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Abordagens distintas à Interação entre *Anopheles* spp. e *Plasmodium* spp.: Estabelecendo um modelo murino de laboratório, estudando o escape dos esporozoítos e a microbiota

por

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Resumo

Nas Américas, a região Amazônica é a área com o maior risco de transmissão de malária. No entanto, a falta de modelos experimentais adequados de anofelinos das Américas tem limitado o progresso para compreender a biologia de transmissão de malária nesta região. Entretanto, com colonização de vetores naturais da Amazônia, como o *A. aquasalis*, abriu pela primeira vez a possibilidade de estudar sua interação com o *Plasmodium* sp. No presente trabalho, questões básicas dessa interação foram investigadas tais como: 1) a susceptibilidade do *A. aquasalis* à diferentes espécies de *Plasmodium* sp. 2) o processo de escape dos esporozoítos dentro do vetor 3) a dinâmica microbiana do *A. aquasalis* em diferentes estágios de vida e fonte alimentar. Como resultados, (1) O *A. aquasalis* se mostrou refratário às infecções com *P. falciparum* (NF54 e 7G8), *P. berghei* e *P. yoelii* 17xnl, e altamente susceptível ao *P. yoelii* N67 tornando a utilização do *P. yoelii* N67/*A. aquasalis* um bom modelo de estudo de laboratório. (2) Nesse trabalho foi descrita a microanatomia do escape dos esporozoítos de oocistos de quatro espécies de *Plasmodium*: *P. gallinaceum* e *P. berghei*, e as duas espécies principais que causam a malária em humanos, *P. vivax* e *P. falciparum*. Verificou-se que os esporozoítos possuem mecanismos específicos de escape do oocisto. O *P. berghei e o P. gallinaceum* têm um mecanismo comum, na qual a parede do oocisto se decompõe antes dos esporozoítos escaparem. Em contraste, os esporozoítos de *P. vivax* e *P. falciparum* possuem um mecanismo de escape ativo, através de propulsão polarizada. (3) A diversidade microbiana descrita demonstrou que as pupas possuem uma riqueza maior de fOTUs comparado aos outros grupos estudados. Foi visto que, a abundância das fOTUs de *Enterobacteriaceae* aumenta nos mosquitos pós infectados com *P. vivax* comparado aos outros grupos, sugerindo uma possível relação com o parasito. Acreditamos que com estes conhecimentos, poderemos abrir novas fronteiras para estudos de controle da malária focado no contexto epidemiológico brasileiro, como, por exemplo, produção de insetos geneticamente modificados potencialmente resistentes ao patógeno, ou mesmo indicar candidatos para estudo de vacinas de bloqueio de transmissão.

Palavras-chave: *Plasmodium*, *Anopheles*, malária, microscopia, modelo de laboratório, infecção

Abstract

In the Americas, the Amazon region is the área with highest risk of malária transmission. However, the lack of adequate experimental models of malaria vectors in the Americas to infect has limited the progress in understanding the biology of transmission in this region. Nevertheless, the colonization of natural vectors of the Amazon as the *A. aquasalis* has been achieved, and for the first opened the possibility of studying their interaction with the *Plasmodium* sp. In this present study, basic questions were investigated such as: 1) the susceptibility of *A. aquasalis* the different species of *Plasmodium* sp. 2) how does the escape process of sporozoites in the mosquito vector occurs using different species of *Plasmodium* and vectors 3) how does the microbial dynamics of *A. aquasalis* varies depending on the life stage and food source. As results: (1)The *A. aquasalis* proved refractory to infection with *P. falciparum* (NF54 and 7G8), *P. berghei and P. yoelii* 17xnl, and high susceptibility to *P. yoelii N67* . This shows that the *P. yoelii* N67 and *A. aquasalis* is a functional study model of malaria transmission outside the endemic areas. (2)We also investigated the microanatomy of escape of sporozoites from oocysts from four *Plasmodium* species: *P. berghei* and *P. gallinaceum*, and the two major species that cause malaria in humans, *P. vivax* and *P. falciparum*. It was found that the sporozoites have specific mechanisms for the oocyst escape. The *P. berghei* and *P. gallinaceum* have a common mechanism in which the oocyst wall decomposes before sporozoites escape. In contrast, the sporozoites of *P. vivax* and *P. falciparum* show an active escape mechanism from the oocyst through polarized propulsion. (3)We also saw that microbial diversity of pupae presented a greater wealth of fOTUs compared to the other groups. However, it was seen that the *Enterobacteriaceae* family increases in mosquitoes post infected with *P. vivax*. We believe that this knowledge can open new frontiers for malaria control studies focused on the epidemiological context, such as, production of genetically modified insect that are potentially resistant to the pathogen, or to indicate candidates for the study of transmission blocking vaccine.

Key words: *Plasmodium*, *Anopheles*, malária, microscopy, lab-model, infection

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1 Introdução

1.1 Aspectos gerais dos anofelinos

Anofelinos são mosquitos pertencentes a ordem Diptera, infraordem *Culicomorpha*, família Culicidae, gênero *Anopheles*. Este gênero compreende aproximadamente 400 espécies, e somente 40 delas transmitem malária no mundo, sendo 10 espécies de importância epidemiológica no Brasil .

No contexto mundial é possível encontrar o *Anopheles maculipennis* (Europa), *A. culicifacies* (Índia), *A. minimus* (Índia até a China e Filipinas), *A. gambiae* e *A. funestus* na África; no neotrópico, *A. albimanus* (México) e *A. nuñeztovari* (Venezuela e Colômbia) (Rebelo et al, 1997) como sendo vetores de maior importância epidemiológica.

No Brasil encontra-se A. darlingi Root, 1926; *A. aquasalis* Curry, 1932; espécies do complexo *A. albitarsis*; *A. marajoara* Galvão & Damasceno, 1942; *A. janconnae* Wilkerson & Sallum, 2009; *A. deaneorum*; espécies do complexo *A. oswaldoi*; A. cruzii, Dyar & Knab, 1908; *A. bellator Dyar & Knab, 1906)* e *A. homunculus* Komp, 1937. O *A. darlingi* é o principal vetor na América do Sul e sua localização está associada com a transmissão de malária na região amazônica do Brasil, Bolívia, Colômbia, Guiana Francesa, Guiana, Peru, Suriname e Venezuela (Zimmerman, 1992; Hiwat et al, 2010) (Figura 1).

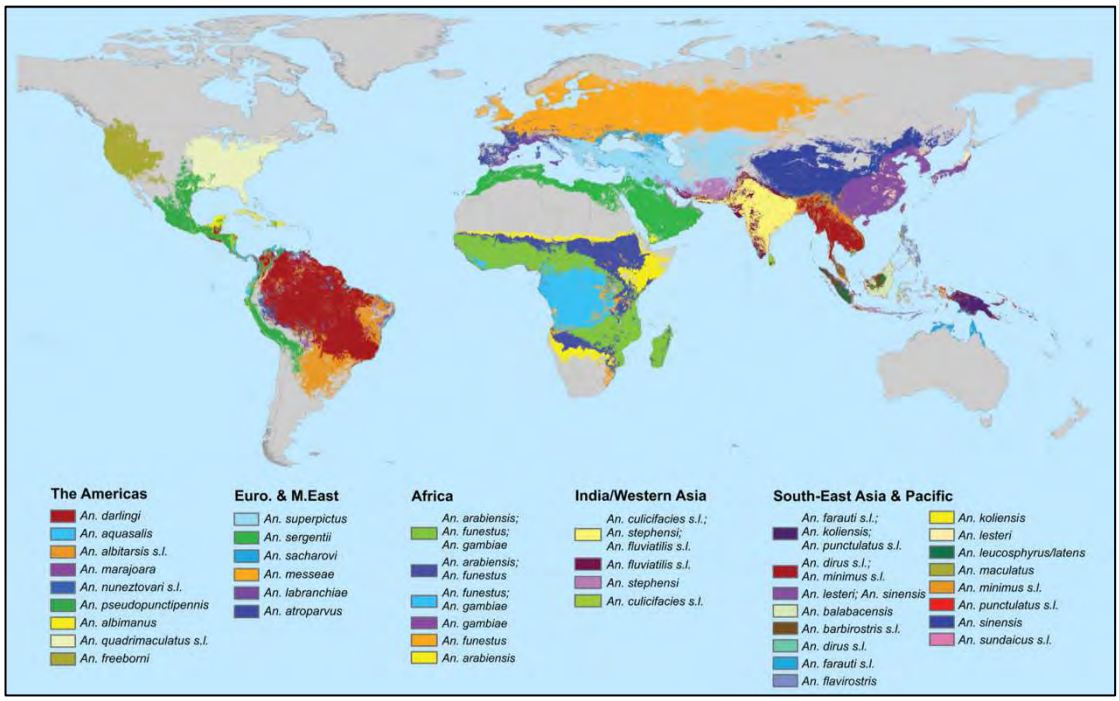


Figura 1: Distribuição de *Anofelinos* no mundo. Fonte: Malária atlas Project disponível em http://www.map.ox.ac.uk.

As principais características dos anofelinos são: palpos de comprimento semelhante ao da probóscide, margem posterior do escutelo arredondada e primeiro tergito abdominal sem escamas. Em repouso, estes insetos apresentam uma posição oblíqua em relação ao substrato. Por essa razão, no Brasil são conhecidos por nomes populares como: mosquito-prego, carapanã ou muriçoca (Consoli & Lourenco-de-Oliveira, 1994).

Como todo mosquito, os anofelinos adultos são alados, possuem pernas e antenas longas e as fêmeas são hematófagas, e nas fases imaturas são encontrados no ambiente aquático. Seu ciclo biológico compreende as fases de ovo, larvas, pupa e adulto. Na fase de pupa, ocorre a metamorfose para o mosquito adulto, o qual por sua vez possui aparelho bucal picador-sugador, tem asas, pernas e genitálias completamente formadas (Consoli & Lourenco-de-Oliveira, 1994; Forattini, 2002). Seu corpo é dividido em cabeça, tórax e abdome. Na cabeça encontram-se os principais órgãos dos sentidos, como os olhos, as antenas e os palpos. No tórax estão os apêndices especializados para locomoção, como as pernas e as asas. O abdômen inclui a maior parte dos órgãos internos do aparelho reprodutor, digestivo e excretor (Consoli & Lourenco-de-Oliveira, 1994) (Figura 2).

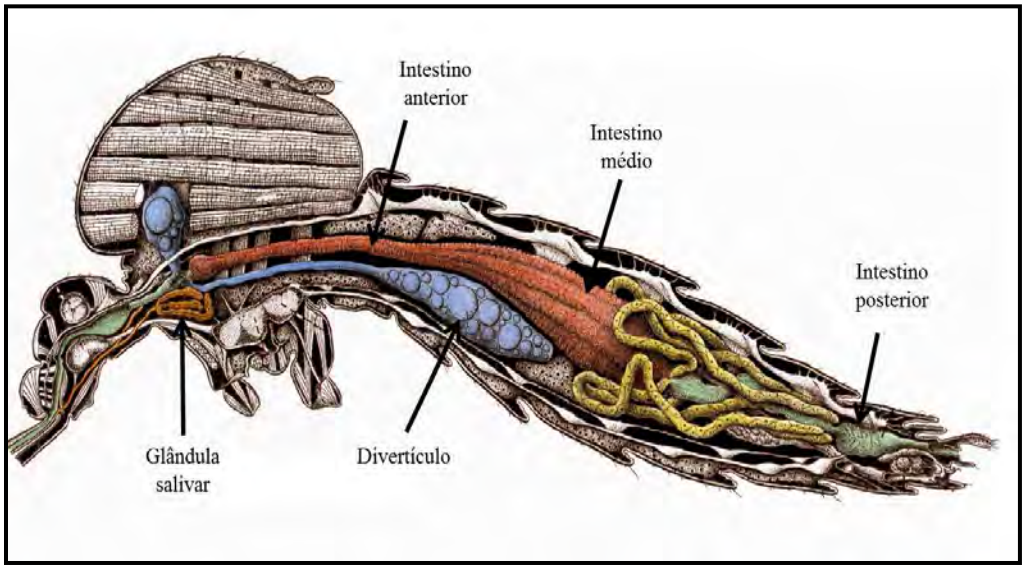


Figura 2: Esquema dos órgãos do mosquito. Modificado de Jobling, 1987

A grande maioria dos mosquitos depende da ingestão de carboidratos, usualmente provenientes de seivas, flores e frutos como sua principal fonte de alimento. Somente as fêmeas são hematófagas e o repasto sanguíneo está relacionado ao desenvolvimento dos ovos (Consoli & Lourenco-de-Oliveira, 1994; Attardo et al, 2005). No momento do repasto sanguíneo, o mosquito injeta sua saliva no vertebrado, e nessa saliva pode ser encontrado parasitos caso esse mosquito esteja infectado.

1.2 Aspectos gerais do *Anopheles aquasalis*

O A. aquasalis é um importante vetor de malária do Brasil, no entanto, é considerado uma espécie oportunista uma vez que é um pobre competidor e possui poucas defesas contra predadores (Sinka et al, 2010). Foi estabelecido como vetor de malária ao longo da costa leste da América do Sul e em algumas ilhas do Caribe.

A sua capacidade de ovipor e crescer em água salina o torna uma espécie dominante na costa da América Central e do Sul, pois essa vantagem de crescer em água salina o permite explorar nichos que outras espécies de anofelinos não são capazes. Essa espécie também pode ser encontrada mais no interior do Brasil em locais onde existe solos ricos em cloreto, como é o caso de algumas áreas do sertão nordestino, ou quando o terreno sofre invasão das marés, como ocorre em Belém-PA no Brasil (Deane et al, 1948).

Na maioria desses territórios, o *A. aquasalis* é exofílico, zoofílico e crepuscular, mas em áreas secas do nordeste ele é frequentemente endofílico e pica hospedeiros humanos. As fêmeas são oportunistas, se alimentando no intra e peridomicilio tanto em animais como em humanos. Eles começam a picar durante o pôr do sol, alcançando atividade máxima no início da noite e diminuindo ao longo dela (Flores-Mendoza et al, 1996). Normalmente antes e depois da alimentação sanguínea o mosquito permanece no peridomicílio (Consoli & Lourenco-de-Oliveira, 1994; Giglioli, 1963; Sinka et al, 2012).

1.3 Aspectos gerais da malária

O termo malária (do italiano Mala-aria), significa “mau ar, porque acreditava-se que a causa da doença eram os vapores vindos dos pântanos. A doença também é conhecida como paludismo, impaludismo, sezão, tremedeira e febre palustre (Garnham, 1966; Neves, 2000).

Os sintomas clássicos da malária são febre intensa, calafrios e sudorese. Os calafrios são frequentemente acompanhados por dor de cabeça, náuseas e fadiga. Nos casos mais graves, os pacientes podem apresentar complicações respiratórias e neurológicas, bem como intensa anemia e insuficiência renal (Neves, 2000; Augustine et al, 2009).

Em relação às doenças infecciosas transmitidas por insetos, a malária é responsável por cerca de 350 a 500 milhões de casos clínicos anuais, permanecendo como um dos maiores problemas de saúde mundial, afetando a saúde e a economia, principalmente nas comunidades mais pobres do globo. Cerca de metade da população mundial está sob risco de contrair a doença, sobretudo crianças menores de cinco anos e mulheres grávidas (World Health Organization., 2015).

Apesar do sucesso da erradicação da doença em diversos países, em 2013, três quartos das mortes causadas por malária ocorreram em crianças abaixo de 5 anos de vida. Estima-se que 15 milhões de mulheres grávidas não recebem uma única dose das drogas preventivas recomendadas, e cerca de 278 milhões de pessoas na África ainda vivem em casas sem mosquiteiros impregnados com inseticidas (World Health Organization., 2015).

De acordo com o relatório de 2015 da Organização Mundial de Saúde, em 2013, a malária esteve presente em 108 países e estima-se que 584.000 pessoas morreram devido a doença (Figura 3) (World Health Organization., 2015), no entanto, existem estudos que demonstram que esses números são subestimados (Murray et al, 2012).

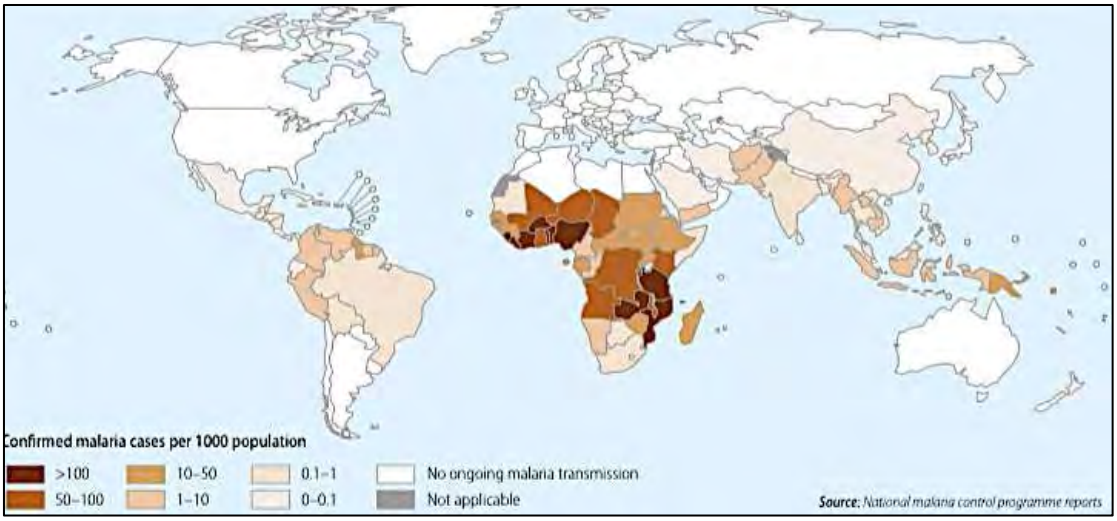


Figura 3: Países com transmissão contínua de malária em 2013. Fonte: (World Health Organization., 2015)

No Brasil, a malária é um grave problema de saúde pública, com predominância de casos na área da Amazônia Legal. Segundo o Ministério da Saúde, em 2011, aproximadamente 99,7% dos casos de malária se concentraram nos sete estados da região amazônica: Acre, Amapá, Amazonas, Mato Grosso, Pará, Rondônia e Roraima (Figura 4) (MS, 2015).

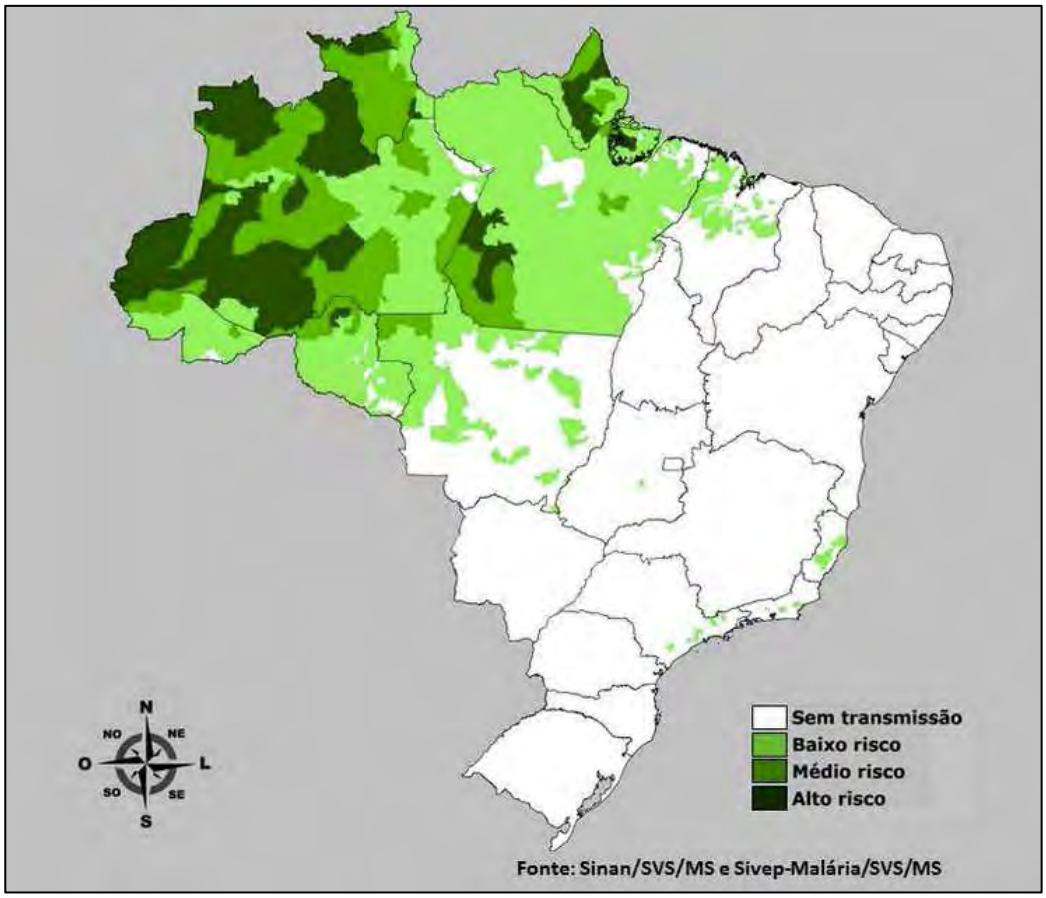


Figura 4: Mapa de risco de transmissão de malária por município de infecção no Brasil em 2014. Fonte: MS (2015).

Os agentes etiológicos da malária são protozoários pertencentes ao filo Apicomplexa, família Plasmodiidae e gênero *Plasmodium*. Em humanos, a doença pode ser causada por cinco espécies: *Plasmodium falciparum, P. vivax*, *P. ovale*, *P. malariae*, e *P. knowlesi*. Os protozoários do gênero *Plasmodium* são transmitidos ao homem pela picada de fêmeas de mosquitos do gênero *Anopheles* (Consoli & Lourenco-de-Oliveira, 1994).

Dentre as espécies de *Plasmodium* que causam malária em humanos, o *P. falciparum* é o responsável pela forma mais grave da doença, devido a sua capacidade de aderir ao epitélio dos capilares, podendo ocasionar falha renal aguda, malária cerebral e edema pulmonar. 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 et al, 2009; Oliveira-Ferreira et al, 2010). O *Plasmodium knowlesi*, parasito que normalmente acomete macacos, ocasionalmente

pode infectar e causar malária em seres humanos, com manifestações clínicas variando entre moderadas a graves (Singh et al, 2004).

1.4 O ciclo da malária

O *Plasmodium* possui o ciclo de vida em duas fases: fase sexual exógena (esporogônica), na qual ocorre a multiplicação dos parasitos no mosquito e uma fase assexuada endógena (esquizogônica), onde ocorre a multiplicação no hospedeiro vertebrado em células parenquimatosas do fígado (esquizogonia hepática) ou nos eritrócitos (esquizogonia eritrocitária) (Sinnis & Coppi, 2007) (Figura 5).

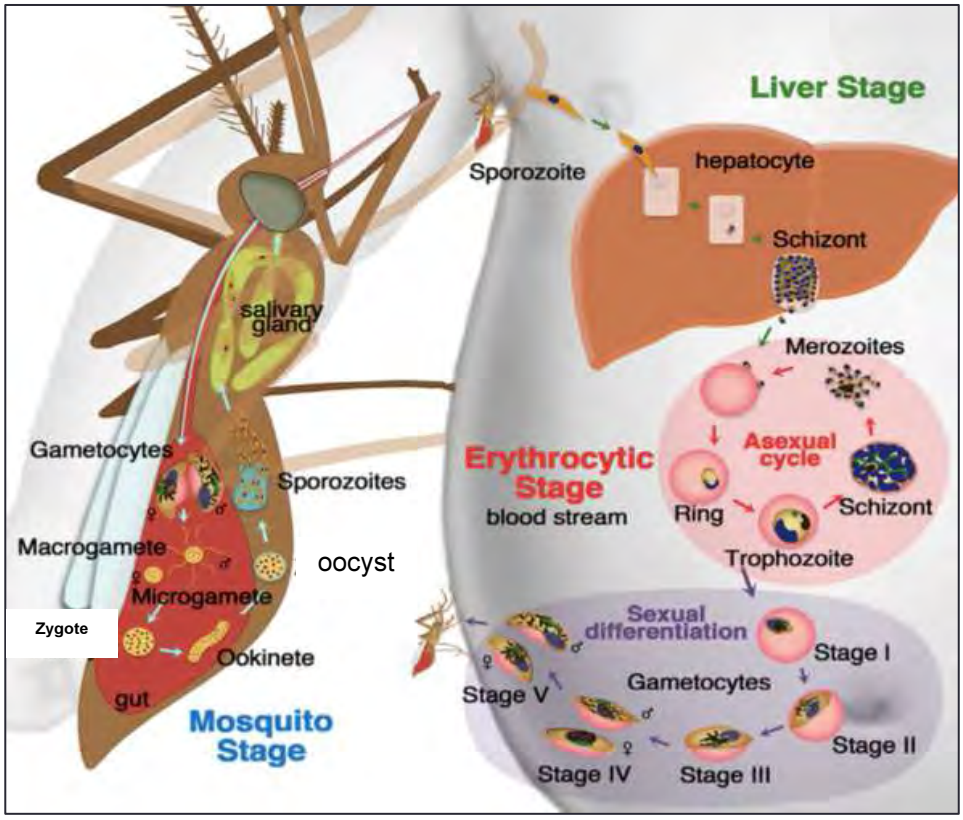


Figura 5: Ciclo de vida do *Plasmodium* spp. Fonte: (Biamonte et al, 2013).

Essa dinâmica do ciclo demonstra que sua grande complexidade está relacionada à habilidade do parasito em alterar suas características celulares e moleculares e em desenvolver-se nos ambientes intra- e extracelular, tanto do hospedeiro vertebrado quanto do mosquito vetor.

Após picarem um hospedeiro infectado, fêmeas de anofelinos ingerem juntamente com o sangue as formas gametocíticas do parasito. No lúmen do intestino se iniciará a fase sexual do ciclo com a diferenciação em microgametócitos

(masculino) e macrogametócitos (femininos) e, após a fecundação, formarão um zigoto diplóide (Sinden, 1999). Esse zigoto diferencia-se em oocineto, uma forma móvel do parasito, e esse desenvolvimento pode durar entre 16 a 24 horas variando de acordo com as diferentes espécies de *Plasmodium* (Ghosh et al, 2000; Dinglasan et al, 2009). O oocineto formado atravessa a matriz peritrófica e atinge a parede do epitélio intestinal onde se transforma em oocisto. Essa travessia estimula o sistema imune do inseto que se manifesta pelo aumento da expressão das espécies reativas de oxigênio (ROS) (Ghosh et al, 2000; Dinglasan et al, 2009).

Esse processo de invasão do epitélio pode ser distinto dependendo do modelo utilizado (Shahabuddin & Pimenta, 1998; Kumar et al, 2004). Estudos demonstraram que em *Anopheles stephensi* infectado com *P. berghei* esse processo de invasão induz a expressão de oxido nítrico sintase (NOS) mediado pela peroxidase, já com o modelo de *A. aegypti* com *Plasmodium gallinaceum* a invasão do epitélio intestinal pelo oocineto ocorre de forma silenciosa sem o aumento da expressão de NOS (Gupta et al, 2005). Após atravessar o epitélio, o oocineto se aloja entre a lamina basal e o epitélio intestinal do mosquito e se diferencia em oocisto, e entre 12 a 15 dias após a infecção ocorre o rompimento do oocisto maduro e a liberação de esporozoítos na hemolinfa (Hillyer et al, 2007). Esses esporozoítos liberados migram e invadem a glândula salivar (Pimenta et al, 1994) tornando esse inseto apto a infectar um novo hospedeiro vertebrado na próxima alimentação sanguínea.

No momento do repasto sanguíneo, o mosquito inocula na pele juntamente com a saliva formas esporozoítas que alcançam a corrente sanguínea do hospedeiro vertebrado, migram para células do fígado e atingem os hepatócitos, iniciando o ciclo exoeritrocítico (Meis & Verhave, 1988; Amino et al, 2006).

Uma vez alcançado o fígado, os esporozoítos migram por várias células hepáticas até se estabelecer no hepatócito final, formando em volta desse parasito um vacúolo parasitóforo (Mota et al, 2001). Com a ocorrência de diferenciação e divisões celulares origina-se as formas esquizontes que ao se romperem liberam milhares de merozoítos (Sturm et al, 2006). Esses merozoitos são liberados em vesículas denominadas merossomos que são capazes de transportar centenas de merozoítos para a corrente sanguínea e ao mesmo tempo protegê-los do ataque das células de Kupfer do fígado (Sturm et al, 2006). Quando esses parasitos alcançam a corrente sanguínea inicia-se o ciclo eritrocítico da doença. Estudos mostram que em

P. vivax e *P. ovale* alguns esporozoítos podem permanecer latentes no hepatócito por semanas ou mesmo anos e eles seriam responsáveis pelos episódios de recaída tardia da doença (Krotoski et al, 1980; Krotoski et al, 1982). Essas formas latentes, denominadas hipnozoítos, são resistentes a maioria dos fármacos utilizados (Wells et al, 2010) e representam um fator agravante no controle da doença (Mueller et al, 2009; White et al, 2014).

Estudos recentes têm mostrado que em mamíferos roedores infectados com *P. berghei* e *P. yoelii* aproximadamente 10% dos parasitos permanecem ativos na pele por até 42 horas após a picada e se associam ao folículo piloso afim de evitar o reconhecimento e ação pelo sistema imune do vertebrado. Esses parasitos remanescentes ainda são capazes de completar o ciclo exoeritrocítico na pele, e não somente nos hepatócitos, com a formação de merossomos (Gueirard et al, 2010).

Ao alcançar a corrente sanguínea os merozoítos interagem com as proteínas presentes na superfície dos eritrócitos e por adesão o parasito invade ativamente as hemácias. Após a invasão, os merozoítos se desenvolvem em trofozoítos e estes dão origem a esquizontes por meio de múltiplas divisões nucleares. Novos merozoítos são formados no interior dos esquizontes e são liberados pela ruptura destes, dando início a uma nova etapa de invasões de eritrócitos e completando-se o ciclo assexuado do parasito (Miller et al, 2002). Alguns trofozoítos passam por um desenvolvimento diferencial resultando na formação de células sexuais especializadas, os gametócitos masculino (microgametócito) e o feminino (macrogametócito), que ao serem ingeridos pelos mosquitos seguirão seu desenvolvimento sexuado no vetor (Gaur et al, 2004).

1.5 Malária experimental

Uma etapa crucial para o entendimento do ciclo de vida do *Plasmodium* é desenvolver infecções em mosquitos vetores em laboratório, possibilitando estudos para o entendimento dessa interação. Quando se analisa modelos de infecção, a susceptibilidade dos mosquitos à invasão pelo *Plasmodium* é um fator determinante na eficiência dos vetores na transmissão da malária. Sabe-se que algumas espécies de mosquitos podem se infectar com esse parasito enquanto outras não, e que, dentro da mesma população de mosquitos, alguns são mais susceptíveis do que outros (Molina-Cruz et al, 2012). Essa susceptibilidade depende de fatores genéticos como variação entre espécies e cepas da mesma espécie, fenotípicos tais como:

tamanho do mosquito, digestão sanguínea e nutrição e fatores externos como temperatura, umidade, ação de inseticidas e drogas (Ichimori, 1989).

A capacidade de adaptação de muitas linhagens de *P. falciparum* aptas a produzir gametócitos em laboratório permitiu a infecção de vetores colonizados em laboratório e ampliou a capacidade de entendimento da doença (Trager & Jensen, 1976; Carter & Miller, 1979). Igualmente o *P. berghei*, *P. yoelii* e *P. chabaudi* são parasitos que têm sido adaptados no laboratório e são considerados bons modelos para estudo de malária em mamíferos e também para estudos de interações parasito-hospedeiro. Essas espécies de *Plasmodium* têm sido usadas por muitos anos em laboratório para infectar *A. gambiae, A. funestus, A. quadrimaculatus e A. stephensi*, todos vetores de malária na África e Ásia (Yoeli et al, 1964; Vaughan et al, 1991; Sinden et al, 2002; Alavi et al, 2003; Akaki & Dvorak, 2005; Frischknecht et al, 2006; Hume et al, 2007; Lo & Coetzee, 2013; Xu et al, 2013).

Tanto os modelos de malária murina quanto humana têm sido de grande ajuda na avaliação de intervenções no controle de malária e também para gerar e testar hipóteses sobre a biologia da malária humana e para testes de drogas (Killick-Kendrick, 1974; Jaramillo-Gutierrez et al, 2009; Xu et al, 2013).

1.6 Sistema imune dos mosquitos

Os insetos são expostos a uma grande variedade de agentes infecciosos no ambiente em que vivem. Com o intuito de diminuir o risco de infecção a diferentes patógenos, os insetos, desenvolveram barreiras físicas e eficientes mecanismos de defesa celular e humoral

A primeira linha de defesa contra micro-organismos são as barreiras físicas compostas pelo exoesqueleto, matriz peritrófica do intestino e um revestimento quitinoso da traquéia. O exoesqueleto protege os órgãos do inseto e a hemolinfa da exposição direta aos micro-organismos do ambiente (Soderhall & Cerenius, 1998; Theopold et al, 2002). A matriz peritrófica é uma estrutura quitinosa que facilita a digestão, é a maior barreira física do mosquito que além de proteger o epitélio intestinal do contato direto com o sangue, protege da flora microbiana que pode aumentar em até 16 vezes depois da alimentação sanguínea em alguns insetos hematófagos (Demaio et al, 1996; Pimenta et al, 1997; Shao et al, 2001; Secundino et al, 2005). Curiosamente, para atravessar a matriz peritrófica, o plasmódio desenvolveu um mecanismo específico de secreção de quitinase e outras enzimas

hidrolíticas causando um buraco enzimático na matriz peritrófica (Huber et al, 1991; Shahabuddin et al, 1993; Vinetz et al, 2000; Tsai et al, 2001).

Após quebrar as barreiras físicas o patógeno terá que lidar com uma resposta imune mais direcionada. Sabe-se que o inseto é capaz de construir uma eficiente defesa contra micro-organismos, a imunidade inata. E nos anos de 1930 descobriu-se que essa defesa envolve aspectos humoral e celular direcionada a alvos específicos de uma variedade de micro-organismos e macroparasitos (Hoffmann, 1995; Richman & Kafatos, 1996). Respostas humorais incluem peptídeos antimicrobianos, cascatas de profenoloxidases que regulam a coagulação e melanização na hemolinfa e a produção de espécies reativas de oxigênio e nitrogênio (Hoffmann et al, 1996; Hoffmann & Reichhart, 2002; Gotz, 1986); E as respostas celulares que referem-se a respostas mediadas por hemócitos como fagocitose, encapsulamento e apoptose (Horton & Ratcliffe, 2001; Kumar et al, 2004; Michel & Kafatos, 2005; Molina-Cruz et al, 2008) (Figura 6).

