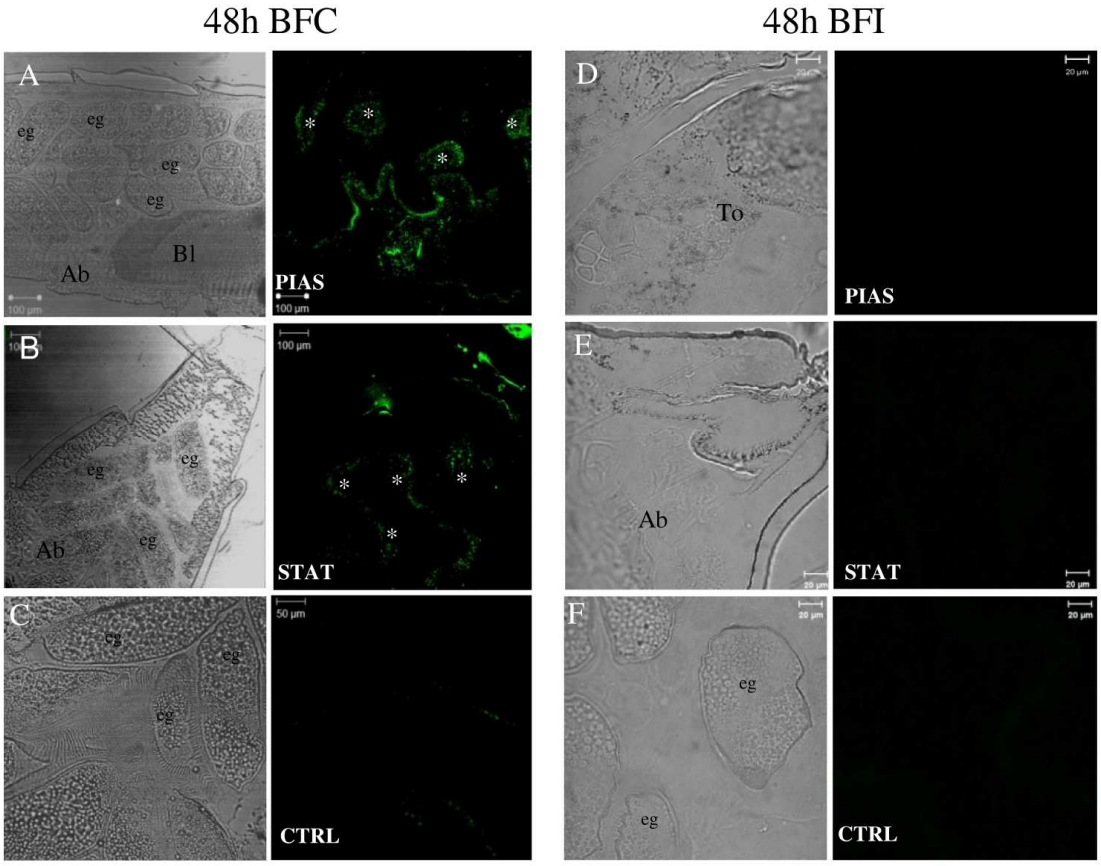
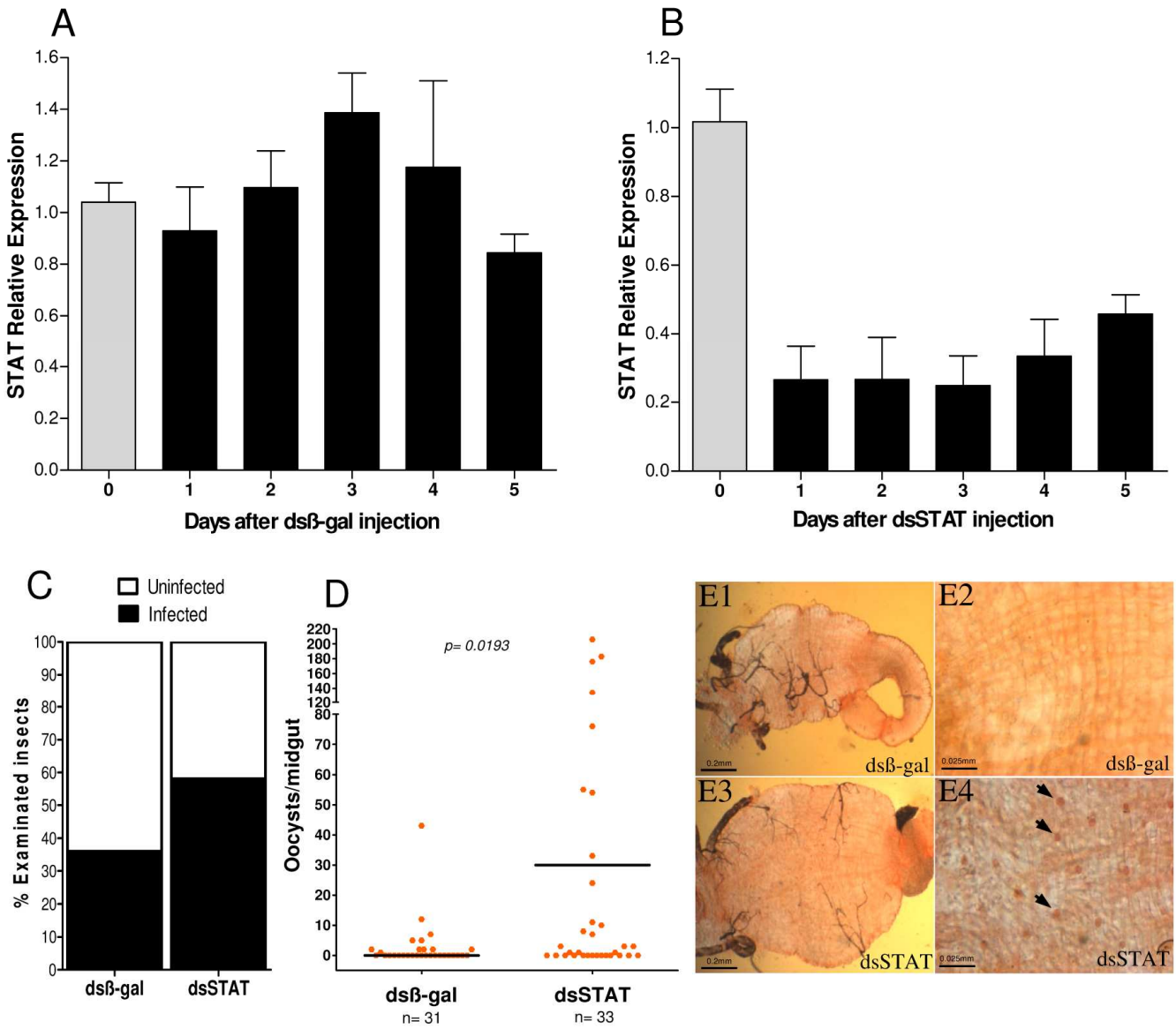

48h BFC
48h BFI
A
eg
eg
eg
eg
Bl
Ab
PIAS
D
To
PIAS
B
eg
eg
Ab
eg
STAT
E
Ab
STAT
C
eg
eg
eg
CTRL
F
eg
eg
CTRL

Figure 11


A
STAT Relative Expression
Days after dsß-gal injection
B
STAT Relative Expression
Days after dsSTAT injection
C
Uninfected
Infected
% Examinated insects
dsß-gal
dsSTAT
D
p= 0.0193
Oocysts/midgut
dsß-gal
n= 31
dsSTAT
n= 33
E1
dsß-gal
E2
dsß-gal
E3
dsSTAT
E4
dsSTAT

Capítulo 3

O papel das espécies reativas de oxigênio na imunidade de *Anopheles aquasalis* contra *Plasmodium vivax*

Manuscrito a ser submetido

Justificativa:

Os insetos possuem um sistema imune eficiente e capaz de curar infecções causadas por diferentes patógenos. Este sistema evoluiu ao longo do tempo e permitiu que este grupo tivesse um grande sucesso evolutivo que é observado nos dias de hoje pela sua diversidade e grande número de espécies. Os radicais livres são moléculas efetoras importantes na resposta imune dos insetos a diferentes patógenos. Contudo, estas moléculas são extremamente perigosas, pois devido a sua alta reatividade com diferentes tipos de moléculas (proteínas, DNA e lipídios) podem causar danos tanto para o microorganismo invasor quanto para o próprio inseto. Para manter a homeostase, os insetos possuem enzimas e moléculas responsáveis por detoxificar estas moléculas. Neste contexto, estudamos a infecção por *P. vivax* e o estresse oxidativo gerado no *A. aquasalis*. Para tanto, a produção de radicais livres, e a expressão e atividade de enzimas antioxidantes foram avaliadas.

The role of reactive oxygen species in *Anopheles aquasalis* response against *Plasmodium vivax*

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Abstract

Malaria affects millions of people worldwide and hundreds of thousands of people each year in Brazil. The mosquito *Anopheles aquasalis* is an important vector of *Plasmodium vivax*, the main human malaria parasite in the Americas. To better understand the interaction mechanisms between these organisms, we are investigating redox metabolism during the interaction between *A. aquasalis* and *P. vivax*. Since the reactive oxygen species (ROS) have been shown to be involved in immune response against a diversity of pathogens, we investigated the mechanisms of free radical production and its modulation after *A. aquasalis* challenge with *P. vivax*. ROS metabolism was evaluated through the expression and activity of three detoxification enzymes, one catalase and two superoxide dismutases (SOD3A and SOD3B). We found that mRNA and activity of catalase and SOD were regulated in *A.*

aquasalis after blood-feeding and infection with *P. vivax.* Both catalase, SOD3A and SOD3B had their expression level up regulated in the midgut after feeding with blood infected with *P. vivax*. However, both enzymes showed reduced activity 24 hours after the infectious meal. Evaluation of ROS production in *A. aquasalis* gut showed that the mosquito maintains the midgut environment in a reduced state after ingestion of blood. RNAi-mediated silencing of catalase reduced enzyme activity in the midgut and strikingly resulted in increased *P. vivax* infection prevalence and intensity. Our finding reveals a previously uncharacterized role of catalase in *A. aquasalis* response to *Plasmodium* infection and may help development of vector-based strategies to block malaria in the Americas.

Introduction

Malaria is an important health problem that affects millions of people and causes almost one million of deaths each year. In Brazil, this disease affects mainly the northern region with approximately 450,000 cases per year (Oliveira-Ferreira *et al.* 2010). Malaria is transmitted by mosquitoes of the *Anopheles* genus. For transmission to occur, the parasite needs to complete a complex cycle inside the insect vector that includes: differentiation of gametes, fertilization, passage through the epithelial cells of the midgut, establishment in the midgut basal lamina as oocysts, cellular division with the production of thousands of new parasites, breakdown of oocysts and release of sporozoites into the hemolymph, invasion of the salivary gland, differentiation and finally inoculation into a new vertebrate host. During these steps the parasite interacts with diverse insect tissues causing activation of the mosquito powerful innate immune system, which is responsible for major parasite losses (Dimopoulos *et al.* 1997, Hoffmann *et al.* 1999). One of the effector molecules implicated in insect innate immunity are the reactive oxygen species (ROS). ROS are multifunctional molecules that have been previously implicated in host defense, mitogenesis, hormone biosynthesis, apoptosis, necrosis and regulation of gene expression (Rada and Leto 2008, Sumimoto 2008). The importance of ROS in immune response was first described in phagocytic cells through ROS production by NADPH oxidases (NOX) leading to pathogen killing.

To date, six human homologues of the NOX protein family (Nox-1, Nox-3, Nox-4, Nox-5, Duox-1 and Duox-2) have been identified in various non-phagocytic cells (reviewed by Sumimoto *et al.* 2008). New homologues of this protein were also identified in organisms such as nematodes, fruit flies, green plants, fungi, and slime molds (reviewed by Bedard *et al.* 2007).

The Dual Oxidases (DUOXs) are important in hormone production, extracellular matrix production and host defense (Donkó *et al.* 2005). DUOX proteins were described in *Drosophila melanogaster* and *Anopheles gambiae* as producers of ROS after pathogen challenges aiming to control the infection (Beutler *et al.* 2004, Kumar *et al.* 2004, Iwagaga *et al.* 2005, Ha *et al.* 2005a, 2009). In *A. gambiae,* DUOX proteins, together with a peroxidase, are also responsible for preventing a strong immune activation by producing a dityrosine network, which decreases gut permeability to immune elicitors (Kumar *et al.* 2010). This mucous protection may prevent the deleterious effect of the powerful immune response to the host itself and to commensal bacteria.

Luckhart and collaborators (1998, 2003) have described an increase of the free radical nitric oxide (NO) as well as of nitric oxide synthase (NOS) in *Anopheles stephensi* after *Plasmodium berghei* invasion of epithelial cells. Also, *A. gambiae* under high oxidative stress was more resistant to *Plasmodium* parasites and bacteria (Kumar *et al.* 2003 and Molina-Cruz *et al.* 2008). This resistance profile was reverted when these insects were subjected to an antioxidant diet, confirming the hypothesis that an oxidant environment might be deleterious by the parasite. Furthermore, after blood ingestion and specially after *Plasmodium* infection the expression of some detoxification enzymes increased significantly.

In spite of ROS being beneficial for parasite clearance, they are potentially toxic to the host. For this reason, the lifespan of these molecules must suffer a fine tuned regulation, which is accomplished through the action of antioxidant enzymes, such as superoxide dismutase (SOD) and catalase, for example, as well as the control of ROS generation. Superoxide dismutases (SODs) transform superoxide ($O_2^{-\bullet}$) in hydrogen peroxide (H_2O_2), and catalase, detoxify hydrogen peroxide into water and oxygen. Other molecules such as uric acid are also antioxidant components utilized by the organisms to neutralize deleterious effects of high levels of ROS (Graça-Souza *et al.* 2006).

Following evidence for a role of ROS in *A. stephensi* and *A. gambiae* immunity, we investigated the recruitment of ROS as an immune defense of the Brazilian vector *A. aquasalis* infected with *P. vivax*, the main human malaria parasite in the Americas. We also investigated the mechanisms used to minimize the harmful effects of ROS generation by this insect.

Material and Methods

Mosquito infection

A. aquasalis reared in controlled temperature and humidity (Horosko e cols. 1997) were blood-fed and infected by artificial feedings. All insect infections were conducted in the endemic city of Manaus, Amazonas state as described in Bahia *et al.* (2010). To prevent exflagellation of *P. vivax* microgametocytes, artificial feeding was performed at 37℃ constant temperature maintain ed using a water circulation system. After the experimental feeding, mosquitoes were transferred to a new cage and fed with 20% sucrose *ad libitum* until the experimental procedures. Infection was evaluated by PCR using a specific *Plasmodium* 18s rRNA gene as described in Gama *et al.* (2007).

PCR using degenerate primers

Degenerate primers designed on conserved regions of SOD and catalase, based in sequences of others insects (*A. gambiae*, *A. stephensi*, *Aedes aegypti* and *D. melanogaster*), were previously described (Barillas-Mury *et al.* 1999). The cycles used in the PCR reaction were: two cycles (1 min steps at 95, 55 and 72^{o}C, and 95, 42 and 72^{o}C) followed by 30 cycles at moderate stringency (1 min steps at 95, 52 and 72^{o}C) and a final 7 min extension at 72^{o}C. All amplicons generated were cloned using pGEM®-T Easy Vector (Promega). The plasmids containing inserts were used to transform high efficiency DH5-α *Escherichia coli* and sequenced. All sequencing was performed using an ABI 3700 sequencer (Applied Biosystems) in the PDTIS/FIOCRUZ Sequencing Platform.

RACE

SOD3A, SOD3B and Catalase 5' and 3' cDNA ends were obtained using the Smart cDNA RACE amplification kit (Becton Dickinson Clontech). High efficiency DH5-α *E. coli* were transformed with vectors containing the cDNA fragments of interest. SODs and catalase full cDNAs were obtained after assembling the sequences using the CAP3 program and aligning the resulting contigs with other insect sequences.

Real time PCR

Real time PCR (RTPCR) was performed with cDNA from whole insects submitted to different experimental conditions (sugar-fed males and females, and females blood-fed or infected with *P. vivax*). Previous to the cDNA synthesis, the extracted RNAs were treated with RQ1 DNAse free-RNAse (Promega). Syber Green fluorescent probe (Applied Biosystems) was used to reveal the amplification rate of the detoxification enzymes catalase and SOD. The RTPCR reactions were performed in an ABI 7000 machine (Applied Biosystems). The PCR cycles used were 50ºC 2 min, 95ºC 10min, 95ºC 15 sec and 63ºC 1 min for 35 times for all reactions. The primer sequences were: SOD3AFwd 5' GTGGAGAGGCAACCCCTTGAGAA 3' and SOD3ARev 5' GGTCGATCTTAGCGTGAAGCAGATT 3', SOD3BFwd 5' GTGGAGAGGCAACCCCTTGAGAA 3' and SOD3BRev 5' CTGATTCCAGGGTACATCGGTG 3', and CatalaseFwd 5' CGGACATGTTCTGGGACTTTATCT 3' and CatalaseRev 5' TTGCCCTCGGCGTTCACCAGCTTAA 3'. The relative expression of the selected genes was based on gene expression CT difference formula (Schefe *et al.* 2006). Quantifications were normalized in relation to the housekeeping gene rp49 (Gentile *et al.* 2005). All experiments were performed using four to six biological replicates. The ANOVA statistical test with multiple comparisons of Tukey or Games-Howell was used in the analysis. When the parametric model was not adequate, the Kruskal-Wallis test with multiple comparisons of Mann-Whitney was utilized. Bonferroni correction was used when necessary. All tests were performed with reliable level of 95% (α= 0.05). The statistical analyses were accomplished using the Graph pad Prism5®, R 2.9.0.

Antioxidant enzymes activity

Three to six samples containing midgut epithelia of *A. aquasalis* females submitted to sugar-feeding, blood-feeding and infected blood-feeding were dissected in 50% ethanol and stored at -70℃ in a cocktail of protease inhibitors (1 mM of Benzamidin, 1 mM of PMSF and 50 µg/µL of SBTI) until assayed. The blood found in the gut of blood-fed insects was removed before freezing. Catalase activity was determined by monitoring hydrogen peroxide consumption at 240 nm at room temperature according to Aebi (1984). SOD activity was measured on the basis of the rate of cytochrome *c* reduction by $O_2^{\cdot\cdot}$ monitored at 550 nm and 25^{o}C using the xanthine-xanthine-oxidase system as the source of O_2^{-} (Flohé & Ötting 1984). Catalase and SOD activities were reported as units per minute per micrograms of protein (U/mg ptn). Data are reported as the mean ± SEM. The ANOVA test with Dunnett's Multiple Comparison Test was used in the analysis. All tests were performed with reliable level of 95% (α= 0.05). The statistical analyses were accomplished using the Graph pad Prism5®, R 2.9.0.

ROS measurement

Guts were dissected from sugar-fed, and 24 hours blood-fed or *P. vivax* infected *A. aquasalis* females, and immediately transferred to a 24 cell plate containing 0.5 mL RPMI medium (Gibco) with the redox sensitive fluorescent probes CM-H2DCFDA [5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester; Molecular Probes]. The amount of free radicals in the insect guts was measured through the fluorescence emitted via immunofluorescence microscopy.

Catalase silencing

The T7 Megascript kit (Ambion) was used to construct double stranded RNAs (dsRNAs) for *Catalase* (dsCAT) and ß-*ga*l (dsß-gal) from PCR-amplified fragments. Amplicons for dsß-gal were produced using plasmid templates and for dsCatalase by RT-PCR products, from sugar-fed female cDNA, giving rise to 544 bp and 466 bp fragments, respectively. Two rounds of PCR were necessary to amplify ß-gal and Catalase. The first PCR round was performed with primers containing a short adaptor sequence at the 5' end (tggcgcccctagatg): ß-galFwd 5' tggcgcccctagatgTGATGGCACCCTGATTGA 3' and ß-galRev 5'

tggcgcccctagatgTCATTGCCCAGAGACCAGA 3' and dsCatalaseFwd 5' tggcgcccctagatgCGTACAATCCGTTCGATCT 3' and dsCatalaseRev 5' tggcgcccctagatgACTGTTGCCTGCGAGAAGTT 3'. The PCR cycles used were 95^{o}C for 3 min, 35 cycles of 95^{o}C for 30 s, 57^{o}C for 45 s and 72^{o}C for 45 s followed by 72^{o}C for 7 min. For the second PCR reaction, two microliters of the first PCR were used. The second round of PCR was utilized to insert the bacteriophage T7 DNA-dependent RNA polymerase promoters to the dsDNA templates. The PCR program of the second round of PCR was the same utilized in the first reaction. The second round PCR primer, which has the T7 (bold letters) and the adaptor sequences, used was 5' ccg**TAATACGACTCACTATAGG**tggcgcccctagatg 3'.

Sixty nine nanoliters of dsRNA from ß-gal and catalase diluted in water to a concentration of 3 µg/µL were introduced into the thorax of cold anesthetized 2–4 day old female mosquitoes by a nano-injector (Nanoject, Drummond) with glass capillary needles. The insects were maintained in an air incubator and fed on sugar solution after the dsRNA injections.

P. vivax infected blood was offered to the inoculated insects two to three days after the dsRNA injections. Oocyst counting was performed three to five days after infection. At least 30 guts of each experimental condition were dissected, stained with 2% mercury chrome and observed under light microscopy. Oocyst numbers in dsCatalase injected insects were compared to dsß-gal, a control for a gene not found in the insect. The significance of gene silencing effect on oocyst loads between the experimental and control groups was determined by Mann-Whitney statistical test.

Results

Cloning and analysis of antioxidant enzymes in *A. aquasalis*

Three antioxidant enzymes (two SODs and one catalase) were amplified by PCR using degenerate primers. After PCR, fragments of 541bp for SOD3A, 268 bp for SODb and 803 bp for catalase were obtained (data not shown). To amplify the full length cDNAs we utilized the Smart Race technique (RACE), which yielded a 1989 bp full-length catalase cDNA (AqCAT), including a 1515 bp coding region, which translates into a 505 amino acid protein, as well as a 161 bp 5' untranslated region (UTR) and 313 bp 3' UTR (Figure 1). AqCAT is very similar to other insect catalases

(Figure 2), giving rise to one long catalase domain (comprising the heme binding pocket and the NADPH binding site), also present in *A. gambiae* and *D. melanogaster* (Figure 2A). In addition, AqCAT bears 94% and 72% identity with *A. gambiae* (XP_314995.4) and *D. melanogaster* (NP_536731.1) catalases, respectively (Figure 2B and 2C), and is not related to the immune-related catalase described in *D. melanogaster* (CG8913) (Data not shown).

Concerning superoxide dismutases, a partial cDNA sequence of SOD3A (AqSOD3A) consisting of 1116 bp, including a 399 bp coding region, which encodes a protein of 133 amino acid residues (Figure 3A), as well as a 254 bp 5' UTR and 470 bp 3' UTR (data not shown), was also obtained by RACE. The full-length SOD3B cDNA (AqSOD3B) is 637 bp long including a 495 bp open reading frame (ORF), encoding a 165 amino acids protein, plus 63 bp and 79 bp 5' and 3' UTRs, respectively (Figure 3B). The deduced AqSOD3A and AqSOD3B proteins have conserved Cu^{2+} and Zn^{2+} binding domains typically found in CuZn-superoxide dismutases (Figure 4A), bearing 94% and 96% identity with putative SOD3A (XP_311594.2) and SOD3B (XP_001230820.1) ortholog genes, from *A. gambiae* (Figure 4B and 4C).

P. vivax infection decreased both catalase and SOD activities in the midgut of *A. aquasalis*

We performed gene expression analyzes using whole body cDNAs from *A. aquasalis* males and females and determined that catalase levels are slightly increased in male mosquitoes (Figure 5A). To explore the putative involvement of this enzyme during malaria infection in the midgut we performed a time course analysis of mosquitoes fed on human blood infected or not with *P. vivax* parasites (Bahia *et al.* 2010) and observed that catalase is significantly up-regulated in infected mosquitoes 36 hours after feeding (Figure 5B). Curiously, enzyme activity at the peak of blood-digestion (24 hours after feeding) was significantly reduced in *P. vivax* infected group (Figure 5C).

The expression of the two SODs was very different between the genders, SOD3A being more expressed in males than females (Figure 6A) while SOD3B had higher expression in females (Figures 6C). SOD3A was significantly up-regulated in the midgut of *P. vivax*-infected group 24 hours feeding (Figure 6B) while SOD3D was

significantly increased 36 hours after the infectious meal (Figure 6D). Both enzymes decreased to almost undetectable levels in the midgut at 48 hours (Figure 6B and 6D). Similar to catalase results, SOD activity was also decreased 24h after infection (Figure 6E) compared to mosquitoes fed with control blood.

ROS production in the *A. aquasalis* midgut

We investigated the production of free radicals in *A. aquasalis* after *P. vivax* infection. We observed a huge amount of free radicals associated with the midgut by fluorescence of the CM-H2DCFDA probe, which is sensible to a redox environment and became fluorescent in mosquitoes fed with sugar solutions alone (Figure 7A). *A. aquasalis* female fed in human blood severely decreased midgut ROS production (Figure 7B). The ingestion of infected blood containing *P. vivax* parasites did not presented any differences towards the insect fed with uninfected blood (Figure 7C).

Catalase silencing enhances *A. aquasalis* susceptibility to *P. vivax* infection

To evaluate the effect of catalase knock-down on *A. aquasalis* infection by *P. vivax*, catalase expression was reduced by dsRNA-mediated silencing in females. A 10-20% reduction of mRNA levels was achieved 2-3 days after mosquito's dsRNA inoculation (Figure 8A). In agreement, enzyme activity was significantly reduced in the midgut epithelia 24 hours after a blood meal (Figure 8B). Surprisingly, catalase knock-down increased the percentage of infected insects (Figure 8C) as well as the number of oocysts in insect midguts (Figures 8D and 8E).

Discussion

Although *A. aquasalis* is an important malaria vector in Brazil (Deane 1986) and *P. vivax* is the most prevalent malaria parasite in the Americas, being responsible for half of the malaria cases outside the African continent, there is a lack of information on this parasite-vector pair (''WHO/HTM/GMP/2008.1''). This is mostly due to the absence of an efficient parasite cultivation system and the wrong assumption that this parasite does not cause severe and lethal malaria (de Lacerda *et al.* 2007, Udomsangpetch *et al.* 2008, Anstey *et al.* 2009), as well as the lack of *A. aquasalis* genome. We are presently investigating the response of *A. aquasalis* to

infection by *P. vivax* (Bahia *et al.* 2010), with emphasis on mosquito redox metabolism.

Mosquito immune system is responsible for healing infections and, in some cases for conferring *Plasmodium* refractoriness (e.g. Kumar *et al.* 2003, Kokoza *et al.* 2010). Most studies related to mosquito immunity were performed on Old World anopheline species and *P. falciparum* or nonhuman malaria parasites (Dong *et al.* 2006, Garver *et al.* 2009). We have recently reported the identification of several up and down-regulated *A. aquasalis* genes in early times of *P. vivax* infection using a strategy based in subtractive libraries from a combination of infected and uninfected mosquitoes (Bahia *et al.* 2010). Surprisingly, few immune genes were identified, using this strategy, what lead us to focus on specific immune targets.

ROS are important effector molecules that participate in the immune responses against various pathogens of organisms as diverse as mammals and insects (Rada and Leto 2008), including mosquito response to *Plasmodium* (Molina-Cruz *et al.* 2008). To test if *P. vivax* infection causes oxidative stress in *A. aquasalis*, the production of free radicals and the expression and activity of antioxidant enzymes were studied. Then, considering the importance of ROS in insect immunity, the production of free radicals in *A. aquasalis* midgut after *P. vivax* infection was investigated. We observed no increase of ROS in the midgut infected insects in relation to blood-fed ones. On the other hand, blood-fed insect midguts (with or without parasites) presented a huge decrease of free radicals in relation to sugar-fed midguts. These results can be explained by the disadvantage of maintaining an oxidative environment in conjunction with heme ingestion. Female mosquitoes normally ingest three or more times their weight in blood in a single meal, ingesting in this processing a huge amounts of hemoglobin (60% of blood protein content) (Graça-Souza *et al.* 2006). The degradation of hemoglobin in the digestive system of the mosquitoes results in the release of very high concentrations of heme, the prosthetic group of hemoglobin. Heme is capable of generating free radicals through Fenton reaction, leading to the oxidation of lipids (Tappel 1955, Gutteridge and Smith, 1988), proteins (Aft and Mueller, 1984) and DNA (Aft and Mueller, 1983), and causing damage to phospholipids membranes (Schmitt *et al.* 1993). For this reason, ROS might be produced locally by the midgut epithelial cells in response to the passage of the parasite, as observed in other mosquito/parasite models, to avoid its

fatal encounter with heme molecules (Luckhart *et al.* 1998, Kumar *et al.* 2004, Gupta *et al.* 2005 and Molina-Cruz *et al.* 2008).

Due the dangerous effects of the ROS, an antioxidant defense system has evolved to minimize or prevent deleterious effects from ROS exposure. Some detoxification enzymes such as catalase, SOD and glutathione peroxidase transform the free radicals into less reactive molecules. The expression of three ROS detoxification enzymes was evaluated in relation to *A. aquasalis* gender and feeding regimens. Three detoxification enzymes of *A. aquasalis,* catalase, SOD3A, and SOD3B, were identified and characterized. The *A. aquasalis* catalase seems to be orthologous with respect to the other mosquito catalase genes, while clearly differing from the immune-related catalase of *D. melanogaster* (Ha *et al.* 2005b). *A. aquasalis* presented two SODs, one more related to SOD3A and the other to SOD3B of *A. gambiae*. The expression of these detoxification enzymes increased after a blood meal. SOD3A and catalase mRNA expression increased 2 and 24 hours, respectively, after blood ingestion compared with sugar-fed females. This increase may be necessary for detoxification of ROS from the mosquito midgut, avoiding the contact with the huge amount of heme molecules released by the red blood cells lysis. In agreement with these results SOD activity increased 24 hours after blood feeding. Differently from SOD, catalase activity decreased compared to sugar-fed mosquitoes. Opposite results were seen in *A. gambiae* and *A. aegypti*, where catalase activity was increased in blood-fed when compared to sugar fed insects (Oliveira 2007, Molina-Cruz *et al.* 2008).

The catalase and SOD activity of *A. aquasalis* decreased 24 hours after *P. vivax* infection, time when *Plasmodium* passes through the mosquito midgut cells. *Molina*-Cruz *et al.* (2008) also observed a decreased in *A. gambiae* catalase mRNA and activity 24 hours after *P. berghei* infection. This phenomenon may be related to the production of high ROS levels in the midgut cells during *Plasmodium* invasion that should be efficient to mount an immune response capable of killing the parasites while preventing self-damage. The expression of SOD3B and catalase mRNA increased 36 hours after *P. vivax* challenge, probably in an attempt to compensate for the reduced enzyme activities observed. The high expression of these enzymes may be correlated to the necessity of detoxification of ROS, which should be induced by the *P. vivax* invasion in *A. aquasalis* midgut cells. The catalase and both SODs

did not present a signal peptide in their sequences (data not shown), which confirms their role in the intracellular environment. High expression of NOS, which produces the free radical NO, was also observed in the cytoplasm of some midgut cells of *A. aquasalis* 24 hours after *P. vivax* infection (Bahia *et al.* 2010b). According with the model proposed by Kumar *et al.* (2004), nitrite formed from the NO together with hydrogen peroxide, which accumulated in the mosquitoes due catalase activity reduction, may be used as substrates generating NO_2 and mediating nitrations effective in parasite clearence. This local ROS production can be effective in maintaining the very low parasite loads observed in infections of *A. aquasalis* with this human parasite. Further investigations are necessary to check this hypothesis.

Catalase is an important detoxification enzyme that transforms hydrogen peroxide (H_2O_2) into water (H_2O) and molecular oxygen (O_2). The catalase knockdown surprisingly exacerbated the infection of *A. aquasalis* by *P. vivax.* In contrast, Molina-Cruz and collaborators (2008) observed a protective effect of catalase silencing on *A. gambiae* infected by *P. berghei.* A possible explanation for the differences observed here could be that, after a *P. vivax* challenge, *A. aquasalis* DUOX proteins produced hydrogen peroxide, which was not removed efficiently due to the catalase mRNA knock-down. This excess of hydrogen peroxide may have been used as substrate by a midgut extracellular peroxidase, similar to IMPer, recently described in *A. gambiae* (Kumar *et al.* 2010), to form excessive dityrosine networks on the gut epithelium. These networks were shown in *A. gambiae* to be responsible for the decreased permeability to immune elicitors (Kumar *et al*. 2010), preventing strong immune activation through NO generation and leading to *Plasmodium* “protection” during its passage through the midgut. This phenomenon might take place in *A. aquasalis* and be important in protecting *P. vivax* from the *A. aquasalis* immune response in the first hours of infection. One possible explanation for the differences observed between *A. gambiae* and *A. aquasalis* could be the level of reduction of catalase mRNA and activity after silencing, when a 10-20% reduction of mRNA and 20% of enzyme activity was observed in *A. aquasalis* compared to 50% of mRNA and 87 to 94% of activity in *A. gambiae* (Molina-Cruz *et al.* 2008). This smaller reduction of catalase might permit low levels of H_2O_2 persistence in the *A. aquasalis* midgut that could be used by the peroxidase to build a dityrosine network and prevent the activation of the mosquito immune response, thus allowing parasite

development. In contrast, the 50% of *A. gambiae* catalase silencing, which results in 87 to 94% of catalase activity reduction (Molina-Cruz *et al.* 2008), could be responsible to the maintenance of high levels of ROS and consequent death of *P. berghei* parasites.

In a previous screening work (Bahia *et al.* 2010), we revealed some immune genes in subtractive libraries of *A. aquasalis* infected with *P. vivax.* Analyses of some of these immune genes such as (bacteria responsive protein, fibrinogen) showed that the presence of the parasite in insect haemolymph 36 hours after infection, rather than its presence in the midgut or during passage through its epithelium 24 hours after infection, appeared to correlate with the induction of an anti-microbial immune response. Our present results show that ROS production can also be important to control the parasite burden in the insect midgut. A slight oxidative stress was also observed in *A. gambiae* infected with the human parasite *P. falciparum* when compared to the murine parasite *P. berghei* (Molina-Cruz *et al.* 2008).

The results here presented show evidence for the existence of a finely regulated free radicals defense system, since small quantitative variations of these molecules can have different effects on the mosquito immune response to pathogens. Thus, molecular manipulations of this system could be targeted in vector control strategies, considering their effects on mosquito susceptibility to malaria parasites. The *A. aquasalis* oxidative response to *P. vivax* infection is presently under investigation.

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Figure legends

Figure 1: **Sequence of *A.aquasalis* Catalase.** Numbers on the left represent nucleotide sequence length and on the right indicate amino acid sequence length; asterisk indicates the stop codon; the aminoacids in bold format indicates the heme binding pocket; the underlined aminoacids represent the tetramer interface.

Figure 2: **Characterization of Catalase.** A: Schematic representation of *A. aquasalis* (AqCAT) catalase protein showing the catalase domain in red. B: Multiple aminoacid sequence alignment of mosquito catalase related proteins. B: Phylogenetic tree for catalase constructed based on the neighbor-joining method. Accession numbers of Catalase sequences from: *A. gambiae* (Ag) (XP_314995.4), *A. aegypti* (Ae) (XP_001663600.1), *Culex quinquefasciatus* (Cq) (XP_001848573.1) and *D. melanogaster* (Dm) (NP_536731.1).

Figure 3: **Sequence of SOD3A (A) and SOD3B (B) obtained PCR using degenerate primers and RACE sequencing.** Numbers on the left represent nucleotide sequence length and on the right indicate amino acid sequence length; asterisk indicates the stop codon; the underlined aminoacids show the P-class dimer interface and in italics the E-class dimer interface; the aminoacids in bold format indicate the zinc binding and active sites; in italics and underlined, the copper binding and active sites, heme binding pocket; the underlined aminoacids represent the tetramer interface.

Figure 4: **Characterization of SOD3A and SOD3B.** A: Schematic representation of SOD of *A. aquasalis*(Aq) and *A. gambiae (*Ag) showing the alpha-hairpin domain (green) and C-terminal domain (blue) of the iron/manganese superoxide dismutases and copper/zinc superoxide dismutase domain (red). C: Multiple aminoacid sequence alignment of mosquito SOD related proteins. B: Phylogenetic tree for SOD constructed based on the neighbor-joining method. Acession numbers of SOD3A and SOD3B sequences from: *A. gambiae* (SOD1 - XP_314490.3, SOD2 - XP_314137.4, SOD3A - XP_311594.2 and SOD3B - XP_001230820.1).

Figure 5: **Expression levels of catalase in *A. aquasalis* following different feeding regimens.** A: mRNA expression of catalase in sugar-fed (SF) males and females; B: sugar-fed (SF) females (dotted line), and blood-fed (control) (BFC) and blood-fed infected (BFI) females; C: catalase activity in SF, 24 hours BFC and BFI females reported as units per minute per micrograms of protein (U/mg ptn). Data are reported as the mean ± SEM. h – hours. * 0.05>p>0.03, ** 0.03>p>0.01, *** p>0.01.

Figure 6: **Expression levels and activity of SOD3A and SOD3B related protein in *A. aquasalis* following different feeding regimens.** A and C: mRNA expression of SOD3A (A) and SOD3B (C) in sugar-fed (SF) males and females, B and D: mRNA expression of SOD3A (B) and SOD3B (D) in sugar-fed females (dotted line), and blood-fed (control) (BFC) and blood-fed infected (BFI) females. C: SOD activity in SF, 24 hours BFC and BFI females reported as units per minute per micrograms of protein (U/mg ptn). Data are reported as the mean ± SEM. * 0.05>p>0.03, ** 0.03>p>0.01, *** p>0.01.

Figure 7: **ROS production in the midgut of *A. aquasalis* submitted to different experimental conditions.** Immunofluorescence staining of *A. aquasalis* midguts in sugar-fed (SF), blood-fed (control) (BFC) and blood-fed infected (BFI) females. The midguts were stained with a redox sensitive fluorescent probe CM-H2DCFDA.

Figure 8: **Molecular analysis of catalase silencing.** A and B – Effect of dscatalase injections on catalase mRNA expression (A) and activity (24 hours after blood-feeding; B). C – Percentage of infected insects after β-gal and catalase dsRNA injection. D and E – Oocysts numbers in the midguts of dsβ-gal and dscatalase injected mosquitoes 3-5 days after *Plasmodium* infection. The significance of gene silencing effect on oocysts loads in experimental samples, compared to water dsβ-gal-treated controls, was determined by Mann-whitney test with Bonferroni correction.

Figure 1

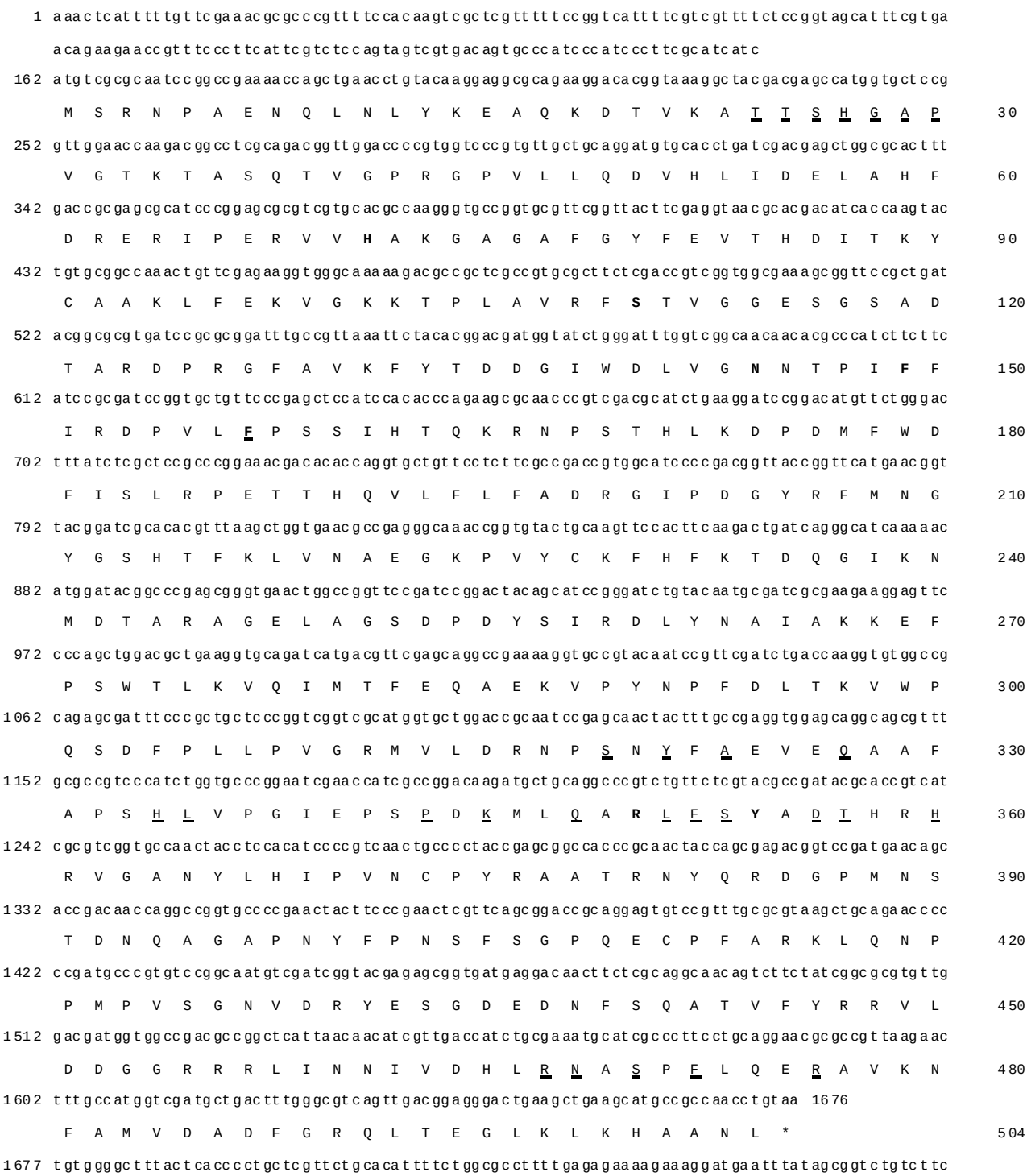
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Figure 2