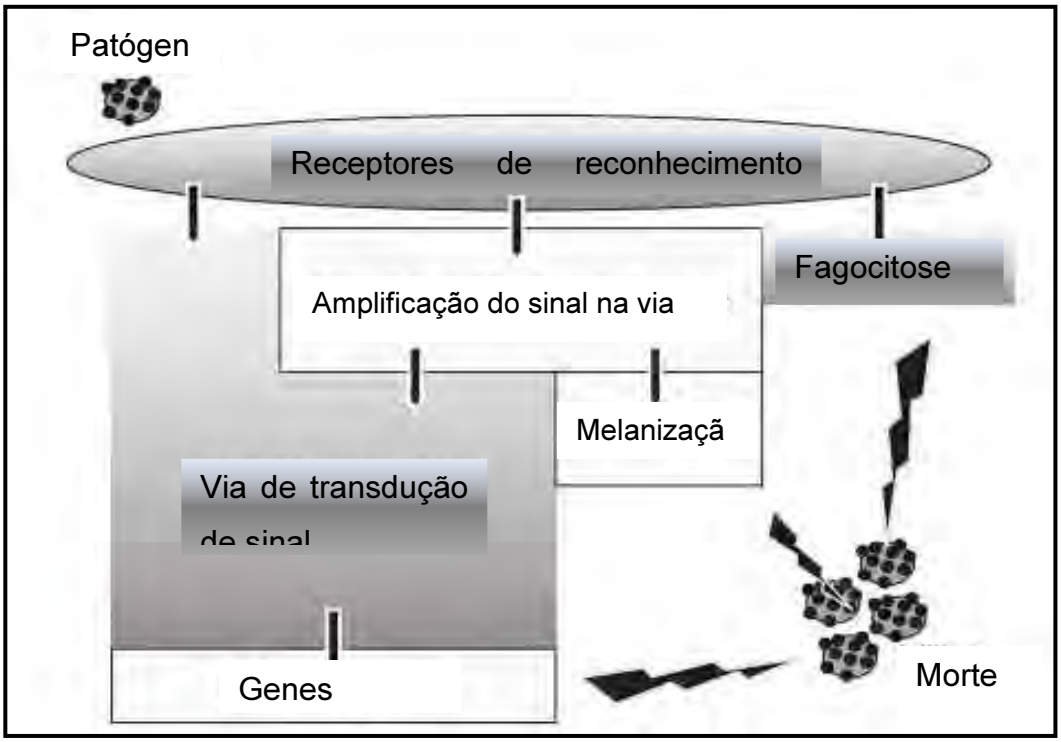


Figura 6: Desenho esquemático da resposta imune em insetos. Receptores de reconhecimento de padrões (PRRs) se ligam especificamente aos padrões moleculares associados a patógenos (PAMP's)(Medzhitov and Janeway Jr., 2002). Esses PRR's podem mediar a morte do patógeno diretamente por fagocitose ou indiretamente desencadeando uma cascata de serina proteases que ativa reações como encapsulamento melanótico ou inicia vias de sinalização intracelulares que regulam genes de peptídeos antimicrobianos e outros genes efetores. Fonte: Adaptado de (Beckage, 2007).

Mosquitos anofelinos estão com o sistema imune intensamente ativado quando os parasitos invadem o tecido epitelial e subsequentemente quando eles migram pelo sistema circulatório. Esses picos de resposta imunológica têm sido associadas com a eliminação do parasito (Dimopoulos et al, 1997; Dimopoulos et al, 1998; Luckhart et al, 1998; Richman et al, 1997; Lowenberger et al, 1999).

A ação do sistema imune em insetos pode ser observado quando mosquitos anofelinos são infectados por parasitos como o *Plasmodium* sp. Após atravessarem pela barreira de escape do tubo digestivo e migrarem pelo sistema circulatório ocorre uma redução no número de parasitos (Korochkina et al, 2006; Hillyer et al, 2007) (Figura 7). Essa diminuição da parasitemia no inseto está associada com os picos de resposta imune. A magnitude dessa perda pode diferir entre infecções com diferentes parasitos e mosquitos (Ghosh et al, 2000; Dimopoulos et al, 2002; Choe et al, 2005).

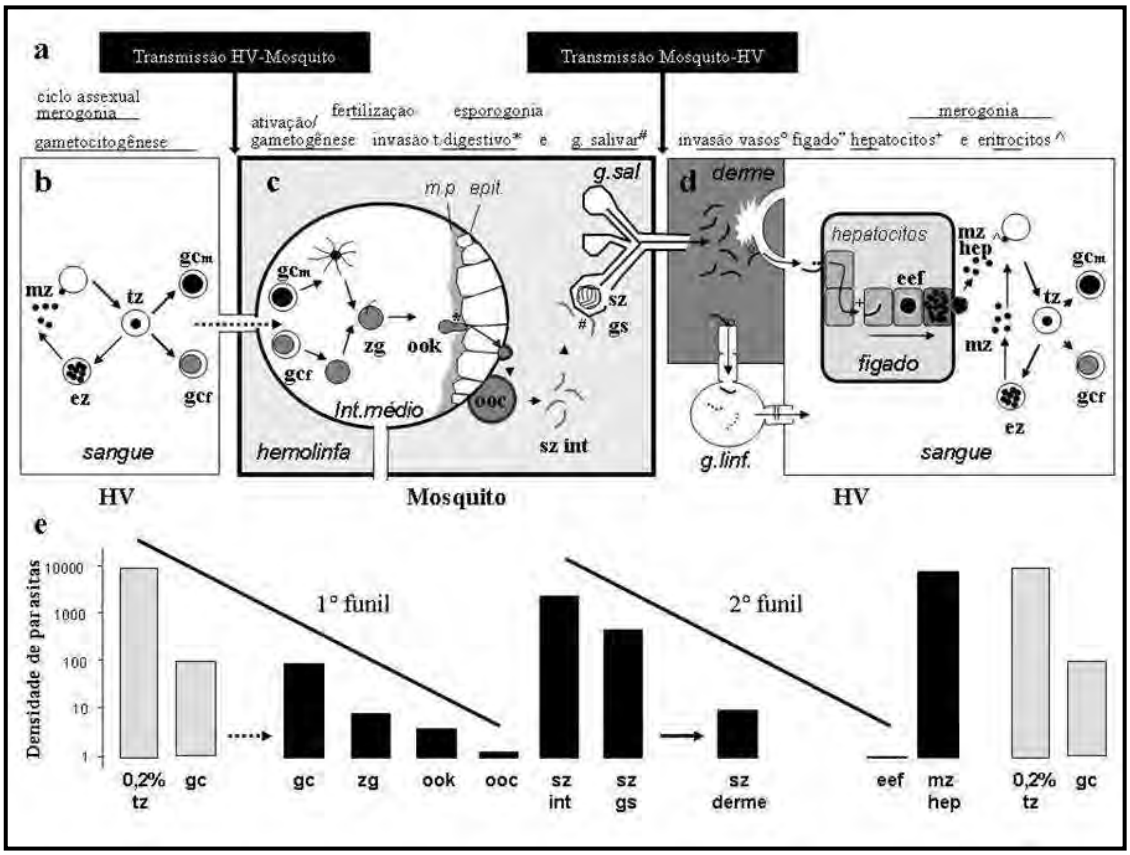


Figura 7: Perda de parasitos ao longo da infecção no inseto e hospedeiro vertebrado. Fonte: (Pimenta et al, 2015) (mz: merozoíto; tz: trofozoíto; ez: esquizonte; gcm: gametócito masculino; gcf: gametócito feminino; zg: zigoto; ook: oocineto; ooc: oocisto; sz int:

esporozoítos no intestino; sz gs: esporozoíto na glândula salivar; mz hep: merozoítos no hepatócito; *: tubo digestivo; #: glândula salivar; ^ : eritrócitos).

1.7 Regulação do sistema imune

As vias de sinalização imune são responsáveis por respostas diretas à patógenos e têm sido as responsáveis por mecanismos de defesa anti-*Plasmodium* em mosquitos. As vias Toll, IMD, JNK e Jak/Stat desempenham papéis fundamentais na regulação dessa defesa contra a infecção pelo *Plasmodium* no inseto, com algum grau de especificidade para as diferentes espécies desse parasito (Christophides et al, 2002; Levashina, 2004; Meister et al, 2004) (Figura 8).

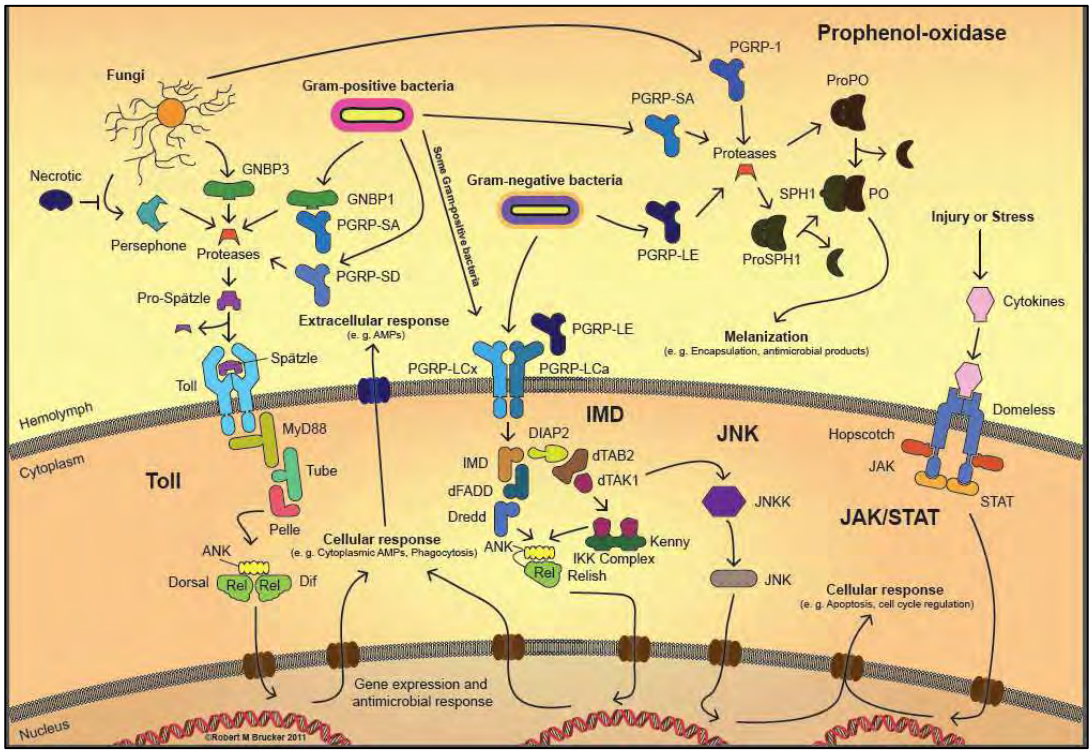


Figura 8: Esquema geral das vias do sistema imune inato em insetos baseado em *D. melanogaster. Disponível em Bordenstein Lab, NSF DEB-1046149*

1.7.1 A via Toll

A via Toll originalmente é ativada pela infecção de bactérias gram-positivas e fungos, no entanto, estudos têm demonstrado que essa via também é ativada nas infecções por *Plasmodium* em mosquitos anofelinos (Frolet et al, 2006; Xi et al, 2008). A resposta de ativação da via Toll frente a uma infecção ocorre via reconhecimento de padrões moleculares associados a patógenos (PAMP's) que

ativam a translocação nuclear do NF-kB os fatores de transcrição Rel1 e Rel2 (via IMD) (Barillas-Mury et al, 1996; Han et al, 1999). Esses fatores de transcrição são regulados negativamente no citoplasma por Cactus e Caspar, respectivamente (Christophides et al, 2002; Frolet et al, 2006). A ativação da via Toll permite que o fator Rel entre no núcleo e acione a transcrição de genes efetores como os peptídeos. Existem quatro principais classes de peptídeos antimicrobianos (AMPs): defensinas, cecropinas, atacinas e gambicinas.

Nas infecções por *Plasmodium*, ocorre a indução de expressão de defensina 1 (Richman & Kafatos, 1996), cecropina 1 (Vizioli et al, 2000) e gambicina 1 (Vizioli et al, 2001) pelo corpo gorduroso e pelos hemócitos do *A. gambiae* e secreção desse peptídeos antimicrobianos na hemolinfa. Usando a técnica de RNA de interferência para silenciar o gene cactus, Frolet e colaboradores (Frolet et al, 2006) demonstraram que a ativação da via Toll diminui significativamente a infecção por *P. berghei*, enquanto ao silenciar Rel1 há aumento nos níveis de infecção no mosquito.

No entanto, a via Toll no controle de infecções pelo *Plasmodium* não é universal como demonstrado por Garver e colaboradores (Garver et al, 2009) que utilizaram múltiplas combinações de *Plasmodium*-mosquito e mostraram que *A. gambiae, A. stephensi,* e *A. albimanus* infectados com *P. berghei* têm a infecção controlada pela ativação da via Toll, enquanto a infecção das mesmas espécies de mosquito pelo *P. falciparum* ocorre independente da ativação dessa via.

1.7.2 Via IMD

É a segunda maior via de sinalização imune, e se assemelha a via de TNF (fator de necrose tumoral) em mamíferos (Aggarwal & Silverman, 2008; Kaneko & Silverman, 2005). A via IMD em mosquitos, quando ativada, controla a translocação do fator de transcrição Rel2, sua regulação negativa é controlada por caspar e sua ativação ocorre de forma semelhante à via Toll. Na ausência de estimulação imune, Rel2 possui duas variantes, uma forma curta (Rel2-S) responsável pela expressão gênica basal, e uma forma longa, “Full- lenght”(Rel2-F) que fica inativa até a estimulação imune ocorrer (Meister et al, 2005; Luna et al, 2006).

A atividade anti-*Plasmodium* da via IMD demonstrou ser mais efetiva contra infecções pelo *P. falciparum,* pois quando se infectou *A. gambiae, A. stephensi* e *A. albimanus* esses insetos apresentaram quase refratariedade completa após o

silenciamento de caspar. Por outro lado, o mesmo não ocorreu quando se silenciaou caspar e infectou as mesmas espécies de mosquitos utilizando o *P. berghei*, sugerindo que o controle da infecção pela via IMD após uma infecção é mosquito-espécie independente mas parasito-espécie dependente (Frolet et al, 2006; Garver et al, 2009; Garver et al, 2012).

Existe inúmeros genes anti-*Plasmodium* regulados pela via IMD, incluindo APL1, TEP1, APL2, FBN9 e LRIM1 além de regular a expressão de muitos AMPs (Cec1, Cec3, Gamb1) (Meister et al, 2005; Mitri et al, 2009; Dong et al, 2006; Riehle et al, 2008; Povelones et al, 2009). Um dos primeiros fatores anti-*Plasmodium* estudados foi a proteína contendo domínio tioéster 1 (TEP1), que se liga e media a morte de oocinetos do *P. berghei* (Blandin et al, 2004). As proteínas com repetições ricas em leucina (LRR), LRIM1 e APL1, juntamente com TEP1 formam um complexo anti-*Plasmodium*. TEP1 foi inicialmente identificado como tendo alta similaridade ao fator C3 do complemento em vertebrados (Baxter et al, 2007; Levashina et al, 2001; Blandin & Levashina, 2004).

TEP1 é secretado na hemolinfa na forma longa “full-lenght”, e sofre clivagem por ação de uma protease ainda desconhecida (Fraiture et al, 2009), dando origem a uma forma madura denominada $TEP1_{cut}$. As proteínas LRR interagem com essa forma $TEP1_{cut}$ estabilizada mantendo-a na circulação, e durante a infecção essas proteínas se deslocam de TEP1 que se comportará como uma opsonina promovendo a fagocitose de bactérias, ou se ligando a superfície do oocineto mediando a lise ou melanização do parasito (Blandin et al, 2004) (Figura 9).

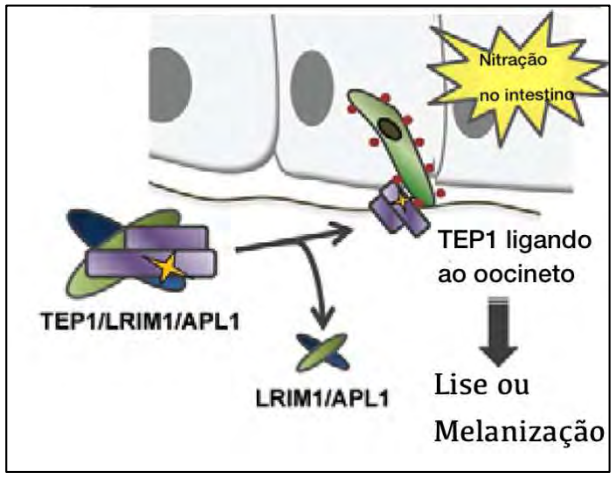


Figura 9: Esquema da ação de TEP1 no oocineto. Modificado de (Smith et al, 2014).

Quando as proteínas LRR são removidas por silenciamento gênico por exemplo, a forma clivada de TEP1 desaparece da circulação e uma massiva deposição de TEP1 é observada nos tecidos do mosquito antes de se ligar à superfície dos parasitos (Fraiture et al, 2009). Portanto, o silenciamento de TEP1, LRIM1 ou APL1 impede a formação do complexo anti-*Plasmodium* que levará ao aumento do número de parasitos no intestino (Fraiture et al, 2009; Povelones et al, 2009).

1.7.3 Via JAK/STAT

A terceira maior via foi descrita em mamíferos como tendo papel importante em respostas antivirais (Dupuis et al, 2003; Karst et al, 2003; Ho et al, 2005), além disso também tem sido associada a resposta por infecção de bactérias patogênicas no intestino de *Drosophila melanogaster* (Buchon et al, 2009; Cronin et al, 2009). É nomeada pela kinase JAK e pelos fatores de transcrição STAT que controlam essa ativação.

A via JAK-STAT media a imunidade do *A. gambiae* a infecções pelo *Plasmodium* através de dois genes STAT-A e STAT-B (Barillas-Mury et al, 1999; Christophides et al, 2002). A transcrição de STAT-A é regulada pelo gene STAT-B. Por sua vez, STAT-A regula os níveis de expressão de SOCS (regulador negativo da via JAK-STAT) e também da óxido nítrico sintase (NOS) em resposta à infecções pelo *Plasmodium* (Gupta et al, 2009).

Ao silenciar o gene STAT-A de *A. gambiae* e infectá-lo com *P. berghei* ou *P. falciparum* observa-se um aumento significativo na quantidade de oocistos maduros (fase tardia: após a invasão do epitélio) para ambos os parasitos. E como esperado, ao silenciar SOCS (regulador negativo da via) ocorre o efeito oposto, diminuindo a infecção pelos mesmos parasitos (Gupta et al, 2009).

Se em *A. gambiae* a ação de STAT-A ocorre na fase tardia de desenvolvimento do oocisto, em *A. aquasalis* infectado com *P. vivax* essa ativação acontece na fase inicial do desenvolvimento, quando o oocineto atravessa o epitélio intestinal e se transforma em oocisto (Bahia et al, 2011). Pois, de acordo com estudo de Bahia e colaboradores (Bahia et al, 2011), o gene STAT de *A. aquasalis* encontra-se super expresso de 24 a 36 horas após a infecção, tempo no qual o oocineto está atravessando o epitélio intestinal (fase inicial). Isso sugere que essa regulação na fase inicial pela via JAK/STAT está relacionada a ativação dos

hemócitos já que essa via também controla a diferenciação dos hemócitos em *D. melanogaster* (Medzhitov & Janeway, Jr., 2002).

1.7.4 Via JNK

A via JNK é uma proteína quinase ativada por mitógenos (MAPK) que é altamente conservada dos mamíferos aos insetos, contudo o papel da JNK na imunidade de insetos ainda é bem limitada (Caffrey et al, 1999; Ragab et al, 2011). As MAPKs são proteínas serina-treonina quinases que regulam uma variedade de processo celulares incluindo apoptose e resposta imune inata (Huang et al, 2009).

Muitos genes ortólogos que mediam a via JNK em vertebrados têm sido identificados em *D. melanogaster* e em *A. gambiae* (Horton et al, 2011; Stronach, 2005). A quinase Jun-N-terminal (JNK) é uma MAP quinase do centro da cascata de sinalização e é ativada por uma MAPK (Hep no caso de *D. melanogaster*) (Boutros et al, 2002; Chen et al, 2002). A quinase JNK fosforilada por sua vez fosforila os fatores de transcrição Jun e Fos dando origem a um dímero Jun/Fos (complexo AP-1) que ativa a transcrição de genes alvos (Kockel et al, 2001) (Figura 10).

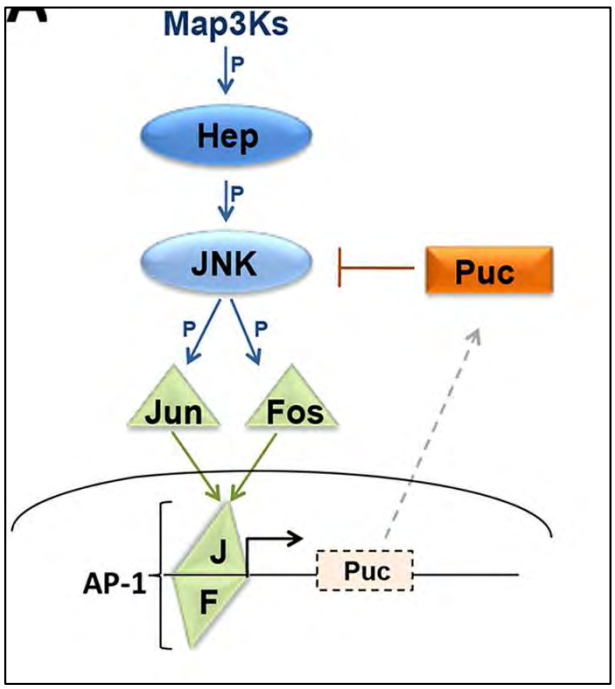


Figura 10: Esquema representando a cascata de sinalização JNK de *A. gambiae* baseado nos estudos funcionais de vertebrados e *D. melanogaster.* Modificado de (Garver et al, 2013).

A ativação dessa via induz o aumento da expressão de genes efetores e do regulador negativo de JNK, *puckered* (Puc) (Glise et al, 1995; Martin-Blanco et al, 1998).

Em *A. gambiae* foi demonstrado que a via JNK regula a expressão de genes que previnem o dano oxidativo como a resistência a oxidação 1 (OXR1), catalase e glutationa peroxidase (Jaramillo-Gutierrez et al, 2010). Silenciando-se esses genes efetores ocorre um aumento dos níveis de espécies reativas de oxigênio (ROS) e a redução da sobrevivência do *Plasmodium*, e silenciando o gene JNK ocorre o efeito oposto culminando no aumento da infecção (Jaramillo-Gutierrez et al, 2010).

A via JNK também é responsável pela regulação basal dos níveis de expressão de TEP1 e FBN9, duas proteínas efetoras produzidas pelos hemócitos que são responsáveis por lisar o oocineto (Blandin et al, 2009; Dong & Dimopoulos, 2009). Estudo de Garver e colaboradores (Garver et al, 2013), propõe que a via JNK faz parte de um sistema de alarme desencadeado pela invasão do parasito, invasão essa que ativa a expressão de NOX5 e HPx2, duas enzimas que catalisam as reações de nitração e marcam os oocinetos para destruição quando eles atravessam as células epiteliais do intestino do mosquito.

1.7.5 A via de RNAi

O RNA de interferência foi descrito primeiramente por Fire e colaboradores (Fire et al, 1998), e consiste de um mecanismo intracelular endógeno dependente de uma dupla fita de RNA (dsRNA) introduzida em um organismo e possui capacidade de silenciar genes pós transcricionalmente (Hannon, 2002; Geley & Muller, 2004), ou seja, o gene é normalmente transcrito dentro da célula, mas não consegue ser traduzido, pois há degradação de seu RNA mensageiro.

O mecanismo de RNAi foi originalmente descoberto em plantas (Matzke et al, 1989; Napoli et al, 1990) porém foi elucidado em estudos com *Caenorhabditis elegans* (Fire et al, 1998; Montgomery et al, 1998) e *D. melanogaster* (Tuschl et al, 1999). É um mecanismo de defesa altamente conservado em eucariotos (Fire, 2007; Terenius et al, 2011).

Existem três classes de pequenos RNAs não codificantes encontrados em insetos e que regulam a expressão gênica: os micro RNAs (miRNA), piwiRNAs, e os pequenos RNAs de interferência (siRNA), porém o mecanismo de ação ocorre de

maneira diferente para cada um deles. Os siRNAs são específicos para um alvo em especial (sequência do gene) enquanto que um único miRNA pode regular a expressão de genes diferentes.

O silenciamento mediado pelo RNAi pode ser dividido em três etapas: (1) os micro ou os pequenos RNAs são gerados a partir de uma grande dupla fita de RNA pela enzima Dicer; (2) esses pequenos RNAs gerados se associam com a proteína Argonauta; tornando-se parte do complexo RISC (3) que irá clivar o mRNA ou reprimir a tradução de genes no organismo (Siomi & Siomi, 2009; Winter et al, 2009) (Figura 11).

A técnica de silenciamento gênico em anofelinos por meio do RNA de interferência vêm sendo utilizada para investigar a função dos genes efetores na resposta imune provocada pelas diferentes espécies de *Plasmodium* (Osta et al, 2004; Jaramillo-Gutierrez et al, 2010; Bahia et al, 2011; Garver et al, 2013; Carissimo et al, 2015).

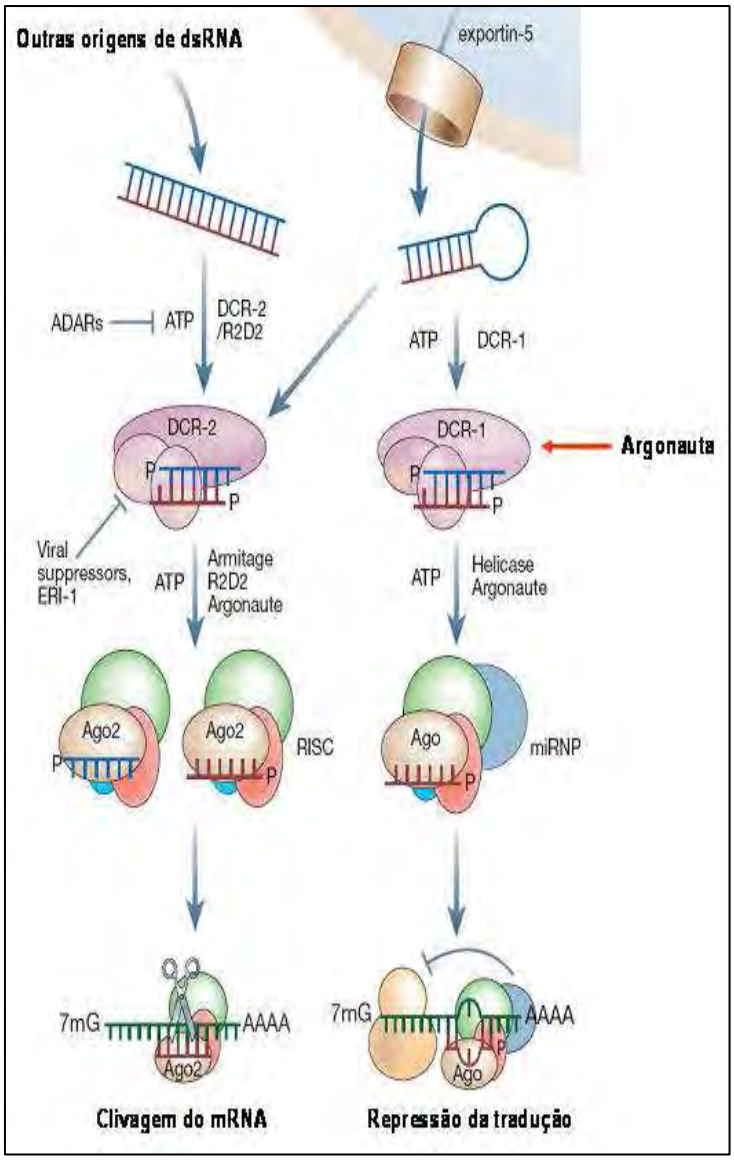


Figura 10: Mecanismo de silenciamento por RNA de interferência. A enzima Dicer corta o RNA de dupla fita formando siRNA ou miRNA. Esse mRNA será degradado pela enzima Argonauta, uma das subunidades de RISC, promovendo a clivagem do mRNA ou inibindo o processo de tradução.

1.8 Microbiota intestinal do mosquito e a interação com o *Plasmodium*

A colonização do intestino por comunidades microbianas comensais ou mutualísticas podem aumentar a resistência do hospedeiro contra a invasão de um parasito. Essas comunidades bacterianas podem estimular a atividade basal da imunidade inata, que consiste na indução de peptídeos antimicrobianos (AMPs) e outros genes imunes específicos que vão combater a infecção por parasitos como o *Plasmodium* sp. (Pumpuni et al, 1996; Ratcliffe & Whitten, 2004; Michel & Kafatos, 2005; Dong & Dimopoulos, 2009). No entanto, muitos pesquisadores ignoraram o impacto causado pela microbiota intestinal na biologia do inseto (Dillon & Dillon, 2004) e somente na última década têm surgido publicações afim de elucidar como a microbiota do intestino interage com o sistema imune do inseto. Sabe-se que os endossimbiontes afetam o processo de infecção no mosquito por uma interação direta com o parasito, que pode ocorrer pela atividade inibitória de enzimas ou toxinas, ou indiretamente pela indução da atividade do sistema imune do hospedeiro (Lowenberger et al, 1999; Dong et al, 2006).

Em anofelinos alguns estudos demonstram que quando infectados pelo *Plasmodium* sp. as bactérias endossimbiontes podem inibir o desenvolvimento esporogônico do parasito (Pumpuni et al, 1993; Pumpuni et al, 1996; Gonzalez-Ceron et al, 2003; Dong & Dimopoulos, 2009; Cirimotich et al, 2011b). Pumpuni e colaboradores, (Pumpuni et al, 1993; Pumpuni et al, 1996), demonstraram que bactérias Gram-negativas tem um papel principal no bloqueio da formação de oocistos seja ele total ou parcial, contudo, a mesma ação não foi observada com bactérias Gram-positivas.

Estudo de Cirimotich e colaboradores (Cirimotich et al, 2011b) demonstraram que a presença de espécies de *Enterobacter* no intestino do *A. arabiensis* originados de uma população da Zâmbia agem diretamente no *P. falciparum* bloqueando seu desenvolvimento, tornando essa população de mosquitos refratária a infecção. Essa refratariedade foi associada à geração de espécies reativas de oxigênio (ROS) que

interferem com o desenvolvimento do parasito e o mata antes da sua invasão no epitélio intestinal (Cirimotich et al, 2011b).

Os estudos de simbiose entre a microbiota e os mosquitos vetores ainda são pouco explorados; porém, a realização de projetos de genoma de insetos e a introdução de novas técnicas moleculares como ensaios de metagenômica estão auxiliando a compreensão da diversidade de micro-organismos que residem nos artrópodes de importância médica. Com isso avançando a compreensão de como os insetos discriminam entre microbiota intestinal não patogênica ou mutualística de um lado das bactérias patogênicas e nocivas do outro (Dillon & Dillon, 2004; Bishop-Lilly et al, 2010; Carpi et al, 2011; Ng et al, 2011a; Ng et al, 2011b; Mokili et al, 2012).

2 Justificativa

Depois de mais de um século de esforços no controle, doenças transmitidas por insetos continuam a ser um sério desafio para a saúde pública mundial. A malária, uma doença transmitida por mosquito, a qual foi considerada capaz de ser extinta há cinco décadas atrás, ainda continua a afetar cerca de 300 milhões de pessoas a cada ano e causar 1,5-2,7 milhões de mortes (World Health Organization, 2015). 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.

Já que a colonização de vetores naturais da Amazônia como o *A. aquasalis* (colonização contínua) tem sido alcançada, e abrindo pela primeira vez a possibilidade de estudar sua interação com o *Plasmodium* spp.

Embora investigações recentes venham contribuindo para o avanço do conhecimento da interação vetor-parasito utilizando modelos como *A. gambiae/ P. falciparum*, *A. gambiae/P. berghei* e *A. gambiae/ P. yoelii*, pouco tem sido os estudos utilizando modelos das Américas. Portanto, pouco se sabe sobre a biologia que envolve vetores e cepas de parasito nativos das Américas. Recentemente, nossos estudos pioneiros (Bahia *et al*.,2010, Bahia *et al*.,2011), definiram vários aspectos da interação do *A. aquasalis* (que é um vetor de malária importante nas regiões litorâneas do Brasil) com *P. vivax*. Também recentemente foi desenvolvido um estudo com *A. albimanus*, um vetor de malária no México e América Central, infectando *P. berghei*, um parasito murino (Herrera-Ortiz et al, 2011). Assim sendo, estudos com vetores americanos são escassos e não existe até o momento conhecimento sobre o processo da interação com o *P. falciparum* e, nem tampouco, com as espécies murinas *P. berghei* e *P. yoelii.* Dados a serem gerados a partir deste tipo de estudo são de grande importância para compreender quão eficiente ou não são os vetores americanos e qual o risco real que eles podem representar para a saúde pública.

Para entender esses mecanismos de interação, questões básicas porém essenciais precisam ser investigadas tais como: 1) de que forma ocorre o processo de invasão e desenvolvimento do parasito; 2) quais as principais vias de regulação na interação vetor-patógeno; 3) qual o papel das bactérias intestinais nos eventos

moleculares que ocorrem durante a alimentação sanguínea e após a infecção com *Plasmodium*. Desta forma, decidimos estudar a interação do *A. aquasalis* com as espécies murinas e humanas causadoras de malária. Acreditamos que com estes conhecimentos, poderemos abrir novas fronteiras para estudos de controle da malária focado no contexto epidemiológico brasileiro, como, por exemplo, produção de insetos geneticamente modificados potencialmente resistentes ao patógeno, ou mesmo indicar candidatos para estudo de vacinas de bloqueio de transmissão.

3 Objetivos

3.1 Objetivo geral

Avaliar diferentes aspectos da Interação entre *Anopheles* spp. e *Plasmodium* spp.: Estabelecer um modelo murino de laboratório, estudar o escape dos esporozoítos e a microbiota do vetor.

3.2 Objetivos específicos

- ✓ Estabelecer a infecção experimental de *A. aquasalis* por *P. falciparum P. yoelli* e *P. berghei.*
- ✓ Avaliar a eficiência da infecção por cepas de *P. falciparum P. yoelli* e *P. berghei* em *A. aquasalis.*
- ✓ Caracterizar o mecanismo de escape dos esporozoítos de quatro espécies de *Plasmodium* em diferentes espécies de vetores.
- ✓ Estudar o papel dos principais genes relevantes das vias de imunidade Toll, JNK e IMD no processo de infecção do *A. aquasalis*.
- ✓ Determinar o papel do gene LRIM1 na resposta imune do *A. aquasalis* durante o processo de infecção pelo *P. falciparum*, *P. berghei* e *P. yoelli.*
- ✓ Predizer a microbiota presente no *A. aquasalis* em diferentes condições alimentares.

4 Material e Métodos

4.1 Manutenção da colônia de *Anopheles aquasalis* e *Anopheles stephensi*

O insetário do Laboratório de Entomologia Médica- CPqRR e do Laboratory of Malaria and Vector Research-NIH são climatizados com uma variação de temperatura de 26-28ºC e umidade relativa do ar em torno de 70-80% e ciclos de 12 horas de claridade e 12 horas de escuridão. As larvas foram mantidas em cubas plásticas contendo água sem cloro e sal marinho na concentração de 0.2%, e foram alimentadas diariamente com ração de peixe (Tetramin®). Após a mudança de fase de larva para pupa, estas foram coletas e transferidas para gaiolas próprias até o surgimento dos adultos que foram utilizados nos experimentos. Os *A. aquasalis* utilizados para infecção receberam solução de ácido úrico 2% a partir de dois dias antes da infecção até o dia de dissecção do intestino com a finalidade de diminuir a taxa de mortalidade causada pelo estresse oxidativo proveniente da alimentação sanguínea (Molina-Cruz et al., 2012).

4.2 Cultivo de parasitos

O cultivo do *P. falciparum* foi desenvolvido no Laboratory of Malaria and Vector Research-NIH. Para o estabelecimento do cultivo de gametócitos, as cepas NF54 e 7G8 de *P. falciparum*, foram separadamente, adicionadas ao meio RPMI. A determinação da parasitemia sexual e assexual foi feita por contagem de células infectadas/campo a partir de esfregaço sanguíneo. Foram adicionados glóbulos vermelhos tipo O^+ somente no primeiro dia do cultivo sexuado e feita troca diária do meio com adição de uma mistura de gases (90% N_2, 5% O_2; 5% CO_2), assegurando que o cultivo se mantivesse a temperatura de 37°C. Gametócitos dos estágios de desenvolvimento I,II e III (gametócitos imaturos) são observados a partir do 8º dia e estágios de desenvolvimento IV e V (gametócitos maduros) observados a partir do 12º dia aptos a infectar o vetor.

4.3 Infecção com *Plasmodium*

As infecções com *P. falciparum* e *P. yoelii* foram realizadas no Laboratory of Malaria and Vector Research-NIH e no Laboratório de Entomologia Médica- CPqRR.

As infecções com *P. vivax* e *P. gallinaceum* foram realizadas no Laboratório de Entomologia Médica- CPqRR.

Para as infecções utilizando *Plasmodium falciparum*, os mosquitos foram artificialmente infectados com estágios V e IV de culturas de gametócitos maduros das cepas NF54 ou 7G8 utilizando alimentador artificial de membrana a 37°C por 30 minutos. O sangue humano utilizado foi obtido do Interstate Blood Bank – USA. Os mosquitos foram mantidos a 26°C por 8 a 10 dias após a infecção, quando os intestinos foram dissecados e corados com mercúrio cromo a 0.1% e os oocistos foram contados por microscopia de luz.