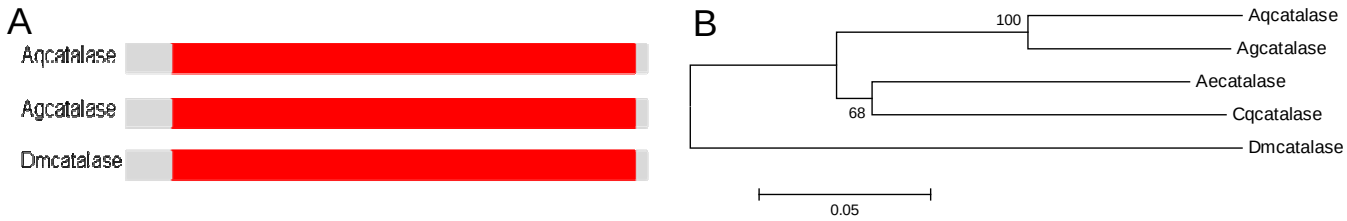

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Figure 3

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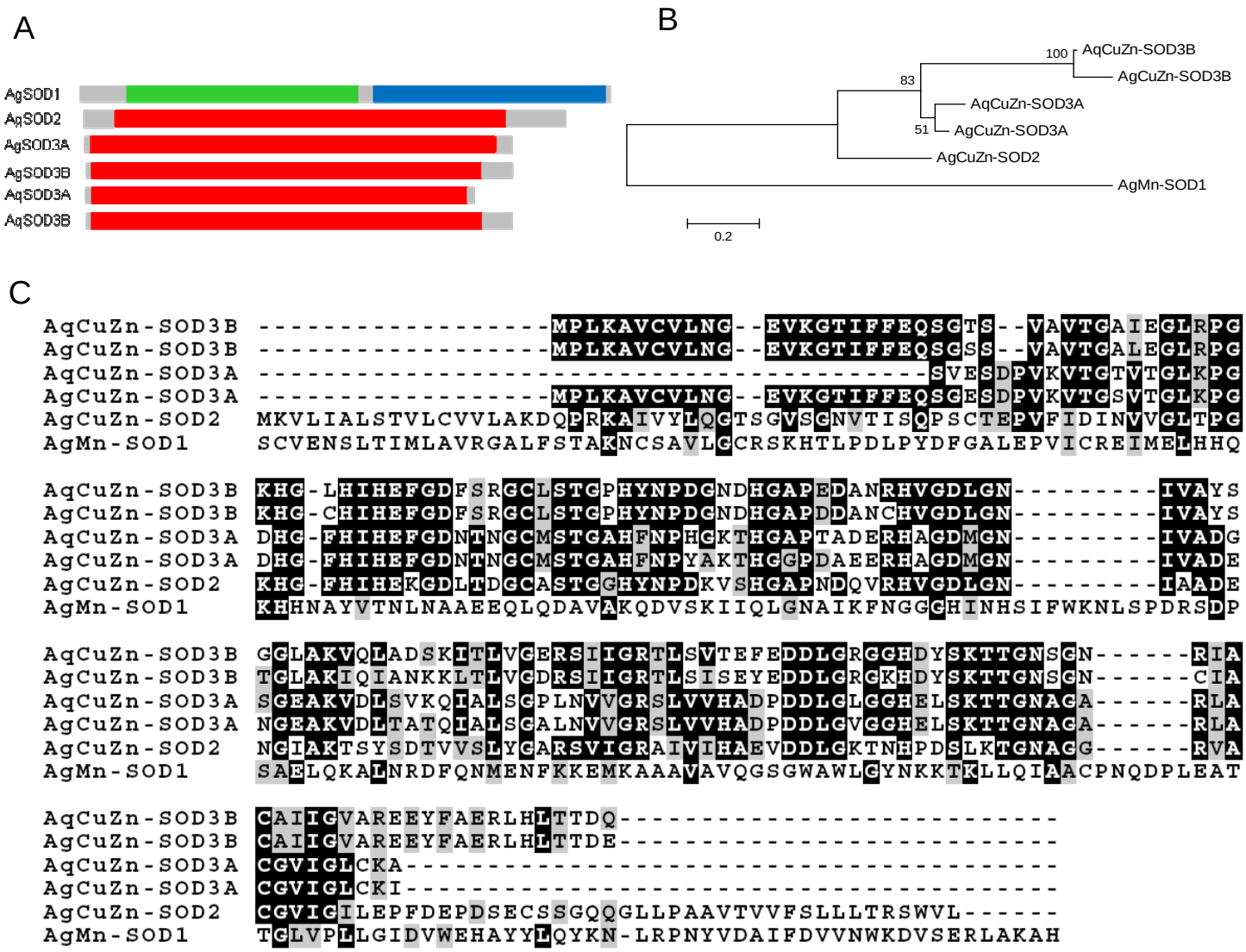
     D  D  L  G  R  G  G  H  D  Y  S  K  T  T  G  N  S  G  N  R  I  A  C  A  I  I  G  V  A  R 150
514 gaagagtatttcgccgaacgattgcatctgaccaccgatcaatga 558
     E  E  Y  F  A  E  R  L  H  L  T  T  D  Q  *
559 gactggacgatattagaaataaaactctatctactgctctgtt[aaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa]637
```

Figure 4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AgSOD1
AqSOD2
AgSOD3A
AgSOD3B
AqSOD3A
AqSOD3B
B
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AqCuZn-SOD3B
AgCuZn-SOD3B
83
AqCuZn-SOD3A
51
AgCuZn-SOD3A
AgCuZn-SOD2
AgMn-SOD1
0.2
C
AqCuZn-SOD3B ----------MPLKAVCVLNG--EVKGTIFFEQSGTS--VAVTGAIEGLRPG
AgCuZn-SOD3B ----------MPLKAVCVLNG--EVKGTIFFEQSGSS--VAVTGALEGLRPG
AqCuZn-SOD3A ----------SVESDPVKVTGTVTGLKPG
AgCuZn-SOD3A ----------MPLKAVCVLNG--EVKGTIFFEQSGESDPVKVTGSVTGLKPG
AgCuZn-SOD2 MKVLIALSTVLCVVLAKDQPRKAIVYLQGTSGVSGNVTISQPSCTEPVFIDINVVGLTPG
AgMn-SOD1 SCVENSLTIMLAVRGALFSTAKNCSAVLGCRSKHTLPDLPYDFGALEPVICREIMELHHQ
AqCuZn-SOD3B KHG-LHIHEFGDFSRGCLSTGPHYNPDGNDHGAPEDANRHVGDLGN---------IVAYS
AgCuZn-SOD3B KHG-CHIHEFGDFSRGCLSTGPHYNPDGNDHGAPDDANCHVGDLGN---------IVAYS
AqCuZn-SOD3A DHG-FHIHEFGDNTNGCMSTGAHFNPHGKTHGAPTADERHAGDMGN---------IVADG
AgCuZn-SOD3A DHG-FHIHEFGDNTNGCMSTGAHFNPYAKTHGGPDAEERHAGDMGN---------IVADE
AgCuZn-SOD2 KHG-FHIHEKGDLTDGCASTGGHYNPDKVSHGAPNDQVRHVGDLGN---------IAADE
AgMn-SOD1 KHHNAYVTNLNAAEEQLQDAVAKQDVSKIIQLGNAIKFNGGGHINHSIFWKNLSPDRSDP
AqCuZn-SOD3B GGLAKVQLADSKITLVGERSIIGRTLSVTEFEDDLGRGGHDYSKTTGNSGN------RIA
AgCuZn-SOD3B TGLAKIQIANKKLTLVGDRSIIGRTLSISEYEDDLGRGKHDYSKTTGNSGN------CIA
AqCuZn-SOD3A SGEAKVDLSVKQIALSGPLNVVGRSLVVHADPDDLGLGGHELSKTTGNAGA------RLA
AgCuZn-SOD3A NGEAKVDLTATQIALSGALNVVGRSLVVHADPDDLGVGGHELSKTTGNAGA------RLA
AgCuZn-SOD2 NGIAKTSYSDTVVSLYGARSVIGRAIVIHAEVDDLGKTNHPDSLKTGNAGG------RVA
AgMn-SOD1 SAELQKALNRDFQNMENFKKEMKAAAVAVQGSGWAWLGYNKKTKLLQIAACPNQDPLEAT
AqCuZn-SOD3B CAIIGVAREEYFAERLHLTTDQ----------
AgCuZn-SOD3B CAIIGVAREEYFAERLHLTTDE----------
AqCuZn-SOD3A CGVIGLCKA----------
AgCuZn-SOD3A CGVIGLCKI----------
AgCuZn-SOD2 CGVIGILEPFDEPDSECSSGQQGLLPAAVTVVFSLLLTRSWVL------
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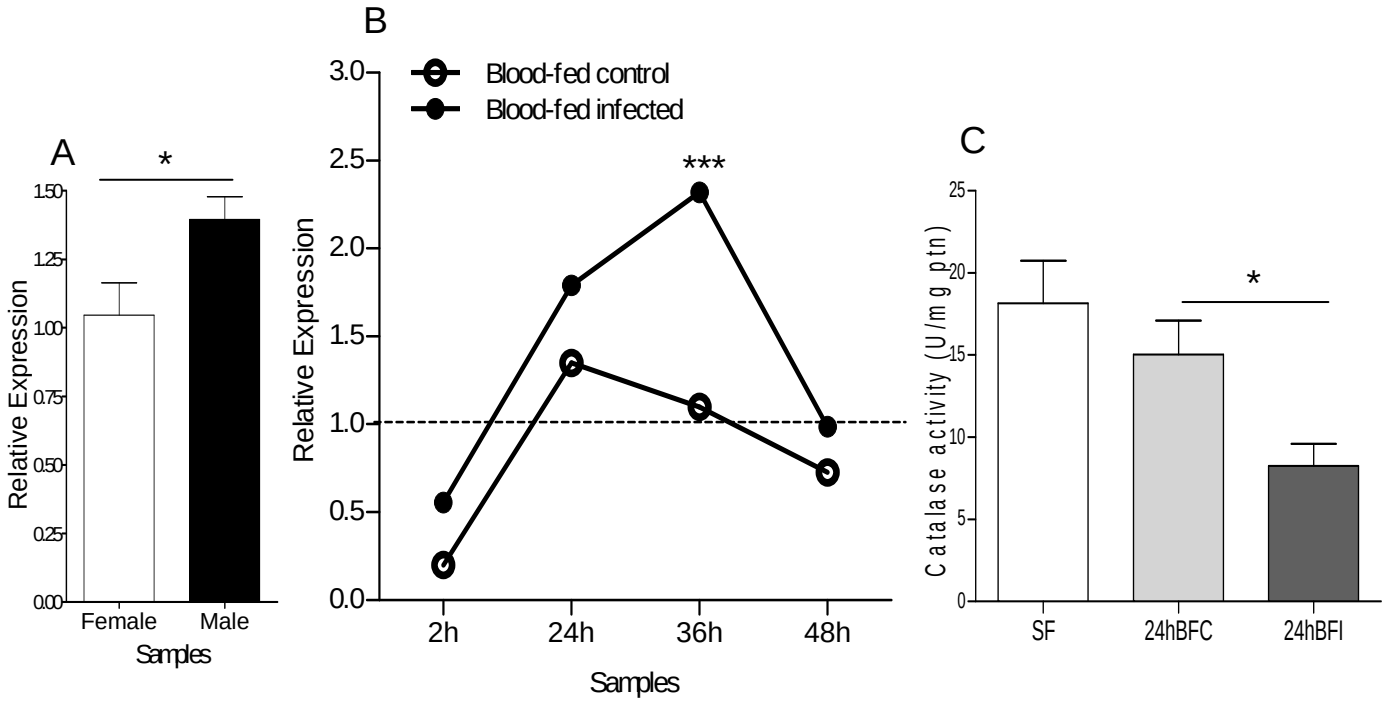
Figure 5


A
*
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1.50
1.25
1.00
0.75
0.50
0.25
0.00
Female
Male
Samples
B
Blood-fed control
Blood-fed infected
***
Relative Expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
2h
24h
36h
48h
Samples
C
*
Catalase activity (U/mg ptn)
25
20
15
10
5
0
SF
24hBFC
24hBFI

Figure 6

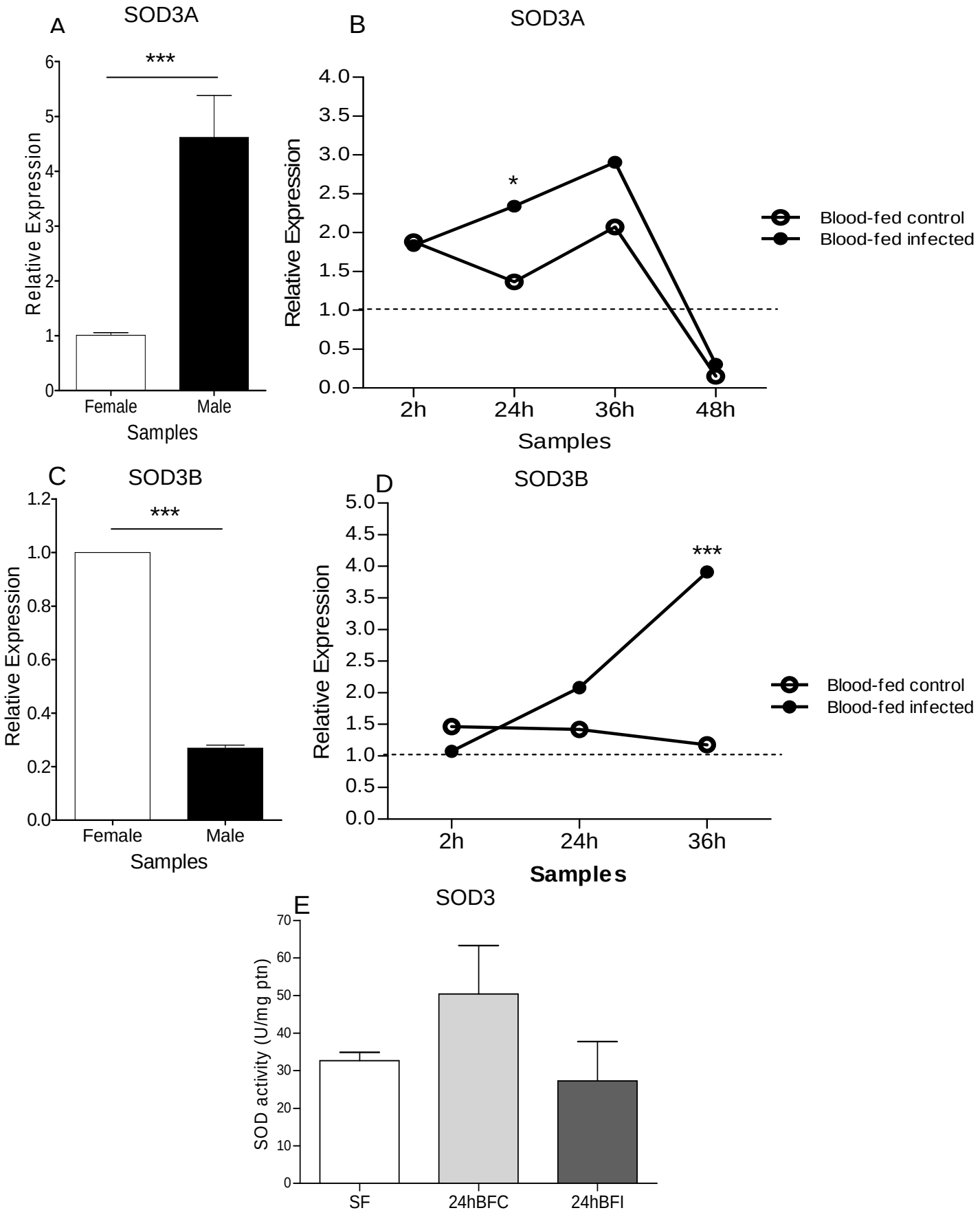

A
SOD3A
***
Relative Expression
Female
Male
Samples
B
SOD3A
*
Relative Expression
2h
24h
36h
48h
Samples
Blood-fed control
Blood-fed infected
C
SOD3B
***
Relative Expression
Female
Male
Samples
D
SOD3B
***
Relative Expression
2h
24h
36h
Samples
Blood-fed control
Blood-fed infected
E
SOD3
SOD activity (U/mg ptn)
SF
24hBFC
24hBFI

Figure 7

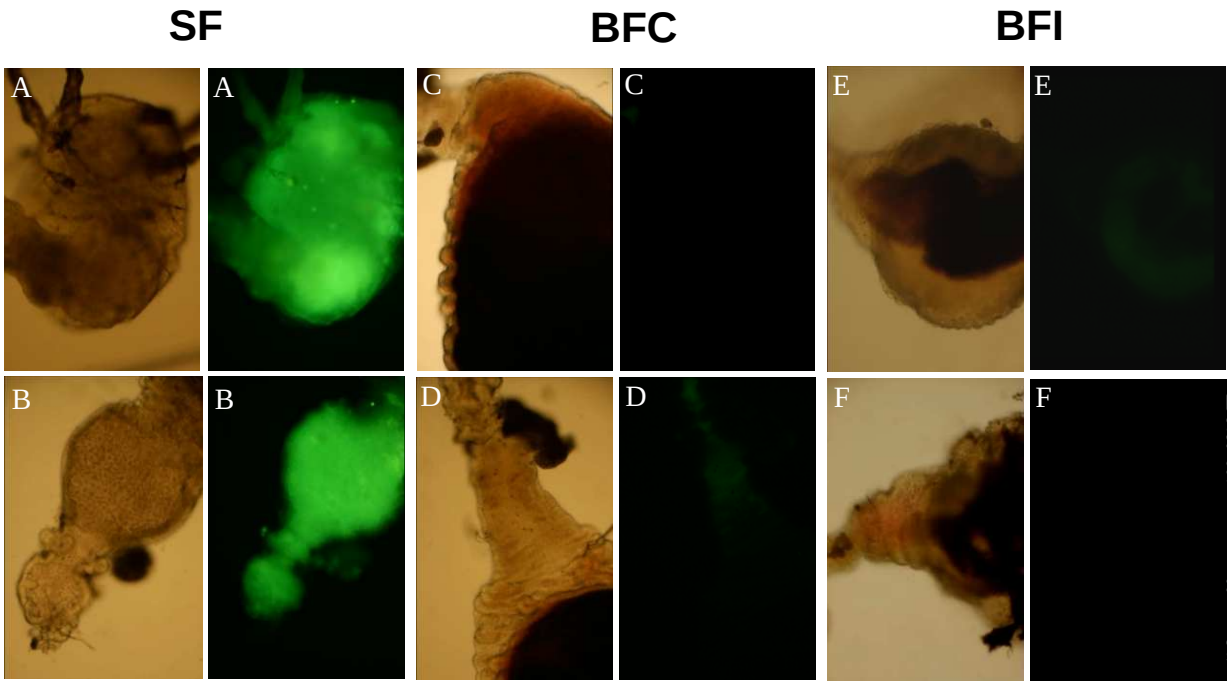
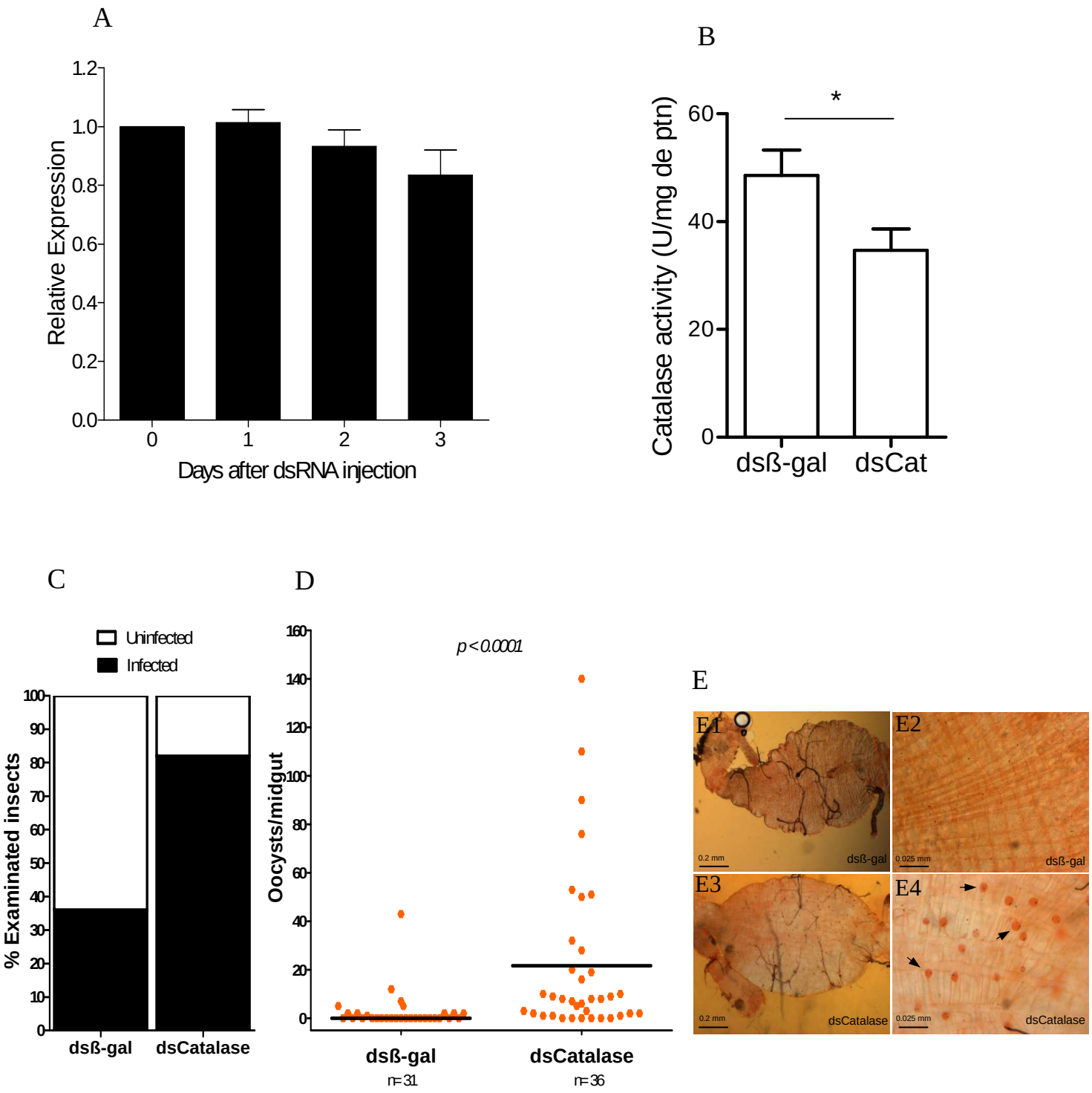


Figure 8


A
Relative Expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
Days after dsRNA injection
B
Catalase activity (U/mg de ptn)
*
60
40
20
0
dsß-gal
dsCat
C
Uninfected
Infected
% Examinated insects
100
90
80
70
60
50
40
30
20
10
0
dsß-gal
dsCatalase
D
p<0.0001
Oocysts/midgut
160
140
120
100
80
60
40
20
0
dsß-gal
n=31
dsCatalase
n=36
E
E1
0.2 mm
dsß-gal
E2
0.025 mm
dsß-gal
E3
0.2 mm
dsCatalase
E4
0.025 mm
dsCatalase

Capítulo 4

O fator de transcrição GATA é requerido para a imunidade de *Anopheles aquasalis* contra *Plasmodium vivax*

Manuscrito a ser submetido

Justificativa:

O sistema imune dos insetos é regido por ativação/desativação de vias de sinalização e produção de genes efetores. A etapa que antecede o final destas vias é a ativação de fatores de transcrição que se direcionam ao núcleo e promovem a transcrição de genes. Muitos genes efetores, como os AMPs, possuem em suas regiões regulatórias sítios de ligação a fatores de transcrição da família NF-kB. Outra família de fatores de transcrição relacionados com aspectos do sistema imune é a GATA. Fatores de transcrição do tipo GATA têm sido descritos em *D. melanogaster* e *Caenorhabditis elegans* como tendo papel na imunidade contra diferentes patógenos. Assim, o possível papel de um fator de transcrição do tipo GATA na resposta imune de *A. aquasalis* ao *P. vivax* foi analisado.

Anopheles aquasalis GATA transcription factor is required for immunity against *Plasmodium vivax*

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Abstract

Innate immunity is an ancient and conserved defense system that provides an effective response against invaders. Many immune genes of *Anopheles* mosquitoes have been implicated in defense against a variety of pathogens, including plasmodia. Nevertheless, only recently *A. aquasalis* immune genes, involved in response against *Plasmodium vivax,* were identified. One such gene is the GATA transcription factor, which is described here. Characterization of this gene revealed that it is closely related to the *Drosophila melanogaster* and *Anopheles gambiae* Serpent GATA transcription factors. Gene expression analysis showed an increase of GATA protein in *P. vivax* infected *A. aquasalis* and functional RNAi experiments identified this GATA as a gene important for the immune response of *A. aquasalis* against *P. vivax* infection. These findings expand our knowledge about the interaction of this poorly studied human malaria parasite and its vector.

Introduction

Insects are continuously exposed to a variety of pathogens, which are inactivated by their efficient immune system. Parasites, however, continuously evolve new surface proteins and virulence mechanisms, which are matched through a constantly evolving immune system, leading to an endless parasite-immune system evolutionary arms race. Nevertheless, some important signaling pathways have remained fairly unchanged in several groups of organisms. Insects have a powerful immune system that includes blood clotting cascades, melanin production, phagocytosis, encapsulation, antimicrobial peptide (AMP) synthesis and free radical production (Lemaitre and Hoffmann 2007). All immune responses are orchestrated by molecular signaling network that leads to the activation of transcription factors (TF). These TFs promote the transcription of effectors genes, which are important to activate immune mechanisms responsible to kill invaders. In anopheline mosquitoes some TFs have been described as key immune molecules. Among these are STAT, Rel1 and Rel2 that are part of the three main immune signaling pathways, JAK-STAT, Toll and IMD, respectively (Garver *et al.* 2009, Gupta *et al.* 2009).

The GATA TFs have one or two zinc fingers that can bind the DNA sequence (AT) GATA (AG). They are conserved among fungi, plants and animals, and are involved in development and differentiation (Patient and McGhee 2002). Mammals present six GATA proteins, of which GATA1, 2 and 3 are crucial for haematopoiesis (Patient and McGhee 2002), and GATA4, 5 and 6 for mesoendodermal development (MolKentin *et al.* 2000). In *Drosophila melanogaster* five GATA factors are known. Only one of these genes, the GATAc (Grain), is an ortholog of GATA1, 2 and 3, and the other four [GATAa (Pannier), GATAb (Serpent), GATAd and GATAe)] are GATA4, 5 and 6 orthologs (Gillis *et al.* 2008). Two of the *Drosophila* GATA proteins, the Serpent and GATAe, which are responsible for early meso- and endodermal development of fat body and midgut, respectively, are also involved in the regulation of transcription of several genes in these tissues, including immune related ones (Petersen *et al.* 1999, Tingvall *et al.* 1999, Senger *et al.* 2006). In *D. melanogaster* the GATA Serpent has been also described as important in the haematopoiesis and differentiation of haemocytes (Artero et al. 2006, Gajewski *et al.* 2007, Muratoglu *et al.* 2007, Frandsen et al. 2008). *Drosophila* haematopoiesis gives rise to three independent haemocyte cell lineages orchestrated by GATA factor signalizations:

plasmatocytes, crystal cells and lamellocytes. These cells are responsible for the cell mediated components of insect immunity (Lemaitre and Hoffmann 2007). Plasmatocytes are small cells involved in phagocytosis and encapsulation and are also involved in production of antimicrobial peptides; crystal cells play a critical role in wound repair and melanization by secreting some components of the phenol oxidases cascade; and lamellocytes are involved in the encapsulation of large invaders (Meister *et al.* 2004, Williams *et al.* 2007).

Based on the importance of the GATA TFs in the insect immune response, we were interested in investigating the putative involvement of these TFs in the activation of the immune system of the Brazilian malaria vector *Anopheles aquasalis* against *Plasmodium vivax*. This vector-parasite pair was chosen for this study due to the prevalence of *P. vivax* as malaria causative agent and the importance of *A. aquasalis* in malaria transmission in Brazil. Furthermore, very few works have been made with *P. vivax* since there is not a continuous cultivation system available for this parasite and due to the wrong belief that this parasite does not cause severe and fatal malaria (Udomsangpetch *et al.* 2008, Anstey *et al.* 2009). In this work, one GATA TF revealed in subtraction libraries of *A. aquasalis* (Bahia *et al.* 2010), was studied in detail. RTPCR experiments showed a 15 fold increase in GATA after *P. vivax* infection. Reverse genetics experiments demonstrated the importance of this gene in the immunity of *A. aquasalis* against *P. vivax.*

Materials and Methods

Insect feeding and maintenance

A. aquasalis were reared at controlled temperature and humidity (Horosko *et al.* 1997). Mosquito infections with *P. vivax* were conducted in Manaus, a Brazilian endemic area for malaria, as described in Bahia *et al.* (2010). All insects were infected with blood from human patients due the lack of a continuous cultivation system of *P. vivax* (Udomsangpetch *et al.* 2008). The experimental feeding of insects with blood of healthy or infected patients was performed at 37^{o}C constant temperature maintained using a water circulation system. After that, the mosquitoes were placed in cages with 20% sucrose *ad libitum* until the experimental procedures.

cDNA RACE Amplification

To obtain the 5' and 3' ends of the GATA cDNA, the SMART cDNA RACE (Becton Dickinson Clontech) amplification technique was used. All amplicons generated by the RACE PCR reaction were cloned into the pGEM®-T Easy Vector (Promega) and used to transform competent *Escherichia coli* DH5α. Plasmids were sequenced in an ABI 3700 sequencer (Applied Biosystems) in the PDTIS/FIOCRUZ Sequencing Platform. The sequences obtained were used to mount the cDNAs of *A. aquasalis* GATA with the CAP3 Sequence Assembly (http://pbil.univ-lyon1.fr/cap3.php) and Clustal W Programs (http://www.ebi.ac.uk/Tools/clustalw2/).

Real Time PCR

Mosquitoes submitted to different experimental feedings [sugar (males and females), and feeding with blood containing or not *P. vivax* parasites (females) were used for real time PCR (RTPCR). Total RNA from whole insects was extracted and treated with RQ1 RNAse-free DNAse (Promega). The synthesis of cDNA was performed with OligodT primer and SuperScript™ II Reverse Transcriptase (Invitrogen). All RTPCR reactions were conducted using the SyberGreen fluorescent probe in an ABI 7000 machine (Applied Biosystems). The PCR cycles used were 50^{o}C 2 min, 95^{o} C 10min, 95^{o} C 15 sec and 63^{o} C 1 min for 35 times for all reactions. To amplify the GATA gene the following primers were used: GATAFwd 5' ATCTGCTACACGCAGCAGGTGCCAT 3' and GATARev 5' TGACGATAGCCCCACTGGAGGGAGT 3'. The calculation of the relative expression of the selected genes was made based on gene expression CT difference formula (Schefe *et al.* 2006). Quantifications were normalized in relation to the housekeeping gene rp49 (Gentile *et al.* 2005). All experiments were performed with four to six biological replicates and three experimental replicates. The statistics method used in the analyses was ANOVA test with multiple comparisons of Tukey or Games-Howell. When the parametric model (ANOVA) was not adequate, we utilized the Kruskal-Wallis test with multiple comparisons of Mann-Whitney. For the male versus female analyses the t-student or the Wilcoxon tests were utilized. All tests were performed with reliable level of 95% (α= 0.05). The statistical analyses were accomplished using the Graph pad Prism5®, R, software.

Gene silencing

Knockdown of the GATA gene was performed by the injection of double stranded RNA (dsRNA) into *A. aquasalis* females. The dsRNAs were produced with the T7 Megascript kit (Ambion) using PCR-amplified fragments. Amplicons for dsß-gal were produced via plasmid templates and for dsGATA by RT-PCR products, from sugar-fed female cDNA, giving rise to 544 bp and 404 bp fragments, respectively. The PCR reactions were performed in two PCR rounds as described (Bahia *et al.* 2010b). The primers used for the first round of PCR were: ß-galdsRNAFwd 5' tggcgcccctagatgTGATGGCACCCTGATTGA 5' and ß-galdsRNARev 5' tggcgcccctagatgTCATTGCCCAGAGACCAGA 3', and GATAdsRNA Fwd 5' tggcgcccctagatgACGAGAGTGCGTCAATTGTG 3' and GATAdsRNA Rev 5' tggcgcccctagatgGATTGTTTCCATCGTTGGCT 3'. The second round PCR primer, used was CCG**TAATACGACTCACTATAGG**TGGCGCCCCTAGATG.

A 69nL volume of GATA or ß-gal dsRNA (3 µg/µL) was introduced into the thorax of cold anesthetized 3–4 day old female mosquitoes by a nano-injector (Nanoject, Drummond) with glass capillary needles. All injected insects were maintained in an air incubator at 28º C and fed on sugar solution *ad libitum*. Two to three days after the dsRNA injections, the insects were fed with *P. vivax* infected blood. The estimation of oocysts numbers was done three to five days after infection. At least 30 mosquitoes were used for each experimental condition. The midguts were dissected under the scope and stained with 2% mercury chrome. Oocyst numbers in dsGATA injected mosquitoes were counted and compared to the control (mosquitoes injected with B-gal dsRNA). The significance of gene silencing on oocysts loads between the experimental and control groups was determined by Mann-Whitney statistical test.

Semi-quantitative RT-PCR

Total RNA was extracted from *A. aquasalis* females either sugar-fed or one to five days after dsRNA injections . Up to 5 µg of RNA were treated with RQ1 RNAse-free DNAse (Promega) and used for first strand cDNA synthesis. The same reaction conditions and primers (GATA and RP49) used for RTPCR were used here and Triplicate experiments were performed. The PCR amplicons were separated in a 2.5% ethidium bromide-stained agarose gel. The intensity of amplified products was

measured using ImageJ 1.34s software (http://rsb.info.nih.gov/ij) and plotted for semi-quantitative analysis. The statistics method used in the analysis was ANOVA test with multiple comparisons of Tukey.

Results

Three GATA sequences (accession numbers GR486699, GR486641, GR486542) were identified in 2 hours after feeding minus 2 hours after infected *A. aquasalis* subtraction library previously published for us (Bahia *et al.* 2010). One single sequence of 174 bp was obtained after the clusterization of these sequences. The SMART cDNA RACE amplification technique was used to obtain the full cDNA sequence for this gene. The full-length 725 bp sequence obtained for the *A. aquasalis* GATA (AqGATA) gene included an open reading frame encoding a 209 amino acid residues protein plus 124bp upstream untranslated region (Figure 1). AqGATA contains two zinc finger binding domains characteristic of the GATA superfamily (Figure 1 and 2A). Phylogenetic analyses showed that this sequence is more related to GATA Serpent protein of *A. gambiae* and *D. melanogaster* than to the other four GATA identified for these insects (Figure 2B and C).

Considering that in *D. melanogaster* GATA has been shown to be evolved in immune induction, we studied in details the expression of AqGATA in male and female insects and in females after feeding on sugar, blood and infected blood. RTPCR results showed that AqGATA is more expressed in males than in females (Figure 3A) and that it is highly induced (almost 15 times) 36 hours after *P. vivax* infection, in contrast with all other times analyzed (Figures 3B and C).

To determine the importance of this GATA factor in *A. aquasalis* immunity against *P. vivax*, reverse genetics experiments using RNA interference were performed. For this, dsRNA from β-gal, a gene not found in the insect genome, and GATA, were injected in *A. aquasalis* females. Semi-quantitative RT-PCR showed a 70-85% decrease of GATA mRNA levels in the first until the fourth day after injection (Figure 4), with levels returning to normal 5 days after inoculation (Figure 4). Two to three days after injection of dsGATA, the mosquitoes were infected with *P. vivax.* Examination and comparison of the mosquito midguts from experimental and control groups revealed that the infection increased after GATA knock-down. It was

observed an increase in the percentage of infection of 39% in the dsβ-gal injected mosquitoes (control group) to 63% in the dsGATA ones (Figure 5A). The median oocyst number per mosquito midgut increased from zero in the control to 11 in the experimental group (Figure 5B and C).

Discussion

GATA is a family of transcription factors known to be important in development and differentiation. In humans, flies and worms these proteins are also important in regulating the expression of many genes which play a role in immunity and inflammation (Petersen *et al.* 1999, Tingvall *et al*. 1999, Senger *et al.* 2006, Shapira *et al.* 2006). Due to this reason, this TF was characterized and assessed in relation to *A. aquasalis* infection by *P. vivax.* The GATA protein of *A. aquasalis* was shown to be more related to the *D. melanogaster* and *A. gambiae* GATA Serpent than the other four GATA TFs presented by these insects. Since in *D. melanogaster* Serpent plays a role in immunity, we tried to investigate if AqGATA plays a role in *A. aquasalis* response against *P. vivax.* The expression of AqGATA was higher in sugar-fed males than females, in agreement with our previous results for other *A. aquasalis* immune genes (Bahia *et al.* 2010). This seems to indicate that male mosquitoes are more prepared for eventual challenges, in opposition to what was observed in vertebrates and some invertebrate species, where females are more immunocompetent than males (reviewed in Nunn *et al.* 2009).

This might be an evolutionary strategy adopted by males to keep healthy during their short lifespan since they live 4 times less than females. Further investigations need to be done to clarify this phenomenon. The expression of AqGATA did not suffer any alterations after ingestion of blood. Nevertheless, GATA mRNA levels increased more than 15 times when this insect became infected by *P. vivax.* The timing of this expression increase, 36 hours after infection, indicated that the activation of this TF was not caused by the simple passage of the parasite through the midgut epithelial cells but by the presence of the parasite in the insect hemolymph. To confirm the immunity role of AqGATA, reverse genetics experiments to knock down the expression of GATA in *A. aquasalis* were performed. These experiments showed an exacerbation of mosquito infection after a 75-80% reduction

in mRNA levels for GATA. *D. melanogaster* Serpent functions as the major GATA TF in the immature stages and adults flies fat body, and is essential for immune response activation in this tissue and for haematopoiesis (Sam *et al.* 1996, Petersen *et al.* 1999, Tingvall *et al*. 1999, Senger *et al.* 2006). We believe in accordance with *D*. *m*elanogaster Serpent, the AqGATA could induce some effectors genes in the fat body or inducing the haemocytes proliferation and differentiation, contributing to mount a robust immune response against *P. vivax* invasion. We also observed that this gene has a role in the immunity against bacteria (data not shown). Hence, the role of this gene in mosquito immunity may be generic and not specific to one pathogen.

In summary, we described here a TF that presents an immune role in controlling *P. vivax* infection. As *A. aquasalis* is a competent malaria vector in nature, we can conclude that this GATA is important to control the *Plasmodium* development or cure infection in some mosquitoes, but not capable of blocking infection in all insects. Our results further encourage exploring the mechanisms that lead to a partial immunity of *A. aquasalis* against *P. vivax.* Finally, due to the apparent importance of GATA in *A. aquasalis* immunity against *P. vivax* and the conservation of this immune signalization pathway shown here, we suggest that this gene as target candidate to be used in control strategies for malaria transmission as production of *A. aquasalis* transgenic mosquitoes more resistant to *P. vivax* parasites.

Acknowledgments

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Figure legends

Figure 1: **Sequence of *A. aquasalis* GATA obtained by RACE sequencing.** Numbers on the left represent nucleotide sequence length and on the right indicate amino acid sequence length; the underlined amino acids show the two GATA zinc finger DNA binding domains, the bold amino acids indicate DNA-binding regions and zinc binding sites are in italics.

Figure 2: **Characterization of *A. aquasalis* GATA.** A: Schematic representation of *A. aquasalis*, *A. gambiae* and *D. melanogaster* GATA Serpent protein showing two GATA zinc finger DNA binding domains. B: Multiple amino acid sequence alignment

of mosquito GATA related proteins. B: Phylogenetic tree for GATA constructed based on the neighbor-joining method. Accession numbers of GATA sequences from: *A. gambiae* (Ag) (Pannier - NW_045682.1, Serpent - NW_045682.1, Grain - NW_045682.1, GATAd - NW_045838.1and GATAe - NW_045682.1), *D. melanogaster* (Dm) (Pannier - NM_057337.2, Serpent - NM_169694.1, Grain - NM_169206.1 , GATAd - NM_135539.3 and GATAe - NM_142259.2).

Figure 3: **Expression levels of GATA transcription factor in *A. aquasalis* following different feeding regimens.** A: mRNA expression of GATA in sugar-fed males and females; B and C: sugar-fed females (dotted line), and blood-fed (BFC) (control) and blood-fed infected (BFI) females. h – hours. +–: s.e.m.; * $p < 0.05$, ** $0.03 > p > 0.01$, *** $p < 0.01$.

Figure 4: **Molecular analysis of GATA silencing.** Effect of dsGATA injections on GATA (A) and on the housekeeping RP49 gene (B) mRNA expression. M – molecular marker, SF – sugar-fed female, 1-5d - females 1 to 5 days after dsRNA injection.

Figure 5: **Analysis of the effect of GATA silencing in mosquito susceptibility to *P. vivax*. A** – Percentage of infected insects after β-gal and GATA dsRNA injection. B and C – Oocyst numbers in the midguts of dsβ-gal and dsGATA injected mosquitoes 3-5 days after *Plasmodium* infection. The significance of gene silencing effect on oocyst loads in experimental samples, compared to dsBgal-treated control, was determined by Mann-whitney test.

Figure 1