Para as infecções com *P. yoelii* nigeriensis (cepa N67), *P. yoelli* 17xnl e *P. berghei* (Anka GFP), parasitos provenientes de estoques congelados foram administrados intraperitonialmente em camundongo. Quando a parasitemia desse camundongo alcançou 5-10%, o sangue foi retirado por punção cardíaca e transferido a outro camundongo por injeção intraperitoneal, utilizado para as infecções. Foram utilizadas fêmeas Balb/c com 5-6 semanas de vida. A parasitemia foi medida por esfregaços obtidos de sangue da ponta da cauda dos camundongos, corados com Giemsa e feita a contagem de hemácias infectadas/campo em microscópio de luz. Após a infecção os mosquitos foram mantidos a 24°C (para *P. yoelli*) e 21°C (para *P. berghei*) por 8-10 dias, até o momento da dissecção.

Para as infecções com *P. gallinaceum,* aproximadamente 200µl de parasitos congelados foram inoculados via intramuscular em pintos híbridos (*Gallus gallus domesticus*) com três dias de vida. O progresso da infecção foi analisado por esfregaço obtido de sangue a partir do corte da extremidade da unha da ave infectada. A lâmina foi fixada e corada com soluções de Panótico Rápido® (Laboclin). O número de células parasitadas foi contado em microscópio óptico, usando aumento de 1000X para estimar a parasitemia. Quando a parasitemia encontrava-se em ascensão (4-10%) e as aves apresentavam uma porcentagem de gametócitos entre 1-2%, as mesmas foram colocadas sobre as gaiolas para a alimentação de mosquitos.

Para as infecções com *P. vivax*, sangue infectado de pacientes foram selecionados dentre as pessoas que foram atendidas no Hospital da Fundação de Medicina Tropical localizadas em Manaus, Brasil. O diagnóstico foi realizada por esfregaço de sangue e coloração Giemsa. Após o diagnóstico positivo e visualização de gametócitos, os pacientes foram entrevistados e foi perguntado sobre a

possibilidade de serem voluntários doando uma pequena quantidade de sangue para fins de investigação. Após acordo verbal, um termo de consentimento foi assinado pelo paciente. Posteriormente, uma amostra de 20 ml do sangue venoso foi retirado de cada paciente e colocado em tubos heparinizados. O sangue infectado com *P. vivax* foi oferecido aos mosquitos através de alimentação artificial. Os critérios de seleção dos pacientes foram: ser *P. vivax* positivo e possuir cerca de 4-8% de gametócitos circulantes.

4.4 Microscopia Eletrônica de varredura

Para a observação do rompimento dos oocistos, os intestinos dos mosquitos foram dissecados diariamente de 8 a 16 dias após a alimentação infectante. Os intestinos dissecados foram fixados por 2 horas em temperatura ambiente em solução de glutaraldeído 4% em 0.1M de tampão cacodilato, pH 7.2. Os intestinos foram lavados em PBS e pós-fixados em tetróxido de ósmio 1% e ferricianeto de potássio 0.8% por 2 horas em temperatura ambiente e ao abrigo da luz. Após a pós-fixação, os intestinos foram lavados em PBS por 3 vezes. As amostras fixas foram desidratadas em solução crescente de acetona (30%-100%) durante 10 minutos cada banho. Na solução de acetona 100% o banho foi repetido mais duas vezes. Em seguida, os intestinos foram secos em ponto crítico (Emitech K850, USA), montados em suportes (*stubs*) e submetidos à metalização com cobertura de ouro por um equipamento metalizador (Emitech K550, USA). As amostras foram observadas e fotografadas usando microscópio eletrônico de varredura Jeol® JSM-5600 do Laboratório de Entomologia Médica do CPqRR.

4.5 Obtenção das sequências dos genes de imunidade de *A. aquasalis* para desenho dos *"primers"* para a síntese de dsRNA

Utilizando os dados brutos do genoma do *A. aquasalis* obtidos pelo nosso laboratório (Villegas et al., não publicado) a sequência de aminoácidos de genes ortólogos de *D. melanogaster* ou *A. gambiae* foram buscadas contra as sequências genômicas de *A. aquasalis* usando a ferramenta TblastN. Para os genes *Cactus*, *Caspar*, *JUN* e *Puckered*, foi utilizado como proteína "query" os genes ortólogos de *D. melanogaster* (números de acesso NP_723960.1, NP_995848.1, NP_724882.1 e NP_524273.1, respectivamente). Para os genes FOS, IMD, JNK, REL, Serpina e TOLL, foram utilizados os genes ortólogos de *A. gambiae* (números de acesso

EAA13539.5, EDO63988.1, EAA05905.3, XP_310177.3, EAA15023.2 e EAA00348.4, respectivamente). Para esta busca, foram utilizados os seguintes parâmetros como critério de corte: e-value 10^{-6} (equivalente a probabilidade de o alinhamento ocorrer por acaso. Quanto mais próximo de zero, maior a chance de o alinhamento ter significado biológico.); Word 28 (número de pares de base utilizados no alinhamento com sequência de referência) e Identidade acima de 80% (porcentagem da sequência que foi similar em relação ao tamanho total do alinhamento). As sequências retornadas foram então utilizadas em sistema de "reciprocal best match" na qual cada sequência genômica (nucleotídeo) de *A. aquasalis* é utilizada em uma pesquisa contra proteínas anotadas de *A. gambiae* com a ferramenta blastX (e-value 10-6, Word 3 e Identidade acima de 80%).

Para a anotação da sequência de nucleotídeos "coding DNA sequence" (CDS) e de aminoácidos, foi utilizada a ferramenta online GeneWise2 (http://www.ebi.ac.uk/Tools/Wise2/advanced.html), na qual a sequência de aminoácidos do ortólogo de *A. gambiae* foi utilizada como molde para a anotação da sequência de nucleotídeo de *A. aquasalis.* Foram alterados os seguintes parâmetros: *Start/End*: "local" e *Splice site model*: "modeled".

4.6 Desenhos dos "primers" para construção da dupla fita de RNA para silenciamento gênico no *A. aquasalis*

Após a obtenção das sequências preditas de cada gene de interesse, essas foram utilizadas para o desenho da dupla fita de RNA, sendo construído "primers" internos para a geração de um fragmento em torno de 400-500 pares de base. Na extremidade de ambos os "primers", foi colocada um sequência do promotor T7. Externamente a essa sequência foi desenhado outro par de "primers" denominados externos, que abrangeria o fragmento interno. Além dos "primers" para dupla fita, também foram desenhados "primers" para qRT-PCR, os quais foram utilizados para verificar o nível de silenciamento de cada gene no mosquito. O programa utilizado para o desenho das sequências foi o Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/).

4.7 Construção da dupla fita de RNA

Para a construção da dupla fita, o RNA total do *A. aquasalis* foi extraído utilizando o reagente Trizol (Invitrogen) e a síntese de cDNA foi realizada usando o kit QuantiTect Reverse Transcription (Qiagen) de acordo com as instruções do

fabricante. Esse cDNA foi utilizado como molde da primeira reação de RT-PCR com os “primers” externos. Com esse produto da primeira reação foi feita uma eletroforese em gel de agarose 1% para verificar a presença do fragmento correspondente ao gene alvo. Após confirmação, esse produto foi utilizado novamente como molde para a segunda reação de RT-PCR com os “primers” internos. Depois da segunda reação esse segundo produto passou novamente por uma verificação por gel de agarose, e como foi obtida somente uma única banda, indicando que não ouve inespecificidade da reação, esse produto foi purificado utilizando o QIAquick PCR Purification Kit (qiagen) seguindo as recomendações do fabricante e enviado para sequenciamento para confirmação da sequência predita na anotação dos genes.

Foi utilizado o serviço da empresa IDT (Integrated DNA Technologies) para o sequenciamento provido pelo LMVR-NIH(Laboratory of Malaria and Vector Research- National Intitutes of Health).

4.8 Silenciamento de genes com RNAi

Sequências de T7 foram adicionadas a “primers” específicos para diferentes genes para obter produtos com 300-500 pares de base. Esse produto amplificado foi utilizado como molde para a produção da dupla fita de RNAi utilizando o kit RNAi Megascript (Ambion), de acordo com as instruções do fabricante. A dupla fita foi eluída em água e a concentração 3 µg/µl foi obtida. Fêmeas de mosquitos de 2-3 dias de idade foram anestesiadas no gelo e 69nl da dupla fita do gene alvo (LRIM1) ou do controle (LacZ) foram injetados no tórax do mosquito usando um nanoinjetor (Nanojet Drummond) com capilares de vidro 3 dias antes de receber alimentação sanguínea infectante. A eficiência do silenciamento foi medida 2-3 dias depois da injeção por qRT-PCR.

4.9 Análise da expressão por qRT-PCR

O RNA total de 15 mosquitos foi isolado utilizando Trizol (Invitrogen) e a síntese de cDNA foi realizada usando o kit QuantiTect Reverse Transcription (Qiagen) de acordo com as instruções do fabricante. A expressão gênica foi medida por SYBR green qRT-PCR (DyNAmo HS; New England Biolabs). Foram utilizadas três replicatas biológicas e experimentais. A proteína ribossomal RP49 foi utilizada

como referência interna para normalizar cada amostra. A diferença de expressão foi calculada utilizando o método $2^{\Delta\Delta}$ CT.

4.10 Análise estatística

Análises estatísticas foram feitas usando o software GraphPad Prism (Prism 5.01; GraphPad Software Inc.). Foi utilizado o Teste Shapiro-Wilk para verificar a normalidade da distribuição. Em caso positivo foi feito o teste T-Student, quando não era possível fazer esse teste, foi feito o teste Mann-Whitney.

4.11 Coleta e preparação dos mosquitos para a metagenômica

Um total de 30 mosquitos para cada grupo sendo, pupa, fêmea adulta alimentada com açúcar, fêmea adulta alimentada com sangue e fêmea adulta alimentada com sangue infectado com *P. vivax*, foram coletados três dias após emergir no caso de alimentado com açúcar ou três dias após a alimentação sanguínea. Estes insetos tiveram as superfícies de seus corpos esterilizadas com lavagens rápidas em hipoclorito de sódio 1%, álcool 70% e PBS estéril (pH 7.2), por um minuto em cada solução. Após a lavagem foi extraído o DNA total de cada grupo utilizando-se DNeasy Blood and Tissue Kit (Qiagen®) seguindo as instruções do fabricante e armazenado a –20ºC para posterior uso. A concentração e qualidade do DNA obtido foram determinadas em espectrofotômetro (Nanodrop ND-1000 Thermo Scientific).

4.12 Sequenciamento de nova geração e predição taxonômica da microbiota associada ao *A. aquasalis*

As amostras indicadas acima foram enviadas à empresa Macrogen (na Coréia do Sul) para sequenciamento utilizando a plataforma Illumina e análise taxonômica. Abaixo uma descrição breve dos procedimentos.

O processamento da sequência, controle de qualidade e predição taxonômica foram feitos pela Macrogen como descrito no anexo 1. A partir desses dados foi construída uma matriz de β-diversidade com o intuito de realizar uma comparação ecológica entre os grupos acima descritos utilizando análises multivariadas de ordenação e “cluster” hierárquico.

A matriz de abundância relativa de f-OTUs (unidades taxonômicas

operacionais ao nível de família), foi transformada para reduzir a dispersão dos dados aplicando a seguinte fórmula: arco seno (√x) (Ramette, 2007).

O estudo de ecologia microbiana comparativa foi feito executando uma análise de escala multidimensional não métrica (NMDS), análises de Bray-Curtis usando o programa R studio versão 0.98.507 equipado com o software estatístico R versão 3.0.2 (R Core Team, 2013) com pacote estatístico versão 2.0-10 (Oksanen et al, 2013). Para testar a significância do NMDS, uma análise não-paramétrica multivariada–NP-MANOVA (Anderson, 2001) foi aplicada à matriz de distância utilizando a função Adonis implementada no pacote MASAME disponível em https://sites.google.com/site/mb3gustame/ (Buttigieg & Ramette, 2014).

Para a construção da matriz foi feito o teste com 5000 permutações. Para isso, uma matriz adicional foi gerada com variáveis artificiais indicando o habitat das amostras (aquático x terrestre) dos grupos testados. A função Adonis calcula um valor de R^2 que representa a porcentagem de variação dentro da matriz de distância que é explicada pela variável artificial.

5 Resultados

Parte 1: O *Anopheles aquasalis* como modelo de estudo de malária

5.1 Susceptibilidade do *A. aquasalis* à cepas de *P. falciparum*

A susceptibilidade do *A. aquasalis* à infecções pelo *P. falciparum* foi avaliada usando cultura de gametócitos provenientes da cepa NF54, uma linhagem de origem africana adaptada ao laboratório. A infectividade dos gametócitos foi confirmada pela alimentação simultânea do *A. stephensi* (Nijmengen Sda500), um vetor asiático selecionado geneticamente para ser altamente susceptível ao *P. falciparum* (Feldmann & Ponnudurai, 1989). O grupo controle, *A. stephensi* foi facilmente infectado com *P. falciparum* NF54, apresentando de 94-100% de prevalência de infecção e mediana de 67-90 oocistos por intestino (Figura10 A e B).

Em contraste, apenas um dos *A. aquasalis* de um total de 53 que se alimentaram na mesma cultura de gametócitos se tornou infectado com um único oocisto (Figura10B). Além disso, uma alta taxa de mortalidade (90%) foi observada nas fêmeas de *A. aquasalis* infectadas com *P. falciparum*, enquanto a sobrevivência do *A. stephensi* não foi afetada com a infecção (Figura10 A e B).

Recentes estudos mostraram que isolados de parasitos de diferentes regiões geográficas podem apresentar grandes diferenças na infectividade do mesmo mosquito (Molina-Cruz & Barillas-Mury, 2014). Baseado nesse estudo, foi testada se uma linhagem brasileira do *P. falciparum* (7G8) que poderia ser mais infectiva ao *A. aquasalis*.

A prevalência do *A. stephensi* foi de 28-72% na infecção por *P. falciparum* (7G8), e ainda assim, nenhuma das fêmeas de *A. aquasalis* se infectou com a cepa brasileira de *P. falciparum* (7G8) (Figura11).

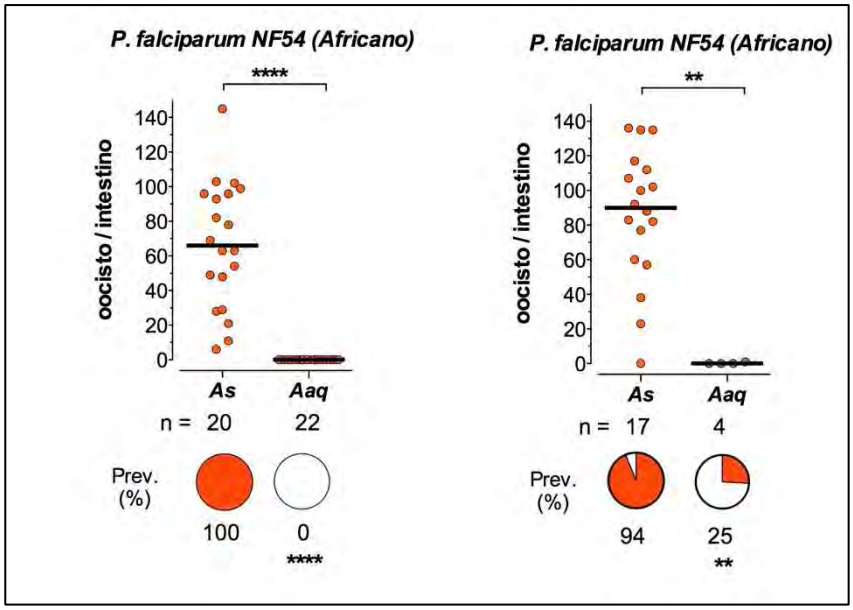


Figura 10: Susceptibilidade do *A. stephensi*(As) e do *A. aquasalis* (Aaq) à infecção com *Plasmodium falciparum* (cepa NF54)(A e B). Cada ponto representa o número de oocistos presentes em um intestino individual 10-12 dias pós infecção e a mediana do número de oocistos está indicado pela linha em preto. As medianas foram comparadas usando o teste Mann-Whitney e a prevalência usando o teste qui-quadrado (** p<0.001, **** p<0.0001).

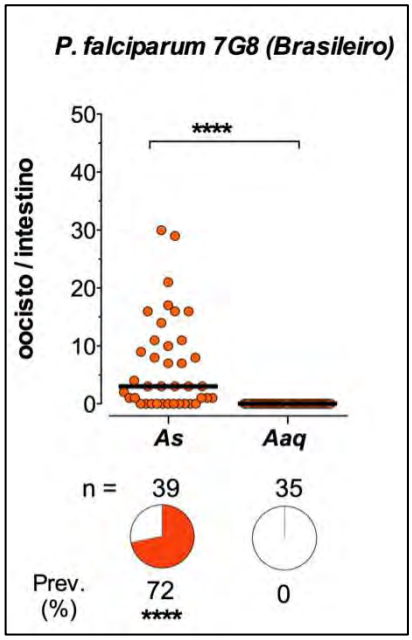


Figura 11: Susceptibilidade do *A. stephensi*(As) e do *A. aquasalis* (Aaq) à infecção com *Plasmodium falciparum* (cepa 7G8). Cada ponto representa o número de oocistos presentes em um intestino individual 10-12 dias pós infecção e a mediana do número de oocistos está

indicado pela linha em preto. As medianas foram comparadas usando o teste Mann-Whitney e a prevalência usando o teste qui-quadrado (**** p<0.0001).

5.2 Efeito da suplementação de antibiótico e ácido úrico na infecção do *A. aquasalis* por *Plasmodium* sp.

Está bem estabelecido que o estresse oxidativo (Molina-Cruz et al, 2012) e a microbiota intestinal (Peterson et al, 2007) podem afetar a sobrevivência do *Plasmodium*. A administração oral de ácido úrico (um potente agente antioxidante) reduz o estresse oxidativo, diminuindo a perda da fecundidade com a idade e evitando a melanização do *Plasmodium* (Dejong et al, 2007; Kumar et al, 2003; Molina-Cruz et al, 2012). O efeito da redução da microbiota intestinal pela administração oral de uma solução de antibióticos (penicilina e estreptomicina) suplementada com ácido úrico, para reduzir o estresse oxidativo foi avaliado nos mosquitos infectados com *P. falciparum* (7G8).

Após oferecer *ad libitum* penicilina/estreptomicina e ácido úrico na solução de açúcar a partir de 24 horas após emergido e mantido até o momento da dissecção dos mosquitos (10-12 dias), foi possível observar que a suplementação da dieta com os antibióticos em conjunto com ácido úrico reduziu a mortalidade após a infecção, no entanto poucos *A. aquasalis* submetidos a esse tratamento se tornaram infectados por *P. falciparum* (7G8) (Figura12A e B), a prevalência foi baixa (10-12%) quando comparado ao *A. stephensi* (28-76%). Nenhuma das fêmeas de *A. aquasalis* que não foram tratadas com a mistura de antibiótico + ácido úrico foram capazes de se infectar com a cepa brasileira.

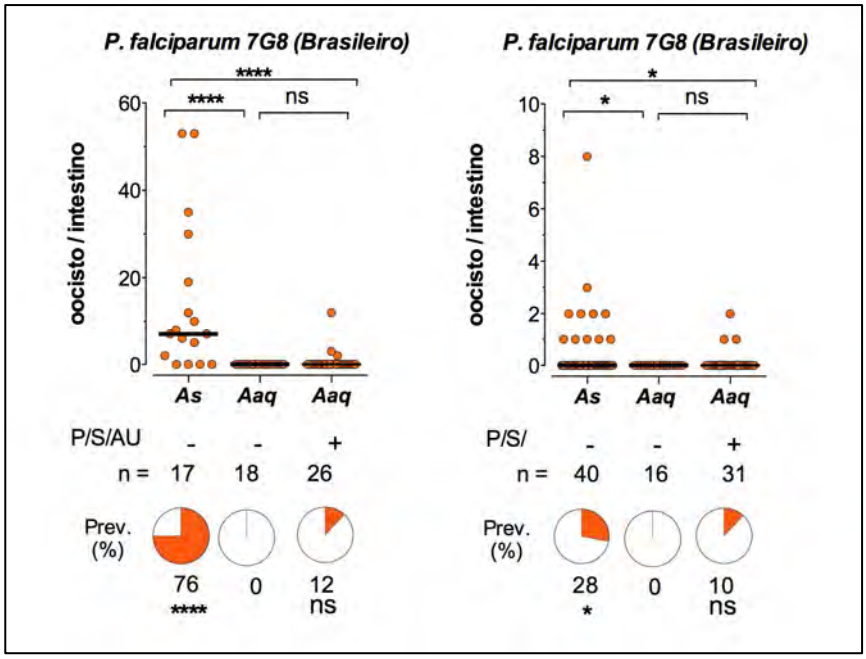


Figura 12: Susceptibilidade do *Anopheles stephensi* (*As*) e do *Anopheles aquasalis* (*Aaq*) à infecção com *Plasmodium falciparum* (cepa 7G8). O efeito da administração oral de antibióticos (Penicilina/streptomicina= P/S) (A) e ácido úrico (AU) (B) nas infecções em Aaq também foi testado. Cada ponto representa o número de oocistos presentes em um intestino individual 10-12 dias pós infecção e a mediana do número de oocistos está indicado pela linha em preto. As medianas foram comparadas usando o teste Mann-Whitney e a prevalência usando o teste qui-quadrado (* $p<0.05$, **** $p<0.0001$, ns= não significativo).

5.3 Susceptibilidade do *A. aquasalis* a infecção com malária murina

Devido à falta de infectividade do *A. aquasalis* pelas culturas de *P. falciparum,* foi testada a susceptibilidade a infecção com diferentes espécies de *Plasmodium* murino. Como esperado, o grupo controle *A. stephensi* foi muito susceptível a infecção com *P. berghei* (Anka 2.34-GFP), com uma prevalência de 100% e mediana de 148 oocistos após 7 dias de infecção. Em contraste, apenas 3 fêmeas de *A. aquasalis* tratados com antibiótico e ácido úrico e injetados com dslacz (controle de dsRNA) se tornaram infectadas e mesmo assim um único oocisto. (Figura 16A).

Com a infecção com *P. yoelii* (17xNL-GFP) novamente como esperado, o *A. stephensi* foi susceptível à infecção com prevalência de70- 90% e mediana de 15 oocistos sete dias após a infecção. Contrariamente, o *A. aquasalis* não se tornou infectado mesmo com o tratamento de antibiótico e ácido úrico (Figura13 A e B).

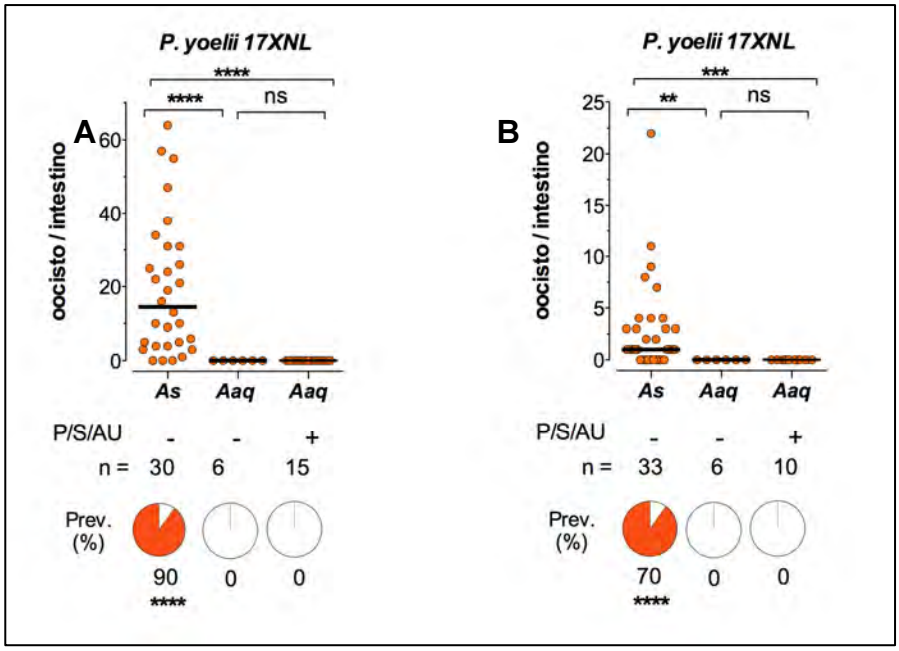


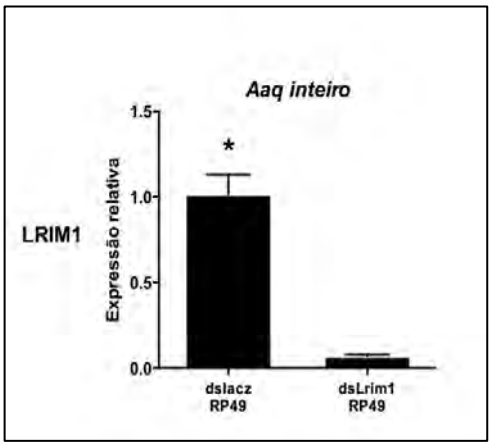
Figura 13: Susceptibilidade do *Anopheles stephensi* (As) e do *Anopheles aquasalis* (Aaq) à infecção com *Plasmodium yoelii* (cepa 17XNL-GFP)(A e B). O efeito da administração oral de antibióticos (Penicilina/streptomicina= P/S) e ácido úrico (AU) nas infecções em Aaq também foi testado. Cada ponto representa o número de oocistos presentes em um intestino individual 7-10 dias pós infecção e a mediana do número de oocistos está indicado pela linha em preto. As medianas foram comparadas usando o teste Mann-Whitney e a prevalência usando o teste qui-quadrado (** p=0.004, ***p=0.0003, **** p<0.0001, ns= não significativo).

5.4 O silenciamento do gene LRIM1 em *A. aquasalis* altera a infecção por *P. falciparum* e *P. berghei*

O LRIM1 juntamente com APL1C são dois receptores de reconhecimento de patógenos de domínios repetidos de leucina que estabilizam TEP1 (thioester containing Protein 1) que é um homólogo estrutural e funcional do sistema do complemento C3 do vertebrado (Baxter et al, 2007; Le et al, 2012). LRIM1 e APL1C circulam na hemolinfa do mosquito e são os maiores antagonistas de infecções com *P. berghei* (Povelones et al.; 2009). Estudos demonstram que silenciando genes que codificam LRIM1, uma cepa de *A. gambiae* passa de refrataria a susceptível a uma infecção por *Plasmodium* (Povelones et al.; 2009, Blandin et al.; 2004).

Para verificar se o mesmo mecanismo ocorre em *A. aquasalis*, foi silenciado o gene LRIM1 e a infecção com *P. berghei* (Anka 2.34-GFP) e *P. falciparum* (NF54) foi realizada. O nível de expressão do mRNA de LRIM1 após o silenciamento foi

verificado no mesmo dia em que a infecção foi feita e estava 94% menos expresso



comparado ao controle (lacZ) (Figura 14).

Figura 14: Efeito da injeção sistêmica de dslacZ ou dsLRIM1 nos níveis de mRNA de LRIM1 nos mosquitos *A. aquasalis* inteiros medidos 3 dias após a injeção de dsRNA. Os dados são mostrados como média do SEM e a diferença significativa é mostrada pelo asterisco (*p<0.05, Teste T).

Com o silenciamento houve um aumento significativo da infecção com a prevalência de 40% e 61% para *P. falciparum* NF54 (Figura15A) e *P. berghei* (Figura16A) respectivamente, mas a intensidade da infecção ainda estava baixa (mediana zero ou próxima a zero) (Figura15 e 16). Além disso, os oocistos estavam com tamanho menores do que os considerados normais com 8-10 dias pós infecção, comparado aos de *A. stephensi*, indicando que os oocinetos que sobreviveram não desenvolveram normalmente no estágio de oocisto (Figura15B e 16B).

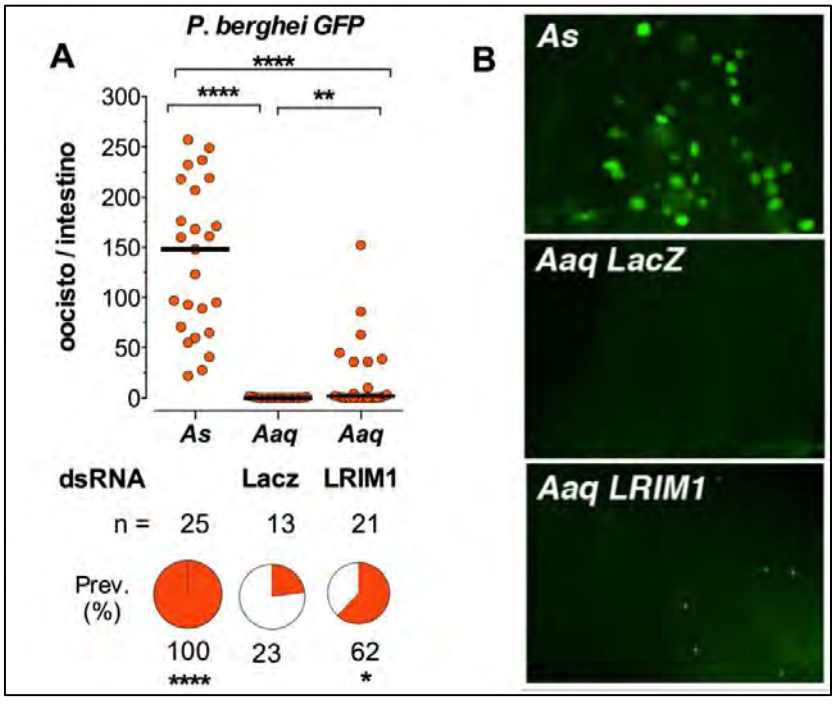
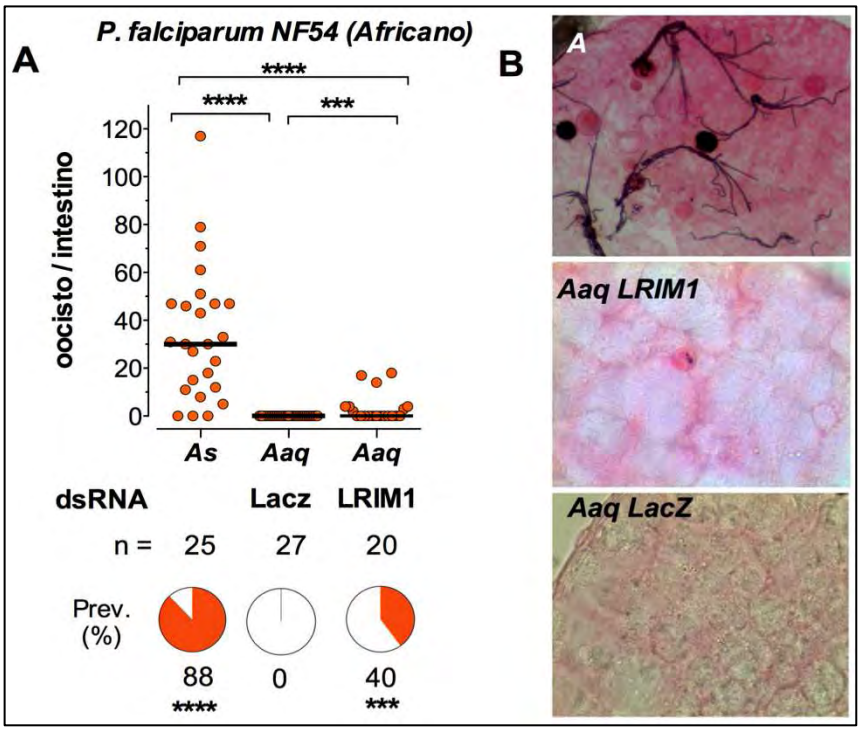


Figura 15: Susceptibilidade do *Anopheles aquasalis* (*Aaq*) à infecção com *P. falciparum*



(cepa NF54) após o silenciamento de LRIM1(A). Oocistos de *P. falciparum* em mosquitos *As* e *Aaq* 10 dias após a infecção (B). Cada ponto representa o número de oocistos presentes em um intestino individual 10 dias pós infecção e a mediana do número de oocistos está indicado pela linha em preto. As medianas foram comparadas usando o teste Mann-Whitney e a prevalência usando o teste qui-quadrado (***p<0.001, **** p<0.0001).

Figura 16: Susceptibilidade do *Anopheles stephensi* (As) e *Anopheles aquasalis* (Aaq) à infecção com *P. berghei* (A) e o efeito do silenciamento de LRIM1 na susceptibilidade do Aaq à infecção (B). Oocistos de *P. berghei* em As e Aaq 8 dias após a infecção. Cada ponto representa o número de oocistos presentes em um intestino individual 7-10 dias pós infecção e a mediana do número de oocistos está indicado pela linha em preto. As medianas foram comparadas usando o teste Mann-Whitney e a prevalência usando o teste qui-quadrado (* p<0.05, **** p<0.0001).

5.5 Susceptibilidade do *A. aquasalis* ao *P. yoelii* (N67)

As diferenças observadas na infecção do *A. aquasalis* ao *P. berghei* (Anka 2.34-GFP) e ao *P. falciparum* (NF54 e 7G8) podem ser refletidas no grau de compatibilidade desse parasitos à cepa/espécie de mosquito utilizada.

Como analisado nos resultados de silenciamento do gene LRIM1 no *A. aquasalis*, os mosquitos podem desencadear diferentes conjuntos de genes efetores em resposta à infecção por diferentes espécies de *Plasmodium*. Afim de explorar essas possibilidades foi avaliada a susceptibilidade do *A. aquasalis* a uma diferente cepa de *P. yoelii*, além da utilizada anteriormente (*P. yoelii* 17xnl-GFP) em que não houve susceptibilidade do *A. aquasalis* à infecção (Figura13A e B).

O *A. aquasalis* se mostrou muito mais susceptível à infecção com o *P. yoelii nigeriensis* (N67), obtendo uma alta prevalência de infecção (68-74%) quando os mosquitos foram tratados com antibiótico + ácido úrico ($p<0.01$) e mediana em torno de 4 e prevalência de 11% para o grupo com açúcar normal (Figura17A e B). Em contraste ao *P. berghei*, o *P. yoelii* N67 se desenvolveu normalmente em *A. aquasalis* e teve tamanho e aparência similares ao controle em *A. stephensi* (Figura17C).

Após 16 dias de infecção foi possível recuperar da glândula salivar do *A. aquasalis* uma modesta quantidade de esporozoítos indicando que o *P. yoelii* N67 é capaz de completar seu ciclo de vida nessa espécie de mosquito.

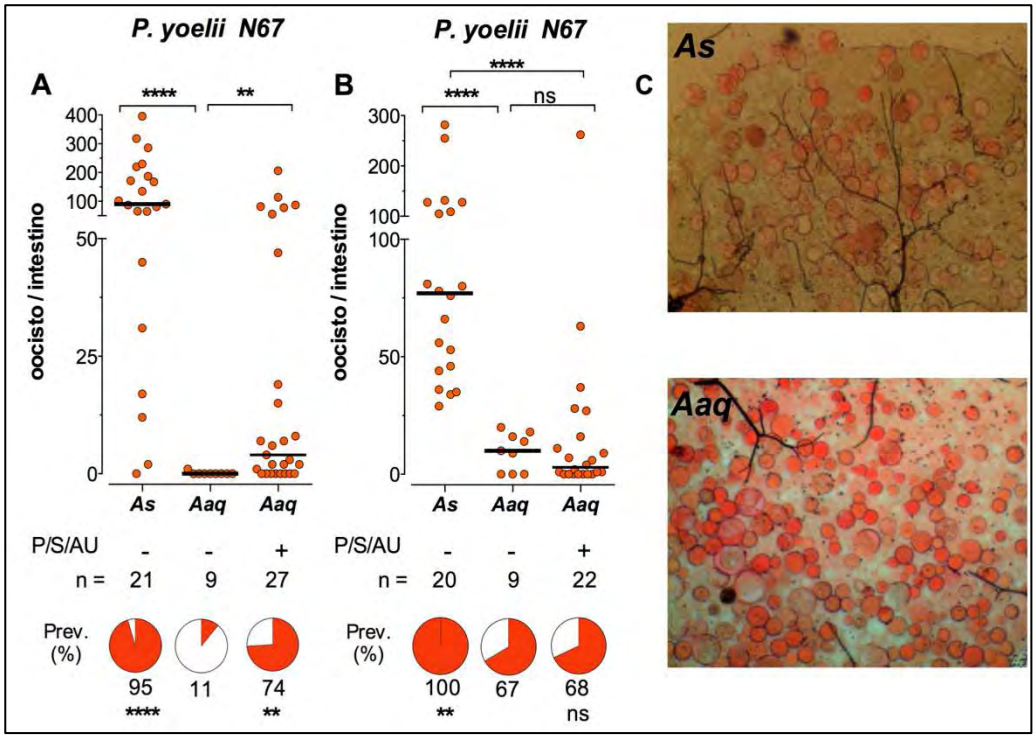


Figura 17*:* Susceptibilidade do *Anopheles stephensi* (*As*) e do *Anopheles aquasalis* (*Aaq*) à infecção com *Plasmodium yoelii nigeriensis* (cepa N67)(A e B). O efeito da administração oral de antibióticos (Penicilina/streptomicina= P/S) e ácido úrico (AU) nas infecções em *Aaq*. Oocistos de PyN67 em *As* e *Aaq* 8-10 dias após infecção. Imagem dos oocistos em *Aaq*

tratados com P/S + AU (C). Cada ponto representa o número de oocistos presentes em um intestino individual 8-10 dias pós infecção e a mediana do número de oocistos está indicado pela linha em preto. As medianas foram comparadas usando o teste Mann Whitney e a prevalência da infecção usando o teste Qui-quadrado (** $p<0.01$, **** $p<0.0001$, ns= não significativo).

Com o intuito de verificar se o silenciamento gênico afetaria o desenvolvimento do *P. yoelii* N67 e tornaria o *A. aquasalis* mais susceptível à infecção, fêmeas de *A. aquasalis* tiveram o gene LRIM1 silenciado. Pode ser observado que existe uma tendência ao aumento da infecção no *Aaq* silenciado (Figura 18). A inibição do LRIM1 no mosquito foi de 80% (Figura 19). Contudo, devido a dificuldades dos mosquitos de se alimentarem no camundongo, os dados ainda são inconclusivos, esses experimentos serão repetidos afim de definir o papel do LRIM1 no *A. aquasalis* infectado por *P. yoelii* (N67).

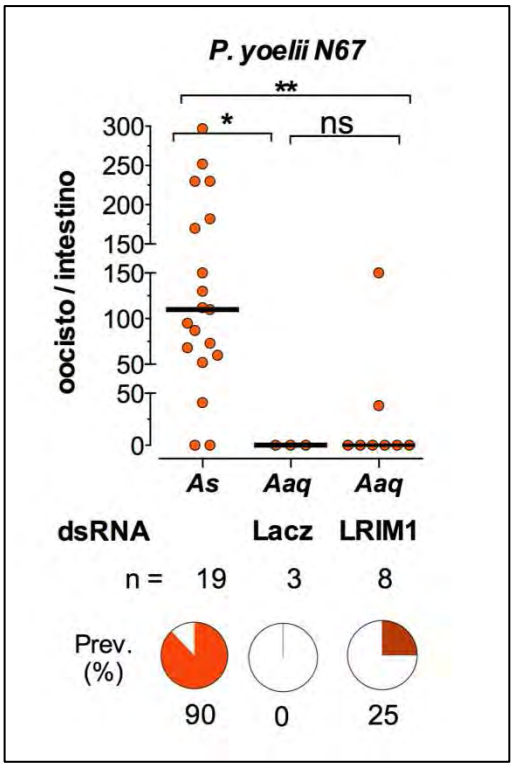
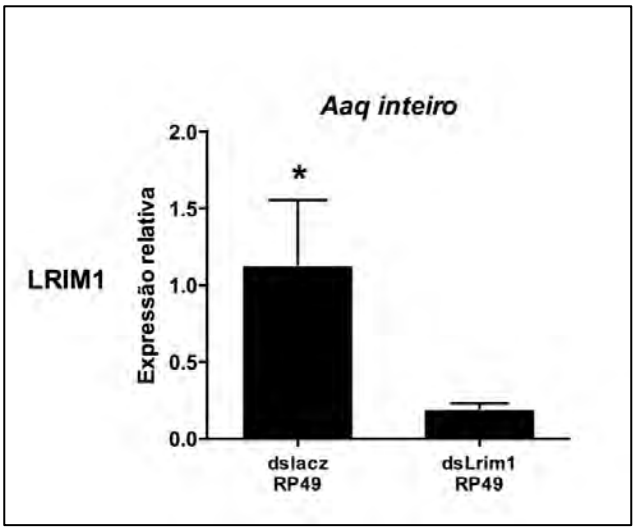


Figura 18: Susceptibilidade do *Anopheles stephensi* (As) e *Anopheles aquasalis* (Aaq) à infecção com *P. yoelii N67* após o silenciamento de LRIM1 no *Aaq.* Cada ponto representa o número de oocistos presentes em um intestino individual 7-10 dias pós infecção e a mediana do número de oocistos está indicada pela linha em preto. As medianas foram



comparadas usando o teste Mann-Whitney e a prevalência usando o teste qui-quadrado (* p<0.01, ** p<0.001, ns= não significativo).

Figura 19: : Efeito da injeção sistêmica de dslacZ ou dsLRIM1 nos níveis de mRNA de LRIM1 nos mosquitos *A. aquasalis* inteiros medidos 3 dias após a injeção de dsRNA. Os dados são mostrados como média do SEM e a diferença significativa é mostrada pelo asterisco (*p<0.05, Teste T).

5.6 Iniciadores para a construção da dupla fita de RNA em *A. aquasalis*

Após obter as sequências preditas de diferentes genes do sistema imune do *A. aquasalis*, foram desenhados “primers” internos e externos (tabela 1) como descrito na metodologia, para a construção da dupla fita de RNA. Os experimentos de silenciamento gênico de alguns genes do *A. aquasalis* através do RNAi ainda estão em andamento.

Gene	Sequência interna 5'-3	Sequência externa 5'-3	Sequência qPCR
Puckered	**PF:** taatacgactcactatagggCAGCCACCGTTCTTCTTGAT **PR:** taatacgactcactatagggGTGGAAGATAGCGAGGATGG	CTCGAGCTGGAACAGAACCT CGTCGAAGATGACGAGGAA	ACCGTACTGCTGCACTGTCA ATACGCCTCCAGTAGCGACA
Fos	taatacgactcactatagggGAACATCGAGCAGAGCATCA taatacgactcactatagggACTGAGGCAGCTCCTACTGC	CAGCGAATTTGCCAACTTTT GACAGATTCGAGGGCTTGTG	CGAGCGGATCATGGACTC GCTGTTACCACCACCAGCAC
Rel1	taatacgactcactatagggGTATCCGGTGGCAAGAACAT taatacgactcactatagggGCAACAGCACCATCATCAAC	TCGGACGTGATCTACGACAA GTGACGTATGTGGCATCTGG	AGCGACTTGAGATCCGTGTT CTCGAGAAACACCTGGAAGC
JNK	taatacgactcactatagggCAAACGAGCCTACAGGGAGT taatacgactcactatagggCCGACCGACCAGATGTCTAC	CACAGCAGAATGTTGCCATC GCGTATCATTTCACCCATGA	TATTGAGCAACTGGGCACAC GAACAGTACGTCGGGGAAGA
Cactus	taatacgactcactatagggGTTCCTTTCCGAGCCTAACC taatacgactcactatagggGGTTTCTCAATCGCTCGAAG	GAAAGTGCCAACACCGATTC CAGGCAAGCTGGGACTTCT	CTTCGATCACCGCAGCTT GGTGTTGGCACTTTCACAGA
TOLL	taatacgactcactatagggCTACGGTGTCCGTGTGTTTG taatacgactcactatagggATGTCGATCACCACGTTTCA	CGGCTGTCCGATACCTACAT GGAGCGCATACCGTAGCTT	CGAATCAGCTGCAAAGTCTG TCGTCGACACAGTACACATCC
IMD	taatacgactcactatagggTGCACACTCATCAACCCGTA taatacgactcactatagggTCTTCGAACGCATCAATCTG	CGATACTGTCCTTGCTCACG CTGCGATACCAATCCGAAAT	ACATAGATTTTTCGACAGTTTGTTAAG TGTTTGAAGTTCTGGCTTGC

Caspar	taatacgactcactatagggCGCACTCAATCAGCAAACAT taatacgactcactatagggCTGGAAAAACATGGGATGCT	CCTTACGACGCCACTCAAAT TTCAGCGCGTCCTCTAGACT	GCAGCGTGCTAACGAATGTA TAAGATCCCACCCGTACAGC
LRIM1	TAATACGACTCACTATAGGGCTGTACGGCACCGTTAACCT TAATACGACTCACTATAGGGCCACGGTAGCTTGTTGTGC		ACCTCAGCGGTAACAAGGTG CTGCGGGTCCTTATTGTTTG

Tabela 1: Sequência dos iniciadores desenhados para a construção da dupla fita de RNA para *A. aquasalis*

PARTE 2: O escape de diferentes espécies de esporozoítos por diferentes vetores

5.7 Caracterização do escape dos esporozoítos no oocisto

As análises dos intestinos infectados foram feitas utilizando o Microscópio Eletrônico de Varredura e revelaram detalhes que determinam características exclusivas para cada espécie de *Plasmodium* estudada.

As imagens de *P. gallinaceum* e *P. vivax* foram utilizados a partir de dados já existentes no laboratório, as imagens foram anexadas aos experimentos realizados com *A. gambiae/P. falciparum* e *A. gambiae/P. berghei* e as análises do escape dos esporozoítos foram realizadas.

5.7.1 Escape dos esporozoítos do *Plasmodium gallinaceum* do oocisto

A microscopia de varredura permitiu a ampliação do intestino médio dissecado e mostrou centenas de oocistos arredondados do *P. gallinaceum* sobre a superfície do intestino médio do *A. aegypti* infectado. A maior parte dos oocistos formaram pequenos grupos na superfície do intestino (Figura 20Ae 20B). Alguns oocistos achatados e oocistos de superfície lisa foram observadas lado a lado, com a presença de alguns hemócitos ligados à superfície (Figura 20C).

No 14° dia após a infecção, foi possível observar o escape dos esporozoítos de dentro dos oocistos no intestino médio. Vários oocistos fragmentados do *P. gallinaceum* foram observados em fases distintas, alguns com pequenas fissuras na superfície, e outros que foram completamente rachados, expondo centenas de esporozoítos escapando (Figuras 20D - 20F). É possível observar milhares de esporozoítos liberados na hemocele a partir dos oocistos completamente rachados (Figura 20D e 20E) . Nas cascas vazias dos oocistos, foi possível observar a superfície porosa do lado interno da parede do oocisto (Figura 20E e 20F).

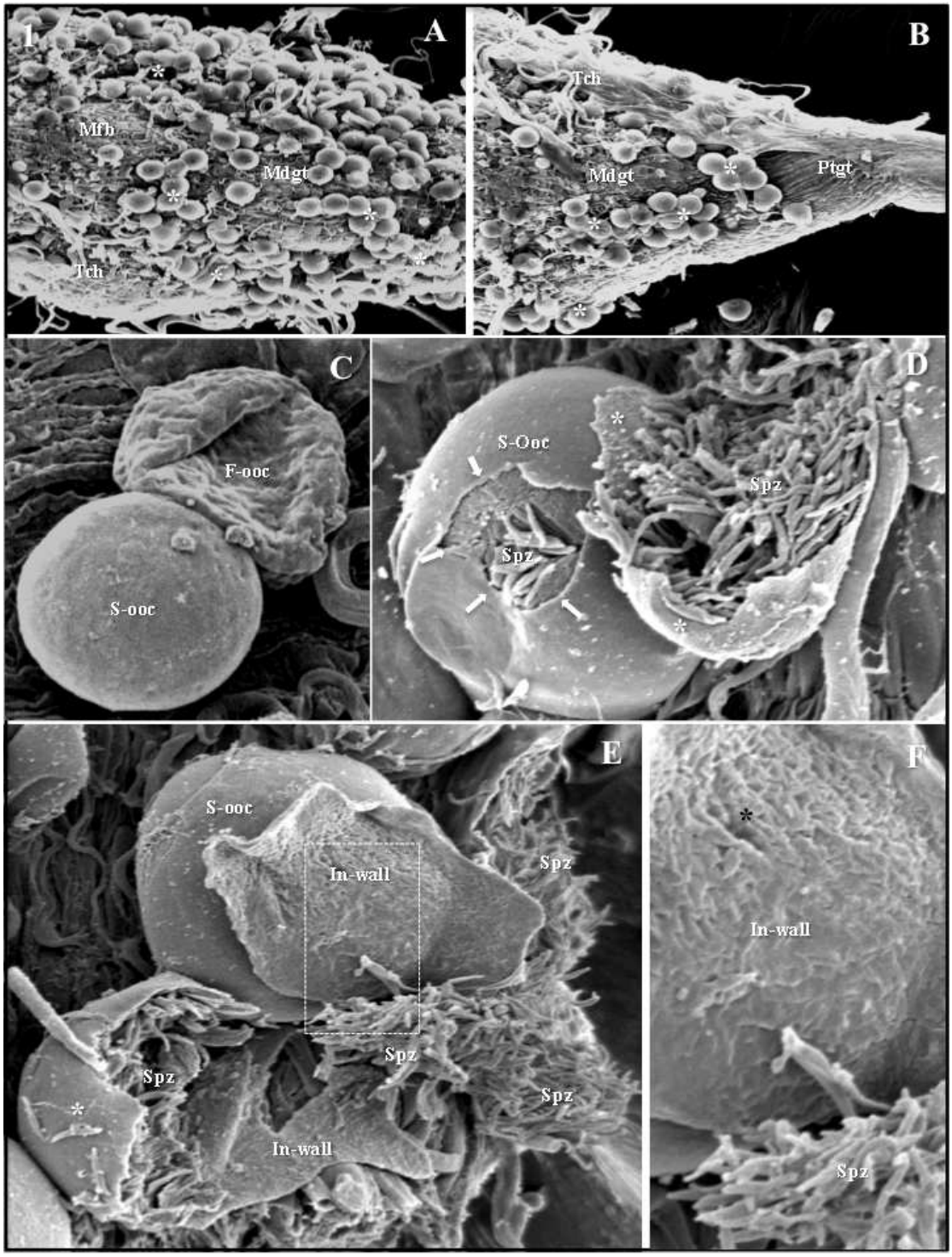


Figura 20: Oocistos de *P. gallinaceum* no *A. aegypti*. (A e B) Visão geral do intestino (Mdgt) com os grupos de oocistos(*) entre as fibras musculares (Mfb). (C) Oocistos liso(S-ooc) e achatado (F-ooc). (D) Oocisto liso (S-ooc) com a parede rachada (seta), e um oocisto parcialmente rachado(*) mostrando centenas esporozoítos(Spz) escapando. (E) Milhares de esporozoítos(Spz) escapando de oocistos parcialmente rachados (*). (F) Visão aumentada da área pontilhada da figura 1E, mostrando a superfície porosa da parede interna (In-wall) do oocisto. Mag: A eB=100x; C=1.400X; D=1.700x; E=1.500x; F= 4.500x.

5.7.2 Escape dos esporozoítos do *Plasmodium berghei* do oocisto

Entre 13 e 14 dias após a infecção do *A. gambiae* com *P. berghei*, foram observados vários oocistos proeminentes entre as fibras musculares que cobrem a superfície do intestino médio em diferentes fases de ruptura (Figuras 21A - 21D). A superfície superior desses oocistos apresentou enrugamentos, e a superfície basal, inserida no tecido do intestino médio, têm a aparência lisa; Além disso, também foi possível observar alguns oocistos achatados (Figura 21A).

Na maior parte dos oocistos, a parede mostrou distintos níveis de "descasque" até os esporozoítos serem libertados. Esta decorticação encontrava-se sempre presente na superfície superior e nas zonas enrugadas da parede do oocisto (Figura 21A - 21C).

Em algumas imagens, é possível observar as fases avançadas de escape do esporozoíto, indicados pela dissolução da parede do oocisto (Figura 21C). No entanto, mesmo depois do oocisto estar completamente aberto, os esporozoítos permaneceram ligados ao lado interno da parede, provavelmente permanecerão até que a parede esteja completamente destruída (Figura 21C e 21D).

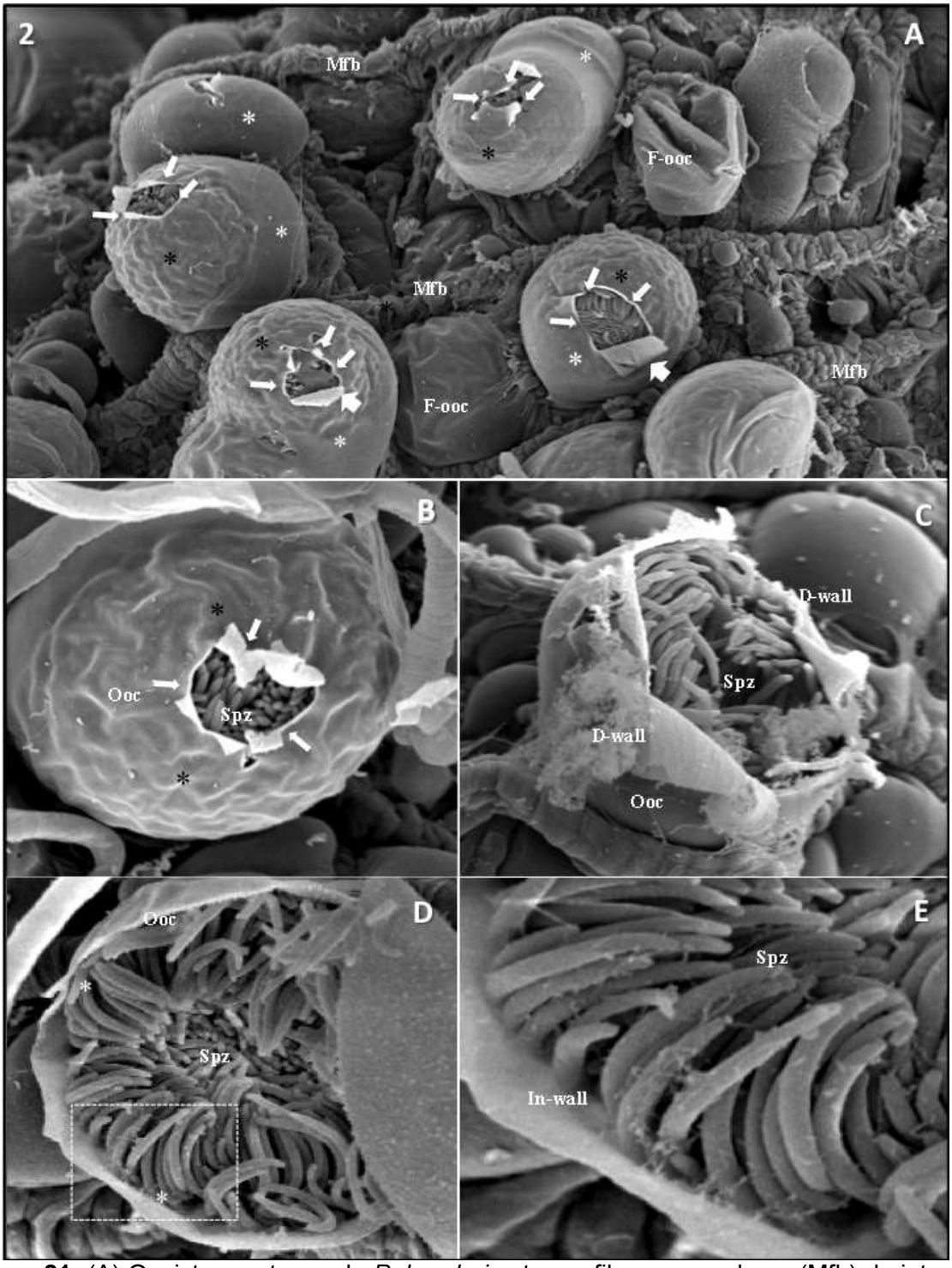


Figura 21: (A) Oocistos protusos de *P. berghei* entre as fibras musculares (Mfb) do intestino do *A. gambiae*. A superfície dos oocistos parcialmente lisas (*branco) e parcialmente rugosas (*), com exceção de alguns oocistos achatados (F-ooc); Esporozoítos vistos dentro do oocisto através da abertura formada (seta branca).(B) Pequena abertura(seta branca) no oocisto mostrando o arranjo ordenado dos esporozoítos (Spz). (C-D) Oocistos com uma grande abertura mostrando parte da parede do oocisto se desfazendo (D-wall) e os esporozoítos prontos para escapar. (E) Visão ampliada da área pontilhada em D, mostrando

o arranjo ordenado dos esporozoítos aderidos na. parede interna do oocisto. Mag: A= 1000x; B e C=1900x; D=1.700x; E=4.300x.

5.7.3 Escape dos esporozoítos do *Plasmodium vivax* do oocisto

O intestino médio dissecado do *A. aquasalis* infectado revelou cerca de dez a algumas centenas de oocistos de *P. vivax* com tamanhos similares presentes sobre a superfície do intestino médio. A maioria dos oocistos foram encontrados isolados ou aos pares, e encontravam-se proeminentes na superfície do intestino médio basal (Figura 22A).

A análise detalhada de intestino infectado usando imagens de alta ampliação permitiu a observação da fuga ativa de um único esporozoíto em uma posição perpendicular rígida (semelhante a um dedo apontando) observada aos 14 dias após a infecção. Este esporozoíto estava forçando a passagem para fora do oocisto, produzindo um buraco na parede desse oocisto (Figura 22B), sugerindo que a liberação de esporozoítos ocorre inicialmente por um único indivíduo ou pequenos grupos de esporozoítos. Imagens adicionais de 15 e 16 dias após a infecção, mostram a fuga de um grupo de esporozoítos a partir de um pequeno orifício, todos foram encontrados em forma de "dedo indicador", mostrando que o escape ocorre de forma ativa através da parede em um primeiro momento da liberação dos esporozoítos dessa espécie (Figuras 22C e 22D).

Por fim, foram observados esporozoítos livres o formato semelhante a uma "vírgula" em regiões distintas da hemocele do mosquito (Figura 22E e 22F). Oocistos vazios, com orifícios por onde o escape dos esporozoítos ocorreu foram também ocasionalmente observadas (Figura 22G), juntamente com alguns oocistos achatados subdesenvolvidos imediatamente adjacentes a oocistos com superfícies completamente lisas (Figura 22H).

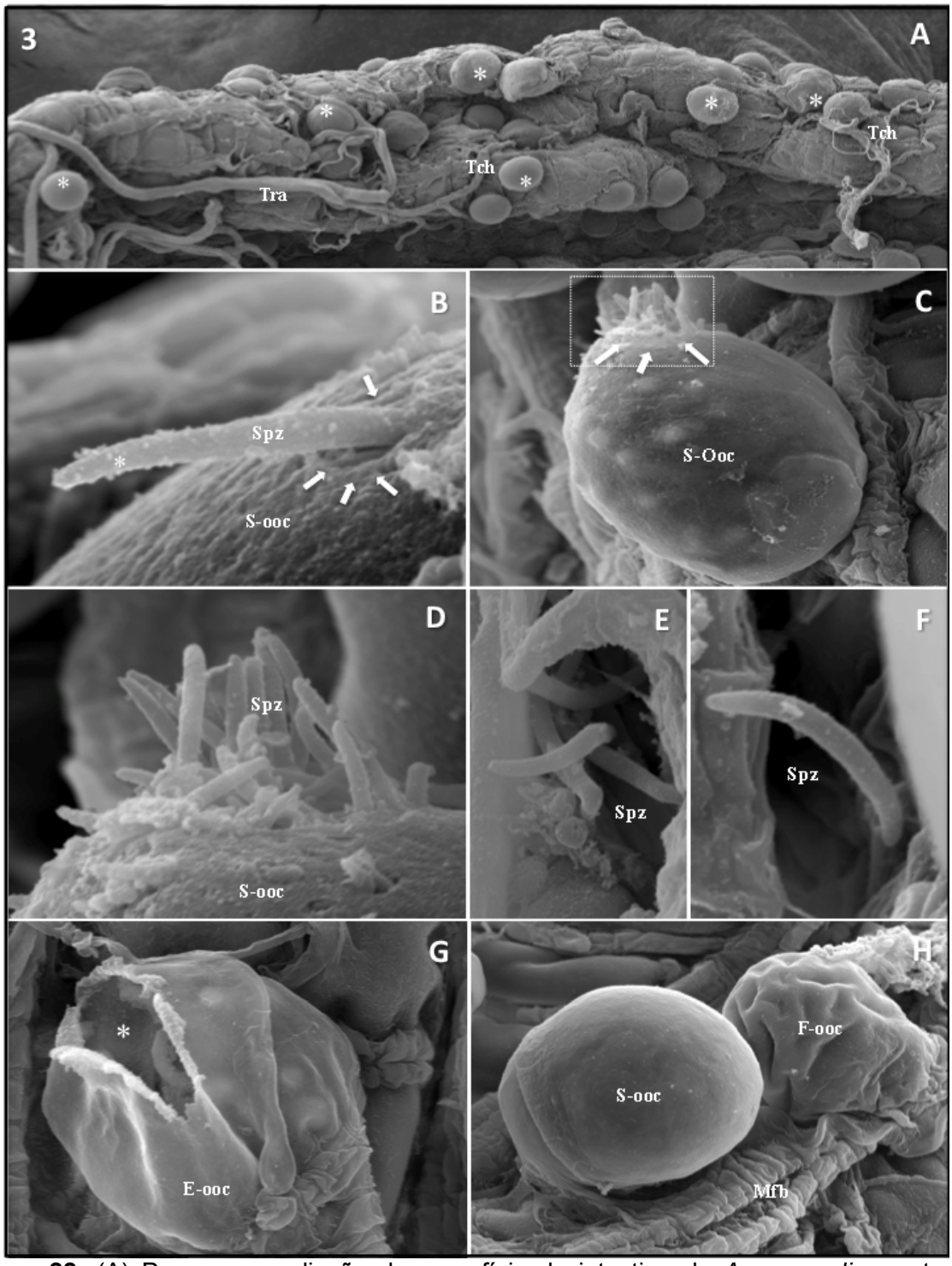


Figura 22: (A) Pequena ampliação da superfície do intestino do *A. aquasalis* mostrando oocistos protusos do *P. vivax* entre as fibras musculares (Mfb), traquéias (Tra) e traqueíolas(Tch). (B) Grande aumento de um único esporozoíto escapando pela abertura(seta) no oocisto liso(S-ooc) utilizando a porção apical (*). (C-D) Grupo de 10 esporozoítos escapando pela lateral do oocistos(seta), note o rígido formato do esporozoíto. (E e F) Esporozoítos livres com o formato semelhante a uma vírgula. (G) Oocisto vazio (E-ooc) exibindo a abertura por onde os esporozoítos escaparam (*). (H) Dois oocistos aderidos as fibras musculares (Mfb) do intestino. Um oocisto de superfície lisa com a parede

completamente esticada (S-ooc) e outro achatado (F-ooc). Mag: A= 200x; B= 8000; C e D=4000x; E e F=1.800x; H= 1.300X.

5.7.4 Escape dos esporozoítos de *Plasmodium falciparum* do oocisto

Os intestinos médio dissecados dos *A. gambiae* infectados revelaram cerca de dez a algumas centenas de oocistos de *P. falciparum* (dados não mostrados), a maioria deles de tamanho semelhante, localizado sobre a superfície do intestino médio. Alguns oocistos se projetavam, isolados ou em grupos de 4-6 indivíduos, na superfície do intestino médio basal.

Os oocistos do *P. falciparum* foram classificados em dois tipos distintos de acordo com a sua superfície: superfícies completamente lisas e superfícies rugosas (Figura 23A e 23B). Observações feitas com 14 dias após a infecção, revelaram o processo inicial de uma única forma ativa de esporozoítos escapando através de um único orifício. Esses escapes ocorreram sempre a partir de um oocisto completamente liso. O escape dos esporozoítos individuais também apresentaram a forma de "apontar o dedo" semelhantes aos observados em *P. vivax* (Figura 23C). No 13° e 14° dias após a infecção alguns oocistos com superfície lisa apresentaram pequenas áreas quebradas por onde ocorreu o escape de alguns esporozoítos (Figura 23D). Estes oocistos passaram a mostrar áreas dobradas na superfície (Figura 23D). Durante a fuga do esporozoíto de *P. falciparum* foi possível observar oocistos achatados com uma abertura lateral, mostrando um aglomerado de esporozoítos escapando (Figura 23E e 23F). Notavelmente, apenas oocistos com superfície completamente lisa mostraram o escape dos esporozoítos de *P. falciparum*, e nunca foram observados escapes a partir de oocistos rugosos.

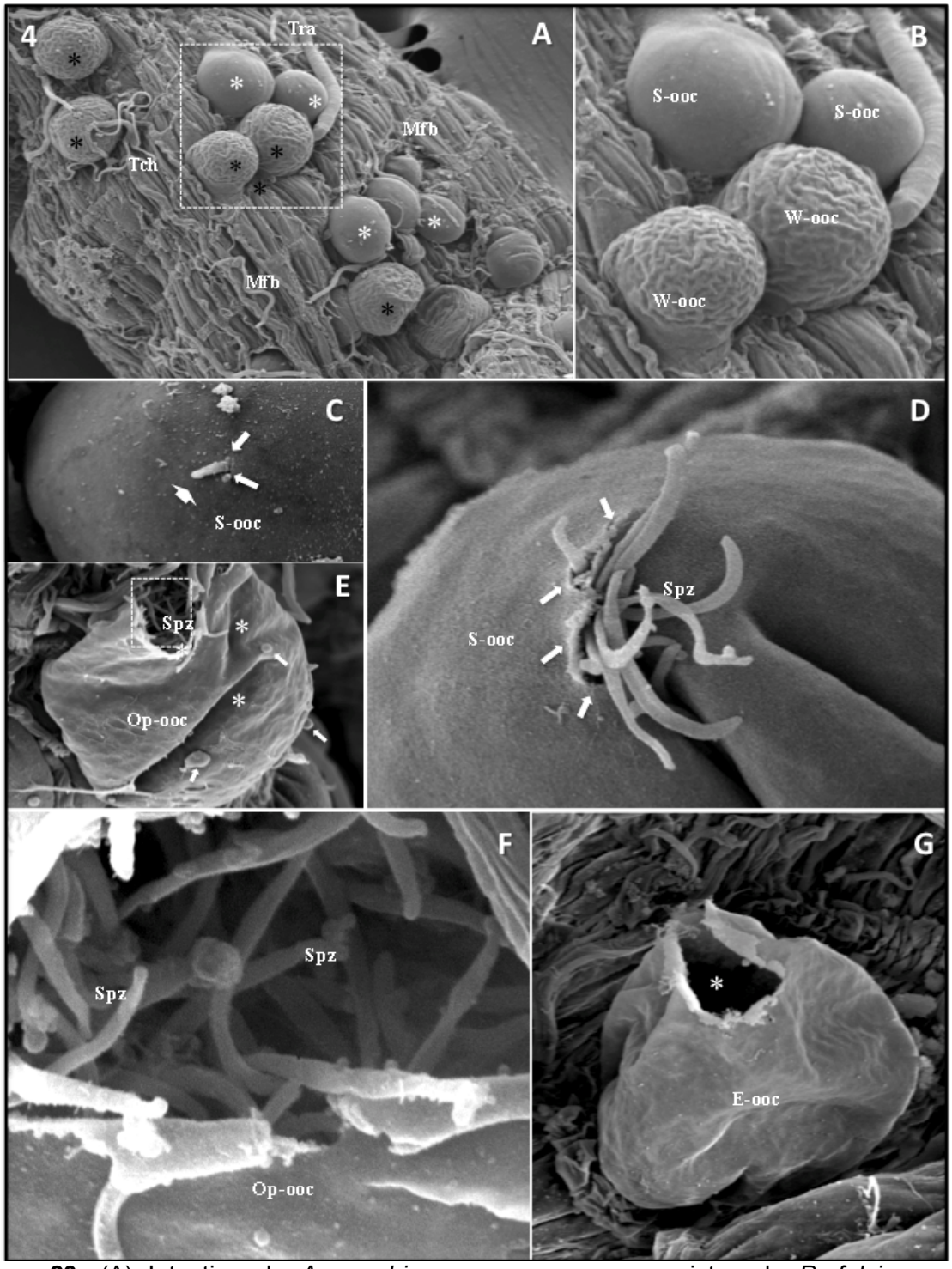


Figura 23: (A) Intestino de *A. gambiae* com numerosos oocistos de *P. falciparum* de superfície lisa (*branco) e superfície enrugada(*) entre as fibras musculares (Mfb). Traqueias (Tra) e traqueíolas(Tch). (B) Grande aumento da área pontilhada em A com um grupo de oocistos de superfície lisa (S-ooc) e rugosa (F-ooc). (C) Um único esporozoíto escapando pela abertura(seta) no oocisto liso(S-ooc) utilizando a porção apical (cabeça de seta). (D) Grupo de esporozoítos escapando pela pequena área quebrada do oocistos(seta). (E) Oocisto achatado e aberto (Op-ooc) na lateral (*) com um grupo de esporozoítos dentro do oocisto. (Seta) hemócitos aderidos ao oocisto. (F) Grande aumento da área pontilhada em E

com esporozoítos escapando (Spz). (G) Oocisto vazio (E-ooc) exibindo a abertura por onde os esporozoítos escaparam (*).Mag: A= 350x; B= 800X; C= 3.000X; D=3.500x; E=1.300X; F=6.000x; G= 1.800X.

Parte 3 A microbiota associada do *A. aquasalis*

5.8 Composição da comunidade bacteriana de *A. aquasalis* em diferentes condições

A composição bacteriana presente no *A. aquasalis* foi classificada utilizando-se OTUs (unidades taxonômicas operacionais) classificadas a nível de família (fOTUs).

Um total de 42 fOTUs (incluindo o grupo de "reads" não classificadas) foram encontradas entre os quatro grupos analisados (pupa, fêmeas alimentadas com açúcar, com sangue, e com sangue infectado) formando a flora bacteriana de *A. aquasalis* (apêndices 1 e 2). O grupo pupas apresentou maior diversidade bacteriana com relação a riqueza de fOTUs (40) seguido pelo grupo sangue (20), açúcar (18) e infectado (16).

Com o intuito de facilitar a representação gráfica da abundância de fOTUs, todas as famílias que correspondiam a menos de 1% do total de OTUs por grupo foram reunidas em "outros" (Figura 24).

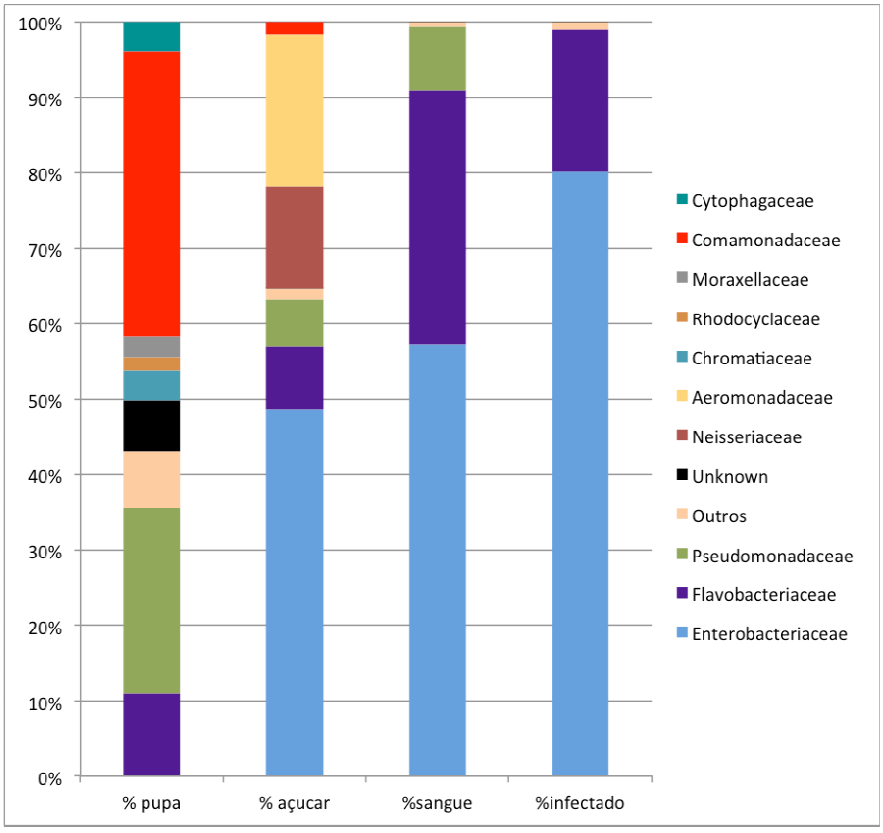


Figura 24: Abundância relativa da comunidade bacteriana (fOTUs) associada a *A. aquasalis* em diferentes condições.