```
  1 accagcaatgtgtgctcctcatcgtcacacatacactgcatacatacagtcatcagcacacactatatcatgatttaatcttatttaaat
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125 ctgttcacggagggacgagagtgcgtcaattgtggcgccatccagacgcccctctggcgtcgcgacggaactggccactacttgtgtaac
     M  F  T  E  G  R  E  C  V  N  C  G  A  I  Q  T  P  L  W  R  R  D  G  T  G  H  Y  L  C  N   30
215 gcgtgcggactctatcacaagatgaacgggatgaatcgccccctggtgaaacagcccagacgtttgagctcggctagacgaacgggactg
     A  C  G  L  Y  H  K  M  N  G  M  N  R  P  L  V  K  Q  P  R  R  L  S  S  A  R  R  T  G  L   60
305 cagtgttcaaactgcaacacgaccaacacttcgctctggcgccgcaatcaggtcggtgaaccggtatgtaacgcttgtgggctgtactac
     Q  C  S  N  C  N  T  T  N  T  S  L  W  R  R  N  Q  V  G  E  P  V  C  N  A  C  G  L  Y  Y   90
495 aaactgcacaacgttaaccgtccgctggctatgaagaaggataacattcagtcgcgcaaacggaagcccaaaggaagcaaaaacagcgat
     K  L  H  N  V  N  R  P  L  A  M  K  K  D  N  I  Q  S  R  K  R  K  P  K  G  S  K  N  S  D  120
585 ggaagcacgacagcgagcaaaaaccagaaggggagcaaagccaacgatggaaacaatcacgctgatcatgatttgaaaataatgcaactg
     G  S  T  T  A  S  K  N  Q  K  G  S  K  A  N  D  G  N  N  H  A  D  H  D  L  K  I  M  Q  L  150
675 ggagaagcttcgacgtacgacaagaatatgcgctcgtccccatccagcgacggtagcaacctgtctccggcgcaccggaatcacatgtcg
     G  E  A  S  T  Y  D  K  N  M  R  S  S  P  S  S  D  G  S  N  L  S  P  A  H  R  N  H  M  S  180
765 ccgatctgctacacgcagcaggtgccatcgccgatcacgagcactccctccagtggggct 824
     P  I  C  Y  T  Q  Q  V  P  S  P  I  T  S  T  P  S  S  G  A  200
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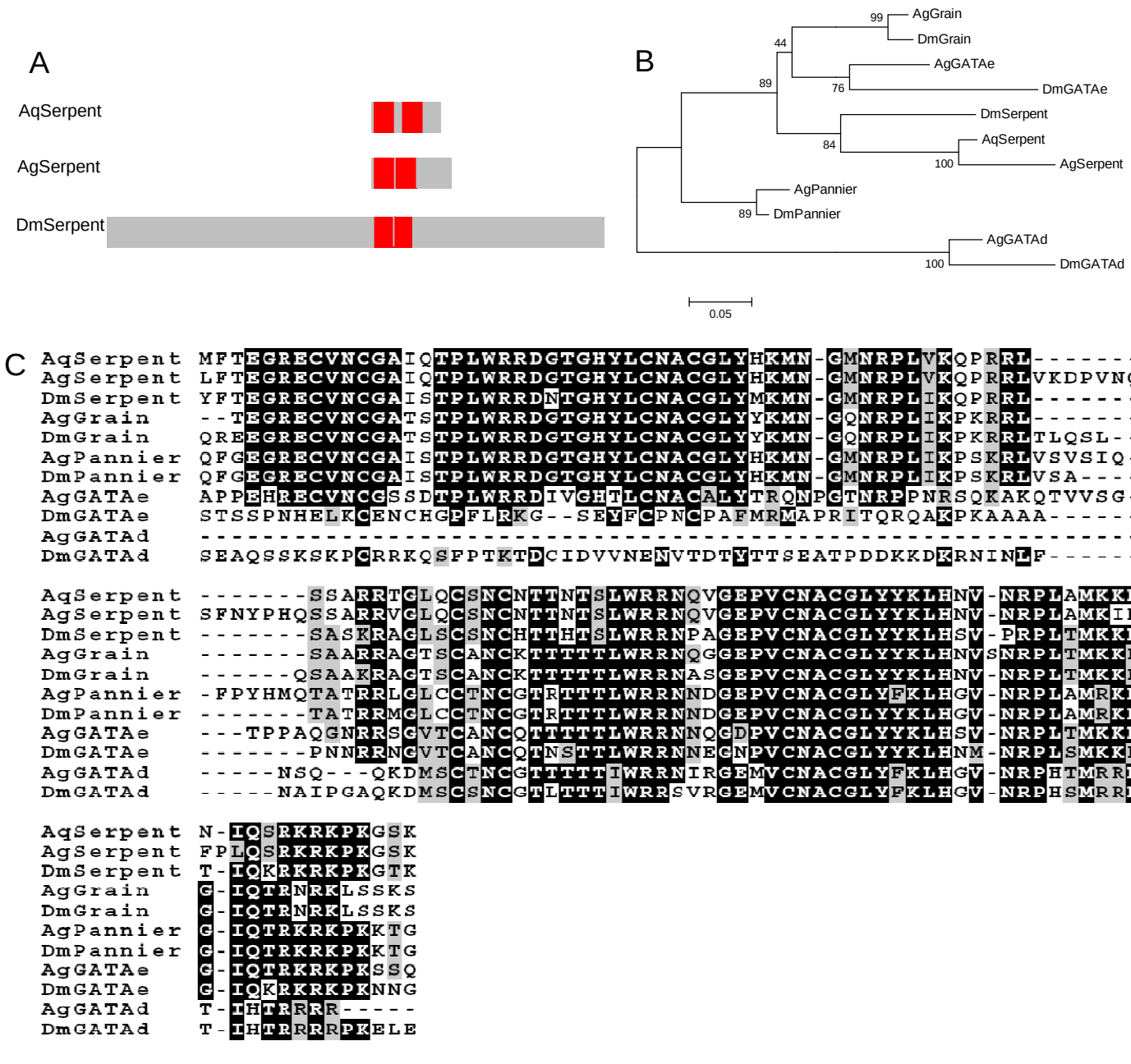
Figure 2


A
AqSerpent
AgSerpent
DmSerpent
B
AgGrain
DmGrain
AgGATAe
DmGATAe
DmSerpent
AqSerpent
AgSerpent
AgPannier
DmPannier
AgGATAd
DmGATAd
99
44
89
76
84
100
89
100
0.05
C
AqSerpent MFTEGRECVNCGAIQTPLWRRDGTGHYLCNACGLYHKMN-GMNRPLVKQPRRL-------
AgSerpent LFTEGRECVNCGAIQTPLWRRDGTGHYLCNACGLYHKMN-GMNRPLVKQPRRLVKDPVNQ
DmSerpent YFTEGRECVNCGAISTPLWRRDNTGHYLCNACGLYMKMN-GMNRPLIKQPRRL-------
AgGrain --TEGRECVNCGATSTPLWRRDGTGHYLCNACGLYYKMN-GQNRPLIKPKRRL-------
DmGrain QREEGRECVNCGATSTPLWRRDGTGHYLCNACGLYYKMN-GQNRPLIKPKRRLTLQSL--
AgPannier QFGEGRECVNCGAISTPLWRRDGTGHYLCNACGLYHKMN-GMNRPLIKPSKRLVSVSIQ-
DmPannier QFGEGRECVNCGAISTPLWRRDGTGHYLCNACGLYHKMN-GMNRPLIKPSKRLVSA----
AgGATAe APPEHRECVNCGSSDTPLWRRDIVGHTLCNACALYTRQNPGTNRPPNRSQKAKQTVVSG-
DmGATAe STSSPNHELKCENCHGPFLRKG--SEYFCPNCPAFMRMAPRITQRQAKPKAAAA------
AgGATAd ----------
DmGATAd SEAQSSKSKPCRRKQSFPTKTDCIDVVNENVTDTYTTSEATPDDKKDKRNINLF------

AqSerpent -------SSARRTGLQCSNCNTTNTSLWRRNQVGEPVCNACGLYYKLHNV-NRPLAMKKD
AgSerpent SFNYPHQSSARRVGLQCSNCNTTNTSLWRRNQVGEPVCNACGLYYKLHNV-NRPLAMKIN
DmSerpent -------SASKRAGLSCSNCHTTHTSLWRRNPAGEPVCNACGLYYKLHSV-PRPLTMKKD
AgGrain -------SAARRAGTSCANCKTTTTTLWRRNQGGEPVCNACGLYYKLHNVSNRPLTMKKE
DmGrain ------QSAAKRAGTSCANCKTTTTTLWRRNASGEPVCNACGLYYKLHNV-NRPLTMKKE
AgPannier -FPYHMQTATRRLGLCCTNCGTRTTTLWRRNNDGEPVCNACGLYFKLHGV-NRPLAMRKD
DmPannier -------TATRRMGLCCTNCGTRTTTLWRRNNDGEPVCNACGLYYKLHGV-NRPLAMRKD
AgGATAe ---TPPAQGNRRSGVTCANCQTTTTTLWRRNNQGDPVCNACGLYYKLHSV-NRPLTMKKD
DmGATAe -------PNNRRNGVTCANCQTNSTTLWRRNNEGNPVCNACGLYYKLHNM-NRPLSMKKE
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DmGATAd -----NAIPGAQKDMSCSNCGTLTTTIWRRSVRGEMVCNACGLYFKLHGV-NRPHSMRRD

AqSerpent N-IQSRKRKPKGSK
AgSerpent FPLQSRKRKPKGSK
DmSerpent T-IQKRKRKPKGTK
AgGrain G-IQTRNRKLSSKS
DmGrain G-IQTRNRKLSSKS
AgPannier G-IQTRKRKPKKTG
DmPannier G-IQTRKRKPKKTG
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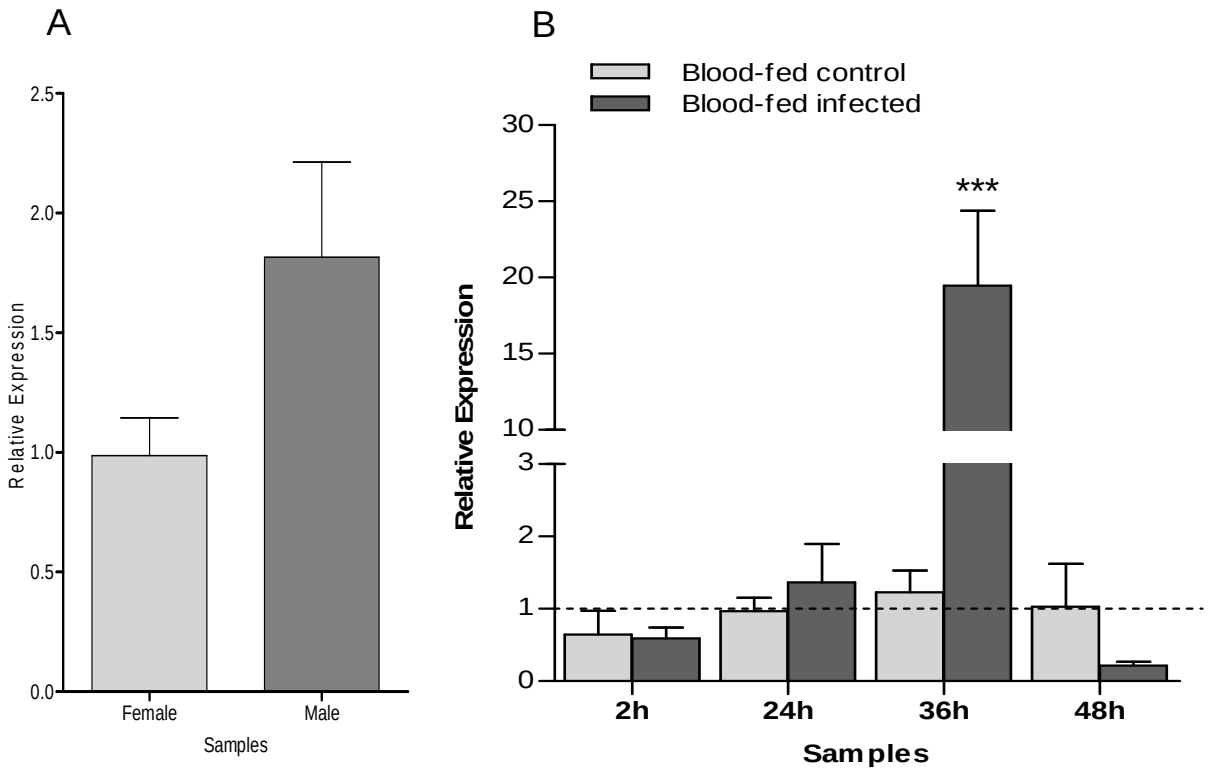
AgGATAd T-IHTRRR-----
DmGATAd T-IHTRRRPKELE

Figure 3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2.0
1.5
1.0
0.5
0.0
Relative Expression
Female
Male
Samples
B
Blood-fed control
Blood-fed infected
30
25
20
15
10
3
2
1
0
***
Relative Expression
2h
24h
36h
48h
Samples

Figure 4

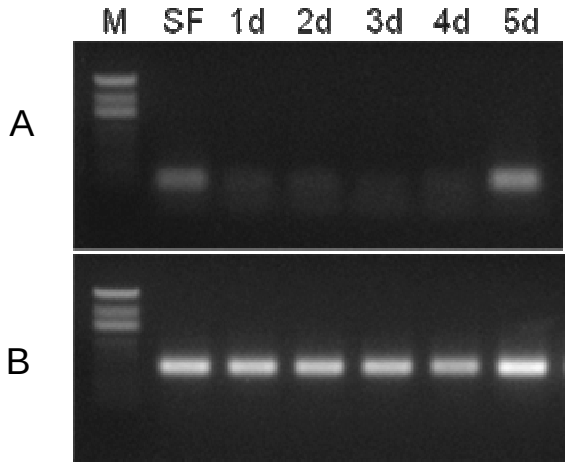
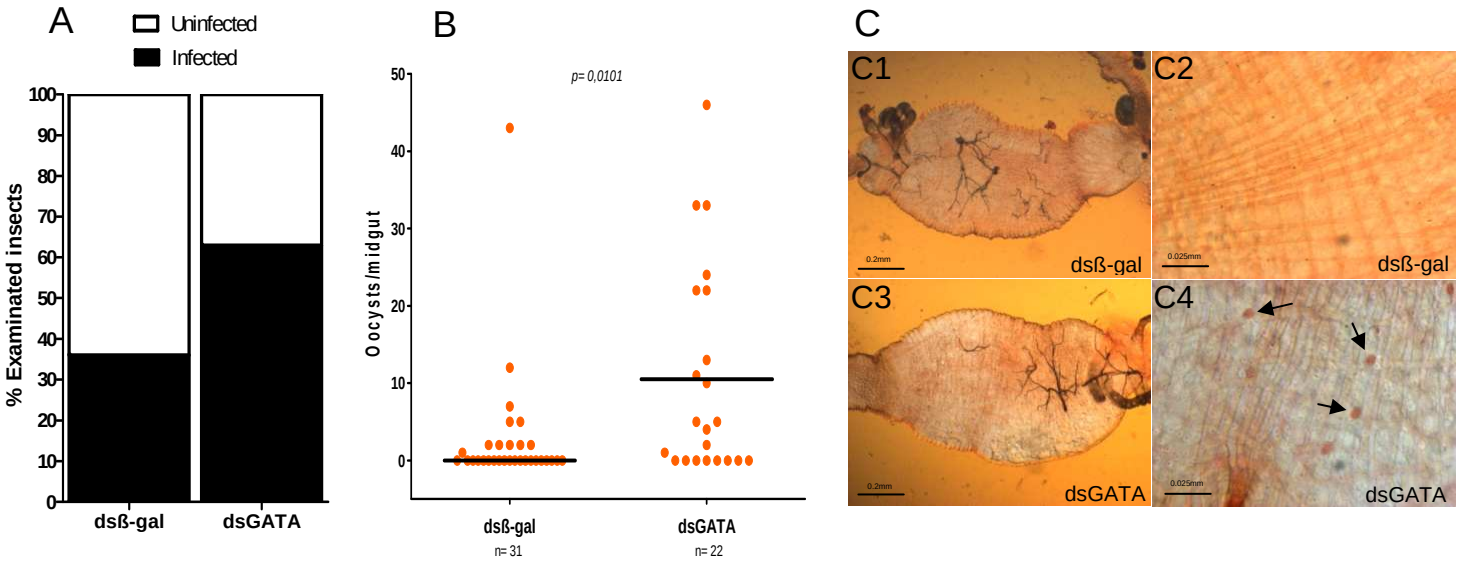


Figure 5


A
Uninfected
Infected
% Examinated insects
100
90
80
70
60
50
40
30
20
10
0
dsß-gal
dsGATA
B
p= 0,0101
Oocysts/midgut
50
40
30
20
10
0
dsß-gal
n= 31
dsGATA
n= 22
C
C1
dsß-gal
C2
dsß-gal
C3
dsGATA
C4
dsGATA

4. Discussão

4. Discussão

4.1 Considerações iniciais

Insetos vetores são responsáveis pela transmissão de patógenos a diversos grupos de animais e plantas. Alguns dos patógenos por eles transmitidos causam doenças de grande impacto em termos de saúde pública mundial como, por exemplo: malária, leishmanioses, tripanossomíases, dengue e filarioses (OMS 2010).