Comamonadaceae (37%), *Pseudomonadaceae* (24%) e Flavobacteriaceae (11%) foram as famílias mais abundantes presentes nas pupas de *A. aquasalis*. Nas fêmeas alimentadas com açúcar as três famílias mais abundantes foram *Enterobacteriaceae* (48%), *Aeromonadaceae* (20%) e *Neisseriaceae* (13%). Em fêmeas alimentadas com sangue houve a predominância de *Enterobacteriaceae* (57%), *Flavobacteriaceae* (33%) e *Pseudomonadaceae* (8%). E finalmente em fêmeas infectadas *Enterobacteriaceae* (80%) e *Flavobacteriaceae* (18%) foram as famílias mais abundantes do grupo (Figura 24).

Adicionalmente, *Flavobacteriaceae* foi a família mais abundante compartilhada entre todos os grupos estudados. Além da classificação a nível de família, um grupo denominado como “Unknown” (desconhecido) representado em maior abundância nas pupas (6,6%) (Figura 24).

Uma árvore taxonômica representando a relação filogenética dos gêneros preditos associados aos grupos de *A. aquasalis* estudados pode ser observada na figura 25. As OTUs obtidas foram agrupadas em 4 filos e 79 gêneros sendo o *Desulfurococos* escolhido como “out-group” (Figura 25). O grupo das pupas foi o único que apresentou 56 gOTUs não compartilhadas pelos outros grupos. Curiosamente 14 gêneros foram compartilhados por todos os grupos (*Catellicoccus*, *Staphylococcus*, *Elizabethkingia*, *Cryseobacterium*, *Flavobacterium*, *Aeromonas*, *Stenotrophomonas*, *Pseudomonas*, *Acinetobacter*, *Thorsellia*, *Serratia*, *Enterobacter*, *Delftia* e *Comamonas*). O grupo açucar teve 19 gêneros porém nenhum exclusivo; o grupo “pós alimentado” com sangue incluiu 20 gêneros e também nenhum em exclusividade e o grupo “pós infectado” se incluiu em 18 gêneros compartilhados também pelos outros grupos. Para compreender essas relações de exclusividade e compartilhamento de gOTUs, foram feitas análises de ecologia microbiana.

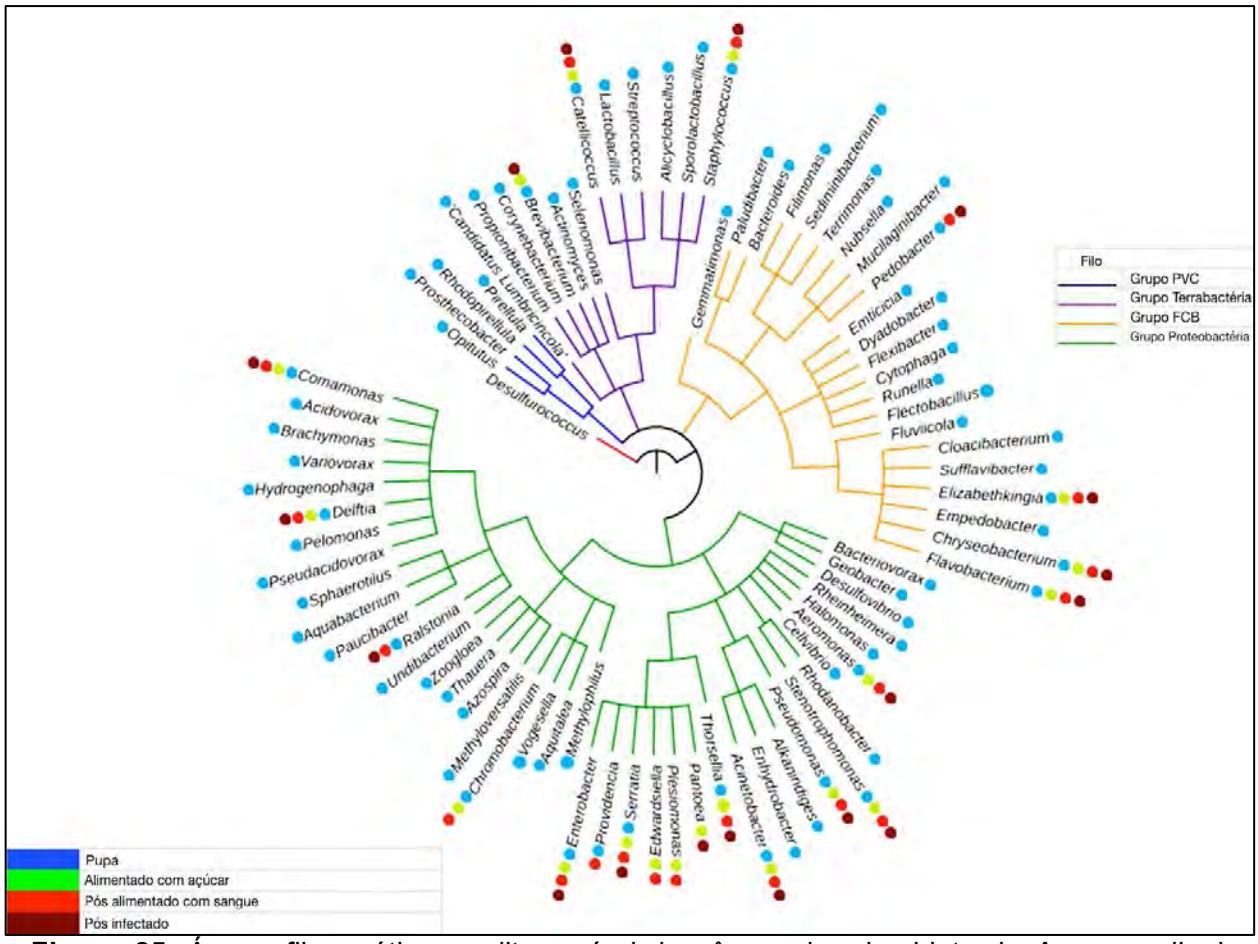


Figura 25: Árvore filogenética predita a nível de gênero da microbiota de *A. aquasalis* de laboratório em diferentes condições alimentares e estágios de vida. Círculo azul representa pupa, verde insetos alimentados com açúcar, vermelho representa insetos pós alimentados com sangue, e marrom insetos pós infecção. As ramificações foram separadas por cores de acordo com o filo que representam, azul grupo PVC (Planctomycetes, Verrucomicrobia e Chlamydiae), roxo o grupo terrabactéria, laranja o grupo FCB (Fibrobacteres, Chlorobi e Bacteroidetes) e em verde o grupo Proteobactéria. A árvore foi construída usando o formato de árvore gerado pelo NCBI taxonomy e visualizado com o iTOL disponível em http://itol.embl.de.

5.9 Exploração de β-diversidade: comparação do perfil bacteriano entre diferentes condições do *A. aquasalis*

As análises exploratórias da diversidade foram feitas afim de determinar se algum padrão biológico ou ecológico poderia ser identificado quando comparadas as quatro diferentes condições alimentares e hábitat do *A. aquasalis* baseado no perfil de abundância bacteriano.

A distância de Bray-Curtis foi utilizada para comparar o perfil de abundância dos grupos testados, e também análises do Cluster hierárquico e NMDS para identificar estruturas dentro da matriz de dados. Como pode ser observado na figura 26, um aparente padrão biológico de agrupamento foi identificado baseado na matriz de abundância microbiana transformada. No espaço NMDS1 observou-se um agrupamento poderia ser relacionado ao habitat do estágio do mosquito, separando-se a pupa (aquático) dos adultos (terrestre). Quando se analisa a distribuição dos grupos no espaço MDS2 observa-se que os adultos alimentados com sangue se agrupam e distanciam-se do adulto alimentado somente com açúcar.

Essa mesma distribuição foi observada com a análise de “Clustering” hierárquico, com fins ilustrativos a figura foi plotada sobrepondo ao espaço NMDS. A correspondência entre as duas análises corrobora a hipótese da existência de um gradiente biológico.

Adicionalmente foi feita uma análise pressupondo que exista um gradiente biológico assim como uma correlação linear entre as variáveis comparadas. Dessa forma com base no apêndice 3, observou-se uma correlação entre a abundância relativa das fOTUs de Enterobacteriaceae, Sinobacteriaceae e Flavobacteriaceae dos grupos alimentados com sangue. Porém a Enterobacteriaceae apresentou uma maior correlação com o grupo alimentado com sangue infectado.

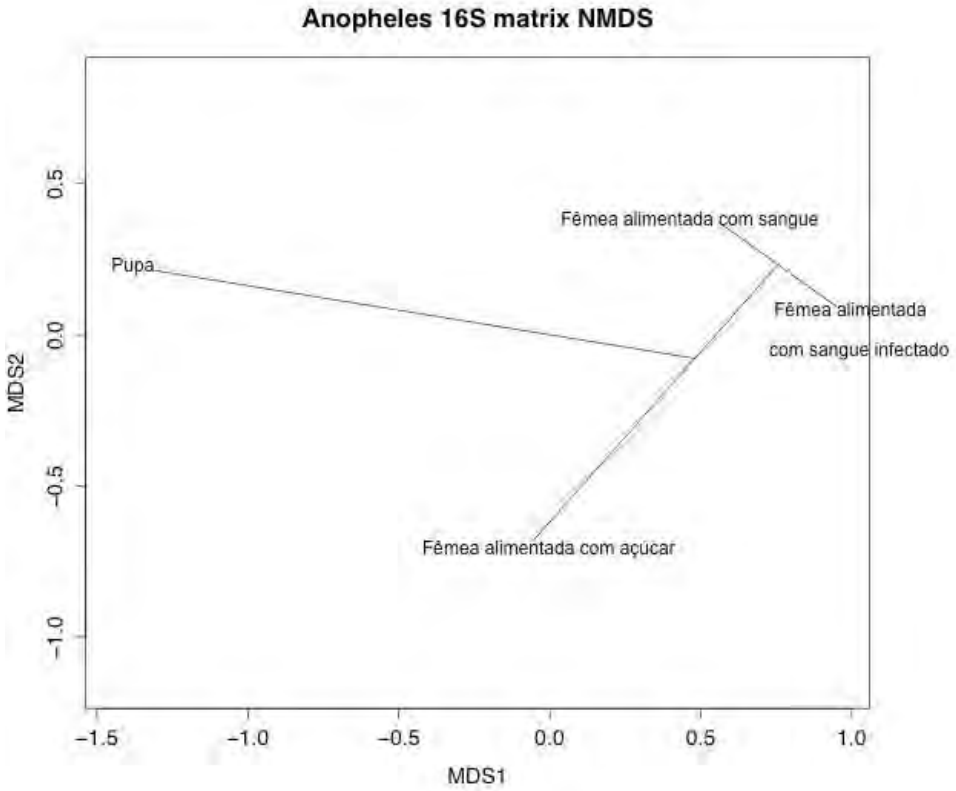


Figura 26: Análise em MNDS comparando o perfil de abundância de fOTUs dos quatro grupos estudados em *A. aquasalis*.

6 Discussão

6.1 *Anopheles aquasalis* como modelo de estudo de malária

6.1.1 Susceptibilidade do *A. aquasalis* à infecções pelo *P. falciparum*

O *A. aquasalis* é naturalmente susceptível as infecções com o *P. vivax* (Povoa et al, 2003; da Silva et al, 2006) e esse sistema experimental têm sido utilizado para investigar o papel da via JAK-STAT (Bahia et al, 2011) e das espécies reativas de oxigênio (Bahia et al, 2013) na susceptibilidade à essa infecção.

Alguns estudos recentes apontaram que a evasão do sistema imune do mosquito pelo *P. falciparum* é mediada pelo gene *pfs47* e é um ponto crítico para a sobrevivência do parasito dentro do vetor (Molina-Cruz et al, 2012; Molina-Cruz et al, 2015). Diferentes haplótipos do gene *pfs47* circulam em diferentes continentes e são os maiores determinantes da compatibilidade vetor e parasito (Molina-Cruz et al, 2015). Sabe-se portanto, que *A. gambiae* é altamente susceptíveis às infecções com duas linhagens africanas de *P. falciparum* (NF54 e MRA1181), enquanto que *A. albimanus* é um vetor altamente refratário à infecções utilizando esses isolados, porém mais susceptível às infecções com a linhagem brasileira de *P. falciparum* (7G8) do que o *A. gambiae* (Molina-Cruz et al, 2015). A falta de compatibilidade entre os isolados de diferentes continentes pode ser alterada, interrompendo o sistema complemento do mosquito, indicando que o sistema imune é selecionado pelo parasito que expressa certos haplótipos de *pfs47* que podem transpor o reconhecimento pelo sistema imune (Molina-Cruz et al, 2015).

Nos resultados obtidos com esse trabalho conclui-se que *A. aquasalis* é quase completamente refratário à infecção pelo *P. falciparum* (NF54), e interrompendo o sistema de complemento por meio do silenciamento do gene LRIM1 ocorre um aumento significativo na prevalência e intensidade da infecção, porém os níveis de infecção foram muitos menores quando comparados ao controle por *A. stephensi*, indicando que apesar da evasão do sistema imune, outro(s) fator(es) em *A. aquasalis* são responsáveis pela baixa infectividade com a linhagem africana NF54 de *P. falciparum*. Essas implicações estão de acordo com as observações de que *A. aquasalis* é também altamente refratário às infecções com a linhagem brasileira de *P. falciparum* (7G8) expressando o mais comum haplótipo de *Pfs47* no

Brasil. A administração oral de antibióticos e ácido úrico permitiu a sobrevivência de poucos parasitos porém a prevalência e intensidade da infecção ainda permaneceu baixa.

Enquanto o *A. aquasalis* é um importante vetor de *P. vivax* no Brasil (Davis & ., 1931; Povoa et al, 2003; da Silva et al, 2006) e na Guiana, nossos resultados indicam que essa espécie não é um vetor competente de malária por *P. falciparum* com as duas linhagens testadas. Na região Amazônica, o *A. darlingi*, *A. albitarsis* e o *A. rondoni* têm sido documentados como vetores de *P. falciparum* por meio de imunodetecção direta de esporozoítos (da Rocha et al, 2008) sendo o *A. darlingi* a espécie com maior prevalência de infecção. Portanto, e baseado nesses dados, não é possível dizer que o *A. aquasalis* é o maior vetor responsável pelos casos de malária por *P. falciparum* no Brasil.

6.1.2 Susceptibilidade do *A. aquasalis* à infecções por parasitos de malária murina

O *A. aquasalis* se mostrou refratário às infecções com *P. berghei* e *P. yoelii* 17xnl, e bastante susceptível ao *P. yoelii* N67. Reduzindo a microbiota com a administração oral de antibióticos e silenciando o gene LRIM1 do sistema imune do *A. aquasalis* foi possível verificar um baixo nível de infecção pelo *P. berghei*, indicando que poucos oocinetos foram capazes de invadir o intestino médio e se desenvolverem em oocistos. No entanto, os oocistos formados foram muito menores do que o comumente esperado sugerindo que o desenvolvimento não ocorreu propriamente até a maturação (Figura 16B). Esse fato pode ter ocorrido devido às condições fisiológicas no *A. aquasalis* que não fornecem um ambiente adequado para o desenvolvimento dos oocistos ou a resposta imune do vetor é direcionada à fase tardia de desenvolvimento do oocisto.

Fëmeas de *A. aquasalis* tratadas com antibiótico e ácido úrico foram muito mais susceptíveis às infecções com *P. yoelii* N67 do que com *P. berghei* e *P. yoelii* 17xnl e os oocistos se desenvolveram até o tamanho normal. Desse modo, é possível concluir que diferentes espécies de anofelinos diferem amplamente na susceptibilidade à infecção com diferentes espécies de parasito. E o *Plasmodium yoelii nigeriensis* (N67) parece ter uma ampla habilidade para infectar diferentes espécies de mosquitos incluindo um vetor do Novo Mundo, tornando a utilização do

par *P. yoelii* N67 e *A. aquasalis* um bom modelo de estudo de transmissão de malária no Brasil fora das áreas endêmicas.

6.2 O escape de diferentes espécies de esporozoítos por diferentes vetores

A esporogonia é o estágio de desenvolvimento mais longo no ciclo de vida do *Plasmodium* spp. no mosquito. Nesse processo um único parasito invade o epitélio intestinal do mosquito e permanece por pelo menos 8 dias e até 14 dias, crescendo e formando um grande globo sincicial por divisões mitóticas, o oocisto, que formará milhares de esporozoítos quando maduro. Esses esporozoítos quando maduros escapam do oocisto e invadem a glândula salivar prontos para serem injetados em um novo hospedeiro vertebrado (revisado em (Smith et al, 2014; Pimenta et al, 2015; Sinden, 1999; Beier, 1998; Vaughan et al, 1991).

As análises da microanatomia revelaram com precisão os aspectos ultraestruturas das superfícies dos oocistos e os processos de fuga dos esporozoítos e demonstraram que em todas as espécies de *Plasmodium* estudadas, os oocistos são estruturas arredondadas que se projetam individualmente ou em pequenos grupos, a partir do exterior da parede do intestino médio do vetor. Contudo, os nossos resultados demonstram que os oocistos das quatro espécies de *Plasmodium* estudadas diferem nas características da superfície da parede externa e também no processo de escape de esporozoítos.

No *P. gallinaceum*, as superfícies exteriores de todos os oocistos encontrados foram completamente lisas e, durante o processo de fuga esporozoíto, os oocistos do *P. gallinaceum* se rompem, sugerindo que a força interna dos esporozoítos quebram a parede do oocisto a partir do interior, de forma semelhante ao que acontece nos ovos, expondo sua superfície interna e com posterior liberação dos grandes grupos de esporozoítos na cavidade do mosquito. Em contraste, todos os oocistos *P. berghei* exibiram uma superfície híbrida, enrugada no topo e lisa na base. Em comparação com o *P. gallinaceum*, os esporozoítos do *P. berghei* parecem ter um mecanismo menos violento para escapar dos oocistos, pois na superfície superior, enrugada, dos oocistos, uma pequena parte da parede começa a decorticar (formação de uma pequena abertura) seguido pela dissolução progressiva da parede do oocisto. Em seguida, os grupos de esporozoítos desprendem-se da parede interna do oocisto. Nas espécies murina e aviária de *Plasmodium*, as etapas

finais do processo de escape esporozoíto, como oocistos vazios, não foram observadas, distintamente do observado nas espécies de *Plasmodium* que infectam o homem. Na literatura, apenas um estudo comparativo de oocistos de *P. berghei* e *P. gallinaceum* foi publicado (Strome & Beaudoin, 1974). Nesse estudo, em ambas as espécies de *Plasmodium*, foram observados oocistos completamente lisos e também oocistos enrugados, que os autores consideraram oocistos maduros ou artefatos de preparação de amostras. Embora eles só tenha mostrado duas imagens, eles sugeriram que estas duas espécies de *Plasmodium* possuem mecanismos de escape dos esporozoítos semelhante (Strome & Beaudoin, 1974).

Todos os oocistos de *P. vivax* exibiram superfícies completamente lisas, morfologicamente semelhantes aos oocistos de *P. gallinaceum*. Em contraste, foram observados dois tipos de oocistos no *P. falciparum*: oocistos completamente lisos e enrugados. Estes oocistos foram distribuídos aleatoriamente, por vezes, lado a lado no intestino médio do mosquito. Estudos anteriores demonstraram que mosquitos infectados com *P. falciparum* continham apenas oocistos enrugados, mas não foram observados esporozoítos escapando desses oocistos (Sinden & Strong, 1978; Meis et al, 1992). Esses autores sugeriram que a superfície rugosa era característica de oocistos maduros. No entanto, embora tivéssemos observado os dois tipos de oocistos em *P. falciparum*, o escape dos esporozoítos foram observados somente nos oocistos que continham a superfície completamente lisa, indicando que esses oocistos contêm esporozoítos maduros e sugerindo que os oocistos enrugados pode ser oocistos imaturos ou ovócitos que não produziram esporozoítos maduros e saudáveis.

A característica mais notável presente nas duas espécies de *Plasmodium* humano, é o mecanismo de fuga ativa de esporozoítos que ocorre de forma distinta das espécies de *Plasmodium* aviária e murina. A fuga inicial do esporozoíto é idêntica para o *P. vivax* e o *P. falciparum*: fuga se inicia com um único esporozoíto, numa posição perpendicular rígida forçando uma saída através da parede do oocisto, abrindo um pequeno orifício na parede do oocisto com a extremidade anterior do parasito. Embora a primeira etapa ocorre de forma semelhante entre as duas espécies, as etapas seguintes diferem-se entre elas: Em *P. vivax*, um pequeno grupo de esporozoítos continuam na mesma posição perpendicular rígida movimentando ativamente para aumentar a abertura inicial causada na parede do oocisto. Em *P. falciparum*, pequenos grupos de esporozoítos em forma de vírgula

escapam da estreita abertura por meio de um movimento aleatório do parasito, como caracterizado em estudos de motilidade dos esporozoítos (Vanderberg, 1974; Kappe et al, 2004; Munter et al, 2009). Battista e colaboradores (Battista et al, 2014) demonstraram por meio de um modelo geométrico que o esporozoíto se autopropulsiona e possui uma curvatura rígida que facilita seu deslocamento por rígidos obstáculos como o oocisto. As espécies de *Plasmodium* pertencem ao filo Apicomplexa que é definido por conter organelas secretoras especializadas denominadas micronemas e róptrias na porção apical do parasito. As proteínas secretadas por esses organelas tem um papel essencial na adesão e invasão de células alvo, bem como na motilidade e mudanças morfológicas (Dubremetz et al, 1998; Kappe et al, 2004; Hliscs et al, 2010; Besteiro et al, 2011; Sharma & Chitnis, 2013). Estudos demonstraram que em *P. berghei* alteração de algumas regiões da proteína CS afeta a formação e maturação dos esporozoítos, escape do oocisto e subsequente progressão do ciclo de vida do *Plasmodium* (Wang et al, 2005; Ferguson et al, 2014).

Nossos dados sugerem que os mecanismos de escape do esporozoíto não é dependente das espécies *Anopheles*, mas sim regulado pelas espécies de *Plasmodium* diferente do que se pensava anteriormente.

6.3 A microbiota associada do *A. aquasalis*

Nossos resultados forneceram uma descrição detalhada da diversa comunidade microbiana encontrada no *A. aquasalis* levando em consideração a dinâmica entre os estágios de vida e também a fonte alimentar. Ao analisar a diversidade bacteriana desses grupos foi observado uma maior riqueza de fOTUs presente no grupo “pupas“, sendo superior a todos os outros grupos estudados. Possivelmente essa diversidade é adquirida devido ao ambiente aquático em que ela se desenvolve. Contudo, as famílias Pseudomonadaceae, Flavobacteriaceae e Aeromonadaceae estiveram presentes em todos os grupos estudados sugerindo uma ocorrência de transmissão transestadial de algumas espécies. Isso já foi descrito ocorrendo em outros anofelinos (Dana et al, 2005; Damiani et al, 2010; Boissiere et al, 2012), pois apesar da “esterilização” que ocorre no mosquito com a metamorfose de pupa a adulto essa não é completa. A família Flavobacteriaceae, o grupo mais abundante e sendo compartilhada entre todos os grupos, é uma família

com membros comumente isolados em anofelinos de diferentes insetários do mundo (Dong & Dimopoulos, 2009; Chouaia et al, 2010; Kampfer et al, 2011).

Foi visto que, a família Enterobacteriaceae está presente em grande abundância nos mosquitos infectados com *P. vivax* (80%). Essa relação têm sido reportada em diversos outros estudos (Cirimotich et al, 2011a; Boissiere et al, 2012; Eappen et al, 2013) que sugerem que o desenvolvimento do parasito pode ser modulado pela abundância de bactérias do gênero *Enterobacter*. Por exemplo, Cirimotich e colaboradores isolaram uma população de bactérias do gênero *Enterobacter* de *A. gambiae* da Zâmbia na África e esses isolados produziam espécies reativas de oxigênio que impediam o desenvolvimento do *P. falciparum* no inseto (Cirimotich et al, 2011a). Acredita-se que as bactérias do gênero *Enterobacter* induzem à expressão de componentes do sistema imune do mosquito e secundariamente interferem no desenvolvimento do parasito no momento em que ocorre a invasão do oocineto no epitélio intestinal ou na diferenciação de oocineto para oocisto (Eappen et al, 2013). Essa correlação entre a família Enterobacteriaceae e os insetos infectados se mantêm nos estudos de ecologia microbiana como mostrado na figura 26, em que há uma correspondência entre a comunidade bacteriana presente nas fêmeas alimentadas com sangue e com sangue infectado. Resultados semelhantes foram observados anteriormente em (Boissiere et al, 2012), considerando o modelo de infecção *A. gambiae* e *P. falciparum*.

Um grupo denominado de “reads” não conhecidas foi responsável por de 6,6% da abundância bacteriana encontrada nas pupas, pode também ser observado nos estudos de (Wang et al, 2011), que mostrou “reads” não classificadas a nível de família em pupas de *A. gambiae* de laboratório e campo com 3.89% e 14.99% respectivamente. Essas observações em pupas sugerem uma maior diversidade microbiana desconhecida do ambiente aquático refletindo a limitação do banco de dados utilizado na predição do perfil taxonômico, que possivelmente estão incompletos, isto deve-se a bactérias que co evoluíram com o hospedeiro as quais não foram ainda descritas.

A análise metagenômica da microbiota revelou diferenças entre os estágios de vida do *A. aquasalis*. Os resultados sugerem que bactérias gênero *Enterobacter*, mais abundante em fêmeas pós alimentadas e fêmeas pós infectadas, podem ter uma correlação direta com a dieta de sangue, influenciando o processo de

desenvolvimento das espécies de *Plasmodium* dentro desse vetor brasileiro determinando a sua capacidade vetorial. É necessário para a compreensão da dinâmica de transmissão da malária no Brasil não somente o conhecimento da interação do parasito com o seu vetor, considerando as linhagens de parasitos brasileiros, como também da microbiota nativa do mosquito modelo. A relevância desse trabalho auxiliará na construção de conhecimento referente a dinâmica dessa tríade. Conhecimento esse, chave no desenvolvimento ou adaptação de estratégias de controle da malária como a paratransgênesene.

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Apêndices

Classe	FOTUs	Pupa	açúcar	sangue	Sangue infectado
Actinobacteria	*Actinomycetaceae*	+	-	-	-
	Brevibacteriaceae	+	+	+	+
	Corynebacteriaceae	+	-	-	-
	Propionibacteriaceae	+	+	+	+
Bacilli	*Alicyclobacillaceae*	+	-	-	-
	Bacillaceae	+	-	-	-
	Enterococcaceae	+	+	+	+
	Lactobacillaceae	+	+	+	-
	Sporolactobacillaceae	+	-	-	-
	Staphylococcaceae	-	+	+	+
	Streptococcaceae	-	-	+	-
Bacteroidia	*Bacteroidaceae*	-	+	+	-
	Porphyromonadaceae	+	-	-	-
	Rikenellaceae	+	-	-	-
Betaproteobacteria	*Burkholderiaceae*	-	+	+	+
	Comamonadaceae	-	+	+	+
	Methylophilaceae	+	-	-	-
	Neisseriaceae	-	+	+	+
	Oxalobacteraceae	+	-	-	-
	Rhodocyclaceae	-	+	-	-
Cytophagia	*Cytophagaceae*	+	-	-	-
Deltaproteobacteria	*Desulfovibrionaceae*	+	-	-	-
	Geobacteraceae	+	-	-	-
Flavobacteria	*Cryomorphaceae*	-	+	-	+
	Flavobacteriaceae	-	+	+	-
Gammaproteobacteria	*Aeromonadaceae*	-	+	+	+
	Chromatiaceae	+	-	-	+
	Enterobacteriaceae	-	+	+	+
	Halomonadaceae	-	-	+	+
	Holophagaceae	-	-	-	-
	Moraxellaceae	-	+	+	+
	Pseudomonadaceae	-	+	+	+
	Sinobacteraceae	-	-	+	-

	Xanthomonadaceae	-	+	+	+
Mollicutes	*Mycoplasmataceae*	+	-	-	+
Negativicutes	*Veillonellaceae*	+	-	-	-
Planctomycetacia	*Planctomycetaceae*	+	-	-	-
Sphingobacteria	*Sphingobacteriaceae*	-	+	+	+
Spirochaetia	*Spirochaetaceae*	-	-	+	-
Verrucomicrobiae	*Opitutaceae*	+	-	-	-
	Verrucomicrobiaceae	+	-	-	-
	Unknown	+	+	+	+

Apêndice 1: Tabela agrupando as OTUs encontradas do *A. aquasalis* em nível de classe e família. A presença e ausência de cada família foi determinada para cada grupo estudado. (+) presença da família bacteriana; (-) ausência da família bacteriana.

	Família	pupa	açúcar	sangue	sangue infectado
1	Streptococcaceae	0,026056479	0	0,004449002	0
2	Unknown	0,261448539	0,002654329	0,001816292	0,002262419
3	Bacillaceae	0,004106623	0	0	0
4	Actinomycetaceae	0,01026671	0	0	0
5	Neisseriaceae	0,082712734	0,377345967	0,019897789	0,002770888
6	Veillonellaceae	0,008213316	0	0	0
7	Pseudomonadaceae	0,518501187	0,253024765	0,292389892	0,068358
8	Burkholderiaceae	0,082815083	0,003753792	0,007705948	0,007154453
9	Aeromonadaceae	0,082173333	0,46371395	0,015730194	0,015998389
10	Staphylococcaceae	0,067058524	0,067984432	0,020469995	0,04129698
11	Corynebacteriaceae	0,017664123	0	0	0
12	Cryomorphaceae	0,072425948	0	0	0
13	Verrucomicrobiaceae	0,052292463	0	0	0
14	Sinobacteraceae	0	0	0,002568627	0
15	Holophagaceae	0,059474626	0	0	0
16	Lactobacillaceae	0,015089227	0,002654329	0,001816292	0
17	Mycoplasmataceae	0,020119564	0	0	0,00452485
18	Spirochaetaceae	0,010470038	0	0	0
19	Geobacteraceae	0,012657765	0	0	0
20	Halomonadaceae	0,03880581	0	0,001816292	0,001599771
21	Rikenellaceae	0,013147951	0	0	0
22	Opitutaceae	0,022958674	0	0	0
23	Propionibacteriaceae	0,009847468	0,00216725	0,005137271	0,003199547
24	Alicyclobacillaceae	0,026853685	0	0	0
25	Desulfovibrionaceae	0,017783088	0	0	0
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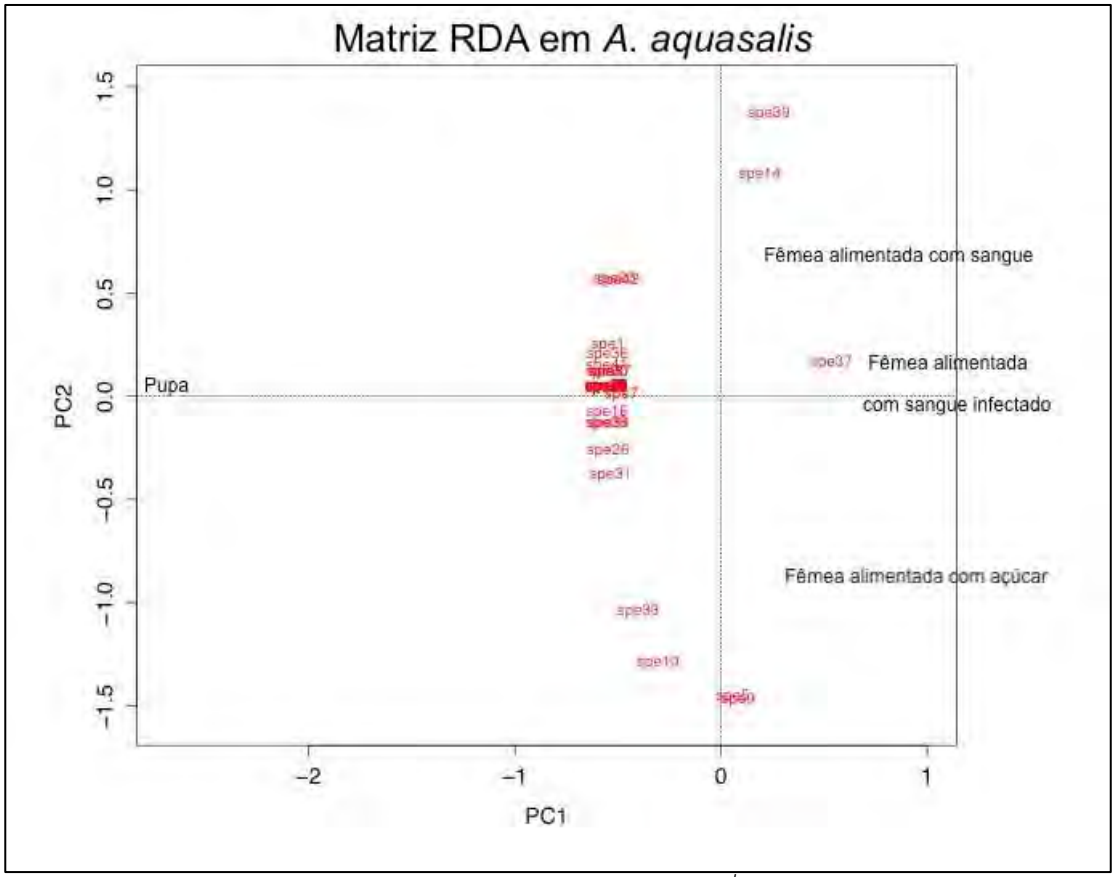
28	Chromatiaceae	0,198242113	0,001532476	0	0
29	Methylophilaceae	0,044812842	0	0	0
30	Rhodocyclaceae	0,138811357	0,001532476	0	0
31	Moraxellaceae	0,165855869	0,070016009	0,031725488	0,018241204

32	Porphyromonadaceae	0,030667202	0	0	0
33	Xanthomonadaceae	0,076295632	0,06833007	0,033050544	0,033024897
34	Bacteroidaceae	0,016427186	0,004334509	0,003145914	0
35	Comamonadaceae	0,661557997	0,13142033	0,055804191	0,041759714
36	Enterococcaceae	0,038368332	0,005308677	0,008710738	0,008615135
37	Enterobacteriaceae	0,086125	0,77315078	0,857771494	1,107761304
38	Cytophagaceae	0,19840581	0	0	0
39	Flavobacteriaceae	0,338706941	0,290908634	0,620668671	0,450279305
40	Planctomycetaceae	0,058145304	0	0	0
41	Sphingobacteriaceae	0,067592424	0,004334509	0,008122792	0,010367885
42	Sporolactobacillaceae	0,00711292	0	0,003145914	0

Apêndice 2: Tabela com as abundancias relativas de fOTUs associadas a anofelinos de



diferentes condições. Dados transformados de arco seno (√x) para reduzir a dispersão.

Apêndice 3: Matriz em RDA mostrando a correlação entre as fOTUs encontradas e os grupos estudados.

Apêndice 4: Artigo 1: Species-specific escape of *Plasmodium* sporozoites from oocyst of avian, rodent and human malarial parasites.

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Alessandra S Orfano, Rafael Nacif-Pimenta, Ana PM Duarte, Luis M Villegas, Nilton B Rodrigues, Luciana C. Pinto, Keillen MM Campos, Yudi T Pinilla, Bárbara Chaves, Maria GV Barbosa, Wuelton M Monteiro, Ryan C. Smith, Alvaro Molina-Cruz , Marcus VG Lacerda , Nágila FC Secundino, Marcelo Jacobs-Lorena , Carolina Barillas-Mury, Paulo FP Pimenta.

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Species-specific escape of *Plasmodium* sporozoites from oocysts of avian, rodent, and human malarial parasites

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Abstract

Background: Malaria is transmitted when an infected mosquito delivers *Plasmodium* sporozoites into a vertebrate host. There are many species of *Plasmodium* and, in general, the infection is host-specific. For example, *Plasmodium gallinaceum* is an avian parasite, while *Plasmodium berghei* infects mice. These two parasites have been extensively used as experimental models of malaria transmission. *Plasmodium falciparum* and *Plasmodium vivax* are the most important agents of human malaria, a life-threatening disease of global importance. To complete their life cycle, *Plasmodium* parasites must traverse the mosquito midgut and form an oocyst that will divide continuously. Mature oocysts release thousands of sporozoites into the mosquito haemolymph that must reach the salivary gland to infect a new vertebrate host. The current understanding of the biology of oocyst formation and sporozoite release is mostly based on experimental infections with *P. berghei*, and the conclusions are generalized to other *Plasmodium* species that infect humans without further morphological analyses.

Results: Here, it is described the microanatomy of sporozoite escape from oocysts of four *Plasmodium* species: the two laboratory models, *P. gallinaceum* and *P. berghei*, and the two main species that cause malaria in humans, *P. vivax* and *P. falciparum*. It was found that sporozoites have species-specific mechanisms of escape from the oocyst. The two model species of *Plasmodium* had a common mechanism, in which the oocyst wall breaks down before sporozoites emerge. In contrast, *P. vivax* and *P. falciparum* sporozoites show a dynamic escape mechanism from the oocyst via polarized propulsion.

Conclusions: This study demonstrated that *Plasmodium* species do not share a common mechanism of sporozoite escape, as previously thought, but show complex and species-specific mechanisms. In addition, the knowledge of this phenomenon in human *Plasmodium* can facilitate transmission-blocking studies and not those ones only based on the murine and avian models.

Keywords: Sporozoite escape, Oocyst, Mosquito vector, *Plasmodium*, Human, Murine, Avian

Background

Malaria remains a life-threatening disease that threatens approximately 3.4 billion people in 104 tropical countries, mainly in Africa, Asia, and South America, with an estimated 207 million cases and half a million deaths reported per year [1]. This vector-borne disease is caused by protozoa of the genus *Plasmodium*, of which *Plasmodium falciparum*, endemic to Africa, is the most prevalent species, followed by *Plasmodium vivax* in Asia and the Americas [1]. Other *Plasmodium* species infect other animal species, such as *Plasmodium gallinaceum* and *Plasmodium berghei*, responsible for avian and murine

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malaria, respectively [2, 3]. Many experimental studies have used *P. berghei* and *P. gallinaceum* as laboratory models to investigate the interactions between the parasites and their vectors. These two *Plasmodium* species are easily maintained in experimental animals, facilitating investigative research in laboratories [4–8].

The *Plasmodium* life cycle begins in a permissive vector when a female mosquito takes a blood meal from an infected vertebrate host that contains gametocytes, the stage of the parasite that can infect the invertebrate vector. Only a few minutes after the infective blood meal enters the midgut lumen of the susceptible mosquito, these gametocytes undergo activation to generate micro- and macro-gametes that fertilize to produce a diploid zygote. After DNA replication and the production of a 4N parasite, the zygote will differentiate into an ookinete over the next 18-24 h depending on the respective parasite species. Ookinetes are a motile form of the parasite that invade and pass through the midgut epithelium until they reach the midgut basal lamina towards the haemocoel of the mosquito. At this location, between the epithelial cells of the midgut and the basal lamina, the ookinete differentiates into a protruding rounded oocyst facing the mosquito haemocoel [8–12]. The presence of well-developed protruding oocysts in the midgut wall is indicative of infection by *Plasmodium* [13–15], and is a reliable measurement to determine the infection rate and the susceptibility of a mosquito species to a particular *Plasmodium* species. In the midgut wall, the oocysts progress to the asexual phase of multiplication known as sporogony, which is completed in approximately 1–2 weeks, the longest phase of the *Plasmodium* life cycle in the mosquito vector. Ultimately, this biological process produces thousands of sporozoites, the final form of *Plasmodium* in the vector. The sporozoites are motile sickle forms that escape from the oocysts into the mosquito hemocoel and invade the salivary gland. Once inside the salivary gland, the sporozoites are ready to be injected into a new vertebrate host via a mosquito bite, completing the *Plasmodium* life cycle in the invertebrate vector [16–18].

Completion of the *Plasmodium* life cycle in the vector requires passage through several barriers inside and outside the midgut. One important and poorly studied barrier is the exit of sporozoites from the oocyst, a critical step that allows sporozoite release into the haemolymph and subsequent invasion of the mosquito salivary gland. Knowledge of the escape mechanism of various *Plasmodium* species is largely unknown for the human malaria parasites, and only a few reports using the laboratory models have previously been published. Studies of the development of *P. berghei* oocysts using a scanning electron microscope (SEM) showed a single small hole in the oocyst wall, inside which sporozoites could be seen [19]. Sinden and Strong reported a torn oocyst from which several *P. falciparum* sporozoites had been released [20]. Meis and collaborators studying the sporogony of *P. falciparum* and *P. berghei*, reported some details of sporozoite escape and concluded that the two species showed similar mechanisms of escape, i.e., the oocysts burst and sporozoites were released into the hemocoel of the mosquito vector [21]. Although published studies have provided some details, knowledge of sporozoite escape from the oocysts of distinct *Plasmodium* species remains incomplete and is primarily based on *P. berghei*, a classical murine malarial parasite used as an experimental model in several laboratories. Moreover, most of the studies on the molecular mechanism of oocyst formation and sporozoite escape have been done using murine *P. berghei* mutant parasites, resulting in conclusions that have been generalized to human *Plasmodium* species without further morphological study.

Understanding the mechanisms of sporozoite escape in various *Plasmodium* species as well as correlations with molecular findings, may contribute to our knowledge of the parasite life cycle in the mosquito vector. Scanning electron microscopy analysis of the external side of the dissected midguts of infected mosquitos is a valuable tool for studying sporozoite escape from oocysts and has not been well explored. Here, this study provides comprehensive insight into the microanatomy of the mechanism of sporozoite escape from oocysts in four species of *Plasmodium*: the two laboratory models, avian *P. gallinaceum* and rodent *P. berghei*, and the two primary causative agents of human malaria, *P. vivax* and *P. falciparum*. It was showed that sporozoite escape is not a common biological process, as previously thought, but the mechanism is complex and species-specific.

Methods

Mosquito rearing

Mosquitoes of *Anopheles gambiae, Anopheles aquasalis* and *Aedes aegypti* were reared at 27 °C with 80 % humidity on 12 h light/dark cycle under insectary conditions. They were provided with 10 % sucrose solution ad libitum until 1 day before the infective blood meal, as described previously [8, 15].

Infection of mosquitoes with *Plasmodium*

Susceptible female mosquitoes (4–5 days old) were chosen to be experimentally infected with one of the four *Plasmodium* species through a membrane feeder device at 37 °C for 30 min, as described previously [8]. *Anopheles gambiae* were infected with stage IV and V gametocytes of the cultured *P. falciparum* NF54 strain. The mature gametocytes were mixed with type O^+ blood and

offered to the mosquitoes [22–24]. *Anopheles gambiae* were also infected with *P. berghei* by direct skin feeding on infected Swiss Webster female mice with a parasitaemia level of 4–8 % and containing 2–3 gametocyte exflagellations per field when observed at 400× under a light microscope. *Aedes aegypti* were infected with *P. gallinaceum* by direct skin feeding on an infected chicken (*Gallus domesticus*) with a 10 % parasitaemia level and at least 2 % circulating gametocytes [25]. *Anopheles aquasalis* were fed on *P. vivax*-infected blood collected from patients diagnosed with malaria, as described in the Ethics statement.

Ethics statement

For the acquisition of *P. vivax* infected human blood, patients were selected among the people visiting the Hospital at the Foundation of Tropical Medicine located in Manaus, 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 ml sample of venous blood was drawn from each patient and placed in heparinized tubes. Blood samples were kept under refrigeration in an icebox (at approximately 15 °C) for about 15 min, taken to the laboratory. The infected *P. vivax* blood samples were offered to mosquitoes through membrane feeder devices. Patient selection criteria were: to be *P. vivax* positive, to have about 4–8 % of circulating gametocytes as determined by the National Institutes of Health international protocols, and to consent to be part of the research consent form that was approved by the Brazilian Ministry of Health, National Council of Health, National Committee of Ethics in Research (CONEP—Approval Number 3726). All patients were treated in accordance with the Brazilian Malaria National Control Programme guidelines.

Also, mice and chickens were maintained at the Animal Care Facility of the FIOCRUZ-MG under specific pathogen-free conditions and were used in accordance to a study protocol approved by the FIOCRUZ Ethical Committee for Animal Use (CEUA; license number LW30/10). It was followed the Public Health Service Animal Welfare Assurance #A4149-01 guidelines according to the National Institutes of Health (NIH) Office of Animal Care and Use (OACU) since these studies were done according to the NIH animal study protocol (ASP) approved by the NIH Animal Care and User Committee (ACUC), with approval ID ASP-LMVR5.

Scanning electron microscopy of infected mosquito midgut

The mosquito midguts were dissected daily, from day 8 to day 16 after the infective blood meal. The dissected midguts were fixed for 2 h in 4 % glutaraldehyde solution in 0.1 M cacodylate buffer, pH 7.2 and then post-fixed with 1 % osmium tetroxide for 2 h. The fixed samples were dehydrated using a graded acetone series, CO_2-dried in a critical-point drying device (Emitech K850, USA) and gold-coated in a sputter coater (Emitech K550, USA) as detailed previously [26]. The samples were analyzed and imaged using a JSM-5600 scanning electron microscope (Jeol USA, Inc).

Results

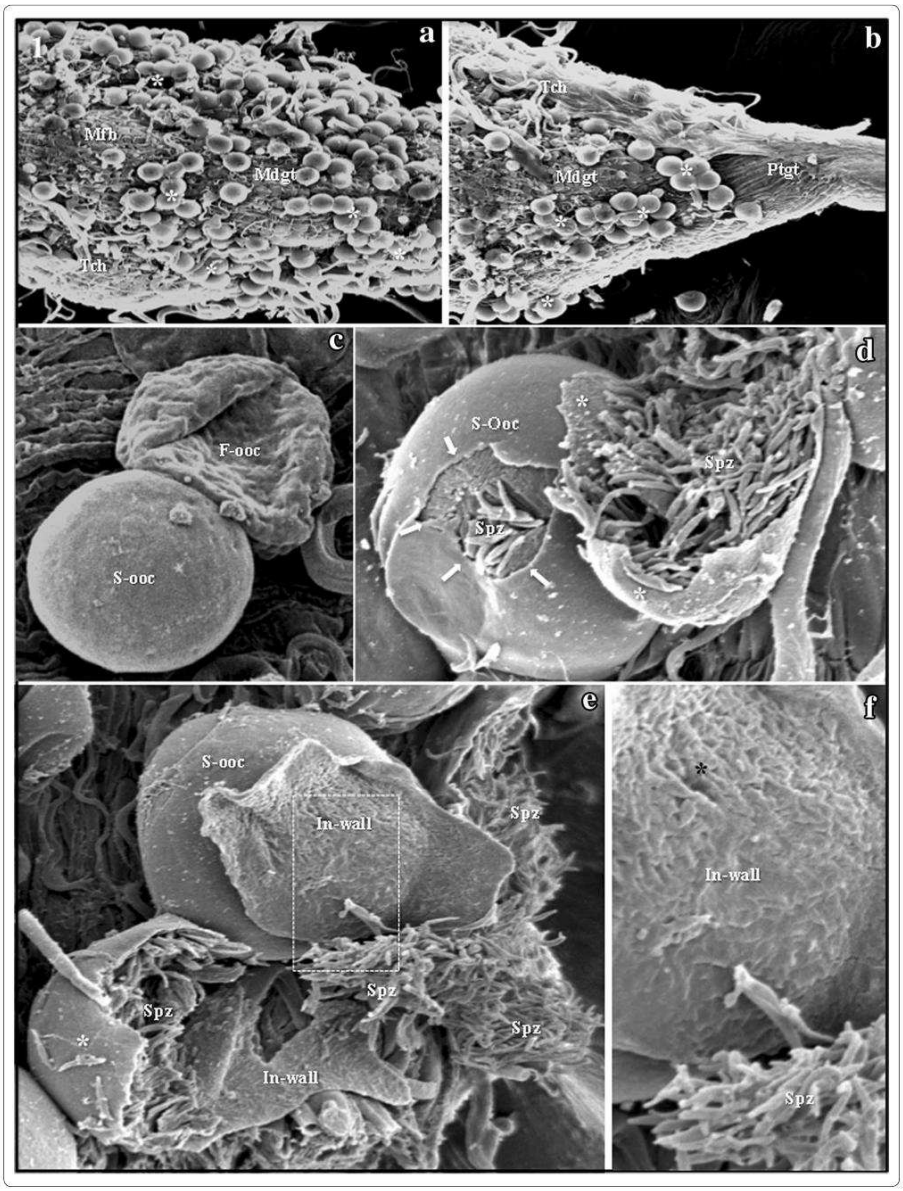
Careful comparative SEM analyses of infected midguts dissected from susceptible mosquito vectors, containing distinct *Plasmodium* species, revealed several new details of the oocyst surface and the sporozoite escape process that are unique to each *Plasmodium* species.

Escape of *Plasmodium gallinaceum* sporozoites from oocysts

Magnification of dissected midguts showed hundreds of rounded avian *P. gallinaceum* oocysts on the midgut surface of the infected *Ae. aegypti*. Most of the oocysts formed small groups on the midgut surface (Fig. 1a, b). Flattened oocysts and completely smooth oocysts were observed side by side, some with haemocytes attached to the surface (Fig. 1c). On the 14th day after infection, it was possible to observe sporozoites escaping from oocysts in the dissected midguts. These dissected midguts were carefully scrutinized for the presence of oocysts, in order to observe the details of sporozoite escape. Several cracked oocysts of *P. gallinaceum* were observed at distinct stages, from some with small cracks in the surface, to some that were completely broken, exposing hundreds of escaping sporozoites (Fig. 1d–f). The completely cracked oocysts liberated thousands of sporozoites into the mosquito haemocoel (Fig. 1d, e). In empty oocyst shells, it was possible to observe the porous surface of the internal side of the oocyst wall (Fig. 1e, f).

Escape of *Plasmodium berghei* sporozoites from oocysts

At 13 and 14 days after infection of *An. gambiae* with *P. berghei*, several oocysts were observed to be protruding between the muscle fibers covering the midgut surface, at different stages of rupture (Fig. 2a–d). The upper surface of these oocysts was wrinkled, and the basal surface, inserted in the midgut tissue, was smooth; it was also possible to observe some flattened oocysts (Fig. 2a). In most of the oocysts, the wall showed distinct stages of "decortication" until the sporozoites were liberated. This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(See figure on previous page.)
Fig. 1 *Plasmodium gallinaceum* sporozoites escaping from oocysts. **a** and **b** Hundreds of rounded *P. gallinaceum* oocysts of similar size protruding from the external surface of the midgut (Mdgt) among the muscle fibers (Mfb) and tracheoles (Tch). Most oocysts form clusters of a few individuals (*asterisks*). All oocysts have a completely smooth surface. Magnification = ×100. **c** Two oocysts attached side by side to the midgut surface; one is completely smooth (S-ooc) and the other is flattened (F-ooc). Note the single hemocyte attached over the completely smooth oocyst wall (*arrow*). Magnification = ×1400. **d** One completely smooth oocyst (S-ooc) with a cracked wall (*arrows*) and a partly cracked oocyst (*asterisks*) showing hundreds of escaping sporozoites (Spz). Magnification = ×1700. **e** Thousands of clustered sporozoites (Spz) can be seen inside and escaping from a partly cracked oocyst (*white asterisk*). In the upper portion of the image, an empty half-shell of a broken oocyst can be seen, in which it is possible to observe details of the internal wall (In-wall). S-ooc = completely smooth oocyst. Magnification = ×1500. **f** Magnified view of the dashed area of Fig. 1d, showing the porous surface of the internal wall (In-wall) of the oocyst. Spz = clusters of escaping sporozoites. Magnification = ×4500

decortication was always present in the upper surface and in the wrinkled areas of the oocyst wall (Fig. 2a–c). In some images, it is possible to observe the advanced stages of sporozoite escape, indicated by dissolution of the oocyst wall (Fig. 2c). However, even after the oocyst had opened completely, the sporozoites remained attached to the internal side of the wall, probably until the wall was completely destroyed (Fig. 2d, e).

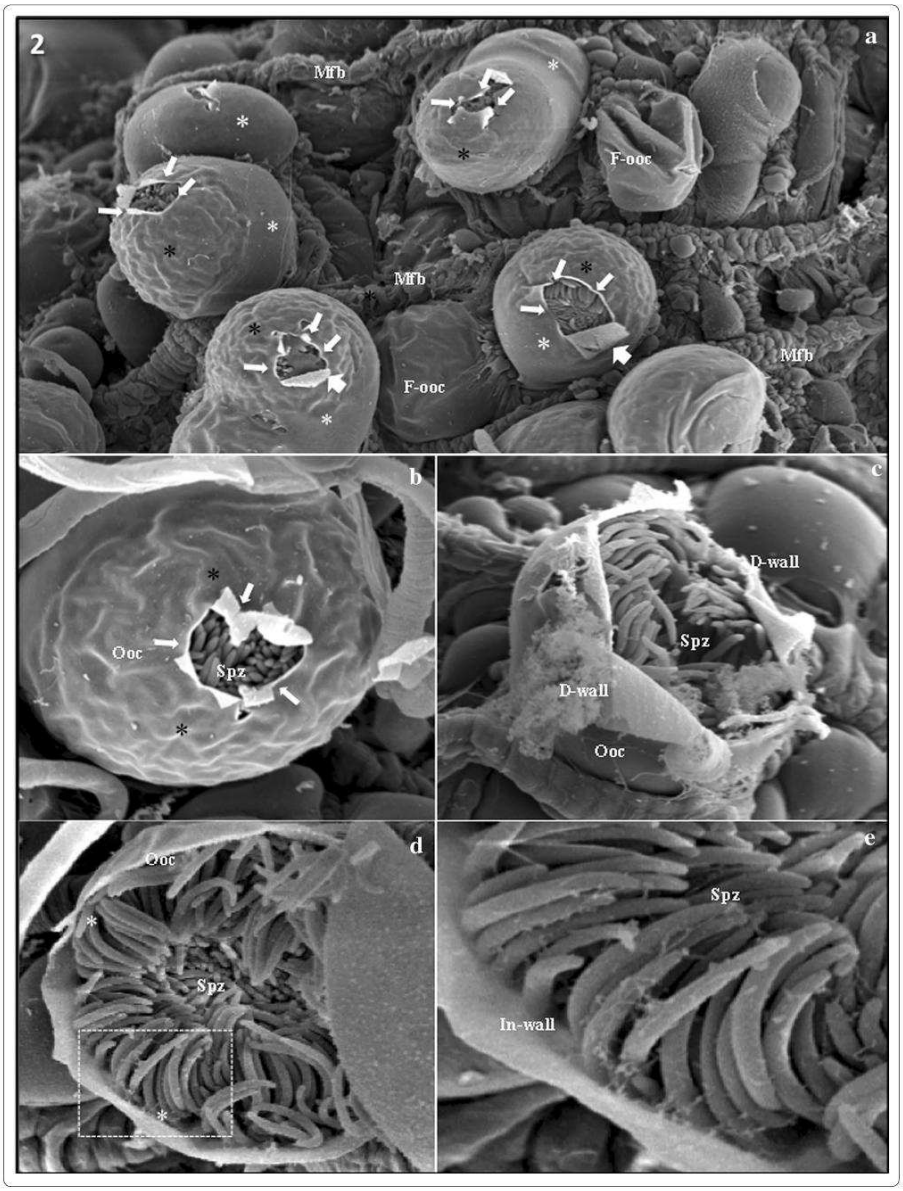
Escape of *P. vivax* sporozoites from oocysts

The dissected midgut of the infected *An. aquasalis* revealed approximately ten to a few hundred *P. vivax* oocysts of similar size on the midgut surface. Most oocysts were isolated or in pairs and they were protruding from the basal midgut surface (Fig. 3a). Detailed analysis of infected midgut using high-magnification images allowed the observation of active escape of a single sporozoite in a rigid perpendicular position (resembling a pointing finger) at 14 days after infection. This sporozoite was forcing its way out of the oocyst by making a hole in the oocyst wall with its anterior tip (Fig. 3b), arguing that sporozoite release is directly initiated by individual or small groups of sporozoites. Additional images of 15 and 16 days after infection show the escape of a group of few sporozoites from a small hole, all with a "pointing finger" shape, indicating that they were actively forcing themselves through the wall in a striking first step to sporozoite release (Fig. 3c, d). Finally, it was observed free sporozoites with the characteristic "comma-shape" in distinct regions of the mosquito hemocoel (Fig. 3e, f). Empty oocysts, with discernible holes where sporozoites had escaped, were occasionally observed (Fig. 3g), along with some undeveloped flattened oocysts immediately adjacent to completely smooth oocysts (Fig. 3g).

Escape of *Plasmodium falciparum* sporozoites from oocysts

The dissected midgut sections of the infected *An. gambiae* revealed approximately ten to a few hundred *P. falciparum* oocysts (data not shown), most of them of similar size, located on the midgut surface. Some oocysts protruded, isolated or in groups of 4–6 individuals, on the basal midgut surface. *Plasmodium falciparum* oocysts could be classified into two distinct types according their surface: completely smooth and wrinkled surfaces (Fig. 4a, b). At 14 days after infection, detailed analysis revealed the initial process of a single sporozoite actively escaping through a unique hole, always from a completely smooth oocyst. These escaping single sporozoites also presented the "pointing finger" shape similar to those seen with *P. vivax*, leading with the anterior tip (Fig. 4c). At 13 and 14 days after infection some completely smooth oocysts showed small broken areas from which a few sporozoites were escaping. These oocysts were beginning to show folded areas on the surface (Fig. 4d). During *P. falciparum* sporozoite escape, it was possible to observe a flattened oocyst with a lateral opening, showing a cluster of escaping sporozoites inside (Fig. 4e, f). Notably, only completely smooth oocysts appeared to produce escaping *P. falciparum* sporozoites, and were never observed escaping from wrinkled oocysts.

(See figure on next page.)
Fig. 2 *Plasmodium berghei* sporozoites escaping from oocysts. **a** *P. berghei* oocysts protruding among muscle fibers (Mfb) on the external surface of the midgut. The surfaces of the oocysts are partly smooth (*white asterisks*) and partly wrinkled (*black asterisks*), with the exception of a few flattened oocysts (F-ooc). Several oocysts have openings on their wrinkled surfaces, which appears as if the wall has peeled away, and sporozoites can be seen inside the hole (*arrows*). Muscle fibers = Mfb. Magnification = ×1000. **b** Small opening (*arrowhead*) in the oocyst (ooc) wall showing the orderly arrangement of several sporozoites (Spz) inside the oocyst. Note the wrinkled surface of the oocyst. Magnification = ×1900. **c** Oocyst (ooc) with a large opening, approximately half-size, showing a sponge-like part of the dissolving oocyst wall (D-wall). Note cluster of sporozoites (Spz) ready to escape from the oocyst. Magnification = ×1900. **d** A single oocyst (ooc) showing a large opening with a "cap" (*asterisk*) that appears to allow sporozoite escape. Note cluster of sporozoites (spz) attached to the internal side of the oocyst wall (*arrows*). Magnification = ×1700. **e** Magnified view of the dashed area from figure **d**, showing a large opening with several orderly arranged sporozoites (Spz) attached to the internal side of the oocyst wall (In-wall). Magnification = ×4300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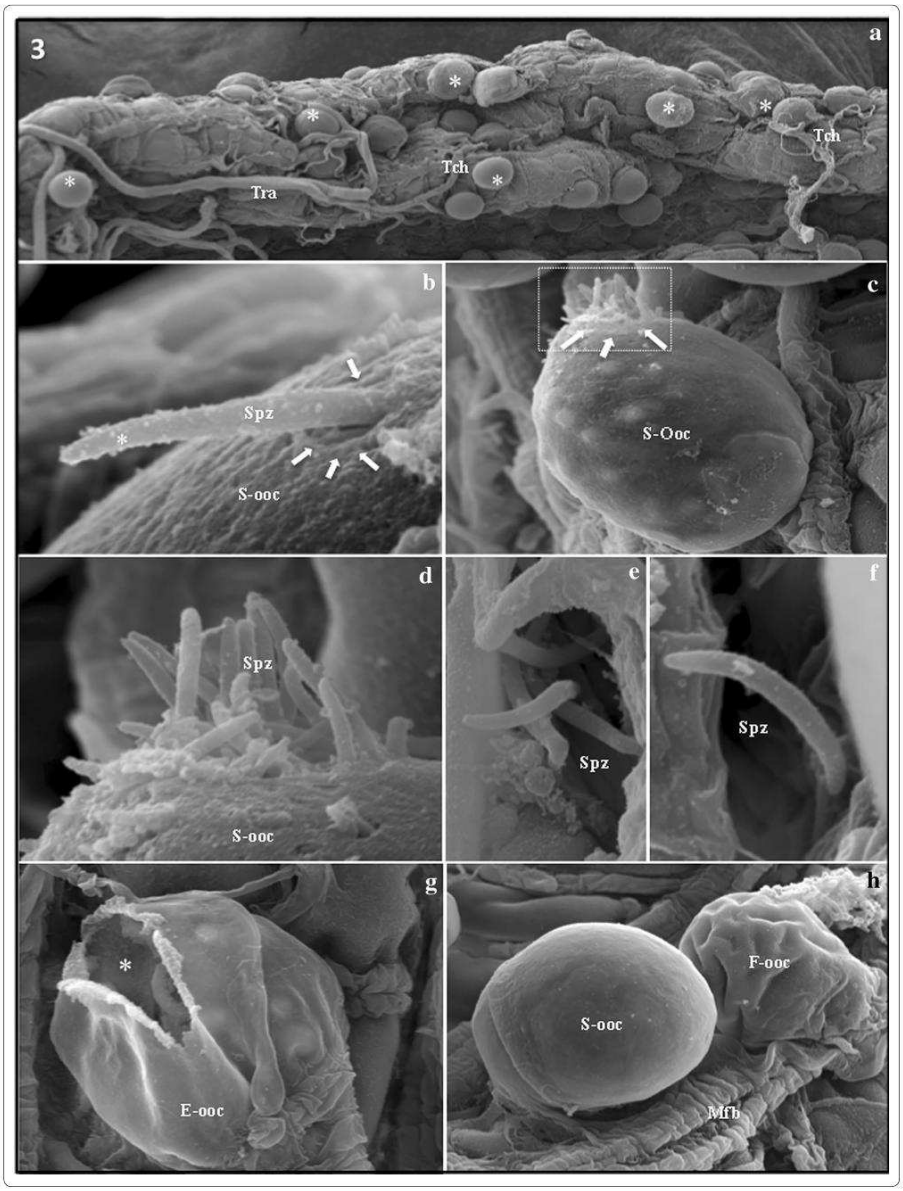
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F-ooc
Mfb

(See figure on previous page.)
Fig. 3 *Plasmodium vivax* sporozoites escaping from oocysts. **a** Low-power magnification of the external surface of the midgut showing protruding *P. vivax* oocysts (*asterisks*) of similar size. The rounded oocysts are arranged individually or in pairs protruding among muscle fibers (Mfb), trachea (Tra), and tracheoles (Tch) that rest on the external surface of the midgut. Magnification = ×200. **b** High-power magnification of a single sporozoite actively escaping by creating a hole (*arrows*) in the smooth oocyst (S-ooc) wall with its anterior end (*asterisk*). Note the rigid perpendicular shape of the escaping sporozoite (Spz). Magnification = ×8000. **c** and **d** Tens of grouped sporozoites (arrowheads) escaping from the lateral wall of an oocyst (S-ooc). Figure **d** is an enlarged image of the dashed area from figure **c**. Note the "rigid perpendicular shape" of the escaping sporozoites (Spz). Magnifications D = ×1400 and E = ×4000. **e** and **f** Free sporozoites (Spz) with the characteristic "comma-shape," as seen in the mosquito hemocoel. Magnifications E = ×4100 and F = ×4300. **g** Empty oocyst (E-ooc) showing a hole through which the sporozoites escaped from the oocyst (*asterisk*). Magnification = ×1800. **h** Two side-by-side oocysts attached to the muscle fibers (Mfb) of the midgut. One smooth oocyst shows a completely stretched wall (S-ooc) and the other shows a flattened wall (F-ooc). Magnification = ×1300

The Table 1 shows the proportion of the distinct *Plasmodium* oocysts according to their main microanatomical aspects of their surfaces, as described in details above.

Discussion

The longest developmental stage of the *Plasmodium* life cycle in the mosquito vector is sporogony, the process of formation of thousands of sporozoites. A single parasite invades the epithelium of the midgut of a mosquito vector and remains in the gut wall for several days. This single-celled protozoan remains outside the mosquito cells, and grows into a large-lobed syncytial nucleus by mitotic division, inside a structure named the oocyst, which forms mature sporozoites. These mature sporozoites escape from the oocysts into the mosquito cavity, after which they invade the salivary gland in preparation for injection in a new vector. The duration of this stage of the *Plasmodium* life cycle varies according to the species, but usually lasts 8–14 days after the mosquito vector has ingested the infective blood meal [7–9, 13, 27].

In this study, to examine the microanatomy of sporozoite escape from oocysts of the four *Plasmodium* species, 10–20 midgut sections were dissected daily from infected mosquito vectors, 6–16 days after the infective blood meal. The midgut samples were dissected, fixed, and processed in the same laboratory, following an identical rigorous protocol to facilitate comparative analyses. The microanatomical analyses presented here clearly and accurately show the ultrastructural aspects of the oocyst surfaces and the processes of sporozoite escape. Preliminary analyses revealed that in all *Plasmodium* species, the oocysts are rounded structures that protrude individually or in small groups from the exterior of the midgut wall of the mosquito vector. However, the oocysts of the four *Plasmodium* species differ in surface features of the external wall and in the process of sporozoite escape.

In the avian parasite *P. gallinaceum,* the outer surfaces of all oocysts were completely smooth. During the process of sporozoite escape, *P. gallinaceum* oocysts were cracked, suggestive of internal forces disrupting the oocyst wall from the inside. The broken oocysts were similar to broken eggs, exposing their internal surface, with subsequent release of large groups of sporozoites into the mosquito cavity. In contrast, all murine *P. berghei* oocysts showed a hybrid surface, wrinkled on the top and smooth on the base. Compared to *P. gallinaceum, P. berghei* sporozoites appear to have a less violent mechanism of escape from the oocysts. On the upper, wrinkled surface of the oocysts, a small part of the wall begins to decorticate, creating a small opening, followed by progressive dissolution of the oocyst wall. Then, the highly structured clusters of sporozoites detach from the internal oocyst wall. In the murine and avian species of *Plasmodium,* the final steps of the sporozoite escape process, no empty oocysts were observed, distinct from the species of *Plasmodium* that infect humans. Only one comparative study of *P. gallinaceum* and *P. berghei* oocysts has been published [19]. In both *Plasmodium* species, both completely smooth

(See figure on next page.)
Fig. 4 *Plasmodium falciparum* sporozoites escaping from oocysts. **a** *P. falciparum* oocysts, with numerous completely smooth oocysts (*white asterisks*) and wrinkled oocysts (*black asterisks*) protruding among muscle fibers (Mfb), trachea (Tra), and tracheoles (Tch) that rests on the external surface of the midgut. Magnification = ×350. **b** High-power magnification of the dashed area from figure **a** showing a group of four oocysts attached to the midgut. It is possible to observe surface details of two completely smooth oocysts (S-ooc) and two wrinkled oocysts (W-ooc). Magnification = ×800. **c** Enlarged image of a small portion of the initial process of a single sporozoite actively escaping from a completely smooth oocyst (S-ooc) by creating a hole (*arrows*). Note the rigid perpendicular shape of the escaping sporozoite and the anterior tip (*large arrow*) of the parasite (*large arrow*). Magnification = ×3000. **d** One completely smooth oocyst (S-ooc) with small broken areas (*arrows*) showing a group of twelve escaping sporozoites (Spz). Magnification = ×3500. **e** Flattened opened oocyst (Op-ooc) showing a lateral opening (*asterisk*) with a cluster of escaping sporozoites (Spz) that remain inside. Note hemocytes attached to the oocyst wall (*arrows*) and folded areas (*asterisks*) of the oocyst surface. Magnification = ×1300. **f** Enlarged image of the dashed area from figure **e** showing the oocyst opening and several escaping sporozoites (Spz). Note the "comma-shaped" sporozoites. Oocyst = Op-ooc. Magnification = ×6000. **g** Flattened empty oocyst (E-ooc) showing the lateral opening (*asterisk*). No sporozoites can be seen inside or around the oocyst opening. Magnification = ×1800

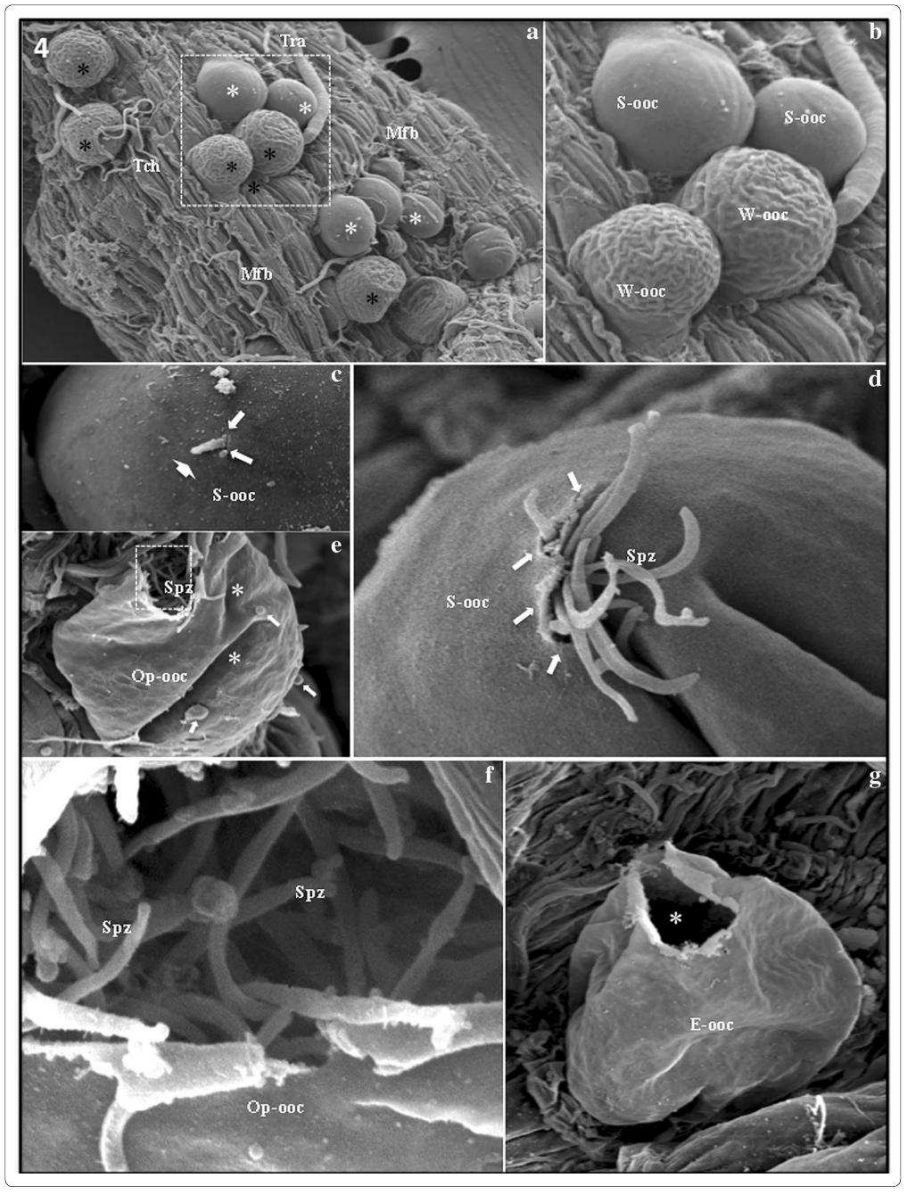
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Table 1 Proportion of the oocysts according to their surface microanatomical details

	P. gallinaceum (n = 138)	*P. berghei* (n = 325)	*P. vivax* (n = 160)	*P. falciparum* (n = 162)
Flattened	7.9 %	2.1 %	11.8 %	6.8 %
Smooth	83.4 %	20 %	85 %	24.8 %
Wrinkled	–	–	–	66.4 %
Wrinkled + smooth[a]	–	58 %	–	–
Cracked	8.7 %	–	–	–
Small openings[b]	–	15.6 %	7.5 %	2.4 %
Large openings[b]	8.7 %	4 %	7.5 %	–

– Oocyst aspects not present in the *Plasmodium* species

[a] Wrinkled/smooth occysts present both characteristic aspects in their surface

[b] Small openings or large openings by our definition was related with small or large fissures considering 1/3 of the oocyst surface

oocysts and rare, wrinkled oocysts were observed, which the authors considered matured oocysts or sample preparation artifacts. Although they only showed two images, they suggested these two *Plasmodium* species have similar sporozoite escape mechanisms.