A utilização do inseticida DDT, no inicio da década de 60, levou a uma expectativa de que vetores de doenças pudessem ser eliminados fácil e definitivamente. Porém, no começo da década seguinte constatou-se que os vetores não haviam sido erradicados, pois determinadas populações haviam desenvolvido resistência ao DDT. O controle da malária nos dias de hoje enfrenta quatro problemas principais: (1) a indisponibilidade de uma vacina antimalárica, (2) a carência de sistemas de diagnóstico que atinjam toda a população mundial, (3) a escassez de métodos para eliminar o contato entre humanos e mosquitos, e (4) o aumento crescente de resistência dos patógenos às drogas utilizadas no tratamento da doença (Hoffman e cols. 2002). Devido a estas dificuldades, o desenvolvimento de novas alternativas de controle, prevenção e tratamento tem sido largamente fomentadas.

O estudo da malária encontra-se em uma situação antes inimaginável: a da disponibilidade simultânea dos genomas dos três organismos que participam do ciclo da doença humana, o plasmódio (com quatro espécies sequenciadas; Carlton e cols. 2002, Gardner e cols. 2002, Carlton e cols. 2008, Pain e cols. 2008), o mosquito (com uma espécie sequenciada; Holt e cols. 2002) e o humano (Lander e cols. 2001). Indiscutivelmente, estes avanços em estudos de genômica propiciam a criação de novas linhas de pesquisa visando o controle de doenças transmitidas por insetos (Hill e cols. 2005).

Consequentemente, estudos de biologia celular e molecular enfocando a relação parasito-hospedeiro sofreram grande incremento. Ainda assim, mais estudos são necessários para a melhor compreensão desta interação, a fim de revelar pontos mais susceptíveis do ciclo de desenvolvimento do parasito que possam ser

utilizados mais eficientemente em estratégias de bloqueio de transmissão da malária.

Estudos de interação anofelinos-plasmódios vêm sendo realizados a várias décadas. No entanto, praticamente todos utilizaram anofelinos da Ásia e África e *P. falciparum* (além de outros plasmódios que não infectam humanos). Apesar da importância do *P. vivax* como agente etiológico responsável por mais de 50% dos casos de malária fora do continente africano (sobretudo nas Américas e na Ásia), poucos estudos foram realizados com este parasito. Há duas explicações para este fato. A primeira diz respeito à crença (equivocada) de que este plasmódio não causaria malária grave (Anstey e cols. 2009, Oliveira-Ferreira e cols. 2010); e a segunda, à dificuldade de se estabelecer, em laboratório, um sistema de cultivo contínuo do parasito (Udomsangpetch e cols. 2008).

Em função das informações apresentadas acima, o objetivo central deste trabalho foi estudar moléculas participantes da interação entre *P. vivax* e *A. aquasalis*, com o intuito de revelar possíveis alvos para serem usados em estratégias de bloqueio da transmissão da malária no Brasil. Como o genoma deste vetor ainda não foi sequenciado e somente poucos genes eram conhecidos, duas estratégias experimentais foram adotadas: (1) construção de bibliotecas de subtração de cDNA; e (2) PCR com iniciadores degenerados.

Todas as infecções dos insetos foram realizadas na cidade de Manaus, com sangue de pacientes locais infectados. Para tal, pacientes diagnosticados com malária (com 4-8% de gametócitos circulantes) eram convidados a participar do estudo. Após o consentimento do doente, seu sangue era coletado e oferecido aos insetos através de alimentação artificial (Bahia e cols. 2010).

4.2 Subtração de cDNAs

A técnica de subtração de cDNAs foi escolhida para obtenção do transcriptoma de *A. aquasalis*, após diferentes horários de alimentação sanguínea e infecção por *P. vivax*, por sua eficiência comprovada na identificação de genes diferencialmente expressos entre duas condições experimentais (Diatchenko e cols. 1996).

Esta técnica possibilitaria revelar genes importantes situados na interface vetor-parasito e evitaria o sequenciamento redundante de cDNAs (Diatchenko e cols. 1996, Rebrikov e cols. 2004). Bibliotecas de cDNA foram construídas em duas direções com o intuito de revelar genes induzidos e suprimidos pela infecção. Das quatro bibliotecas geradas, duas tinham a finalidade de revelar genes suprimidos pela infecção: *duas horas* e *vinte quatro horas* após alimentação sanguínea, menos *duas horas* e *vinte quatro horas* após infecção por *P. vivax* (2 e 24 hF-I); enquanto as outras duas tinham o objetivo de gerar sequências de cDNA induzidas pela infecção: *duas horas* e *vinte quatro horas* após infecção por *P. vivax* menos *duas horas* e *vinte quatro horas* após alimentação sanguínea (2 e 24 hI-F).

Os tempos de 2 h e 24 h após a infecção foram escolhidos para a geração das bibliotecas, pois são anteriores à diferenciação e multiplicação destes parasitos, e, portanto, deveriam fornecer dados mais relevantes para o desenvolvimento de estratégias de bloqueio da transmissão desta enfermidade. Além disso, o tempo de 2 h foi escolhido em função do desconhecimento acerca das etapas iniciais do desenvolvimento do parasito (diferenciação de gametócitos, fecundação e formação do zigoto); já o de 24 h foi escolhido por ser próximo a uma etapa de interação estreita entre o parasito e o inseto (a travessia do oocineto pelo epitélio intestinal do mosquito).

Com a produção das mini-bibliotecas de cDNAs de *A. aquasalis* (Bahia e cols. 2010), cerca de 500 sequências foram geradas e se encontram disponíveis nos bancos de dados públicos, para estudos subsequentes com este importante vetor de malária.

Os resultados aqui apresentados também demonstram claramente que a presença do *P. vivax* no *A. aquasalis* leva a alterações importantes em sua fisiologia, representadas por um atraso na embriogênese do inseto. Genes relacionados ao desenvolvimento embrionário do inseto foram negativamente regulados após a infecção, baixando de 30% na biblioteca de cDNAs de insetos 24 hF-I para 1,9% na biblioteca de insetos 24 hI-F. Os resultados de microscopia confocal vieram a corroborar estes achados. Foi observado que fêmeas de *A. aquasalis* apresentavam desenvolvimento ovariano 36 horas após alimentação sanguínea. Porém, em fêmeas infectadas com *P. vivax*, o desenvolvimento ovariano só foi observado 48 horas após alimentação. Vários trabalhos já evidenciaram alterações fisiológicas do

organismo hospedeiro após a infecção (e.g. Gustafsson e cols. 1994). Em mosquitos, já foi demonstrado que a infecção por *Plasmodium* leva apoptose de células epiteliais do folículo ovariano (e.g. Ahmed e Hurd 2006). Estas alterações na *fitness* (adaptabilidade) do inseto podem ser devido às seguintes razões: (1) custo de se montar uma resposta imune que leve a um balanço funcional entre a imunidade e a reprodução; (2) deficiência nutricional gerada pela regulação negativa de genes de metabolismo, como a enzima prolina oxidase (somente observada na biblioteca 2 hF-I); (3) aquisição de nutrientes pelo parasito; ou (4) alteração na expressão de algumas enzimas digestivas (observado para a enzima quimiotripsina).

Como comentado anteriormente, bibliotecas subtrativas são geradas a partir de subtração de cDNAs de amostras distintas, e, portanto, só devem apresentar genes diferencialmente expressos entre as duas condições analisadas. Curiosamente, vários genes considerados constitutivos por desempenharem papéis chave na fisiologia das células, como genes constituintes do citoesqueleto e da lâmina basal, foram encontrados nas bibliotecas subtrativas. Uma análise mais detalhada de uma actina, proteína componente do citoesqueleto, corroborou estes resultados. Esta proteína de *A. aquasalis* teve sua expressão regulada após alimentação e infecção por *P. vivax.* De acordo com estes achados, podemos supor que a infecção do *A. aquasalis* pelo *P. vivax* leva a uma desregulação não só da fisiologia, mas também do ambiente celular do hospedeiro invertebrado.

Baseado na importância que as enzimas digestivas têm na degradação do alimento ingerido e consequentemente, no desenvolvimento do parasito, duas enzimas foram escolhidas para serem estudadas. A primeira pertence à classe das carboxipeptidases e a segunda à classe das serinoproteases. As carboxipeptidases são exopeptidases que atuam liberando aminoácidos da região carboxi terminal livre de peptídeos ou proteínas. A carboxipeptidase encontrada em *A. aquasalis* apresenta-se mais relacionada com as carboxipeptidases A de insetos. As serinoproteases são endopeptidases que possuem no seu sítio ativo uma serina. Um tipo particular de serinoprotease são as quimiotripsinas. Estas enzimas são específicas para ligações peptídicas contendo resíduos de aminoácidos com cadeias laterais hidrofóbicas, como a fenilalanina, tirosina e triptofano. Análises da serinoprotease de *A. aquasalis* revelaram que esta enzima era similar às quimiotripsinas de insetos. Experimentos de PCR em Tempo Real (*Real Time PCR* -

RTPCR) revelaram que a expressão de RNAm para a carboxipeptidase A não sofre alteração após infecção com *P. vivax.* Em contrapartida, a infecção pelo *P. vivax* regulou negativamente a expressão da quimiotripsina. Alterações na atividade de diversas enzimas digestivas de mosquitos após desafio com patógenos, já foram demonstradas (Jahan e cols. 1999, Somboon e Prapanthadara 2002). Porém, nenhum trabalho descreveu estas mudanças como consequências de alterações expressão de RNAm. A interferência do parasito em alguma via de sinalização que leva à transcrição deste gene pode explicar esta observação. Recentemente, Brandon e colaboradores (2008) demonstraram que a via de sinalização TOR (*target of rapamycin* (TOR) *kinase* – alvo de rapamicina), implicada na sensibilidade a nutrientes, estaria envolvida na transcrição e síntese de tripsina no *A. aegypti* em resposta à alimentação sanguínea. É possível que a modulação desta via pelo *P. vivax* possa ser responsável pela regulação negativa da quimiotripsina de *A. aquasalis*. Do ponto de vista do parasito, a modulação da transcrição desta quimiotripsina de *A. aquasalis* pelo *P. vivax* pode aumentar sua sobrevivência, uma vez que formas iniciais de *Plasmodium* são susceptíveis à digestão por proteases (e.g. Gass e Yeates 1979).

A anotação dos cDNAs destas bibliotecas revelou poucos genes relacionados à imunidade de *A. aquasalis*. Além disso, não foi observada diferença no número de genes de imunidade ao se comparar as bibliotecas de insetos alimentados e infectados em ambos os tempos estudados. A ausência de uma resposta imune robusta de *A. aquasalis* contra os estágios iniciais de desenvolvimento do *P. vivax* pode ser responsável pelo sucesso na colonização do mosquito por este parasito. Uma resposta imune fraca, como a obtida no presente trabalho, foi observada em *A. stephensi* infectado por *P. berghei* (parasito causador de malária murina; Srinivasan e cols. 2004). Em contraste, uma resposta imune forte foi observada em *A. gambiae* infectado por *P. falciparum* (Dong e cols. 2006a). Embora os resultados encontrados já tenham sido observados em outros insetos e da técnica de subtração ter como objetivo o enriquecimento de RNAs raros, não se pode excluir completamente a possibilidade da técnica não ter detectado alguns genes de imunidade que eram pouco expressos (Diatchenko e cols. 1996).

Apesar de poucos genes relacionados com a imunidade terem sido revelados pelas bibliotecas, alguns destes genes podem ser bons candidatos para o

desenvolvimento de estratégias para interrupção da transmissão da malária. Atualmente, uma das estratégias mais promissoras para o combate a doenças transmitidas por insetos está relacionada à manipulação genética desses organismos visando a redução de sua capacidade vetorial (Hill e cols. 2005). Em laboratório, genes relacionados com o sistema imune já foram utilizados para criar mosquitos transgênicos refratários à malária (e.g. Kim e cols. 2004, Kokoza e cols. 2010).

As FREPs são proteínas com um ou dois domínios do tipo fibrinogênio que desempenham diversas funções no sistema imune inato dos vertebrados e invertebrados (Middha e Wang 2008, Dong e Dimopoulos 2009). Proteínas com este domínio têm sido implicadas em respostas imunes de *Anopheles* contra plasmódios e bactérias (Dimopoulos e cols. 2002, Dong e cols. 2006b). Nas bibliotecas de *A. aquasalis* foram encontradas três FREPs (cDNAs). Uma dessas três proteínas apresentou alta similaridade com as tequilectinas do caranguejo-ferradura *Tachypleus tridentatus*, que reconhecem moléculas não-próprias (Gokudan e cols. 1999), e foi, portanto, escolhida para ter sua expressão confirmada por RTPCR. A tequilectina de *A. aquasalis* não apresentou diferenças significativas na expressão de RNAm entre fêmeas alimentadas e infectadas nos três tempos estudados (2, 24 e 36 horas). No entanto, um aumento modesto na expressão de RNAm foi observado 36 horas após infecção. Resultados similares foram também encontrados em outras espécies de mosquito. Em *A. gambiae*, os níveis de FREPs aumentaram imediatamente após o desafio com bactérias Gram-negativas e Gram-positivas e também 24 horas após a infecção por *P. berghei* (Dimopoulos e cols. 2002). Os níveis de RNAm para outra FREP também aumentaram 48 horas após a infecção de *A. stephensi* com *P. berghei* (Srinivasan e cols. 2004). O aumento da expressão de RNAm para a FREP de *A. aquasalis* 36 horas após a infecção pode ser importante no reconhecimento dos parasitos na hemolinfa e na ativação do sistema imune do inseto. Outras duas FREPs, ficolina e fibronectina, apareceram nas bibliotecas 2 hI-F, indicando uma possível regulação positiva destas pela infecção. Ficolinas são moléculas extremamente eficientes no reconhecimento de PAMPs e no desencadeamento de respostas imunes como fagocitose e ativação do complemento (Matsushita e Fujita 2002). Domínios do tipo fibronectina são encontrados nas proteínas Dscam de diversos insetos. Dscams, proteínas

hipervariáveis formadas a partir de combinações de éxons de um único gene, são envolvidas no desenvolvimento e função do sistema nervoso dos insetos bem como no reconhecimento de patógenos (Graveley e cols. 2004). Portanto, o aumento na expressão de RNAm para estas proteínas nas primeiras horas após infecção pode ser uma forma de o inseto aumentar o repertório de moléculas de reconhecimento de patógenos.

Uma proteína com alta similaridade genética às BRPs de *A. gambiae* foi encontrada nas bibliotecas de *A. aquasalis*. De acordo com Shi e Paskewitz (2004), proteínas BRP podem promover a proliferação celular ou regular a migração celular e agregação. Estudos de RTPCR mostraram que o RNAm para esta proteína é induzido 24 e 36 horas após infecção por *P. vivax*. Estes resultados indicam que a passagem do parasito pelo intestino e sua permanência na lâmina basal podem estar estimulando o sistema imune do mosquito. Portanto, o aumento de RNAm para BRP2 em *A. aquasalis* pode promover funções imunes secundárias na hemolinfa do inseto em resposta à presença do *P. vivax*.

Cecropina é um potente AMP com uma grande abrangência de alvos microbianos. Este AMP tem sido implicado em diversas respostas imunes de insetos a microorganismos. Em *A. aegypti* e *A. gambiae* ocorre um aumento na expressão de cecropina após infecções com bactérias, fungos filamentosos, leveduras e plasmódio (Lowenberger e cols. 1999 e Vizioli e cols. 2000). Além disso, *A. gambiae,* manipulados geneticamente para expressar altos níveis de cecropina, apresentaram uma redução de 60% na infecção por *P. berghei* (Kim e cols. 2004). A superexpressão da cecropina combinada ao AMP defensina gerou um fenótipo de completa resistência de *A. aegypti* ao *P. gallinaceum*, parasito causador da malária aviária (Kokoza e cols. 2010). No presente trabalho, observamos que a expressão de RNAm para uma cecropina foi reduzida no *A. aquasalis* 24 horas após infecção. Portanto, este AMP parece não possuir um papel central na resposta imune deste mosquito contra *P. vivax*, visto que o tempo de 24 horas é um período em que o sistema imune do mosquito é ativado fortemente devido à passagem do plasmódio através das células intestinais do inseto (e.g. Dimopoulos e cols. 1998). Estas observações sugerem que a infecção por *P. vivax* pode: (1) regular a carga de bactérias dentro do inseto e, em consequência, diminuir a ativação do sistema imune e o aumento dos níveis deste AMP; ou (2) suprimir alguma via de sinalização, como

a via NF-κB, que leva ao aumento deste AMP. Essa regulação negativa de cecropina em *A. aquasalis* infectados por *P. vivax* deve ser importante para a sobrevivência e desenvolvimento do parasito dentro do vetor. Além disso, foi observado um aumento na expressão de cecropina no *A. aquasalis* após a ingestão de sangue. A indução deste AMP logo após o repasto sanguíneo pode ser uma estratégia adotada pelo inseto para controlar a expansão da microflora gerada pelo aumento de nutrientes e diminuição de ROS (em resposta à toxicidade da molécula de heme) após a ingestão de sangue (Luckhart e cols. 1998, Graça-Souza e cols. 2006, Oliveira e cols. 2007).

As serpinas são moléculas importantes na regulação do sistema imune de invertebrados. Alguns membros desta família são essenciais para o desenvolvimento de *Plasmodium* em seus vetores (Michel e cols. 2005, Abraham e cols. 2005, Danielli e cols. 2005). A expressão de RNAm para a serpina 4 de *A. aquasalis* sofreu um aumento 36 horas após a infecção por *P. vivax*. Estes resultados contrastam com os observados para a Serpina 4 de *A. gambiae* que é regulada positivamente após infecção por bactéria, mas não por plasmódio (Christophides e cols. 2002). Nossos resultados indicaram que o aumento da expressão da serpina em *A. aquasalis* pode ser desencadeado pela passagem do parasito através do epitélio intestinal e pela permanência do parasito na hemolinfa. Além disso, o aumento deste regulador negativo pode ser responsável pela susceptibilidade do *A. aquasalis* ao *P. vivax,* devido ao potencial da serpina de suprimir mecanismos imunes do mosquito como, por exemplo, a melanização. Estudos funcionais são necessários para comprovar se esta serpina atua como imunomodulador negativo. Se a serpina do *A. aquasalis* possui realmente uma função protetora para o parasito, ela pode ser um alvo interessante para estratégias de bloqueio da transmissão da malária.

Outros genes possivelmente relacionados com o sistema imune dos insetos foram também identificados nas bibliotecas subtrativas. Uma proteína inibidora de apoptose (*apoptosis inhibtor protein* 5 - IAP-5) foi encontrada nas bibliotecas de insetos 2 hF-I, indicando possível regulação negativa pela infecção. As IAPs previnem a morte celular programada ligando-se às caspases e inibindo-as. Em insetos, a apoptose está intimamente relacionada ao desenvolvimento embrionário e à defesa imune contra uma gama de patógenos. Em anofelinos, foi demonstrado

que a passagem do plasmódio através das células epiteliais do intestino médio causa diversos danos intracelulares que levam à apoptose da célula invadida (Han e cols. 2000 e Kumar e cols. 2003 e 2004). Especula-se que a apoptose da célula do intestino seja importante no controle da carga de plasmódios dentro do inseto. Além disso, em mosquitos, a apoptose é também utilizada na prevenção e redução de infecções virais (Vaidyanathan e Scott 2006). Em *D. melanogaster,* a IAP-2 foi associada ao controle da resposta imune inata através da regulação da via IMD (Leulier e cols. 2006). Portanto, é provável que a regulação negativa da IAP-5 no *A. aquasalis* infectado seja importante no aumento da apoptose das células do mosquito e, consequentemente, no controle da carga parasitária do *P. vivax.*

Uma V-ATPase foi descoberta na biblioteca subtrativa 2 hF-I de *A. aquasalis*. Estas enzimas encontram-se presentes em organelas e membranas de invertebrados e vertebrados, e funcionam como bombas de prótons, sendo responsáveis pela acidificação do citoplasma como outros compartimentos das células (Nelson 2003). Em insetos, estas enzimas também promovem a acidificação do intestino médio. Em *A. stephensi* foi demonstrado que a invasão do intestino pelo *P. berghei* ocorre preferencialmente em células expressando baixos níveis de V-ATPase (Han e cols. 2000). Em *Lutzomyia longipalpis*, a re-acidificação do intestino médio logo após a alimentação sanguínea parece favorecer a metaciclogênese de *Leishmania* (Gontijo e cols. 1998). Portanto, a regulação desta V-ATPase logo após a infecção, modificando a acidez intestinal e a atividade das enzimas digestivas, pode ter um efeito determinante no estabelecimento das primeiras formas de *P. vivax* dentro do intestino do *A. aquasalis*.

Os insetos não produzem imunoglobulinas como os vertebrados, porém, nos genomas de *D. melanogaster* e *A. gambiae* existem entre 140 e 150 genes com domínios de imunoglobulinas, respectivamente. Dong e colaboradores (2006) demonstraram que o *A. gambiae* desafiado com diferentes patógenos era capaz de produzir milhares de proteínas Dscam singulares, através de combinações dos seus exóns. Moléculas com domínios similares a imunoglobulinas (*alpha-2-macroglobulin receptor-associated protein* e *integral transmembrane protein 2-related with immunoglobulin domain*) foram reguladas no *A. aquasalis* após a infecção por *P. vivax* e capturadas pela técnica de subtração de cDNA. A regulação positiva destas

moléculas pode ser crucial para o reconhecimento do *P. vivax* e para a ativação do sistema imune do *A. aquasalis*.

4.2.1 Fator de transcrição GATA e imunidade em *A. aquasalis*

Um fator de transcrição da família GATA foi também descoberto nas bibliotecas de *A. aquasalis.* Fatores de transcrição desta família encontram-se amplamente distribuídos entre animais e plantas e têm sido implicadas no desenvolvimento, diferenciação, proliferação e imunidade (Petersen e cols. 1999, Tingvall e cols. 1999, Patient and McGhee 2002, Senger e cols. 2006, Shapira e cols. 2006). Análises filogenéticas do gene GATA de *A. aquasalis* revelaram que este gene é mais similar geneticamente ao GATA serpent de *D. melanogaster* e de *A. gambiae.* Em *D. melanogaster,* o GATA serpent atua nos tecidos endodermais como o corpo gorduroso, promovendo seu desenvolvimento e a transcrição de moléculas efetoras do sistema imune (proteína 1 do corpo gorduroso e o AMP cecropina A1), e é importante na hematopoiese e diferenciação de hemócitos (Petersen e cols. 1999, Brodu e cols. 2001, Senger e cols. 2004, Artero e cols. 2006, Gajewski e cols. 2007, Muratoglu e cols. 2007, Frandsen e cols. 2008). Experimentos de RTPCR mostraram que o RNAm para o gene GATA é induzido cerca de 15 vezes após desafios de *A. aquasalis* com *P. vivax.* O silenciamento deste fator de transcrição no *A. aquasalis* gerou um fenótipo de maior susceptibilidade ao plasmódio, demonstrando que este gene é importante para a resposta imune deste vetor ao *P. vivax.* Novos estudos são necessários para revelar os mecanismos imunes desencadeados pelo aumento da expressão do GATA em *A. aquasalis* infectados por *P. vivax.* Portanto, devido à sua similaridade com o fator GATA serpent de *D. melanogaster* e sua importância na imunidade de *A. aquasalis* contra *P. vivax,* acreditamos que este gene também possa participar do desenvolvimento do corpo gorduroso em *A. aquasalis.*

4.3 PCR com iniciadores degenerados

A outra estratégia metodológica de PCR usando iniciadores degenerados levou à descoberta de outros genes importantes na interação *A. aquasalis-P. vivax*. Foram estudados genes relacionados com a via de sinalização JAK-STAT e envolvidos com o sistema de defesa baseado em radical livres.