All *P. vivax* oocysts showed similar completely smooth surfaces, and in this respect, they are morphologically similar to *P. gallinaceum* oocysts. In contrast, two types of *P. falciparum* oocysts were observed: completely smooth and wrinkled oocysts. These oocysts were randomly distributed, sometimes side-by-side, in the mosquito midgut at a 50:50 ratio. Previous studies found that infected *P. falciparum* mosquitoes contained only wrinkled oocysts, but no escaping sporozoites were observed [20, 21]. The authors suggested that the wrinkled surface was characteristic of mature oocysts. However, although we also observed two types of oocysts in *P. falciparum*, sporozoites were only observed escaping from completely smooth oocysts, indicating that completely smooth oocysts contain mature sporozoites. The wrinkled oocysts may be immature oocysts or oocytes that cannot produce healthy, mature sporozoites.

The most noteworthy feature of the two human *Plasmodium* species, *P. vivax* and *P. falciparum,* is the dynamic mechanism of sporozoite escape from oocysts, distinct from that of the laboratory model *Plasmodium* species. Careful observation showed that the first signals of sporozoite escape are identical for the two human *Plasmodium* species: escape begins with a single sporozoite, in a rigid perpendicular position, forcing an exit from through the oocyst wall. The rigid perpendicular sporozoite opens a tiny hole in the oocyst wall with its anterior end. The oocyst wall is composed of two layers; the internal layer is of *Plasmodium* origin and the external thick layer that is derived from the basal lamina of the mosquito midgut [28, 29]. Moreover, in addition to allowing for growth, the capsule must have an ordered structure to allow for precursors and nutrients that support parasite growth and differentiation to enter the oocyst and metabolites to exit it [30, 31]. Subsequently, this tiny hole in the oocyst wall grows larger and allows other sporozoites to escape. Although this first step, with a single sporozoite making a tiny hole in the oocyst wall, is identical between the two species, the subsequent steps of sporozoite escape differ between *P. vivax* and *P. falciparum*. In *P. vivax,* a small group of sporozoites continue, in the same rigid perpendicular position as the first, to actively move forward to enlarge the hole in the oocyst wall. In *P. falciparum* oocysts, small groups of sporozoites escape, and individual sporozoites are flexible comma shapes, characteristic of random motion of the parasite [32–34]. A geometrical model of malaria parasite migration demonstrated that sporozoites could be modeled as self-propelled individuals that can have curved or rigid structures for motion in distinct environments [35]. This programmed rigidness and flexibility of the human *Plasmodium* sporozoites appears to act distinctly in the two species of *Plasmodium,* since it plays a role in opening the oocyst wall, allowing escape.

Molecular mechanisms related to oocyst formation and sporozoite escape have been demonstrated, mainly using mutants of murine *P. berghei*, which infects rodents, but not in *Plasmodium* species that infect humans. It is important to note that these analyses demonstrate that *P. berghei* sporozoites escape from oocysts by a process that harms the oocyst wall. The circumsporozoite (CS) protein, secreted by sporozoites, covers the internal layer of the oocyst wall [36, 37]. It was demonstrated in *P. berghei* that the disruption or deletion of some regions of the CS protein affects the formation and maturation of sporozoites, escape from the oocyst, and subsequent progression of the *Plasmodium* life cycle [38, 39]. Likewise, several other gene deletions have been described that affect *P. berghei* oocyst formation and consequent sporozoite

escape: an oocyst-specific papain-like cysteine protease, known as the egress cysteine protease (ECP1), oocyst capsule protein (PbCAP380), fertilization gene (Pb GEX), lectin adhesive proteins (PbLAPs), protein kinases (PbCDLK), and nuclear forming-like protein (PbMISFIT) [40–48]. The results showed that *P. berghei* sporozoites are liberated from the oocyst by decortication and subsequent dissolution of the oocyst wall, which is consistent with a mechanism involving a proteolytic activity as has been proposed for *P. berghei* [42]. Thus, these findings indicate that proteins that act on the oocyst wall, rather than in the sporozoite, should be considered as target candidate molecules to stop transmission.

Analyses of the sporozoite escape processes in the *Plasmodium* species that infect humans clearly showed the action of the actively protunding sporozoites is dissimilar from that of murine and avian *Plasmodium* species. *Plasmodium* belongs to the phylum Apicomplexa, which is well defined by polarized extracellular stages, which contain specialized secretory organelles named micronemes and rhoptries in their anterior edge. Proteins secreted by these organelles play essential roles in attachment and invasion of target cells, as well as gliding motility, locomotion, and morphological changes [33, 49–52]. The main mode of active locomotion of the sporozoite is an actomyosin-dependent motility that is important for forward locomotion, and penetration and invasion of target cells [53]. In addition, during sporozoite motility, TRAP may coordinate the formation of contact sites and the dissociation of these contact sites from the substrate, including involvement of actin filaments [54, 55]. This raises the possibility that secretory proteins that are involved in the interplay of adhesion molecules and the invasion mechanism, well studied in invasion of host cells, can also play roles in the initial active stage that guides the escape of *P. vivax* and *P. falciparum* from the oocyst.

Careful comparative microanatomical analyses of midguts of mosquitos infected with four distinct *Plasmodium* species allowed us to make novel observations of sporozoite escape from oocysts. The key findings of this study are the morphological features that reveal for first time the mechanisms of sporozoite escape from oocysts of four *Plasmodium* species, including avian, murine, and human malarial parasites. Sporozoites of the four *Plasmodium* species exit oocysts using different mechanisms. The avian *P. gallinaceum* and murine *P. berghei* have been used as experimental models in several laboratories for infection of vertebrates and mosquito vectors. Mice infected with *P. berghei* have been used as laboratory models for human malaria [56–58] and to investigate interaction of the parasite with vectors of human malaria such as *An. gambiae* and *An. stephensi* [59–61]. It is important to state that the findings of the escape of *P. berghei* and *P. falciparum* sporozoites from oocysts were obtained from experimental infections of the same mosquito species, the *An. gambiae*. This fact suggests that the distinct mechanisms of the sporozoite escape is not dependent of the *Anopheles* species but is regulated by the *Plasmodium* species. Nevertheless, it is noteworthy to consider that these *Plasmodium* species differ in the oocyst microanatomical appearance and in the process of the sporozoite escape. Although the molecular mechanism that regulates sporozoite escape remains largely unknown, this study clearly indicates that *Plasmodium* species do not share a common mechanism, as previously thought.

Conclusions

It was demonstrated that sporozoites of the human malarial parasites *P. vivax* and *P. falciparum* escape from the oocyst via a more active process than those of the avian and murine malarial parasites, *P. gallinaceum* and *P. berghei*. Detailed analysis showed that all four have distinct escape mechanisms. Sporozoites that infect humans actively create a hole in the oocyst wall, and are not dependent on the breakdown or dissolution of the oocyst wall for escape. These findings provide a strong basis for future studies of how to block sporozoite escape from oocysts in order to prevent transmission of malaria.

Authors' contributions

ASO, RNP, LCP, KMMC, YTP, BC, RCS carried out the infection experiments of the mosquitoes, participated in the microscopy analysis and drafted the manuscript. LMV, APMD, NBR, MGVB, WMM, AMC participated in the analysis of the results and drafted the manuscript. MVGL, NFCS, MJL, CBM, participated in the design of the study, performed the analysis and helped to draft the manuscript. PFPP conceived the study, in its design and coordination and write the manuscript. All authors read and approved the final manuscript.

Authors' information

ASO and RNP are Ph.D. students of the Graduate Program in Health Sciences of the FIOCRUZ-Minas Gerais. KMMC, YTP and BC are Ph.D. students of the Graduate Program in Tropical Medicine of the UEA/FMT-HVD. APMD, LMV, NBR and LCP are pos-doctoral fellows. PFPP is a senior visiting fellow at the FMT-HVD supported by Amazonas State Research Support Foundation (FAPEAM). PFPP, NFCS, MGVB and MVGL are senior fellows from the Brazilian Council for Scientific and Technological Development (CNPq).

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

Availability of data and material
All data will be made available upon request to all interested researchers.

Consent for publication
All authors read and approved the final manuscript for publication.

Ethics approval and consent to participate
Patient selection criteria were determined by the National Institutes of Health international protocols, and to consent to be part of the research consent form that was approved by the Brazilian Ministry of Health, National Council of Health, National Committee of Ethics in Research (CONEP—approval number 3726).

Also, mice and chickens were maintained at the Animal Care Facility of the FIOCRUZ-MG were used in accordance to a study protocol approved by the FIOCRUZ Ethical Committee for Animal Use (CEUA; License Number LW30/10). It was followed the Public Health Service Animal Welfare Assurance #A4149-01 guidelines according to the National Institutes of Health (NIH) Office of Animal Care and Use (OACU) and NIH animal study protocol (ASP) approved by the NIH Animal Care and User Committee (ACUC), with approval ID ASP-LMVR5.

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Apêndice 5: Artigo 2 *Plasmodium yoelii* nigeriensis (N67) is a robust animal model to study malaria transmission by South American anopheline mosquitoes.

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Alessandra S. Orfano, Ana Paula M. Duarte1, Alvaro Molina-Cruz, Paulo F. Pimenta, Carolina Barillas-Mury.

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RESEARCH ARTICLE

Plasmodium yoelii nigeriensis (N67) Is a Robust Animal Model to Study Malaria Transmission by South American Anopheline Mosquitoes

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Abstract

Malaria is endemic in the American continent and the Amazonian rainforest is the region with the highest risk of transmission. However, the lack of suitable experimental models to infect malaria vectors from the Americas has limited the progress to understand the biology of transmission in this region. *Anopheles aquasalis*, a major vector in coastal areas of South America, was found to be highly refractory to infection with two strains of *Plasmodium falciparum* (NF54 and 7G8) and with *Plasmodium berghei* (mouse malaria), even when the microbiota was eliminated with antibiotics and oxidative stress was reduced with uric acid. In contrast, *An. aquasalis* females treated with antibiotics and uric acid are susceptible to infection with a second murine parasite, *Plasmodium yoelii nigeriensis* N67 (PyN67). *Anopheles albimanus*, one of the main malaria vectors in Central America, Southern Mexico and the Caribbean, was more susceptible to infection with PyN67 than *An. aquasalis*, even in the absence of any pre-treatment, but was still less susceptible than *Anopheles stephensi*. Disruption of the complement-like system in *An. albimanus* significantly enhanced PyN67 infection, indicating that the mosquito immune system is mounting effective antiplasmodial responses. PyN67 has the ability to infect a broad range of anophelines and is an excellent model to study malaria transmission by South American vectors.

Introduction

Malaria, a parasitic disease transmitted by mosquitoes, has a major impact on global public health and threatens the economy of one third of the world's population. In the Americas, 22 countries are affected. In 2015, 660,000 cases of malaria were estimated and 120 million people in the Americas live in areas at risk of malaria [1]. The Amazonian rainforest is the region with the highest risk of transmission, however, the malaria threat extends throughout the Northern regions of South America and across Central America, the Caribbean and Mexico [2]. In

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Abbreviations: APL1, *Anopheles Plasmodium*-responsive leucine-rich repeat 1 protein; LRIM1, leucine-rich repeat immune protein 1; TEP1, Thioester-containing Protein.

Brazil, malaria affects thousands of people every year accounting for 10% of all cases reported outside Africa [1].

Anopheles mosquitoes are the vectors of several *Plasmodium* species that cause malaria in humans, with *Plasmodium vivax* (71%) and *Plasmodium falciparum* (29%) being the most prevalent infections in the Americas [1]. Despite the relative abundance of malaria in the New World, little is known about the biology of the mosquito vectors in this region, when compared to the vast knowledge available for vectors from Africa and Asia. Anopheline mosquitoes diverged from culicines approximately 217 million years ago (MYA;) [3] in the Pangea supercontinent. The three major subgenera of *Anopheles* (*Anopheles*, *Cellia*, and *Nyssorhynchus)* that transmit malaria to humans exhibit differing geographical ranges. Malaria vectors in Africa and Asia belong to the subgenus *Cellia* or *Anopheles* and diverged from the subgenus *Nyssorhynchus* about 125–115 MYA [4] as the landmasses that would become South America and Africa drifted apart. The subgenus *Cellia* is restricted to the Old World, while *Nyssorhynchus* is limited to tropical regions of the New World [5]. Several malaria vectors of the subgenus *Kerteszia* are also present in South America (*e.g. An. cruzzi*, *An. bellator* and *An. neivai*).

The laboratory colonization of American vectors of the subgenus *Nyssorhynchus*, such as *An. aquasalis* and *An. albimanus*, opened the possibility of studying their interactions with *Plasmodium* parasites. *An. aquasalis* is an important vector in coastal areas of South America, and its colonization was achieved in 1995 [6]. This was followed by the colonization of *An. albimanus*, one of the main malaria vectors in Central America, Southern Mexico and the Caribbean [7]. *An. aquasalis* and *An. albimanus* are the only two long-term colonized Central and South American malaria vectors maintained in laboratories that have been used for experimental infections mostly by feeding them on blood of patients from endemic regions infected with *P. vivax*, demonstrating that they can be good models to study the interaction of American vectors with *Plasmodium* species [8]. More recently, *An. darlingi* has been colonized and successfully infected with *P. vivax* [9].

Studies in *An. gambiae* infected with *Plasmodium berghei*, a murine malaria model, revealed that the mosquito complement-like system can greatly limit *Plasmodium* infection [10]. The Thioester-containing Protein TEP1 is a major effector molecule that is stabilized in the mosquito hemolymph by interacting with two leucine-rich proteins, the leucine-rich repeat immune protein 1 (LRIM1) and the *Anopheles Plasmodium*-responsive leucine-rich repeat 1 protein (APL1) [11–13]. LRIM1 silencing results in premature activation of TEP1 and disrupts complement-mediated mosquito antiplasmodial responses, greatly increasing *An. gambiae* infection with *P. berghei* [12, 13], and with some strains *P. falciparum* strains[14] [15].

The establishment of robust animal models has been key to our current understanding of the biology of malaria transmission and the mosquito responses to *Plasmodium* infection. Understanding the parasite/vector interactions that affect vectorial capacity is indispensable for the development of new drugs or vaccines to disrupt transmission. The most widely used laboratory models to study malaria transmission are the *in vitro* production of *P. falciparum* gametocytes, a human malaria parasite, and *in vivo* infection with two murine parasites, *P. berghei* and *P. yoelii*. The two mosquito species most widely studied are the African vector *An. gambiae*, and the Asian vector *An. stephensi*. Mosquitoes can differ widely in their susceptibility to infection with specific *Plasmodium* parasite species [16] and great differences have been documented even between parasite strains [17, 18]. *An. albimanus* can be infected by direct feeding on *P. berghei*-infected mice [19] but, in general, infections are much lower when compared to *An. gambiae* or *An. stpehensi*. *An. albimanus* is readily infected with a *P. falciparum* strain of Brazilian origin (7G8) but is highly resistant to infection with African and Asian strains [18].

The main goal of this study was to establish robust experimental models to study malaria transmission by two colonized New World vectors, *An. aquasalis* and *An. albimanus*. Their susceptibility to laboratorial infections with the human malaria parasite *P. falciparum* and the rodent parasites *P. berghei* and *P. yoelii* was investigated. The role of the mosquito complement-like system in their susceptibility to infections was also evaluated by silencing LRIM1. Our studies show that *Plasmodium yoelii nigeriensis* is a viable model system to study malaria transmission by New World vectors.

Results

Plasmodium falciparum infection in *An. aquasalis*

A major limitation in establishing experimental models for New World vectors is the impossibility of using mosquito vectors that are widely used elsewhere for laboratory infections (such as *An. gambiae* or *An. stephensi*) to ensure the quality of the gametocytes (positive controls) when performing experiments in laboratories located in malaria endemic areas. This problem was circumvented by transiently establishing an *An. aquasalis* colony at the National Institutes of Health (NIH) in the USA. The susceptibility of *An. aquasalis* to *P. falciparum* infection was evaluated using gametocyte cultures from the NF54 strain, a laboratory-adapted line of putative African origin. The quality of the gametocyte cultures was confirmed by simultaneously feeding *An. stephensi* (Nijmengen Sda500) mosquitoes, a laboratory strain that has been genetically selected to be highly susceptible to *P. falciparum* infection [20]. The *An. stephensi* control groups were readily infected with *P. falciparum* NF54, reaching infection prevalences of 88–100% and medians of 30–90 oocysts/midgut (Fig 1A and S1 Fig). In contrast, only one of the *An. aquasalis* mosquitoes out of 53 that fed on the same gametocyte cultures became infected with a single oocyst ($p<0.0001$, Fig 1A and S1 Fig).

We investigated whether the lack of infectivity of *An. aquasalis* was due to activation of the mosquito immune system by silencing LRIM1 expression. We were able to clone a 1,501bp partial sequence of the *An. aquasalis* LRIM1 cDNA (S2 Fig) using primers designed based on the annotated *An. albimanus* LRIM1 transcript (AALB005865-RA). This region has 81% identity to the *An. albimanus* LRIM1 transcript, and the 571bp 3'-end of our partial *A. aquasalis* cDNA has 100% homology to a shorter non-annotated transcript in Vector Base labeled GAMD01000377.1_Aaquasalis_Anoaqua-4332_mRNA. Our partial cDNA sequence (S2 Fig) was used to design primers to generate dsRNA and to evaluate the silencing efficiency.

None of the mosquitoes injected with the dsLacZ control were infected, while 40% of the *An. aquasalis* mosquitoes in which LRIM1 was silenced became infected ($p<0.001$, Fig 1B). However, even after silencing LRIM1 the infection intensity was still low (median of 0) in *An. aquasalis*, when compared to *An. stephensi* with median of 38 oocysts ($p<0.0001$), and a prevalence of 88% ($p<0.001$); indicating that only some of the parasites are eliminated by the *An. aquasalis* immune system.

Recent studies have shown that parasite isolates from different geographic origin can exhibit dramatic differences in infectivity to the same mosquito vector. For example, a *P. falciparum* line of Brazilian origin (7G8) is more effective infecting *An. albimanus* mosquitoes than parasite lines of African origin [18]. It is also well established that oxidative stress [21] and the gut microbiota [22] can affect *Plasmodium* survival. Oral administration of uric acid reduces oxidative stress, decreasing loss of fecundity with age and preventing *Plasmodium* melanization [21, 23]. We tested the susceptibility of *An. aquasalis* to infection with the *P. falciparum* 7G8 line and the effect of reducing the gut microbiota by oral administration of antibiotics solution (Penicillin and Streptomycin). The solution was also supplemented with uric acid, to reduce oxidative stress in the mosquito. Although a few *An. aquasalis* subjected to

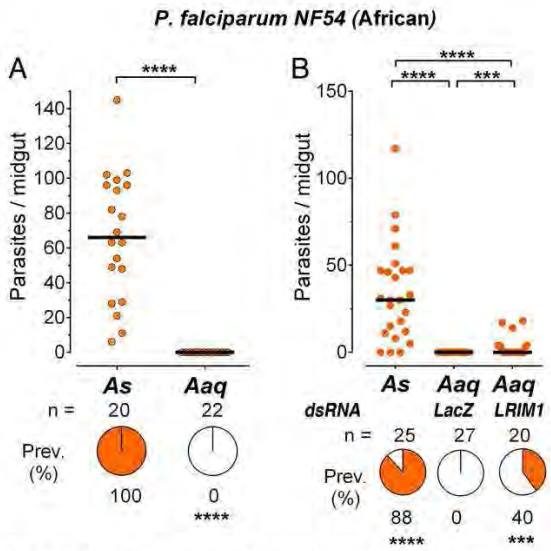

Fig 1. Infection of *A. aquasalis* with *P. falciparum* NF54. (A) Susceptibility of *Anopheles stephensi* (As) and *Anopheles aquasalis* (Aaq) mosquitoes to infection with *Plasmodium falciparum* NF54 strain. (B) Effect of disrupting the mosquito immune system by silencing LRIM1 on Aaq susceptibility to infection. Each dot represents the number of oocysts present on an individual midgut 10–12 days post-infection and the median number of oocysts is indicated by the black line. The medians were compared using the Mann-Whitney test and the infection prevalence using Chi-square (*** p<0.001, **** p<0.0001).

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this treatment became infected with *P. falciparum* 7G8 (Fig 2 and S3 Fig), the prevalence was low (10–12%) when compared to *An. stephensi* (28–76%, p<0.001). None of the *An. aquasalis* females that were not treated with the antibiotic + uric acid mixture became infected (p<0.0001).

Susceptibility of *An. aquasalis* to infection with murine malaria parasites

The *P. berghei* ANKA 2.34 strain can effectively infect *An. albimanus* when mosquitoes are fed ookinetes cultured *in vitro*, and a high prevalence (>90%) and intensity of infection (20–30 oocysts/midgut) can be obtained [19]. Multiple times we attempted to infect females from a *An. aquasalis* colony established in Brazil by direct feeding on *P. berghei* (Anka 2.34-GFP)-infected mice with no success, even when mice parasitemias and exflagellations were optimal (data not shown). We decided to confirm this lack of infectivity at the Laboratory of Malaria and Vector Research (NIH), by infecting *An. stephensi* and *An. aquasalis* using the same mouse, under optimal conditions. As expected, the *An. stephensi* control group was very susceptible to infection with *P. berghei* (Anka 2.34-GFP) with a prevalence of 100% and median of 148 oocysts 7 days post-feeding. In contrast, only three of the *An. aquasalis* females treated with the antibiotic + uric acid solution and injected with a dsLacZ control dsRNA became

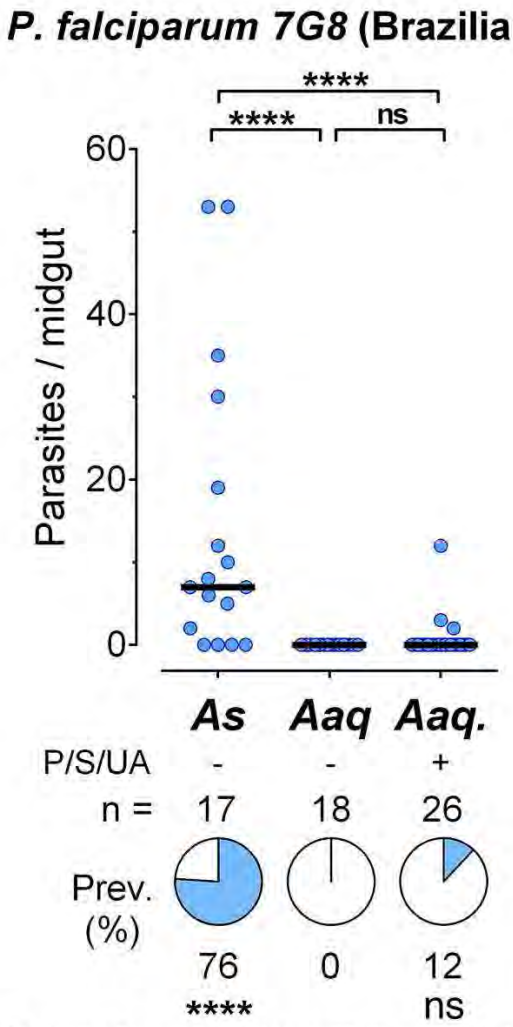

Fig 2. Infection of *A. aquasalis* with *P. falciparum* 7G8. Susceptibility of *Anopheles stephensi* (As) and *Anopheles aquasalis* (Aaq) mosquitoes to infection with *Plasmodium falciparum* 7G8 strain. The effect of oral administration of antibiotics (Penicillin/Streptomycin = P/S) and uric acid (UA) on Aaq infection was also tested. Each dot represents the number of oocysts present on an individual midgut 10–12 days post-infection and the median number of oocysts is indicated by the black line. The medians were compared using the Mann-Whitney test and the infection prevalence using Chi-square (**** p<0.0001).

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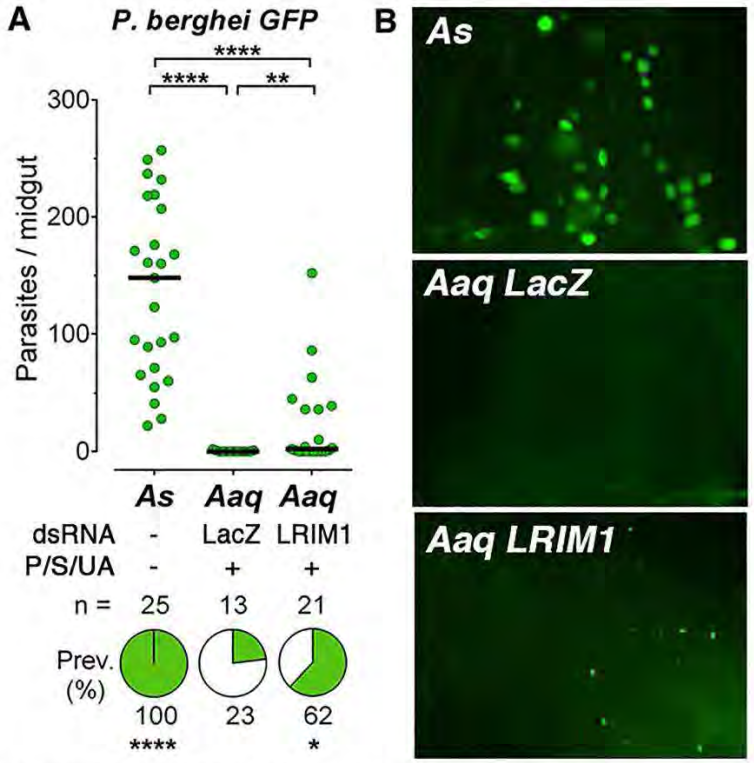

Fig 3. Infection of *A. aquasalis* with *P. berghei*. Susceptibility of *Anopheles stephensi* (As) and *Anopheles aquasalis* (Aaq) mosquitoes to infection with *P. berghei*. (A) Effect of disrupting the mosquito immune system by silencing LRIM1 on Aaq susceptibility to infection. (B) *P. berghei* oocysts in As and Aaq mosquitoes 8 days post infection. Each dot represents the number of oocysts present on an individual midgut 10–12 days post-infection and the median number of oocysts is indicated by the black line. The medians were compared using the Mann-Whitney test and the infection prevalence using Chi-square (* $p<0.05$, **** $p<0.0001$).

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infected with a single oocyst ($p<0.0001$, Fig 3A). Silencing LRIM1 significantly enhanced the infection prevalence to 62% ($p<0.04$), but the intensity of infection remained low (median of 1) ($p<0.01$, Fig 3A). Furthermore, the oocysts that developed were very small at 7 days post-feeding, relative to those in *A. stephensi*, (Fig 3B) indicating that the ookinetes that survived in *An. aquasalis* did not develop normally into the oocysts stage.

Given the extremely low infectivity of *P. berghei*, we decided to test a different murine malaria parasite. We found that *An. aquasalis* was much more susceptible to infection with *P. yoelii nigeriensis* N67 (PyN67) parasites, reaching a consistent high prevalence of infection

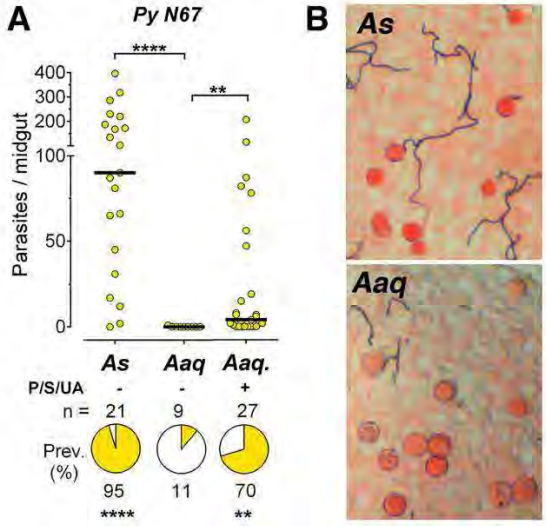

Fig 4. Infection of *A. aquasalis* with *P. yoelii nigeriensis N67*. Susceptibility of *Anopheles stephensi* (*As*) and *Anopheles aquasalis* (*Aaq*) mosquitoes to infection with *P. yoelii nigeriensis N67* (PyN67). (A) Effect of oral administration of antibiotics (Penicillin/Streptomycin = P/S) and uric acid (UA) on *Aaq* infection. (B) PyN67 oocysts in *As* and *Aaq* mosquitoes 8 days post infection. Image of oocysts in *Aaq* mosquitoes treated with P/D + UA. Each dot represents the number of oocysts present on an individual midgut 10–12 days post-infection and the median number of oocysts is indicated by the black line. The medians were compared using the Mann-Whitney test and the infection prevalence using Chi-square (** p<0.01, **** p<0.0001).

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(54–70%) when mosquitoes were treated with antibiotic + uric acid solution ($p<0.01$), (Figs 4A and 5A, and S4 Fig). In contrast to *P. berghei*, PyN67 oocysts developed normally in *An. aquasalis* and had a similar size and appearance as in *An. stephensi* controls (Fig 4B). We were able to recover sporozoites from the salivary glands of *An. aquasalis* (17,000–19,000 spz/mosquito) indicating that PyN67 can complete its life cycle in this mosquito species. The infectivity of these sporozoites to mice was not tested.

Susceptibility of *An. albimanus* to infection with *P. yoelii* N67

An. albimanus is an important malaria vector in Mexico, Central America and the Northern regions of South America. We explored the potential of *P. yoelii* N67 as a model of malaria transmission in this mosquito species. *An. albimanus* is more susceptible to infection with PyN67 than *An. aquasalis* (Fig 5A), reaching a high prevalence (73%) of infection and a median of 36 oocysts/midgut without administration of antibiotics or uric acid, while no oocysts could be detected in *An. aquasalis* females ($p<0.0001$, Fig 5A). However, the intensity and prevalence of infection was significantly lower than in *An. stephensi* ($p<0.0001$, Fig 5B and S5 Fig). PyN67 oocysts also developed normally in *An. albimanus* (Fig 5C). Injection of

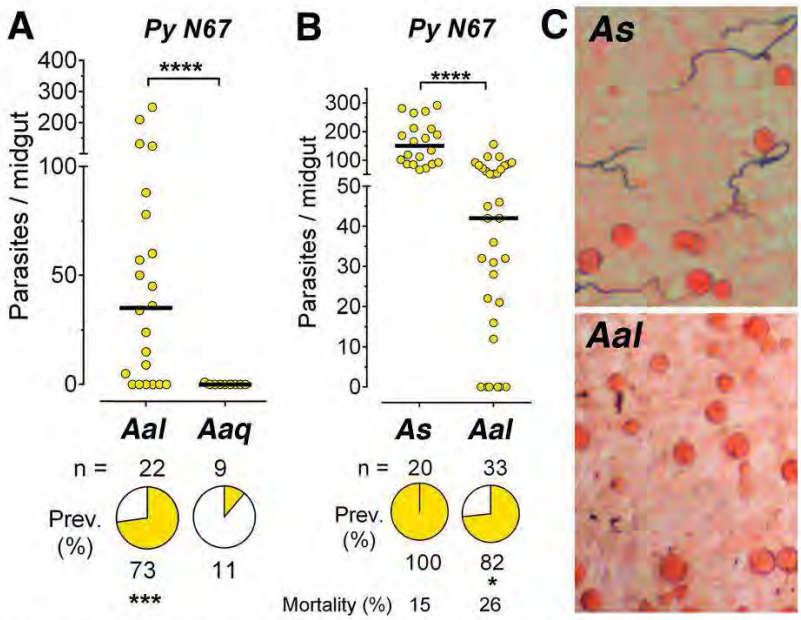

Fig 5. Infection of *A. albimanus* with *P. yoelii nigeriensis N67*. (A and B) Susceptibility of *Anopheles albimanus* (Aal), *Anopheles aquasalis* (Aaq) and *Anopheles stephensi* (As) mosquitoes to infection with *P. yoelii nigeriensis N67* (PyN67), without antibiotics or uric acid. (C) PyN67 oocysts in *As* and *Aal* mosquitoes 8 days post infection. Each dot represents the number of oocysts present on an individual midgut 10–12 days post-infection and the median number of oocysts is indicated by the black line. The medians were compared using the Mann-Whitney test and the infection prevalence using Chi-square (* $p<0.05$, *** $p<0.001$, **** $p<0.0001$).

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dsLacZ control dsRNA had no effect, while silencing LRIM1 significantly increased the intensity of infection ($p<0.001$, Fig 6A and S6 Fig), indicating that the *An. albimanus* complement-like system limits PyN67 infection.

We were able to recover a small number of sporozoites from infected salivary glands of *An. albimanus* mosquitoes (200–500 spz/mosquito), and they were used to infect a recipient mouse. The infection was successful when 1000 PyN67 sporozoites extracted from *A. albimanus* salivary glands were injected IV into a BalbC mouse, indicating that PyN67 parasites can complete their development in *An. albimanus* and generate infectious sporozoites. In general, after completing one developmental cycle in *An. albimanus*, mosquito infections were high, often reaching medians of more than 100 oocysts in *An. stephensi* (Fig 6B and 6C and S7 Fig). Interestingly, these parasites seemed to be more pathogenic to *An. albimanus* mosquitoes, as they caused 6–9% mortality in *A. stephensi*, while in *An. albimanus* mortality was significantly higher 20–39% ($p<0.01$).

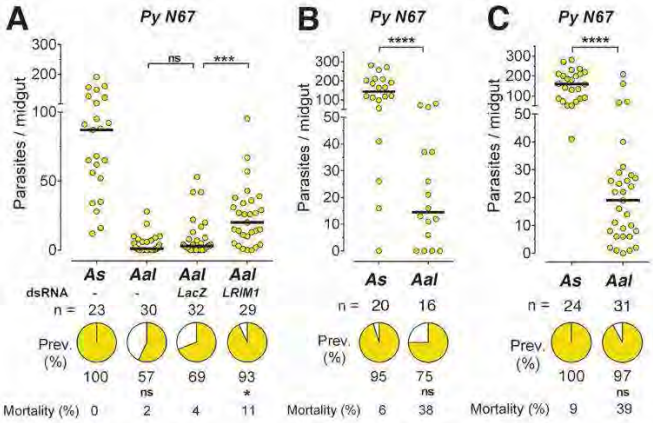

Fig 6. Effect of disrupting the mosquito immune system on *PyN67* infection in *A. albimanus*. Susceptibility of *Anopheles stephensi* (*As*) and *Anopheles albimanus* (*Aal*) mosquitoes to infection with *P. yoelii nigeriensis N67* (PyN67). (A) Susceptibiltiy to infection and effect of disrupting the mosquito immune system by silencing LRIM1. (B and C) Susceptibility of infection and mortality after PyN67 completed one developmental cycle in *An. albimanus*. Each dot represents the number of oocysts present on an individual midgut 10–12 days post-infection and the median number of oocysts is indicated by the black line. The medians were compared using the Mann-Whitney test and the infection prevalence using Chi-square (*p<0.05, *** p<0.001, **** p<0.0001, ns = not significant).