4.3.1 Via JAK-STAT

Três genes relacionados com a via JAK-STAT (o FT STAT, a sua proteína inibitória PIAS e a enzima NOS) foram amplificados utilizando as técnicas de iniciadores degenerados e de amplificação rápida das porções finais dos cDNAs (*Rapid Amplification of cDNA Ends* - RACE) e posteriormente clonados, sequenciados e caracterizados. O STAT de *A. aquasalis* mostrou-se mais similar à proteína STAT-A de *A. gambiae* e de *A. aegypti*, porém não apresentou todos os domínios encontrados na STAT destes outros mosquitos. Este transcrito encontrado em *A. aquasalis* deve ser uma forma de RNAm gerado a partir do *splicing* alternativo do gene STAT. Um transcrito de STAT com esta organização já foi descrito em *D.* melanogaster (Henriksen e cols. 2002). A proteína PIAS de *A. aquasalis* é altamente similar às proteínas PIAS de dípteros. Experimentos de expressão de RNAm e de proteína revelaram que STAT e PIAS são induzidos 24 horas após infecção por *P. vivax* e têm o seu pico de expressão 36 horas após infecção.

Genes efetores (possivelmente ativados pela via JAK-STAT) foram procurados com o intuito de se confirmar a relação entre esta via e a resposta imune do mosquito contra o plasmódio. Sequências de DNA com sítios de ligação a STAT foram descritas na região regulatória da enzima NOS de *A. stephensi*, responsável pela produção do radical NO (Luckhart e cols. 1998). Em *A. aquasalis*, a expressão da enzima NOS foi ativada somente 36 horas após infecção por *P. vivax*. Contudo, experimentos de imunocitoquímica revelaram que algumas células do intestino médio de *A. aquasalis* expressavam fortemente a enzima NOS 24 horas após infecção por *P. vivax*. A expressão de NOS é ativada precocemente em diversas espécies de anofelinos após infecção por parasitos da malária. O pico de expressão

de RNAm para este gene condiz com o que foi observado para os genes STAT e PIAS, isto é, alta expressão 36 horas após infecção. O tempo de indução de NOS, um pouco postergado com relação à indução de STAT e PIAS, levanta indícios de que este gene pode estar sendo regulado por esta via. Desta forma, podemos supor que a ativação da via JAK-STAT levou à transcrição da enzima NOS e à produção da molécula efetora NO em *A. aquasalis* infectados por *P. vivax*, como observado para outros modelos de anofelinos-plasmódios. Em *A. gambiae* a expressão de NOS aumentou 24 horas após a infecção por *P. berghei* (Gupta e cols. 2009) e 14 horas após a infecção por *P. falciparum* (Tahar e cols. 2002); já em *A. stephensi* os aumentos ocorreram 6, 24, 48 e 72 horas após a infecção por *P. berghei* (Luckhart e cols.1998, 2003). Em alguns modelos de vetor-parasito como *A. stephensi-P. berghei*, as células epiteliais do intestino do inseto sofrem vários danos após invasão por plasmódio que levam à produção de NO e, posteriormente, à morte da célula infectada (Han e cols. 2000, Kumar e cols. 2005b). A resposta imune destas células epiteliais é importante no controle da carga parasitária e na eliminação da infecção. No entanto, este mecanismo não é universal, pois a indução da NOS não foi observada em outras combinações de vetor-parasito, como *A. aegypti-P. gallinaceum* e *A. stephensi-P. gallinaceum* (Gupta e cols. 2005). A falta de uma completa ativação da enzima NOS em *A. aquasalis* 24 horas após infecção pode ser um dos motivos que confere susceptibilidade deste vetor ao *P. vivax*.

Experimentos de imunocitoquímica realizados em cortes longitudinais de *A. aquasalis* mostraram que o principal órgão que expressa STAT e PIAS é o corpo gorduroso. Estes resultados condizem com o papel do corpo gorduroso como principal órgão imune dos insetos. Além disso, o pico de indução destes genes (36h após infecção) coincide com o encontro do parasito na hemocele do inseto (local onde estão localizados os corpos gordurosos periféricos).

Para confirmar o papel da via JAK-STAT na imunidade de *A. aquasalis* contra *P. vivax* foram realizados experimentos de genética reversa através da via de RNAi. O silenciamento gênico do FT STAT causou um agravamento na infecção de *A. aquasalis* por *P. vivax,* comprovando a importância desta via no combate a este parasito.

Os picos de indução de RNAm para os genes STAT, PIAS e NOS (36h após infecção) indicam que a via JAK-STAT encontra-se completamente ativada somente

após o estabelecimento do *P. vivax* na hemocele do inseto. A falta de uma ativação imune forte no intestino pode ser importante na susceptibilidade deste inseto ao parasito da malária humana, pois se a via estivesse super ativada no momento de travessia do parasito pelo epitélio intestinal, estes insetos poderiam ser capazes de manter um fenótipo de resistência a infecção.

Os resultados acima mostram que a via JAK-STAT é importante para limitar a infecção do *A. aquasalis* por *P. vivax*. Em *A. gambiae,* a infecção por *P. falciparum* e *P. berghei* leva a uma ativação da via JAK-STAT que também é eficiente no controle do desenvolvimento destes parasitos. Entretanto, diferentemente de *A. aquasalis,* a resposta anti-plasmódio do *A. gambiae* através desta via ocorre tardiamente (oito dias após infecção; Gupta e cols. 2009). Novos experimentos precisam ser feitos com o intuito de investigar se a via JAK-STAT é também importante na resposta imune tardia do *A. aquasalis* contra *P. vivax*.

4.3.2 Mecanismo de defesa por radicais livres

Desde a década de 70 vem sendo mostrado que os radicais livres são utilizados pelo sistema imune inato de vertebrados como forma de destruir microorganismos fagocitados (e.g. Babior e cols. 1976). Recentemente, foi descoberto que uma linhagem de *A. gambiae* era refratária ao plasmódio, pois se encontrava em um estado crônico de estresse oxidativo, o que reduzia a infecção (Kumar e col. 2003, Gupta e cols. 2009) e a fecundidade dos mosquitos (DeJong e cols. 2007). Após a invasão do epitélio intestinal de *A. gambiae* pelo plasmódio, foi observada a indução da expressão da enzima DUOX que, junto com a produção de NO, leva à nitração das proteínas da célula invadida, à sua apoptose e à morte do parasito (Kumar e cols. 2004). Em *D. melanogaster,* foi também demonstrada que a expressão de DUOX no epitélio intestinal era importante para controlar o desenvolvimento de bactérias no tubo digestivo (Ha e cols. 2005a, b). Apesar do papel protetor dos radicais livres, estas moléculas são extremamente perigosas, pois podem reagir com muitas biomoléculas (com o objetivo de se tornarem estáveis), causando enormes danos ao hospedeiro que as produz. Logo, o processo de produção e detoxificação destas moléculas precisa acontecer de forma bastante

sincronizada. Para tanto, os insetos produzem moléculas capazes de prevenir ou retardar a oxidação de outros componentes por estes radicais livres. Enzimas, como a catalase e a superóxido dismutase, e agentes de baixo peso molecular, como a vitamina C e o ácido úrico, são exemplos de antioxidantes.

Considerando a importância de ROS na imunidade de insetos e os efeitos nocivos destas moléculas, um dos objetivos deste trabalho foi o de investigar o papel dos radicais livres na resposta imune de *A. aquasalis* contra *P. vivax*. Para isso, a produção de ROS e a expressão e atividade de algumas enzimas de antioxidantes foram avaliadas.

Três enzimas de detoxificação (uma catalase e duas SOD) foram descobertas através das técnicas de PCR utilizando iniciadores degenerados e RACE. A catalase de *A. aquasalis* mostrou-se muito similar à catalase de outros mosquitos. As SODs apresentaram bastante similaridade à SOD3 de insetos e, mais especificamente, com a SOD3A e SOD3B de *A. gambiae*.

A expressão de RNAm para a SOD3A, SOD3B e catalase não variou após a alimentação sanguínea do *A. aquasalis.* Contudo, a atividade enzimática da catalase e SOD sofreu uma diminuição 24 horas após a infecção do mosquito por *P. vivax*. A redução da atividade destas enzimas pode ser uma resposta das células epiteliais do intestino do *A. aquasalis* à invasão pelo *P. vivax,* com o objetivo de matar o parasito e impedi-lo de se disseminar para outros tecidos. A diminuição da atividade destas enzimas poderia permitir a manutenção de níveis mais altos de ROS nas células invadidas e, consequentemente, criar um ambiente mais hostil e possivelmente letal para o parasito. Molina-Cruz e cols. (2008) também observaram uma diminuição da atividade de catalase em *A. gambiae* infectado por *P. berghei*. A expressão de RNAm para as enzimas SOD3B e catalase foi aumentada 36 horas após infecção. Este aumento pode ser explicado como uma tentativa do inseto de regular os níveis de radicais livres (gerados pela passagem do parasito pelas suas células) com o intuito de se proteger dos eventuais danos causados pela ação destas moléculas. Resultados semelhantes foram encontrados para diversas enzimas como a SOD3A, catalase e SOD1 no corpo gorduroso de *A. gambiae* infectados por *P. berghei* (Molina-Cruz e cols. 2008). Análises *in silico* da catalase e das SODs de *A. aquasalis* foram incapazes de revelar peptídeo sinal para estas

enzimas (dados não mostrados), fato que corrobora a possível ação destas proteínas no ambiente intracelular do intestino médio do inseto.

Experimentos de microscopia de fluorescência foram realizados em intestinos de *A. aquasalis* com o objetivo de observar a produção de radicais livres após a alimentação dos insetos com açúcar, sangue e sangue infectado. Os experimentos revelaram uma redução dos radicais livres em intestinos de *A. aquasalis* alimentados com sangue e infectados, quando comparados com alimentados com açúcar, sugerindo que o *A. aquasalis* regula a quantidade de ROS no intestino após a ingestão de sangue. Essa regulação pode acontecer em resposta à presença da molécula de heme no sangue, que em um ambiente oxidativo é capaz de causar efeitos bastante danosos para o organismo (Graça-Souza e cols. 2006). Resultados similares já foram descritos para o mosquito *A. aegypti* (Oliveira 2007). Esta regulação negativa da quantidade de radicais livres no intestino dos insetos pode ter possibilitado o desenvolvimento das formas iniciais do parasito e, consequentemente, sua sobrevivência dentro do mosquito. Apesar desta regulação de radicais livres no intestino dos mosquitos após a ingestão do sangue, o aumento de radicais livres em insetos infectados foi indiretamente inferido através do aumento da expressão de RNAm para a enzima óxido nítrico sintase 36 horas após infecção e pela expressão da enzima NOS em células intestinais de mosquitos 24 horas após infecção. Estes resultados, somados à redução da atividade das enzimas de detoxificação 24 h após infecção, sugerem uma estratégia do inseto de produção local dos radicais livres com o intuito de minimizar os possíveis danos causados por estas moléculas durante a alimentação sanguínea. Han e cols. (2000) e Gupta e cols. (2005 e 2009) mostraram que as células de *A. gambiae* e *A. stephensi* invadidas por *P. berghei* e *P. gallinaceum* eram capazes de produzir radical NO como estratégia de defesa. A presença de ROS no intestino de mosquitos alimentados com açúcar comprova que estas moléculas possuem um papel muito importante para o inseto. Uma explicação aceitável seria uma possível função destas moléculas na manutenção da homeostase local como sistema de defesa contra a proliferação da microbiota.

Para confirmar o papel dos radicais livres como moléculas importantes na imunidade de *A. aquasalis* contra o *P. vivax*, experimentos de silenciamento da enzima catalase foram realizados. Surpreendentemente, o silenciamento da catalase

teve um efeito intensificador da infecção do *A. aquasalis.* Outros experimentos estão sendo realizados com o intuito de desvendar os mecanismos envolvidos neste fenômeno. Recentemente, um mecanismo protetor para o plasmódio, gerado pelo aumento de radicais livres no intestino médio de *A. gambiae,* foi descoberto. Foi observado que moléculas de peróxido de hidrogênio, geradas no lúmen do intestino do inseto através da ação da enzima DUOX, eram utilizadas por uma peroxidase para formar uma rede de ditirosina que reduzia a permeabilidade de moléculas ativadoras do sistema imune. Por esta razão, o sistema imune do *A. gambiae* permanecia em forma latente o que possibilitava o desenvolvimento dos parasitos *P. berghei* e *P. falciparum* (Kumar e cols. 2010). A formação destas redes no intestino médio destes insetos poderia ser uma das razões pelas quais observamos um aumento do número de oocistos em insetos que tiveram o gene da catalase silenciado.

4.4 Considerações finais

Todos os resultados reportados nesta tese mostram que o *A. aquasalis* responde imunologicamente à infecção por *P. vivax* e que a resposta imune gerada é capaz de controlar a infecção e até tornar alguns espécimes refratários ao parasito causador da malária em humanos. A maior parte dos genes de *A. aquasalis* estudados teve sua expressão alterada em função da infecção por *P. vivax*. Isto demonstra que a ativação do sistema imune do *A. aquasalis* parece ocorrer de forma integrada para formar uma resposta imune robusta com a finalidade de controlar o desenvolvimento do *P. vivax.* Os genes imunes que foram regulados e que podem ser importantes no controle da infecção do *P. vivax* pelo *A. aquasalis* foram: BRP, fibrinogênio, GATA, STAT, PIAS, NOS, catalase e SOD3. Outros genes estudados aparentemente não tiveram participação na montagem da resposta imune observada em *A. aquasalis,* pois após a infecção por *P. vivax* sua expressão ou (1) não sofreu alteração, como na carboxipeptidase; ou (2) foi regulada negativamente, como na cecropina e na serino protease; ou (3) foi regulada positivamente de forma a gerar uma regulação negativa da resposta imune, como no caso da serpina. A alteração da expressão dos genes da carboxipeptidase, cecropina e serpina pode acontecer

devido à sua manipulação pelo parasito ou pelo inseto. A fina regulação de genes de imunidade pelo inseto é necessária para prevenir uma reação exagerada do sistema imune que seria prejudicial ao próprio organismo. A manutenção de uma resposta imune eficaz é energeticamente custosa para o organismo e, muitas vezes, causa desregulações fisiológicas (Hurd e cols. 2005, DeJong e cols. 2007). Em nosso modelo, como discutido anteriormente, mostramos que a infecção por *P. vivax* gera um retardo na embriogênese do inseto (Bahia e cols. 2010). A regulação negativa por parte destes genes pode contribuir para a susceptibilidade do *A. aquasalis* ao *P. vivax.*

Os experimentos de subtração de cDNAs não revelaram muitos genes de imunidade, enquanto os de RTPCR mostraram que etapas iniciais da infecção por *P. vivax* (2 e 24 horas) não parecem ativar o sistema imune do *A. aquasalis* de forma tão eficiente, como observado em outros modelos (e.g. Dimopoulos e cols. 1998, Han e cols. 2000, Kumar e cols. 2005). No caso do *A. aquasalis*, a presença do parasito na hemocele do inseto 36 horas após infecção parece ser mais importante no desencadeamento da resposta imune do inseto, do que sua presença no intestino (ou sua passagem pelo epitélio). Estes dados, associados com resultados de microscopia, mostram que o tecido responsável pela maior parte da resposta imune do *A. aquasalis* contra *P. vivax* é o corpo gorduroso. Contudo, ativação nas células epiteliais do intestino médio também foi observada. Ativação imune de múltiplos órgãos do inseto (intestino médio, corpo gorduroso e glândula salivar) foi também reportada na interação entre *A. gambiae-P. berghei* (e.g. Dimopoulos e cols. 1998). A falha na montagem de uma resposta imune robusta no intestino médio do inseto pode ser preponderante para o sucesso de colonização do *P. vivax,* pois este órgão é responsável pelas maiores reduções de carga parasitária (Christophides 2004).

Apesar do *A. aquasalis* apresentar uma resposta imune forte (com alta expressão de vários genes de imunidade) 36 h após infecção, esta resposta é efêmera. Nossos dados apontam que a resposta imune deste inseto é ativada 24 horas após a infecção por *P. vivax*, atinge seu máximo às 36 horas e às 48 horas encontra-se completamente desativada. A desativação precoce do sistema imune do inseto coincide com a mudança de fase do parasito, na qual este vira um oocisto e provavelmente se torna irreconhecível para o sistema imune do inseto. Resultados

similares já foram reportados para outros pares de vetor-parasito, como *A. gambiae*-*P. berghei* (Dimopoulos e cols. 1998).

Os machos de *A. aquasalis* apresentaram níveis mais elevados de RNAm para genes de imunidade BRP, cecropina, STAT, PIAS, NOS e GATA e mais baixos para os reguladores negativos de imunidade serpina e SOD3B, indicando que encontram-se imunologicamente prontos para a ingestão de açúcar. Outra possível explicação para este fenômeno seria a de que, como os machos não se alimentam de sangue, eles não precisariam manter um número alto de bactérias no lúmen do intestino como as fêmeas, que precisam destes microorganismos para realizar uma parte da digestão do sangue. Outra explicação plausível seria a de que os machos expressariam altos níveis de genes imunes com o intuito de responder rapidamente às infecções e se manter saudável para inseminar o maior número possível de fêmeas durante o seu curto período de vida. Resultados similares foram também observados para o mosquito *A. aegypti* (Nascimento-Silva 2008). Por outro lado, foi observado que as fêmeas, diferentemente dos machos, encontram-se prontas para a ingestão do alimento sanguíneo poucos dias após sua emergência da pupa, pois expressam em grandes quantidades RNAm para enzimas digestivas, como carboxipeptidase e quimiotripsina.

Os genes e os mecanismos moleculares de imunidade apresentados e discutidos nesta tese são de importância fundamental para a compreensão dos processos envolvidos direta ou indiretamente na defesa de *A. aquasalis* contra *P. vivax*. Todas as informações geradas neste trabalho podem ser utilizadas na geração de novas estratégias de controle da malária transmitida pelo *A. aquasalis* nas regiões litorâneas de países da América do Sul. A manipulação genética destes insetos visando a superexpressão de genes como STAT e GATA, ou redução na expressão de genes como a serpina, pode ser uma estratégia interessante no desenvolvimento de mecanismos de interrupção do ciclo do *P. vivax* no *A. aquasalis.* Para uma maior eficácia, a expressão destes genes deve ser realizada nas fases iniciais da infecção, nas quais o plasmódio encontra-se restrito ao intestino do vetor, evitando assim a etapa de multiplicação do parasito que ocorre na hemocele do inseto. Estudos de manipulação para superexpressar um gene de imunidade já foram realizados em outros insetos e apresentaram uma redução parcial da infecção dos mosquitos (Kim e cols. 2004). A regulação simultânea de dois genes efetores

aumentou consideravelmente a refratariedade dos mosquitos aos parasitos causadores da malária (Kokoza e cols. 2010). Portanto, em *A. aquasalis* (assim como em outros mosquitos), a superexpressão conjunta de um grupo de genes de imunidade, com o intuito de montar uma resposta imune integrada, pode trazer resultados mais eficazes, uma vez que foi observado que este inseto utiliza diversos mecanismos imunes para responder à infecção por *P. vivax.*

5. Conclusões

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A resposta imune de *A. aquasalis* contra *P. vivax* não é completamente ativada durante a fase em que o parasito encontra-se restrito ao intestino do inseto. Uma resposta imune forte é somente gerada após o estabelecimento dos parasitos de plasmódio na hemocele do inseto;

A resposta imune do *A. aquasalis* a infecções pelo *P. vivax* é transitória. Sua ativação "começa" 24h após a infecção, atinge o seu máximo às 36 e às 48 é desativada;

O corpo gorduroso de *A. aquasalis* é o principal órgão que responde imunologicamente a infecções pelo *P. vivax;*

A via JAK-STAT possui papel importante no controle da carga parasitária do *P. vivax* em *A. aquasalis*, possivelmente através da ação da enzima NOS;

Os radicais livres são moléculas importantes na imunidade do *A. aquasalis* contra *P. vivax* e são produzidos localmente nas células epiteliais do intestino médio do inseto com o intuito de matar o parasito e proteger o hospedeiro invertebrado;

O *A. aquasalis* regula a quantidade de ROS no lúmen do seu intestino após a alimentação sanguínea, possivelmente de forma a evitar o contato entre estas moléculas e as de heme;

Pequenas alterações nas quantidades de radicais livres mudam o fenótipo de susceptibilidade-resistência do *A. aquasalis* ao *P. vivax*;

O fator de transcrição GATA é importante no controle da carga parasitária do *P. vivax* em *A. aquasalis*.

6. Referências

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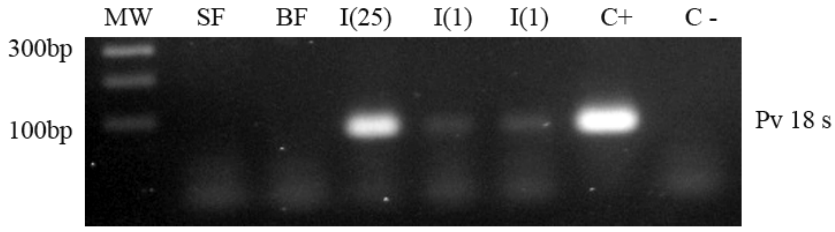
7. Anexos

ANEXO 1

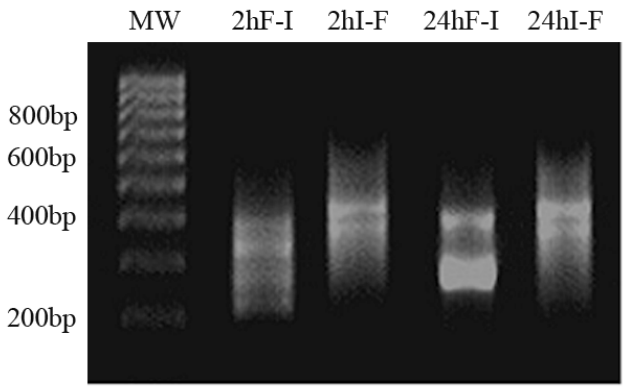
Figura Suplementar 1 do capítulo 1



PCR para confirmar a infecção experimental por *P. vivax*. MW – "molecular weight marker" – marcador de peso molecular, Pv18s – gene RNA ribosomal de *P. vivax*, I – insetos infectados, C- - controle negativo, C+ - amostra de sangue de pacientes humanos infectados com *P. vivax*, I (25) – grupo de 25 insetos infectados por *P. vivax*, I (1) – um inseto infectado por *P. vivax.*

ANEXO 2

Figura Suplementar 2 do capítulo 1



cDNAs diferencialmente expressos amplificados após diferentes subtrações de cDNAs 2hF-I , 2hI-F, 24hF-I e 24hI-F). MW - *molecular weight marker* ou marcador de peso molecular), F-I - cDNA de insetos após alimentação sanguínea menos após infecção com *P. vivax* e I-F - cDNA de insetos após infecção com *P. vivax* menos após alimentação sanguínea.

ANEXO 3

Tabela Suplementar 1 do capítulo 1

(Anotação das sequências obtidas com a biblioteca de *A. aquasalis* 2 horas após alimentação sanguínea menos 2 horas após infecção com *P. vivax)*

Accession number	Number of reads	G+C Content	CDS Length	Annotated Description	E-value	Score	Organism /Database	Gene Accession nº.
Signal transduction mechanism								
GR486343	1	59%	87	Rhodopsin receptor 3	1.0e-08	54	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP001178-PA
GR486481	1	46%	243	14-3-3 protein	6.0e-41	163	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP007643-PB
GR486499	1	57%	63	Phosrestin i (arrestin b or 2)	2.0e-06	47	Aedes_aegypti.AaegL1.50.pep.all.fa	AAEL003116-PA
Biomolecules degradation								
GR486394	1	54%	93	Neprilysin	8.0e-14	71	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP001791-PA
GR486445	1	51%	132	Proteasome activator subunit	2.0e-19	89	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP000308-PA
GR486421	1	58%	462	Arginine methyltransferase-interacting protein	1.0e-74	275	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP008075-PA
GR486410	2	58%	177	Metalloprotease	1.0e-27	116	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP000935-PA
GR486444	1	56%	330	Arginine methyltransferase-interacting protein	4.0e-66	246	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP008075-PA
GR486476	1	56%	330	E3 ubiquitin ligase interacting with arginine methyltransferase	5.0e-66	246	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP008075-PA
GR486472	2	52%	222	Lipase 1	7.0e-20	91	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP002353-PA
GR486479	1	47%	210	Ubiquitin specific protease family C19-related	1.0e-34	140	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP006652-PA
GR486446	3	53%	417	Alpha-amylase	4.0e-51	196	Aedes_aegypti.AaegL1.50.pep.all.fa	AAEL000667-PA
GR486569	1	53%	339	Dipeptidyl peptidase 4	3.0e-58	219	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP008176-PA
GR486703	1	56%	180	Acid phosphatase	2.0e-18	86	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP007400-PA
GR486555	1	49%	147	Serine protease	7.0e-34	129	anopheles_aquasalis_EST.fasta	EX809821
Replication, translation and transcription								
GR486346	1	44%	84	28S large subunit ribosomal RNA	7.0e-15	70	anopheles_darlingi_nucleotideo.fasta	AF417805
GR486357	1	43%	87	28S large subunit ribosomal RNA	3.0e-14	68	anopheles_darlingi_nucleotideo.fasta	AF417805
GR486366	1	56%	288	ATP-binding cassette, sub-family E (OABP), member 1	1.0e-47	183	Aedes_aegypti.AaegL1.50.pep.all.fa	AAEL010059-PA
GR486347	1	44%	84	28S large subunit ribosomal RNA	7.0e-15	70	anopheles_darlingi_nucleotideo.fasta	AF417805
GR486367	1	43%	87	28S large subunit ribosomal RNA	3.0e-14	68	anopheles_darlingi_nucleotideo.fasta	AF417805
GR486332	3	51%	234	40S ribosomal protein S11	1.0e-40	153	anopheles_darlingi_ptna.fasta	ACI30064
GR486392	1	47%	249	50S/60S ribosomal protein L14/L23	1.0e-44	167	anopheles_darlingi_ptna.fasta	ACI30146

GR486337	1	54%	318	60S ribosomal protein NHP2/L7A	8.0e-47	181	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP012204-PA
GR486328	1	53%	150	Ubiquitin/60S ribosomal protein L40 fusion	7.0e-10	57	apis_mellifera_ptna.fa	XP_397323
GR486344	1	47%	129	60S ribosomal protein L14/L17/L23	8.0e-22	91	anopheles_darlingi_ptna.fasta	ACI30146
GR486409	2	52%	267	NEFA-interacting nuclear protein NIP30	2.0e-18	86	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP010768-PA
GR486464	2	54%	129	Histone acetyltransferase gcn5	2.0e-17	83	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP004434-PA
GR486490, GR486416	3	53%	1179	DEAD box ATP-dependent RNA helicase	1.0e-167	583	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP008578-PA
GR486423	2	56%	621	Transient receptor potential channel 4	2.0e-83	304	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP010630-PA
GR486452	3	54%	285	Mago nashi protein	6.0e-49	187	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP010755-PA
GR486469	1	53%	150	Proliferation-associated 2g4	2.0e-19	90	Aedes_aegypti.AaegL1.50.pep.all.fa	AAEL012312-PA
GR486467	1	53%	285	Endoribonuclease XendoU	1.0e-46	181	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP002925-PA
GR486448	1	53%	291	U4/U6-associated splicing factor PRP4				
GR486487	1	59%	114	Ribosomal protein S15p/S13e	3.0e-15	75	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP005947-PA
GR486580	1	49%	129	60S ribosomal protein L14/L17/L23	5.0e-21	88	anopheles_darlingi_ptna.fasta	ACI30146
GR486621	1	54%	177	40S ribosomal protein S11	2.0e-39	116	anopheles_darlingi_ptna.fasta	ACI30064
GR486558	1	56%	135	Transcriptional Coactivator p15 (PC4)	3.0e-14	73	Aedes_aegypti.AaegL1.50.pep.all.fa	AAEL008736-PB
GR486591	1	54%	204	40S ribosomal protein S7	2.0e-31	129	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP010592-PA
GR486601	1	59%	141	40S ribosomal protein S4	2.0e-24	99	anopheles_darlingi_ptna.fasta	ACI30066
GR486576	2	49%	291	Nonsense-mediated RNAm decay 2 protein				
GR486699	1	62%	117	Transcription factor GATAa2	2.0e-07	50	cpipiens.PEPTIDES-CpipJ1.1.fa	CPIJ008350-PA
GR486701	1	47%	276	60S ribosomal protein L14/L17/L23	6.0e-35	134	anopheles_darlingi_ptna.fasta	ACI30146
GR486632	1	54%	225	Ribosomal protein L28	2.0e-15	78	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP008376-PA
GR486641	1	60%	141	Transcription factor GATA-4	7.0e-10	58	Aedes_aegypti.AaegL1.50.pep.all.fa	AAEL010222-PB
GR486542	1	63%	117	Transcription factor GATA	1.0e-08	54	cpipiens.PEPTIDES-CpipJ1.1.fa	CPIJ008350-PA
GR486604	1	57%	228	60S ribosomal protein L29	3.0e-41	155	anopheles_darlingi_ptna.fasta	ACI30089
GR486570	1	58%	69	Translationally-controlled tumor protein	4.0e-08	52	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP002667-PA

Metabolism								
GR486454	1	60%	174	Aspartate ammonia lyase	1.0e-33	144	cpipiens.SUPERCONTIGS-Johannesburg.CpipJ1.fa	DS231997.1
GR486439	1	61%	144	Glycogenin	8.0e-17	83	cpipiens.PEPTIDES-CpipJ1.1.fa	CPIJ013596-PA
GR486511	1	51%	615	Proline oxidase	8.0e-96	345	cpipiens.PEPTIDES-CpipJ1.1.fa	CPIJ009461-PA
GR486431	2	52%	255	Alanine aminotransferase	7.0e-42	164	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP000901-PA
GR486459	3	56%	279	Threonine dehydrogenase	2.0e-51	195	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP011948-PA
GR486681	2	52%	354	Choline/ethanolamine kinase	3.0e-29	118	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP007957-PA
GR486674	1	54%	126	Farnesoic acid O-methyltransferase-like protein	1.0e-16	80	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP006103-PA
GR486638	1	51%	336	Choline/ethanolamine kinase	1.0e-22	100	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP007957-PA
GR486566	2	53%	201	HAD-superfamily hydrolase	3.0e-17	83	Aedes_aegypti.AaegL1.50.pep.all.fa	AAEL007703-PA
Defense and detoxification								
GR486377	1	45%	207	Fibrinogen (techylectin-5B)	3.0e-33	135	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP004917-PA
GR486417	1	56%	192	V-type ATP synthase beta chain	1.0e-32	133	Aedes_aegypti.AaegL1.50.pep.all.fa	AAEL005798-PA
GR486477	1	54%	213	Integral transmembrane protein 2-related with immunoglobulin domain	1.0e-29	125	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP009156-PA
GR486461	2	53%	465	Apoptosis inhibitory protein 5 (API5)	3.0e-51	196	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP009645-PA
GR486572	2	53%	369	Serine protease inhibitor	1.0e-23	103	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP009670-PB
GR486610	1	54%	180	Antimicrobial peptide cecropin	2.0e-22	92	anopheles_darlingi_ptna.fasta	ACI30166
GR486626	1	50%	210	Internalin A	5.0e-29	121	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP004458-PA
GR486612	2	54%	180	Antimicrobial peptide cecropin	2.0e-22	92	anopheles_darlingi_ptna.fasta	ACI30166
Structural genes								
GR486532	1	57%	507	Myosin heavy chain	3.0e-33	137	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP007523-PB
GR486523,	22	58%	549	Myosin	2.0e-33	137	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP007523-PB
GR486397	1	58%	372	Myosin light chain 1	3.0e-62	234	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP007806-PA
GR486468	1	58%	372	Myosin light chain 1	3.0e-62	234	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP007806-PA
GR486530	1	56%	252	Zeelin1	8.0e-32	132	Anopheles_gambiae.AgamP3.50.pe	AGAP004161-PA

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GR486552	1	51%	153	Brain chitinase and chia	2.0e-12	66	Aedes_aegypti.AaegL1.50.pep.all.fa	AAEL012467-PA
GR486516	1	52%	162	Thymosin	2.0e-10	68	uniprot_trembl.fasta	8343_uniprot_trembl.
GR486496	1	52%	162	Thymosin	2.0e-10	68	uniprot_trembl.fasta	8343_uniprot_trembl.
GR486492	1	52%	162	Thymosin	2.0e-10	68	uniprot_trembl.fasta	8343_uniprot_trembl.
GR486549	1	59%	105	Gelsolin	2.0e-11	63	cpipiens.PEPTIDES-CpipJ1.1.fa	CPIJ004628-PA
GR486683	1	50%	456	CLIP-associating protein	8.0e-47	181	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP007623-PA
GR486603	1	59%	177	Mucin-like peritrophin	2.0e-32	125	anopheles_darlingi_ptna.fasta	ACI30179
GR486538	1	57%	204	Myosin light chain 1	2.0e-29	122	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP001569-PA
Energy metabolism								
GR486534	4	58%	402	AMP dependent coa ligase	7.0e-52	199	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP008557-PA
GR486462	1	61%	393	Dihydrolipoamide succinyltransferase	1.0e-38	154	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP004055-PA
GR486403, GR486413	7	61%	408	Dihydrolipoamide succinyltransferase	1.0e-41	164	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP004055-PA
GR486418, GR486414	6	59%	399	AMP dependent coa ligase	4.0e-51	197	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP008557-PA
GR486435	1	55%	267	Phosphoenolpyruvate carboxykinase	3.0e-46	178	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP003350-PA
GR486453	1	57%	396	Dihydrolipoamide succinyltransferase	3.0e-25	110	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP004055-PA
GR486498	1	57%	174	Pyruvate/2-oxoglutarate dehydrogenase complex	3.0e-21	96	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP003136-PA
GR486597	1	58%	207	Glutaryl-CoA dehydrogenase	8.0e-33	134	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP008501-PA
Embryogenesis								
GR486396	1	55%	189	Apolipophorins / vitellogenin	3.0e-25	108	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP001826-PA
Transport and secretion								
GR486335	2	50%	270	Calmodulin and related proteins	5.0e-26	111	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP006182-PA
GR486503	1	59%	687	Monocarboxylate transporter	6.0e-87	315	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP002587-PA
GR486527	1	55%	708	Coatomer	1.0e-118	421	Aedes_aegypti.AaegL1.50.pep.all.fa	AAEL011650-PA
GR486429	2	49%	96	Clathrin coat assembly protein AP17	3.0e-11	64	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP001703-PA
GR486457	1	42%	177	Vacuolar sorting protein PEP3/VPS18	4.0e-26	111	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP000983-PA

GR486535	1	54%	129	Antigen 5-related salivary protein	1.0e-21	90	anopheles_darlingi_ptna.fasta	AAQ17073
GR486671	1	55%	198	Translocon-associated protein alpha	7.0e-29	120	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP001721-PA
GR486702	1	57%	291	Calcium-transporting atpase sarcoplasmic/endoplasmic reticulum type (calcium pump)	2.0e-52	199	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP006186-PD
Unknown protein								
GR486339	1	53%	171	Unknown protein				
GR486386	1	37%	459	Unknown protein				
GR486334	3	47%	633	Unknown protein with coiled-coil domain				
GR486331	1	44%	624	Unknown protein				
GR486342	1	45%	87	Unknown protein				
GR486333	4	45%	159	Unknown protein				
GR486382	1	51%	180	Unknown protein				
GR486398	1	45%	168	Unknown protein				
GR486390	1	46%	420	Unknown protein				
GR486690	2	50%	471	Unknown protein				
GR486488	1	50%	813	Unknown protein				
GR486482	1	45%	519	Unknown protein with 2Fe-2S ferredoxin-type iron-sulfur binding region signature				
GR486405	1	39%	540	Unknown protein				
GR486447	1	53%	309	Unknown protein				
GR486406	1	51%	102	Unknown protein				
GR486393	2	53%	801	Unknown protein				
GR486415	1	48%	768	Unknown protein				
GR486463	1	54%	705	Unknown protein				
GR486480	1	51%	126	Unknown protein				
GR486515	1	50%	402	Unknown protein				
GR486474	3	43%	282	Unknown protein				
GR486424	1	39%	288	Unknown protein				
GR486521	1	44%	279	Unknown protein				
GR486550	1	46%	294	Unknown protein				
GR486513	1	39%	612	Unknown protein				
GR486545	1	43%	258	Unknown protein				
GR486685	1	51%	174	Unknown protein				
GR486599	1	46%	450	Unknown protein with EGF-1 domain				
GR486584	4	44%	489	Unknown protein				
GR486553	1	47%	216	Unknown protein				
GR486595	2	45%	384	Unknown protein				
GR486533	1	61%	135	Unknown protein				
GR486578	2	33%	372	Unknown protein				
GR486548	1	49%	261	Unknown protein				
GR486559	1	56%	162	Unknown protein				
GR486592	2	48%	162	Unknown protein				
GR486679	1	40%	402	Unknown protein				
GR486593	1	36%	174	Unknown protein				
GR486666	1	50%	195	Unknown protein				
GR486571	2	54%	171	Unknown protein				
GR486607	1	39%	282	Unknown protein				
GR486551, GR486588	2	55%	282	Unknown protein				
GR486573	1	34%	489	Unknown protein				
GR486547	1	45%	333	Unknown protein				

				with Kringle-like domain				
GR486546	1	43%	285	Unknown protein				
GR486636	1	46%	300	Unknown protein				
GR486544	1	37%	615	Unknown protein				
GR486589	1	42%	150	Unknown protein				
GR486598	1	54%	201	Unknown protein				
GR486633	1	54%	114	Unknown protein				
GR486792	1	53%	90	Unknown protein				
GR486781	1	55%	210	Unknown protein				
GR486721	1	55%	150	Unknown protein				
Unknown conserved protein								
GR486340	1	46%	414	Unknown conserved protein	5.0e-28	119	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP006195-PA
GR486336	5	50%	60	Unknown conserved protein	7.0e-09	40	anopheles_darlingi_EST.fasta	FK704551
GR486374	1	40%	267	Unknown conserved protein	7.0e-26	82	A.stephensi_EST.fasta	EX225359
GR486341	1	44%	114	Unknown conserved protein	1.0e-14	68	anopheles_darlingi_EST.fasta	FK703975
GR486378	2	50%	153	Unknown conserved protein	2.0e-27	112	anopheles_darlingi_EST.fasta	FK705369
GR486329	1	57%	204	Unknown conserved protein	1.0e-26	113	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP007745-PA
GR486327	1	50%	189	Unknown conserved protein	1.0e-08	51	anopheles_darlingi_EST.fasta	FK705188
GR486400, GR486419	2	49%	957	Unknown conserved protein with coiled-coil domain	5.0e-12	78	aaegypti.CONTIGS-Liverpool.AaegL1.fa	AAGE02021470.1
GR486460	1	56%	159	Unknown conserved protein	5.0e-21	95	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP010723-PA
GR486512	1	56%	828	Unknown conserved protein	4.0e-11	47	anopheles_darlingi_EST.fasta	FK704111
GR486522	1	51%	441	Unknown conserved protein	7.0e-09	51	agambiae.EST-CLIPPED.mar08.fa	BX615325.1
GR486510	1	58%	147	Unknown conserved protein	2.0e-14	75	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP008438-PA
GR486441	1	51%	441	Unknown conserved protein	7.0e-09	51	agambiae.EST-CLIPPED.mar08.fa	BX615325.1
GR486485	2	53%	123	Unknown conserved protein	2.0e-16	79	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP012926-PA
GR486470	1	51%	477	Unknown conserved protein	4.0e-08	49	agambiae.EST-CLIPPED.mar08.fa	BX615325.1
GR486438	1	44%	204	Unknown conserved protein	5.0e-19	50	anopheles_darlingi_EST.fasta	DV729753
GR486440	1	36%	576	Unknown conserved protein	1.0e-44	84	anopheles_darlingi_EST.fasta	DV729447
GR486668	1	52%	468	Unknown conserved protein	4.0e-45	135	anopheles_darlingi_EST.fasta	DV729843
GR486634	1	39%	75	Unknown conserved protein	2.0e-18	62	anopheles_darlingi_EST.fasta	FK704481
GR486619	1	37%	84	Unknown conserved protein	5.0e-20	77	anopheles_darlingi_EST.fasta	FK703921
GR486574	3	48%	183	Unknown conserved protein	3.0e-28	118	Anopheles_gambiae.AgamP3.50.pe	AGAP002010-PA

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GR486623	1	52%	402	Unknown conserved protein with NUC173 domain	2.0e-42	167	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP002961-PA
GR486536	2	54%	162	Unknown conserved protein	5.0e-13	36	anopheles_darlingi_EST.fasta	FK705500
GR486757	1	70%	141	Unknown conserved protein	5.0e-06	45	cpipiens.EST-CLIPPED.mar08.fa	EV302468.1
GR486691	1	50%	114	Unknown conserved protein	7.0e-10	60	aaegypti.EST-CLIPPED.mar08.fa	DV322465.1
Bacterial protein								
GR486501, GR487733	4	57%	87	Bacterial protein	1.0e-07	53	refseq_protein	ZP_00630616
GR486543	1	53%	78	Bacterial protein	4.0e-06	47	refseq_protein	ZP_00630616
GR486657	1	72%	225	Bacterial protein	1.0e-10	69	uniprot_trembl.fasta	25737_uniprot_trembl
GR486648	1	55%	78	Bacterial protein	2.0e-06	47	refseq_protein	ZP_00630616
GR486678	1	49%	168	Bacterial protein	3.0e-11	70	uniref90.fasta	UniRef90_Q3BKI4
GR486725	1	53%	194	Bacterial protein	8.0e-06	46	uniref90.fasta	UniRef90_UPI00005545
GR486773	1	50%	225	Arabinose-proton symporter bacterial protein	1.0e-14	82	refseq_protein	ZP_02038208
GR486780	1	56%	87	Bacterial protein	1.0e-07	53	refseq_protein	ZP_00630616

ANEXO 4

Tabela Suplementar 2 do capítulo 1

(Anotação das sequências obtidas com a biblioteca de *A. aquasalis* 2 horas após infecção com *P. vivax* menos 2 horas após alimentação sanguínea)

Accession number	Number of reads	G+C Content	CDS Length	Annotated Description	E-value	Score	Organism /Database	Gene Accession nº.
Signal transduction mechanism								
GR486807	2	52%	327	Phosrestin ii (arrestin N)	6.0e-57	214	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP010134-PA
GR486806	1	64%	108	Rhodopsin receptor 3	3.0e-13	69	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP001178-PA
GR487139	1	54%	183	Adenylate and Guanylate cyclase	3.0e-31	129	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP002998-PA
GR486987	1	56%	189	LIM domain-binding protein	4.0e-37	148	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP006901-PC
Biomolecules degradation								
GR486815	1	56%	216	Zinc carboxypeptidase A 1	3.0e-36	132	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP009593-PA
GR486794	1	62%	168	Zinc carboxypeptidase A 1	4.0e-16	79	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP009593-PA
GR486817	5	53%	633	Chymotrypsin-like protein	1.0e-72	268	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP001198-PA
GR486928	1	53%	165	Fructose-1,6-bisphosphatase	3.0e-25	108	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP009173-PA
GR486820	1	49%	267	Alpha-amylase	2.0e-41	162	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP012401-PA
GR486808	1	49%	153	Chymotrypsin 1	9.0e-27	103	anopheles_aquasalis_ptna.fasta	AAD17491
GR486875	2	55%	249	Ubiquitin-activating enzyme E1	2.0e-40	159	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP011872-PA
GR486905	1	52%	267	Alpha-amylase / Maltase-like protein Agm2	3.0e-45	175	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP012400-PA
GR486957	2	57%	114	Chymotrypsin	2.0e-10	60	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP010547-PA
GR486935	1	56%	291	Serine protease SP24D	2.0e-26	112	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP005065-PA
GR487069	1	28%	162	Subtilisin-like serine protease	2.0e-27	99	anopheles_darlingi_EST.fasta	FK703921
GR487148	1	56%	249	Ubiquitin-activating enzyme E1	3.0e-41	161	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP011872-PA
GR487150	1	53%	219	Protease M1 zinc metalloprotease	4.0e-08	52	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP012745-PA
GR487054	1	57%	228	Insulinase family metalloproteinase	6.0e-32	130	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP006099-PA
GR487131	2	49%	204	Metalloprotease	8.0e-22	97	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP004747-PA
GR486970	1	60%	96	Ubiquinol-cytochrome c reductase complex core protein	4.0e-10	59	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP006099-PA
GR487046	1	61%	96	Insulinase (Peptidase family	2.0e-10	59	Anopheles_gambiae.AgamP3.50.pep.a	AGAP006099-PA

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GR487040	3	54%	330	Chymotrypsin A	1.0e-34	139	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP001199-PA
GR486963	1	57%	183	Hsp70 chaperones	2.0e-28	119	cpipiens.PEPTIDES-CpipJ1.1.fa	CPIJ008915-PA
GR486979	1	58%	114	Chymotrypsin	3.0e-10	59	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP010547-PA
Replication, translation and transcription								
GR486798	2	59%	405	60S ribosomal protein L21	1.0e-75	270	anopheles_darlingi_ptna.fasta	ACI30091
GR486793	1	50%	345	Proliferating Cell Nuclear Antigen (PCNA)	2.0e-57	215	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP010220-PA
GR486810	1	40%	564	Extensin 2	2.0e-16	86	aaegypti.EST-CLIPPED.mar08.faEG009251.1	EG009251
GR486879	1	55%	255	5' nucleotidase apyrase	2.0e-43	162	anopheles_darlingi_ptna.fasta	ACI30113
GR486831	1	59%	183	40S ribosomal protein S23	5.0e-30	117	anopheles_darlingi_ptna.fasta	ACI30052
GR486969	2	57%	93	60S ribosomal protein L10	1.0e-13	70	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP011298-PA
GR486986	38	28%	276	Mitochondrial large ribosomal RNA	4.0e-36	149	dmel-all-gene-r5.8.fasta	FBgn0013686
GR486974	1	57%	75	60S ribosomal protein L22	1.0e-10	53	anopheles_darlingi_ptna.fasta	ACI30068
GR486882	1	66%	198	60S ribosomal protein L7a	3.0e-20	86	anopheles_darlingi_ptna.fasta	ACI30081
GR486956	1	61%	342	40S ribosomal protein S2/30S ribosomal protein S5	1.0e-58	212	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP003768-PA
GR487127	1	51%	174	Ribosomal protein L22	3.0e-07	43	anopheles_darlingi_nucleotideo.fasta	EU934314
GR487124	1	55%	294	60S ribosomal protein L24	1.0e-21	91	anopheles_darlingi_ptna.fasta	ACI30074
GR487092	2	53%	240	DNA/RNA repair protein	5.0e-38	151	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP002472-PA
GR487026	7	55%	213	60S ribosomal protein L5	6.0e-30	124	cpipiens.PEPTIDES-CpipJ1.1.fa	CPIJ010112-PA
GR487048	2	54%	216	RUVB-related reptin and pontin	2.0e-33	132	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP009746-PA
GR486866	1	55%	102	Ubiquitin/60S ribosomal protein L40 fusion	4.0e-15	68	anopheles_darlingi_ptna.fasta	ACI30049
GR486949	1	52%	195	Homeobox protein extradenticle	3.0e-27	115	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP004696-PA
GR486983	1	62%	147	Translation elongation factor 2	2.0e-28	89	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP009441-PA
GR487063	1	58%	186	Elongation factor 1-gamma	6.0e-32	130	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP000883-PA
GR486868	2	59%	231	Eukaryotic translation elongation factor	7.0e-59	154	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP009441-PA
GR487025	4	47%	504	Homeobox protein extradenticle	6.0e-89	322	Anopheles_gambiae.AgamP3.50.pep.a	AGAP004696-PA

							ll.fa	
GR487081	2	54%	165	Mediator of RNA polymerase II transcription subunit 22	2.0e-22	100	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP004191-PA
Metabolism								
GR487021	1	61%	138	Creatine kinase	6.0e-20	91	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP005627-PB
GR487113	1	46%	279	Coproporphyrinogen III oxidase	6.0e-49	187	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP004749-PB
GR486965	1	62%	249	Bifunctional purine biosynthesis protein	1.0e-37	150	Aedes_aegypti.AaegL1.50.pep.all.fa	AAEL012825-PA
GR487008	3	58%	318	Fatty acid desaturase	2.0e-59	222	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP001713-PA
GR487129	2	52%	270	Ribose-phosphate pyrophosphokinase	5.0e-48	185	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP004890-PB
GR486994	1	55%	66	Haloacid dehalogenase-like hydrolase	6.0e-06	45	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP002841-PA
Defense and detoxification								
GR486800	2	61%	150	Bacteria responsive protein 2 / imaginal disc growth factor	5.0e-22	98	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP008060-PA
GR486898	1	55%	138	Fibronectin	5.0e-21	95	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP002579-PA
GR487115	1	54%	96	Alpha-2-macroglobulin receptor-associated protein	1.0e-14	56	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP003521-PA
GR487133	1	42%	336	Fibrinogen (ficolin)	1.0e-19	90	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP010811-PA
GR487128	3	51%	141	Alpha-2-macroglobulin receptor-associated protein	3.0e-21	96	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP003521-PA
Structural genes								
GR486812	3	47%	99	Profilin	3.0e-13	69	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP009861-PA
GR486936	3	58%	276	Actin	2.0e-47	182	Aedes_aegypti.AaegL1.50.pep.all.fa	AAEL004631-PA
GR486917	3	57%	189	Actin	3.0e-34	138	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP005095-PA
GR486912	3	53%	129	Myotonin-protein kinase	1.0e-18	87	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP012090-PA
GR486947	2	49%	147	Cuticular protein 76	4.0e-19	89	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP009874-PA
GR486927	1	47%	237	Actin				
GR487080	1	56%	147	Collagen IV alpha 1 chain	5.0e-25	108	cpipiens.PEPTIDES-CpipJ1.1.fa	CPIJ005294-PA
GR487057	1	57%	138	Actin	1.0e-21	97	Aedes_aegypti.AaegL1.50.pep.all.fa	AAEL005961-PA
GR487024	1	55%	339	Laminin subunit gamma-1	5.0e-27	114	Anopheles_gambiae.AgamP3.50.pep.a	AGAP007629-PA

							ll.fa	
Energy metabolism								
GR486805	2	56%	195	Fumarylacetoacetate hydrolase	6.0e-31	127	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP005865-PA
GR486939	2	56%	258	Succinyl-coa:3-ketoacid-coenzyme a transferase	2.0e-43	169	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP006096-PA
GR486946	2	56%	174	Mitochondrial phosphate carrier protein	1.0e-29	123	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP003586-PA
GR486887	2	45%	210	Succinyl-coa synthetase beta chain	1.0e-33	136	Aedes_aegypti.AaegL1.50.pep.all.fa	AAEL011746-PA
GR487019	2	61%	75	3-hydroxyacyl-coa dehydrogenase	1.0e-06	48	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP007784-PA
GR487121	1	63%	75	Multifunctional fatty acid oxidation complex	9.0e-06	45	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP007784-PA
GR487114	1	51%	246	Glucosidase II beta subunit-like protein	6.0e-31	127	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP009546-PA
GR487090	2	54%	273	S-adenosylmethionine synthetase	1.0e-21	97	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP009447-PA
GR487137	1	55%	165	Mitochondrial Aconitase	1.0e-26	113	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP007852-PA
GR487100	1	51%	210	Succinyl-coa synthetase beta chain	5.0e-18	85	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP004744-PA
GR487117	1	62%	126	Cytochrome c oxidase subunit IV	1.0e-17	84	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP008727-PA
GR487116	1	52%	312	Phosphorylase kinase alpha/beta	1.0e-39	156	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP009278-PA
GR486860	3	52%	243	Dihydrolipoamide succinyltransferase	7.0e-39	154	Aedes_aegypti.AaegL1.50.pep.all.fa	AAEL002764-PB
Embryogenesis								
GR486841	10	54%	231	Vitellogenin	3.0e-16	87	uniref90.fasta	UniRef90_Q49MF2
GR486827	1	61%	186	Apolipophorins / vitellogenin	3.0e-19	89	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP001826-PA
GR487097	1	55%	237	Vitellogenin	1.0e-39	164	uniref90.fasta	UniRef90_Q49MF2
GR487034	2	59%	285	Apolipophorins	3.0e-43	168	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP001826-PA
GR486937	1	52%	147	Maternal protein exuperantia	2.0e-21	96	Aedes_aegypti.AaegL1.50.pep.all.fa	AAEL010097-PA
Transport and secretion								
GR486819	2	54%	123	Ferritin light chain-like protein precursor	1.0e-14	67	anopheles_darlingi_ptna.fasta	ACI30191
GR486929	1	52%	315	Ferritin heavy chain	2.0e-27	115	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP002465-PA
GR486950	1	43%	75	Amino acid permease	4.0e-07	49	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP011386-PA
GR487007	1	55%	303	Importin alpha	5.0e-45	174	Anopheles_gambia	AGAP001273-

							e.AgamP3.50.pep.all.fa	PA
GR487035	3	52%	522	Importin alpha	5.0e-90	325	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP001273-PA
GR486988	1	58%	117	Pmp22 peroxisomal membrane protein	1.0e-14	74	Aedes_aegypti.AaegL1.50.pep.all.fa	AAEL004577-PA
GR486894	1	56%	135	Nuclear pore complex protein Nup107	1.0e-18	87	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP001685-PA
GR486954	1	54%	198	Importin alpha	2.0e-25	109	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP001273-PA
GR487027	5	59%	309	Calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type	8.0e-54	203	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP006186-PD
GR487052	4	49%	198	Retinal degeneration b beta	5.0e-33	135	cpipiens.PEPTIDES-CpipJ1.1.fa	CPIJ009191-PA
Unknown protein								
GR486816	2	44%	375	Unknown protein				
GR486802	1	49%	315	Unknown protein				
GR486797	1	53%	114	Unknown protein				
GR486930	1	43%	330	Unknown protein				
GR486931	1	48%	162	Unknown protein				
GR486845	1	40%	339	Unknown protein				
GR486811	1	42%	468	Unknown protein				
GR486821	1	40%	240	Unknown protein				
GR486828	1	48%	228	Unknown protein				
GR487154	1	50%	192	Unknown protein				
GR487161	1	46%	291	Unknown protein				
GR486899	61	52%	498	Unknown protein				
GR486854	1	50%	384	Unknown protein				
GR486952	1	49%	183	Unknown protein				
GR487056	1	32%	183	Unknown protein				
GR486863	1	53%	180	Unknown protein				
GR486964	1	56%	240	Unknown protein				
GR486903	1	56%	141	Unknown protein				
GR486862	1	43%	522	Unknown protein				
GR486865	2	51%	438	Unknown protein				
GR486942	1	51%	243	Unknown protein				
GR486980	2	49%	168	Unknown protein				
GR486891	5	48%	150	Unknown protein				
GR486995	1	51%	345	Unknown protein				
GR486967	1	17%	180	Unknown protein with coiled-coil domain				
GR486848	1	42%	459	Unknown protein				
GR487079	1	44%	207	Unknown protein				
GR487041	1	44%	378	Unknown protein				
GR486921	2	52%	246	Unknown protein				
GR486872, GR486977	2	44%	522	Unknown protein				
GR486960	1	44%	213	Unknown protein				
GR487047	1	49%	183	Unknown protein				
GR486889	3	48%	288	Unknown protein				
GR486858	1	32%	183	Unknown protein				
GR487125	1	41%	192	Unknown protein				
GR487029	6	51%	174	Unknown protein / ribosomal protein				

				L22				
GR487006	2	47%	135	Unknown protein				
GR487077	1	49%	162	Unknown protein				
GR487152	6	42%	441	Unknown protein				
GR487106	1	44%	210	Unknown protein				
GR487083	1	49%	339	Unknown protein				
GR487134	1	45%	198	Unknown protein				
GR486989	1	50%	129	Unknown protein				
GR487039	1	45%	243	Unknown protein				
GR487017	1	33%	174	Unknown protein				
GR487144	1	50%	327	Unknown protein				
GR487020	1	52%	168	Unknown protein				
GR487142	1	34%	180	Unknown protein				
GR487013	1	44%	321	Unknown protein				
GR487143	1	48%	420	Unknown protein				
GR487051	1	52%	132	Unknown protein				
GR487078	1	48%	96	Unknown protein				
GR487109	3	45%	432	Unknown protein				
GR487055	1	43%	228	Unknown protein				
GR487105	1	48%	420	Unknown protein				
GR487003	1	57%	138	Unknown protein				
GR487107	1	47%	336	Unknown protein				
GR486998	2	50%	339	Unknown protein				
GR486958	1	50%	96	Unknown protein				
GR486968	1	50%	222	Unknown protein				
GR487049	1	51%	153	Unknown protein				
GR487060	1	44%	174	Unknown protein				
GR487111	1	48%	180	Unknown protein				
GR486886	2	49%	147	Unknown protein				
GR487110	1	32%	180	Unknown protein				
Unknown conserved protein								
GR486838	1	59%	141	Unknown conserved protein	1.0e-11	74	aaegypti.CONTIGS-Liverpool.AaegL1.fa	AAGE02020711.1
GR486823	1	52%	63	Unknown conserved protein	4.0e-06	49	aegypti.EST-CLIPPED.mar08.fa	DV370849.1
GR486871	1	51%	135	Unknown conserved protein	2.0e-09	52	anopheles_darlingi_EST.fasta	DV729374
GR486918	1	49%	87	Unknown conserved protein	1.0e-10	55	aaegypti.EST-CLIPPED.mar08.fa	DV321842.1
GR487160	1	48%	81	Unknown conserved protein	3.0e-10	54	aaegypti.EST-CLIPPED.mar08.fa	DV322465.1
GR486878	1	50%	111	Unknown conserved protein	2.0e-12	68	aaegypti.EST-CLIPPED.mar08.fa	DV322465.1
GR487009	1	53%	153	Unknown conserved protein	2.0e-08	56	raaegypti.EST-CLIPPED.mar08.fa	DV321842.1
GR487172	1	58%	156	Unknown conserved protein	2.0e-11	58	aaegypti.EST-CLIPPED.mar08.fa	DV321842.1
GR486940	1	37%	261	Unknown conserved protein with coiled-coil domain	2.0e-15	50	anopheles_darlingi_EST.fasta	FK704481
GR486984	2	45%	156	Unknown conserved protein with Leucine-rich repeat (LRR)	2.0e-06	47	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP007453-PA
GR487075	1	52%	417	Unknown conserved protein	4.0e-64	238	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP007851-PA
GR487044	1	48%	162	Unknown conserved protein	4.0e-08	52	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP003939-PA
GR486907	2	42%	389	Unknown conserved protein	2.0e-35	83	anopheles_darlingi_EST.fasta	FK704163
GR486938	2	43%	387	Unknown	6.0e-28	118	Anopheles_gambia	AGAP006275-

				conserved protein			e.AgamP3.50.pep.all.fa	PA
GR487122	1	45%	111	Unknown conserved protein	4.0e-07	53	cpipiens.SUPERCONTIGS-Johannesburg.CpipJ1.fa	DS231941.1
GR486962	1	50%	231	Unknown conserved protein	2.0e-08	57	agambiae.EST-CLIPPED.mar08.fa	BX605447.1
GR487099	1	50%	294	Unknown conserved protein	2.0e-11	62	rprolixus.EST-CLIPPED.mar08.fa FD777574.1	FD777574
GR486892	1	55%	351	Unknown conserved protein	2.0e-47	183	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP011476-PA
Bacterial protein								
GR487168	1	55%	192	Bacterial protein	2.0e-09	59	uniref90.fasta	UniRef90_UPI00005545
GR487162	1	56%	87	Bacterial protein				

ANEXO 5

Tabela Suplementar 3 do capítulo 1

(Anotação das sequências obtidas com a biblioteca de *A. aquasalis* 24 horas após alimentação sanguínea menos 24 horas após infecção com *P. vivax)*

Accession number	Number of reads	G+C Content	CDS Length	Annotated Description	E-value	Score	Organism /Database	Gene Accession nº.
Signal transduction mechanism								
GR487233	1	53%	168	Rhodopsin receptor 1	6.0e-19	88	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP001178-PA
GR487190	1	57%	114	Ultraviolet-sensitive opsin	3.0e-29	57	cpipiens.PEPTIDES-CpipJ1.1.fa	CPIJ013408-PA
GR487344	1	60%	171	Rhodopsin receptor 1	3.0e-25	108	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP001178-PA
GR487351	3	64%	108	Rhodopsin receptor 3	3.0e-13	69	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP001178-PA
Biomolecules degradation								
GR487200	5	50%	165	Chymotrypsin 1	1.0e-28	110	anopheles_aquasalis_ptna.fasta	AAD17491
GR487219	1	61%	135	Cathepsin 1	3.0e-21	96	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP012577-PA
GR487348	3	62%	90	Cathepsin 1	7.0e-13	68	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP011828-PA
GR487377	1	56%	126	Chymotrypsin 1	2.0e-23	93	anopheles_aquasalis_ptna.fasta	AAD17491
GR487341	15	57%	126	Chymotrypsin 1	2.0e-23	93	anopheles_aquasalis_ptna.fasta	AAD17491
GR487388	1	43%	192	Rhomboid 1 (intramembrane serine protease)	2.0e-21	96	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP000832-PA
Replication, translation and transcription								
GR487203	1	57%	69	Translationally-controlled tumor protein	4.0e-08	52	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP002667-PA
GR487236	4	55%	294	60S ribosomal protein L24	1.0e-21	91	anopheles_darlingi_ptna.fasta	ACI30074
GR487363	1	54%	96	60S ribosomal protein L10	4.0e-11	62	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP011298-PA
GR487217	2	56%	165	40S ribosomal protein S5	3.0e-13	69	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP008329-PA
GR487235	12	57%	93	60S ribosomal protein L10	1.0e-13	70	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP011298-PA
GR487342	4	50%	261	DEAD box ATP-dependent RNA helicase	2.0e-35	142	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP009863-PA
GR487335	2	58%	198	40S ribosomal protein S4	8.0e-35	133	anopheles_darlingi_ptna.fasta	ACI30066
Metabolism								
GR487184	1	61%	138	Creatine kinase	6.0e-20	91	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP005627-PB
GR487358	3	57%	162	Phosphoserine phosphatase	4.0e-22	99	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP012247-PA
GR487209	1	54%	321	Dimethylaniline monooxygenase	2.0e-54	205	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP010398-PA
Defense and detoxification								
GR487220	1	57%	114	Cu-Zn Superoxide Dismutase	1.0e-07	52	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP001623-PA

Structural genes								
GR487244	2	57%	297	Fibulin 1	1.0e-48	186	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP011322-PA
Energy metabolism								
GR487211	2	55%	168	Cytochrome c oxidase subunit IV	3.0e-17	83	Aedes_aegypti.AaegL1.50.pep.all.fa	AAEL005170-PA
Embryogenesis								
GR487194	4	56%	231	Vitellogenin	7.0e-14	79	uniref90.fasta	UniRef90_Q49MF2
GR487227	1	56%	153	Vitellogenin	1.0e-25	110	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP004203-PB
GR487240	1	59%	99	Vitellogenin	1.0e-12	67	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP004203-PB
GR487183	1	57%	234	Vitellogenin	4.0e-08	60	uniref90.fasta	UniRef90_Q49MF2
GR487365	1	56%	232	Vitellogenin	7.0e-14	79	uniref90.fasta	UniRef90_Q49MF2
GR487376	1	57%	231	Vitellogenin	6.0e-13	76	uniref90.fasta	UniRef90_Q49MF2
GR487332	35	54%	231	Vitellogenin	3.0e-16	87	uniref90.fasta	UniRef90_Q49MF2
GR487338	1	57%	246	Vitellogenin	3.0e-15	84	uniref90.fasta	UniRef90_Q49MF2
GR487251	1	62%	180	Vitellogenin	1.0e-06	47	uniref90.fasta	UniRef90_Q49MF2
GR487242	1	56%	237	Vitellogenin	7.0e-15	82	uniref90.fasta	UniRef90_Q49MF2
GR487247	1	57%	246	Vitellogenin	3.0e-15	84	uniref90.fasta	UniRef90_Q49MF2
GR487375	1	62%	141	Vitellogenin	2.0e-09	57	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP004203-PB
GR487340	14	59%	132	Vitellogenin	7.0e-20	91	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP004203-PB
GR487396	1	56%	231	Vitellogenin	1.0e-12	75	uniref90.fasta	UniRef90_Q49MF2
GR487414	1	57%	246	Vitellogenin	3.0e-13	77	uniref90.fasta	UniRef90_Q49MF2
GR487438	1	60%	141	Vitellogenin	2.0e-19	90	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP001826-PA
GR487428	1	55%	231	Vitellogenin	2.0e-13	78	uniref90.fasta	UniRef90_Q49MF2
GR487410	1	57%	246	Vitellogenin	1.0e-14	81	uniref90.fasta	UniRef90_Q49MF2
GR487431	1	57%	246	Vitellogenin	4.0e-14	80	uniref90.fasta	UniRef90_Q49MF2
GR487394	1	55%	219	Vitellogenin	3.0e-12	74	uniref90.fasta	UniRef90_Q49MF2
GR487440	1	55%	219	Vitellogenin	6.0e-11	69	uniref90.fasta	UniRef90_Q49MF2
GR487444	1	58%	231	Vitellogenin	4.0e-11	70	uniref90.fasta	UniRef90_Q49MF2
Unknown protein								
GR487197	1	44%	207	Unknown protein				
GR487192	1	52%	159	Unknown protein				
GR487255	4	46%	420	Unknown protein				
GR487204	1	53%	177	Unknown protein				
GR487287	1	56%	189	Unknown protein				
GR487271	2	53%	204	Unknown protein				

GR487199	3	51%	174	Unknown protein / ribosomal protein L22				
GR487260	1	45%	183	Unknown protein				
GR487262	5	48%	198	Unknown protein				
GR487368	1	48%	201	Unknown protein				
GR487253	1	50%	258	Unknown protein				
GR487221	1	61%	207	Unknown protein				
GR487216	1	46%	108	Unknown protein				
GR487369	1	50%	363	Unknown protein				
GR487207	1	49%	336	Unknown protein				
GR487333	1	51%	363	Unknown protein				
GR487243	1	52%	180	Unknown protein				
GR487383	1	49%	195	Unknown protein				
GR487402	1	31%	126	Unknown protein				
GR487439	1	50%	336	Unknown protein				
GR487386	1	53%	180	Unknown protein				
Unknown conserved protein								
GR487373	1	53%	135	Unknown conserved protein	4.0e-07	51	agambiae.EST-CLIPPED.mar08.fa	CD743825.1
GR487350	4	54%	339	Unknown conserved protein	6.0e-12	69	agambiae.EST-CLIPPED.mar08.fa	BM590044.1
GR487330	1	54%	135	Unknown conserved protein	2.0e-08	49	anopheles_darlingi_EST.fasta	DV729374
GR487415	2	57%	141	Unknown conserved protein	8.0e-13	72	agambiae.EST-CLIPPED.mar08.fa	BX006650.1
GR487397	1	58%	189	Unknown conserved protein	5.0e-06	48	agambiae.EST-CLIPPED.mar08.fa	BX766947.1
Bacterial protein								
GR487187	3	55%	78	Bacterial protein	2.0e-08	57	refseq_protein	ZP_00630616
GR487196	10	59%	87	Bacterial protein	2.0e-08	57	refseq_protein	ZP_00630616

ANEXO 6

Tabela Suplementar 4 do capítulo 1

(Anotação das sequências obtidas com a biblioteca de *A. aquasalis* 24 horas após infecção com *P. vivax* menos 24 horas após alimentação sanguínea)

Accession number	Number of reads	G+C Content	CDS Length	Annotated Description	E-value	Score	Organism /Database	Gene Accession nº.
Signal transduction mechanism								
GR487536	1	56%	261	Rhodopsin receptor 1	5.0e-45	174	Aedes_aegypti.AaegL1.50.pep.all.fa	AAEL006498-PA
Biomolecules degradation								
GR487853	1	49%	102	Chymotrypsin 1	9.0e-14	60	anopheles_aquasalis_ptna.fasta	AAD17491
Replication, translation and transcription								
GR487475	1	48%	258	Ribosomal protein S2	1.0e-124	450	aaegypti.CONTIGS-Liverpool.AaegL1.fa	AAGE02025428.1
GR487585	1	48%	258	Ribosomal protein S2	1.0e-124	450	aaegypti.CONTIGS-Liverpool.AaegL1.fa	AAGE02025428.1
GR487592	1	47%	645	18S small subunit ribosomal RNA	1.0e-141	491	anopheles_darlingi_nucleotideo.fasta	AF417770
GR487676	1	47%	663	18S small subunit ribosomal RNA	1.0e-148	515	anopheles_darlingi_nucleotideo.fasta	AF417770
GR487656	1	48%	273	18S small subunit ribosomal RNA	1.0e-119	419	anopheles_darlingi_nucleotideo.fasta	AF417770
GR487750	1	49%	516	18S small subunit ribosomal RNA	1.0e-112	394	anopheles_darlingi_nucleotideo.fasta	AF417770
Defense and detoxification								
GR487564	1	61%	150	Bacteria responsive protein 2 / imaginal disc growth factor	5.0e-22	98	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP008060-PA
Energy metabolism								
GR487489	1	55%	636	Diacylglycerol acyltransferase	1.0e-120	405	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP005949-PB
Embryogenesis								
GR487801	2	55%	381	Vitellogenin	1.0e-44	181	uniref90.fasta	UniRef90_Q49MF2
Transport and secretion								
GR487840	1	53%	228	Thiamine transporter 1 / folate transporter	2.0e-29	122	cpipiens.PEPTIDES-CpipJ1.1.fa	CPIJ007516-PA
Unknown protein								
GR487867	2	53%	348	Unknown protein				
GR487535	1	41%	768	Unknown protein				
GR487529	1	49%	810	Unknown protein of C2H2 and C2HC zinc fingers family				
GR487904	1	41%	159	Unknown protein				
GR487471	6	45%	291	Unknown protein				
GR487568	1	44%	273	Unknown protein with coiled-coil domain				
GR487720	1	44%	273	Unknown protein with coiled-coil domain				
GR487706	1	44%	270	Unknown protein with coiled-coil domain				
GR487511	1	46%	294	Unknown protein with coiled-coil domain				
GR487588	1	47%	294	Unknown protein with coiled-coil domain				

GR487770	1	44%	273	Unknown protein with coiled-coil domain				
GR487755	1	46%	294	Unknown protein with coiled-coil domain				
GR487722	1	46%	294	Unknown protein with coiled-coil domain				
GR487687	1	46%	288	Unknown protein with coiled-coil domain				
GR487476	1	45%	291	Unknown protein with coiled-coil domain				
GR487640	1	46%	291	Unknown protein with coiled-coil domain				
GR487595	1	44%	273	Unknown protein with coiled-coil domain				
GR487714	1	46%	270	Unknown protein with coiled-coil domain				
GR487566	1	46%	291	Unknown protein with coiled-coil domain				
GR487602	1	44%	273	Unknown protein with coiled-coil domain				
GR487649	1	45%	273	Unknown protein with coiled-coil domain				
GR487668	1	42%	252	Unknown protein with coiled-coil domain				
GR487683	1	46%	288	Unknown protein with coiled-coil domain				
GR487516	1	45%	294	Unknown protein with coiled-coil domain				
GR487699	1	46%	291	Unknown protein with coiled-coil domain				
GR487820	1	54%	147	Unknown protein				
GR487821	1	44%	270	Unknown protein with coiled-coil domain				
GR487816	1	43%	240	Unknown protein				
GR487825	1	46%	294	Unknown protein with coiled-coil domain				
GR487828	1	44%	225	Unknown protein				
GR487817	1	47%	291	Unknown protein with coiled-coil domain				
GR487837	1	44%	267	Unknown protein with coiled-coil domain				
GR487754	1	50%	219	Unknown protein				
GR487791	1	51%	306	Unknown protein				
GR487789	1	51%	303	Unknown protein				
GR487696	145	52%	498	Unknown protein				

GR487591	1	42%	177	Unknown protein				
GR487805	1	42%	285	Unknown protein				
Unknown conserved protein								
GR487847	1	49%	180	Unknown conserved protein with coiled-coil domain	2.0e-07	49	Anopheles_gambiae.AgamP3.50.pep.all.fa	AGAP003939-PA
GR487846	1	41%	141	Unknown conserved protein	2.0e-06	43	cpipiens.EST-CLIPPED.mar08.fa	EV343992.1
GR487908	1	46%	247	Unknown conserved protein	2.0e-09	61	agambiae.EST-CLIPPED.mar08.fa	BX035406.1
GR487555	1	54%	147	Unknown conserved protein	4.0e-06	29	aaegypti.EST-CLIPPED.mar08.fa	DV322465.1
GR487819	1	50%	72	Unknown conserved protein	1.0e-10	51	aaegypti.EST-CLIPPED.mar08.fa	DV321842.1
GR487627	2	52%	84	Unknown conserved protein	7.0e-06	46	cpipiens.EST-CLIPPED.mar08.fa	EV304184.1
GR487594	1	35%	246	Unknown conserved protein	6.0e-06	34	A.stephensi_EST.fasta	EX222236
Bacterial protein								
GR487921	1	57%	228	TonB-dependent vitamin B12 receptor	0.0e+00	76	PS00430	TONB_DEPENDENT_REC_1
GR487533	6	42%	177	Bacterial UDP-N-acetylglucosamine 1-carboxyvinyltransferase	3.0e-14	77	uniprot_sprot.fasta	Q8A681
GR487618	1	39%	174	Bacterial protein	2.0e-13	74	uniprot_sprot.fasta	Q8A681
GR487658	1	43%	192	Bacterial protein	1.0e-07	60	Rrna.fasta	AF478100
GR487701	1	43%	99	Bacterial protein	5.0e-11	70	uniref90.fasta	UniRef90_B4WEV8
GR487600	1	64%	198	Bacterial protein	1.0e-47	192	Rrna.fasta	DQ306696
GR487733	1	57%	87	Bacterial protein	6.0e-41	137	Rrna.fasta	DQ306696

ANEXO 7

Tabela Suplementar 5 do capítulo 1

(Iniciadores utilizados no RTPCR)

Primer name	Primer code	Sequence (5' - 3')	Amplicon length
Actin	ACTFwd	GATCTGGCATCACACCTTCTACAAT	104bp
	ACTRev	TCTTCTCACGGTTGGCCTTCGGGTT	
BRP	BRPFwd	CAACAAGGCAGGTTACGTGAA	141bp
	BRPRev	ACATCCGATTACAGCCGATACTT	
Carboxypeptidase	CPFwd	GTAACCCCTGCTCGGACACTT	82bp
	CPRev	GTCTTCACGAACGCAGCCAACGATT	
Cecropin	CECFwd	TGAACTTCACGAAACTCTTCATTGT	127bp
	CECRev	AACACATTCCGACCCAGCTTTTCAA	
Chymotrypsin	SERPROTFwd	CCCGATGAACTGATGAAAATTGATA	95bp
	SERPROTRev	GCACAGATTTCCTTGCTCTCGTCA	
Fibrinogen	FIBFwd	TGGTTGGGTTGTCATTCAGCAT	118bp
	FIBRev	ACGATCAAGACCAAGCCAGAAT	
Serpin	SRPNFwd	TCGTGTCACCTGCCTAAAGGATAAT	115bp
	SRPNRev	GTCCGCAAAATCCATCGTCGTATCA	