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Discussion

An. aquasalis is susceptible to infection with *P. vivax* [24] and this experimental system has been used to investigate the role of the STAT pathway [25] and reactive oxygen species [26] on mosquito susceptibility to infection. Recent studies indicate that *P. falciparum* evasion of mosquito immunity is mediated by the *Pfs47* gene and is critical for parasite survival. Different haplotypes of *Pfs47* are circulating in different continents and they are major determinants of vector/parasite compatibility [18]. *An. gambiae* mosquitoes are very susceptible to infection with two different *P. falciparum* lines of African origin (NF54 and MRA1181), while *An. albimanus* is highly refractory to infection with these isolates. Conversely, *An. albimanus* is more susceptible to infection with a *P. falciparum* line (7G8) of Brazilian origin than *An. gambiae* [18]. The lack of compatibility between isolates from a different continent can be overcome by disrupting the mosquito complement-like system, indicating that the mosquito immune system is selecting for parasites that express certain haplotypes of *Pfs47* and can evade immunity [18]. We found that *An. aquasalis* is almost completely refractory to infection with *P. falciparum* NF54 parasites, however, although disrupting the mosquito complement-like system by silencing LRIM1 significantly increased the prevalence and intensity of infection, the infection levels are much lower than in *An. stephensi* controls or in *An. albimanus* females in which LRIM1 expression was silenced [18]. This suggests that, besides the mosquito immune system, some other factor(s) in *An. aquasalis* is probably responsible for the low infectivity with *P. falciparum* NF54 parasites. This is in agreement with the observation that *An. aquasalis* is also highly refractory to infection with a *P. falciparum* (7G8) line expressing the most common

Pfs47 haplotype in Brazil. Oral administration of antibiotics and uric acid allowed survival of a few parasites, but the prevalence and intensity of infection were still low. While it is clear that *An. aquasalis* is an important vector of *P. vivax* malaria in Brazil (reviewed by [8]) and Guyana [27], our findings indicate that this mosquito species is not a competent vector of *P. falciparum* malaria with the two different lines tested. In the Amazon region, *An. darling*, *An. albitarsis* and *An. rondoni* have been documented as vectors of *P. falciparum* by direct immunodetection of sporozites, with *An. darling* being the most prevalent infected species. This kind of direct evidence for *An. aquasalis* as a major vector of *P. falciparum* malaria is not available.

An. aquasalis is less susceptible to infection with *P. berghei* and *P. yoelii* N67 than *An. albimanus*. This could be due to differences in physiology or in the microbiota, as *An. aquasalis* larvae have adapted to brackish water. Reducing the microbiota by oral administration of antibiotics and disruption of the *An. aquasalis* immune system was able to rescue some low level of *P. berghei* infection, indicating that a few ookinetes were successful in invading the midgut and transformed into oocyts. However, the oocysts that formed were very small, indicating that they did not develop properly (Fig 3B). This could be due to physiological conditions in *An. aquasalis* that do not provide and adequate environment for the developing oocysts or to late phase immune responses that target the oocyst stage of the parasite. *An. aquasalis* females treated with antibiotics and uric acid were much more susceptible to infection with PyN67 than with *P. berghei* and the oocysts that developed were normal in size; indicating that murine PyN67 infection is a good animal model to study malaria transmission by *An. aquasalis*.

An. albimanus is more susceptible to PyN67 infection than *An. aquasalis*, and it is readily infected without the need for oral administration of antibiotics or uric acid. It also supports normal oocyst development. Silencing the *An. albimanus* immune system enhances infection, indicating that mosquitoes are mounting an active immune response to infection. Furthermore, PyN67 salivary gland sporozoites were infectious to mice, making it possible to close the transmission cycle under laboratory conditions. In all experiments, *An. stephensi* was more susceptible to infection than *An. aquasalis* and *An. albimanus*, probably because this line was genetically selected to be highly susceptible to *P. falciparum* infection and these mosquitoes take very large blood meals under laboratory conditions. We conclude that different anopheline mosquito species differ broadly in their susceptibility to infection with different *Plasmodium* species. *P. yoelii* N67 appears to have a broad ability to infect many different mosquito species, including New World vectors, making it an excellent model system to study malaria transmission.

Materials and Methods

Ethics statement

Public Health Service Animal Welfare Assurance #A4149-01 guidelines were followed according to the National Institutes of Health Animal (NIH) Office of Animal Care and Use (OACU). These studies were done according to the NIH animal study protocol (ASP) approved by the NIH Animal Care and User Committee (ACUC), with approval ID ASP-LMVR5. All the animal procedures used in this study have been approved with the NIH Animal Care and User Committee (ACUC).

Mosquito rearing

An. Stephensi, *Anopheles aquasalis* and *Anopheles albimanus* mosquitoes were reared at 27˚C and 80% humidity on a 12-h light/dark cycle under insectary conditions [21]. All mosquito larvae were readed in unchlorinated water, by allowing chorinated water to rest for 48h in an open container. Tetramin Tropical Flakes® fish food was grinded into fine powder using a

coffee mill and used as feeding source for larvae. *An. aquasalis* larvae were reared in water that contained table salt at a final concentration of 2g/L. *An. aquasalis* adult females were fed on cow blood for colony maintenance by membrane feeding (using hog gut sausage casings as membranes), while *An. albimanus* and *An. stephensi* females were fed on live anesthetized chickens.

Mosquitoes were fed a 10% sucrose solution in a cotton ball until two days before the infective blood meals, when some experimental groups were switched to drinking a 1% uric acid solution with Penicillin (100 units/ml) and Streptomycin (0.1 mg/ml) in water, and they obtained sugar from a solid sugar cube placed on top of the cage. They were fed this solution and the sugar cube until the end of the experimental infections. The solution was fed by placing it in an inverted glass test tube with a cotton ball plug and was changed daily.

Plasmodium infections

Female mosquitoes (4–5 days old) were infected by feeding them blood meals with mature stage IV and V *P. falciparum* gametocyte cultures (NF54 or 7G8 strains) through a membrane feeder at 37˚C for 30 min [17]. Human blood was obtained from the Interstate Blood Bank, Memphis, Tennessee. Membrane feedings were done using hog gut sausage casings as membranes.

For *P. yoelii nigeriensis* (N67 strain) and *P. berghei* infections, parasites from frozen stocks were administered via intraperitoneal (IP) injection to 3- to 5-week-old BALB/c female mice. When the parasitemia of the donor mice reached 5–10% (in about 5–7 days), the infected blood was taken by cardiac puncture and transferred to a healthy mouse. Mouse parasitemia was determined by light microscopy inspection of Giemsa-stained thin blood smears obtained by tail snip. Experimental BALB/c mice were infected by intraperitonealy (IP) injection 20–30μl fresh blood from the donor mice. This recipient mouse was used to infect mosquitoes when it reached 3–5% parasitemia 2–3 days after inoculation. Female mosquitoes (5–7 days old) were infected by direct feeding on anesthetized infected mice. For *P yoelii* infections, mosquitoes were maintained at 24˚C, while *P. berghei*-infected mosquitoes were kept at 21˚C (their respective permissive temperatures for gametogenesis). Both were kept at 80% humidity. *P. yoelii* midgut infection was assessed by light microscopy 10–11 days after feeding with mercurochrome staining (0.1% in water) [28]; while *P. berghei* midgut infection was assessed by fluorescence microscopy of GFP parasites 7 days after feeding. Oocysts on individual midguts were counted to determine the prevalence and intensity of infection.

The number of PyN67 sporozoites recovered from *An. aquasalis* salivary glands was between 17,000–19,000 spz/mosquito. Their infectivity to mice was not tested. The number of sporozoites recovered from *An. albimanus* salivary glands was between 200–500 spz/mosquito. We injected 1000 *P. yoelii* N67 sporozoites extracted from *An. albimanus* salivary glands via IV into the tail vein of a Balb/c mouse in a volume of 100 μl of RPMI with 10% mouse serum, and the animal became infected.

RNAi gene silencing

Individual female *A. gambiae* mosquitoes were injected 1–2 d after emergence as previously described [21]. Briefly, mosquitoes were injected with 69 nL of a 3 μg/μL dsRNA solution 3–4 d before receiving a Plasmodium-infected blood meal. The control dsRNA (dsLacZ) was produced as previously described [21]. Double-stranded dsLacZ RNA was generated by introducing T7 promoters (in bold letters) thought PCR amplification of the cloned insert using the following vector primers: M13F 5' GTAAAACGACGGCCAG 3' and M13Rev- T7 5'CTCGAG

TAATACGACTCACTATAGGGCAGGAAACAGCTATGAC3'. The PCR product was used as template to generate dsRNA using T7 RNA polymerase, as described below.

The *An. aquasalis* LRIM1 dsRNA was synthesized with primers designed based on the partial An. aquasialis LRIM1 cDNA sequence (S2 Fig). The sequence of the primers used is: LRIM1_AaqFw, 5′- **TAATACGACTCACTATAGGG**TTGTACGGCACGGTAAACCT-3′, and LRIM1_AaqRv, 5′-**TAATACGACTCACTATAGGG**CCACGGTAGCTTGTTGTGC-3′ (PCR conditions were 94˚C for 3min; 40 cycles of 94˚C for 30 s, 59˚C for 30 s, and 72˚C for 1min; final extension, 72˚C, 5 min).

The primers used for *An. albimanus* LRIM1 were as follows: two external PCR primers were used, and the product of the first amplification was used as template for a second one using internal primers with the following sequences:

LRIM1_AalExFw, 5′-AAGGTTGAGCCGAAGAATGA-3′, andLRIM1_AalExRv,5′-GCACTTCCCATGCTGCTAAT-3′ (PCR conditions were 94˚C for 3min; 35 cycles of 94˚C for 30 s, 55˚C for 30 s, and 72˚C for 1 min; final extension, 72˚C, 5 min); for internal PCR (primers containing T7 promoter): LRIM1_AalInFw, 5′-**TAATACGACTCACTATAGGG**CTGTACGGCACCGTTAACCT-3′, and LRIM1_AalInRv, 5′-**TAATACGACTCACTATAGGG**AGCTTGTTGTGCGAAAGGTC-3′ (PCR conditions were 94˚C for 3min; 40 cycles of 94˚C for 30 s, 59˚C for 30 s, and 72˚C for 1min; final extension, 72˚C, 5 min; using 1 μL of a 1–20 dilution of the external primer PCR).

RNAs were synthesized simultaneously from the template, annealed and purified using the T7 RNAi Mega-script kit (Ambion) following the procedure recommended by the manufacturer. dsRNA products were eluted in water to a final concentration of 3 μg/μl. A volume of 69nl of dsRNA preparation was injected into the thorax of cold anesthetized, 2–3 day-old female mosquitoes using a nano-injector (Nanoject; Drummond Scientific, Broomall, Pennsylvania, USA) fitted with a glass capillary needle. The dsLacZ RNA was used in each experiment to control for any unspecific effect of dsRNA injection. Mosquitoes received a *Plasmodium*-infected blood meal 2–3 day post-dsRNA injection.

qRT-PCR gene expression

Total RNA was isolated from 15 to 20 mosquito midguts using Trizol (Invitrogen) and cDNA synthesis was performed using QuantiTect Reverse Transcription Kit (Qiagen). Gene-expression analysis was measured by SYBR green qRT-PCR (DyNAmo HS; New England Biolabs) in a CFX96 system (Biorad). Gene expression was assessed using two to three technical replicates and three biological replicates. The *An. gambiae* ribosomal protein S7 was used as an internal reference to normalize each sample for the amount of template present. Fold-change was calculated using the 2−ΔΔ Ct method.

An. aquasalis and *An. albimanus* LRIM1 gene silencing was assessed in whole sugar-fed mosquitoes by quantitative real-time PCR (qPCR) using the S7 ribosomal protein gene as internal reference. The primers used for qPCR for *A. aquasalis* were as follows: LRIM1_Aaq_qPFw, 5′-ACCTCAGCGGTAACAAGGTG-3′; LRIM1_Aaq_qPRv; 5′- CTGCGGGTCCTTATTGTTTG-3′; S7_Aaq_qPFw, 5′- ATCCTGGAGCTGGAGATGAA-3′; and S7_Aaq_qPRv, 5′- ACGATGGCCTTCTTGTTGTT-3′.

The primers used for qPCR for *A. albimanus* were as follows: LRIM1_Aal_qPFw, 5′-GACAAAAGTGTGCGCTTTGA-3′; LRIM1_Aal_qPRv; 5′-CACTCCCGGATTAGAGCTTG-3′; S7_Aal_qPFw, 5′-ACCTGGACAAGAACCAGCAG-3′; and S7_Aal_qPRv, 5′-GTTTTCTGGGAATTCGAACG-3′. The silencing efficiency in dsRNA-injected mosquitoes was 94–98% for *An. aquasalis* LRIM1 and 85–98% for *An. albimanus* LRIM1, relative to dsLacZ-injected controls.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 5 (GraphPad software). Analyses derived from at least two independent biological replicates. Gene-expression data were analyzed using Student's t test on mean value of all independent experiments. Infection intensity of oocysts was compared with each other using Mann–Whitney tests, infection prevalence and overall mortality were compared using χ^2 analysis.

Supporting Information

S1 Fig. Susceptibility of *Anopheles stephensi* (As) and *Anopheles aquasalis* (Aaq) mosquitoes to infection with *Plasmodium falciparum* NF54 strain.
(DOCX)

S2 Fig. Partial nucleotide sequence of *An. aquasalis* LRIM1 cDNA.
(DOCX)

S3 Fig. Susceptibility of *Anopheles stephensi* (As) and *Anopheles aquasalis* (Aaq) mosquitoes to infection with *Plasmodium falciparum* 7G8 strain.
(DOCX)

S4 Fig. Susceptibility of *Anopheles stephensi* (As) and *Anopheles aquasalis* (Aaq) mosquitoes to infection with *P. yoelii nigeriensis N67* (PyN67).
(DOCX)

S5 Fig. Susceptibility of *Anopheles stephensi* (*As*) and *Anopheles albimanus* (*Aal*) to infection with *P. yoelii nigeriensis N67* (PyN67), without antibiotics or uric acid.
(DOCX)

S6 Fig. Susceptibility of *Anopheles stephensi* (*As*) and *Anopheles albimanus* (*Aal*) mosquitoes to infection with *P. yoelii nigeriensis N67* (PyN67), and effect of disrupting the mosquito immune system by silencing LRIM1 on *Aal* susceptibility to infection.
(DOCX)

S7 Fig. Susceptibility of *Anopheles stephensi* (*As*) and *Anopheles albimanus* (*Aal*) mosquitoes to infection with *P. yoelii nigeriensis N67* (PyN67) and mortality after PyN67 completed one developmental cycle in *An. albimanus*.
(DOCX)

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Apêndice 6: Artigo 3 An overview of malaria transmission from the perspective of Amazon *Anopheles* vectors

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An overview of malaria transmission from the perspective of Amazon *Anopheles* vectors

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In the Americas, areas with a high risk of malaria transmission are mainly located in the Amazon Forest, which extends across nine countries. One keystone step to understanding the Plasmodium *life cycle in* Anopheles *species from the Amazon Region is to obtain experimentally infected mosquito vectors. Several attempts to colonise* Anopheles *species have been conducted, but with only short-lived success or no success at all. In this review, we review the literature on malaria transmission from the perspective of its Amazon vectors. Currently, it is possible to develop experimental* Plasmodium vivax *infection of the colonised and field-captured vectors in laboratories located close to Amazonian endemic areas. We are also reviewing studies related to the immune response to* P. vivax *infection of* Anopheles aquasalis*, a coastal mosquito species. Finally, we discuss the importance of the modulation of* Plasmodium *infection by the vector microbiota and also consider the anopheline genomes. The establishment of experimental mosquito infections with* Plasmodium falciparum, Plasmodium yoelii *and* Plasmodium berghei *parasites that could provide interesting models for studying malaria in the Amazonian scenario is important. Understanding the molecular mechanisms involved in the development of the parasites in New World vectors is crucial in order to better determine the interaction process and vectorial competence.*

Key words: *Anopheles* - *Plasmodium* - transmission - Amazon vectors

Malaria is an infectious disease that has a major impact on global public health and the economy, with an estimated 3.4 billion people at risk. Currently, malaria threatens almost one third of the world's population in 104 tropical countries and territories where it is considered an endemic disease. The World Health Organization (WHO) estimates that 207 million cases of malaria occurred globally in 2012 and led to 627,000 deaths. Africa, South-East Asia and the Eastern Mediterranean were the regions with the highest numbers of reported cases and deaths reported, mainly in children under five years of age (WHO 2013).

In the Americas, 22 countries are affected by malaria, with approximately 1.1 million cases and 1,100 deaths registered in 2010. In this continent, 30% of the population is considered to be at risk and 8% are classified as being at high risk. Areas with a high transmission risk are mainly located in the Amazonian rainforest, which extends across nine countries including Brazil, Bolivia, Colombia, Ecuador, Peru, Venezuela, Guyana, Suriname and French Guiana. Brazil and Colombia accounted for 68% of the malaria cases in 2011 (PAHO 2011, WHO 2013).

In Brazil, approximately 241,000 clinical cases and 64 deaths were registered in 2012, most of them (99.88%) in the Amazon Region where malaria is endemic in nine states, namely, Acre, Amapá (AP), Amazonas (AM), Mato Grosso, Pará (PA), Rondônia, Roraima, Tocantins and Maranhão. PA and AM registered almost 70% of the cases in 2012; 14.4% were in urban areas, 25% in gold mine exploitation areas and the others were in rural settlements and indigenous areas (MS/SVS 2013, SVS 2013).

A gradual reduction in the overall number of cases has been observed over the last five years, but there has also been a significant increase in the number of cases in the Brazilian Amazon Region in 2012. Factors that contributed to the increased transmission of malaria include intensive and disorganised occupancy on the outskirts of cities, deforestation and artificial fishponds (MS/SVS 2013, SVS 2013).

Outside the Amazon Region, there were 914 cases registered in 2012 in different Brazilian states, mainly in São Paulo (SP) (188), Rio de Janeiro (130), Minas Gerais (105), Goiás (82) and Piauí (72) (SVS 2013). Most of these cases were due to migration from the Amazon

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Region or from the African continent, but a few were autochthonous from the endemic Atlantic Forest endemic region where few foci are maintained (Rezende et al. 2009, Duarte et al. 2013, Neves et al. 2013).

Malaria is due to infection by a parasitic protozoa of the *Plasmodium* genus. Several *Plasmodium* species infect humans and other animals, including birds, reptiles and rodents. In Brazil, three human *Plasmodium* parasites are prevalent. *Plasmodium vivax* is the predominant species (83.81%) and is responsible for cases associated with severe clinical complications and death (Alexandre et al. 2010, Costa et al. 2012, Lacerda et al. 2012). The prevalence of *Plasmodium falciparum* (13.15%) has declined in the last decade, whilst *Plasmodium malariae* is the least prevalent species (0.037%). However, these numbers may be underestimated because the thick blood smear method that is used for routine malaria diagnosis may lead to misidentification of the species (Cavasini et al. 2000).

Plasmodium cycle in the vector

Mosquitoes of the *Anopheles* genus are the vectors of the *Plasmodium* species, the causative agents of malarial disease. More than 400 species of the *Anopheles* mosquito have been described and approximately 70 these species are potential vectors of malaria that affect humans (Sinka et al. 2012). In the natural vector, the life cycle starts when the female *Anopheles* mosquito takes a blood meal from an infected vertebrate host and ingests gametocytic forms of the parasite that are present in the blood (Smith et al. 2014).

One mosquito ingests an average of 10^3 gametocytes in an infected blood meal. Within minutes after the infective blood meal, these gametocytes undergo maturation inside the lumen of the midgut, which generates micro and macrogametocytes that will be fertilised and produce a diploid zygote (Sinden 1999). The mature zygote will differentiate into the mobile form of the parasite known as the ookinete *via* a process that can take up to 16-24 h, depending on the *Plasmodium* species (Ghosh et al. 2000, Dinglasan et al. 2009). This process starts with the exflagellation of the gametocytes in the mosquito's midgut after ingestion of the infected blood meal. Exflagellation will lead to the formation of the micro and macrogametocytes and occurs mainly due to differences in temperature and pH and the production of xanturenic acid by the mosquito (Billker et al. 1997, 1998). The formation of the zygote occurs after fertilisation of the micro and macrogametocytes and will eventually differentiate into an ookinete. This development will only occur if the parasites are able to defeat the action of the digestive enzymes that are secreted by the epithelium and are active throughout the midgut. It is believed that the ookinetes in the outer parts of the blood meal will die first from the actions of these digestive enzymes and the ookinetes that are closer to the interior of the blood meal and consequently farther away from the effects of the enzyme, will have a longer time in which to differentiate and survive the actions of the enzyme (Abraham & Jacobs-Lorena 2004). The ookinete, which is the mobile form of the parasite, will move and penetrate the peritrophic matrix (PM) and pass through the intestinal epithelium before transforming into an oocyst (Smith et al. 2014).

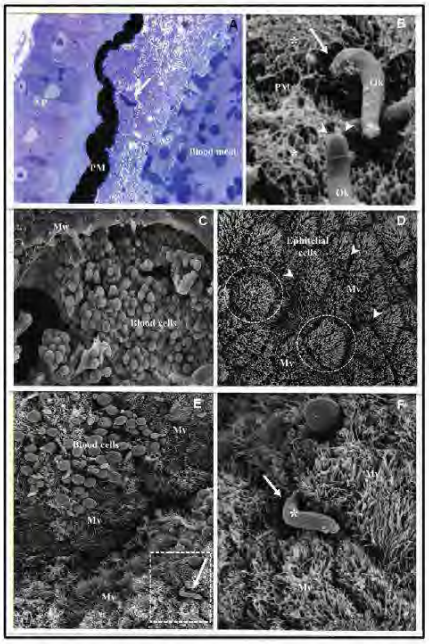

Fig. 1: histology (A) and scanning electron microscopy (SEM) (B-F) of *Anopheles aquasalis* midguts after a *Plasmodium vivax* infective blood meal. A: historesin section of a midgut stained with Giemsa. The peritrophic matrix (PM) sturdily stained in black is separating the midgut epithelium (Ep) from the blood meal. Note an ookinete (Ok) (arrow) close to the PM; B: SEM of an opened midgut showing two Oks over the PM. Observe the fibrous aspect (asterisks) of the internal side of the PM. One Ok is crossing the PM throughout the fibre layer (large arrow). Another Ok is showing details of its anterior extremity (arrowheads); C: small magnification of an opened midgut showing the blood meal containing the numerous blood cells. Note a portion of the midgut wall (Mw); D: large magnification of an opened midgut showing details of the epithelial cells. The epithelial cells have polygonal shapes (circles) and their surfaces are covered by microvilli (Mv). Note the clefts (arrowheads) among the epithelial cells; E: small magnification of an opened midgut with blood cells of the blood meal. Note inside the square area one Ok (arrow) penetrating the Ep Mv; F: large magnification of the square area of E in the Figure showing details of the Ok penetration. Note the Ok (asterisk) extremity inserted in a cleft (asterisk) among the epithelial cell Mv.

The PM is a layer comprised of chitin, proteins and proteoglycans that surround the blood meal that has been ingested (Fig. 1). Physical distension caused by the ingestion of the blood and the blood meal itself are signals for the mosquito's midgut to induce the formation of the PM. This matrix is seen as a physical barrier to many parasites as it prevents their contact with the insect gut (Ghosh et al. 2000). Several studies have suggested that *P. falciparum* and *Plasmodium gallinaceum* may secrete chitinase additional to that already produced by the in-

sect which would allow the parasite to accomplish three crucial steps in the infection of the invertebrate host: (i) penetrate through the PM, (ii) escape the deadly action of digestive enzymes and (iii) successfully invade the epithelial cells of the intestine (Huber et al. 1991, Dessens et al. 1999, Vinetz et al. 1999, 2000). The details of the penetration of the PM by the ookinete are seen in Fig. 1A, B. The recently transformed ookinete moves in the direction of the mosquito epithelium (Fig. 1A) and penetrates the PM by introducing its anterior extremity into the fibrous layer of the internal side of the PM (Fig. 1B).

The penetration of the *Plasmodium* ookinete into the midgut epithelium is an important step in the infection of mosquitoes and has been thoroughly studied previously (Fig. 1B-F). The epithelial cells have polygonal shapes and their surfaces are covered with microvilli (Fig. 1D). The ookinete penetrates the microvilli clefts that exist among the epithelial cells toward their anterior extremity (Fig. 1E, F) in order to initiate the invasion process.

Different theories have arisen regarding the ookinete's strategies for penetration and invasion of the epithelial cells and escaping detection by the host's immune system. After several years without any conclusive studies on how the ookinete invades the mosquito epithelium, Shahabuddin and Pimenta (1998) used an in vitro system to study the interaction of *P. gallinaceum* with *Aedes aegypti*. The methodology consisting of the incubation of the parasites with dissected midgut was successfully applied to a study of the *Leishmania*-vector interaction (Pimenta et al. 1992, 1994). The result suggested the existence of specialised cells in the midgut epithelium of *Ae. aegypti* that the authors called Ross cells, which would serve as a specific entry point for the ookinete (Shahabuddin & Pimenta 1998). Subsequently, Han et al. (2000) proposed a time bomb theory in which parasites invade any epithelial cell in the midgut and this process of penetration triggers an immune response, causing this particular cell to begin apoptosis. However, a conclusive report from Barillas-Mury's group at National Institute of Allergy and Infectious Diseases that was completed with our collaboration (Gupta et al. 2005) indicated that *Ae. aegypti* and *Anopheles stephensi* differ in their mechanisms of epithelial repair after *Plasmodium* ookinete invasion. *An. stephensi* damaged cells *via* an actin-mediated budding-off mechanism when invaded by either *Plasmodium berghei* or *P. gallinaceum*. In *Ae. aegypti*, the midgut epithelium is repaired by a unique actin cone zipper mechanism that involves the formation of a cone-shaped actin aggregate at the base of the cell that closes sequentially, expelling the cellular contents into the midgut lumen as it brings together healthy neighbouring cells. This study had important findings: (i) it determined that the apparent target cells used by *P. gallinaceum* to invade the vector epithelium were in fact an in vitro artifact; the Ross cells are believed to represent cells that have lost their integrity and some of their cytoplasmic contents after parasite invasion and (ii) these studies indicated that the epithelial responses of different mosquito vectors to *Plasmodium* depend on the vector-parasite combinations and are not universal.

After crossing the epithelial layer of the gut, the ookinetes will remain between the intestinal epithelium and the basal lamina, at which point the maturation of the oocyst will occur. A simple method of staining with mercurochrome (Merbromin) solution is useful for the identification of infected midguts. The rounded oocysts can be seen in bright red (Fig. 2A, B). Scanned electron microscope images of the external side of the infected midguts are valuable for showing the morphological aspects of the developing oocysts (Fig. 2C-F). These oocysts appear as protruding structures among the muscle fibres of the midgut wall (Fig. 2D). Some haemo-

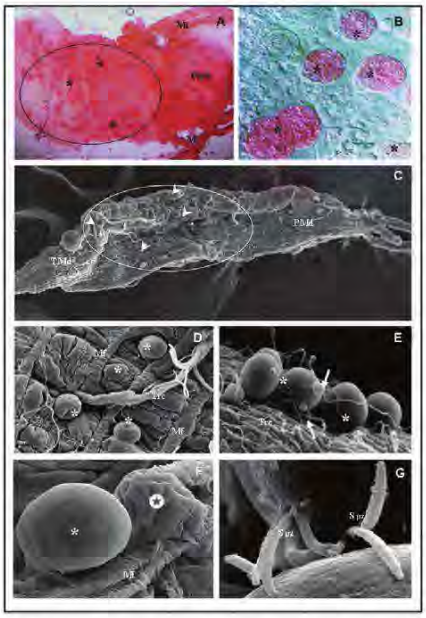

Fig. 2: optical microscopy (OM) and scanning electron microscopy (SEM) of *Anopheles aquasalis* midguts infected with *Plasmodium vivax*. A: small magnification of a dissected infected midgut stained with commercial mercurochrome and visualised by an OM. Note in the elliptical area the presence of numerous oocysts (asterisks); B: large magnification image of the A in Figure. Observe the granular aspects of the developing rounded oocysts (asterisks) in the midgut wall; C: SEM small magnification image of a dissected infected midgut. Note inside the elliptical area the presence of several rounded oocysts (arrowheads) protruding from the midgut wall. The oocysts are concentrated in the transition region between the thoracic midgut (TMd) and the posterior midgut (PMd); D: SEM image of oocysts (asterisks) protruding among the microfibres (Mf) that are presenting outside the midgut wall; E: a group of oocysts (asterisks) are seen protruding on the midgut wall. They are surrounding by small tracheoles (Trc). Two haemocytes (arrows) are attached to one oocyst; F: a large magnification view of two oocysts showing one with a smooth surface (asterisk) and another with shrunk surface (black star) possibly due to the liberation of sporozoites (Spz) into the haemocoel; G: large magnification of SEM images of a group of Spz that already escaped from the oocysts and are free in the mosquito haemocoel; Mt: Malpighian tubules.

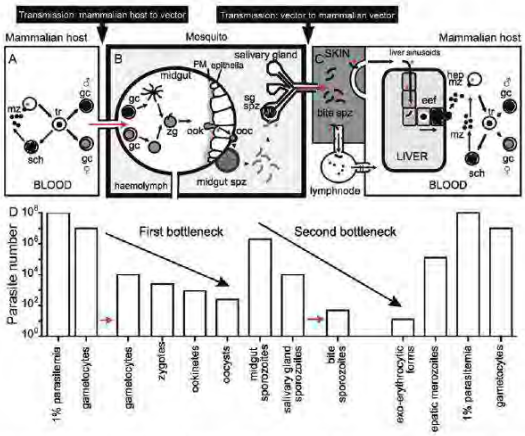

Fig. 3: parasite load inside the vertebrate and invertebrate hosts. Qualitative view of the major steps in the life-cycle of *Plasmodium* parasites inside the mammalian host (A-C) and the mosquito vector (B). Invasive steps are marked with a red asterisks and parasite transmission by red arrows. A: merozoites (mz) invade red blood cells (RBCs) and transform in trophozoites (tr). After asexual division, tr mature in schizonts (sch), which liberate new mz in the blood circulation. Some mz can also differentiate into male or female gametocytes (gc) inside infected RBCs; B: these sexual dimorphic stages are ingested by a mosquito during a blood meal and after activation reproduce sexually generating a zygote (zg). The zg differentiates into the motile ookinete (ook) that crosses the peritrophic matrix (PM) and midgut epithelial cells to develop as an oocyst (ooc) in the laminal basal of the midgut. The ooc then generates midgut sporozoites (spz) that after being released into the haemolymph, invade and are stored in the mosquito salivary glands (sg); C: during the bite the infected mosquito deposits spz (bite spz) in the extravascular parts of the skin. Some spz invade lymph vessels, but are trapped and degraded in the draining lymph nodes. Some spz invade blood vessels and reach the liver sinusoids. After invading the liver parenchyma and traversing host cells, the spz invades and develops as an exoerythrocytic form (eef) in a parasitophorous vacuole inside a hepatocyte. The eef generates hepatic mz (hep mz) that are released inside merosomes in the blood circulation initiating a new cycle of RBC invasion; D: quantitative view of the major steps in the life-cycle of *Plasmodium* parasites. The bars represent the estimated number of *Plasmodium berghei* parasites infecting mice and *Anopheles stephensi* mosquitoes. Data modified from Baton and Ranford-Cartwright (2005), Medica and Sinnis (2005), Amino et al. (2006) and Sinden et al. (2007). Parameters for estimation: 1e10 RBCs/mouse, 1 µL of blood ingested by mosquito, ratio 1 gametocyte: 10 infected RBC, 25% of bite spz infect hepatocyted, 1 eef generates 10,000 hep mz.

cytes can be seen attached to oocysts (Fig. 2E). It is also possible to observe shrunken oocysts due to the rupture of the oocyst wall (Fig. 2F). Oocyst rupture and the subsequent release of sporozoites occur once the maturation is complete (usually within 10-24 days, depending on the *Plasmodium* species). This leads to the release of anywhere from hundreds to thousands of sporozoites into the mosquito haemocoel (Hillyer et al. 2007) (Fig. 1G). Before reaching the salivary gland, the sporozoites still need to overcome the other barriers that is produced by the immune system, including: (i) haemocytes (Fig. 2E), which are cells that are responsible for the internal defense system of the mosquito, (ii) antimicrobial peptides and (iii) other humoral factors (Dimopoulos et al. 2001).

In general, the process of invasion of the salivary gland by sporozoites is very inefficient; usually less than 20% of the total numbers of parasites produced are able to invade the organ (Korochkina et al. 2006, Hillyer et al. 2007). Those sporozoites that survive after overcoming various barriers to reaching the salivary gland are finally able to invade the organ. By means of a specific recognition receptor present in the salivary gland of the vector, these parasites are able to adhere to and penetrate the basal lamina of the gland before penetrating the host plasma membrane of the salivary cells. A number of parasite ligands are necessary for the initial attachment of the sporozoites to the salivary glands, such as some regions of the circumsporozoite protein and thrombospondin-related anonymous protein [see details in Sinden and Matuschewski (2005) and Aly et al. (2009)]. This process of invasion has been well described using the *P. gallinaceum*/*Ae. aegypti* model (Pimenta et al. 1994). The penetration process appears to involve the formation of membrane junctions. Once inside the host cells, the sporozoites are seen within vacuoles attached by their anterior end to the vacuolar membrane. Mitochondria surround and are closely associated with the invading sporozoites. After the disruption of the membrane vacuole, the parasites traverse the cytoplasm, attach to and invade the secretory cavity through the apical plasma membrane of the cells. Inside the secretory cavity, the sporozoites are again seen inside the vacuoles. Upon escaping from these vacuoles, the sporozoites are positioned in parallel arrays, forming large bundles attached by multilamellar membrane junctions. Several sporozoites are seen inside and around the secretory duct. Except for the penetration of the chitinous salivary duct, these observations have morphologically characterised

the entire process of sporozoite passage through the salivary gland (Pimenta et al. 1994). The sporozoites that are now inside the secretory duct of the salivary gland are ready to be injected by the mosquito bite into the skin of a new vertebrate host. An analysis of the amount of parasite that an infected mosquito could inject into the skin of a mouse varied between zero and approximately 1,300 and there appears to be a weak correlation of the number of injected sporozoites with the salivary gland load (Medica & Sinnis 2005).

Considering the entire *Plasmodium* life cycle in the vector and in the vertebrate host, it is fascinating to observe the complexity of distinct developmental forms and the parasite load during the course of infection. There is extraordinary adaptation of the *Plasmodium* parasite to its environment, which is reflected in morphological changes and the parasite load of distinct organs inside the vertebrate host and the mosquito vector (Baton & Ranford-Cartwright 2005, Medica & Sinnis 2005, Amino et al. 2006, Ma et al. 2010, Smith et al. 2014). During the stages that the *Plasmodium* moves from the mammalian host to the vector and *vice versa*, two "bottlenecks" occur that are characterised by a small number of parasites. Fig. 3 shows an animated model that illustrates qualitative and quantitative views of the major steps of the life cycle of the *P. berghei* parasites infecting mice and *An. stephensi* mosquitoes. Murine-*Plasmodium* spp interaction studies are considered to be suitable experimental models to better understand the interaction between malarial parasites and vectors.

The key Amazon *Anopheles* vectors

Among the *Anopheles* mosquito species that inhabit the Amazon, *Anopheles darlingi, Anopheles albitarsis s.l.* and *Anopheles aquasalis* are considered the principle mosquito vectors. Specifically, *An. darlingi* is the main vector in South America and has been associated with the dynamics of malaria transmission in the Amazonian regions of Bolivia, Colombia, French Guiana, Guyana, Peru, Suriname and Venezuela (Zimmerman 1992, Hiwat et al. 2010). *An. albitarsis s.l.* inhabits regions of Venezuela (Rubio-Palis et al. 1992) and *An. aquasalis* is found in Trinidad (Chadee & Kitron 1999), Guyana (Laubach et al. 2001) and Venezuela (Berti et al. 1993).

Other anopheline species can be secondary or occasional malaria vectors because of their population density, anthropophilic behaviour and natural infectivity across their geographical distributions (Deane 1986, Zimmerman 1992, Sinka et al. 2010, 2012). *Anopheles nuneztovari s.l.* and *Anopheles triannulatus s.l.* are commonly collected in the Amazon by researchers and they have been observed to be infected with *P. vivax* and *P. falciparum*, but their role as malaria vectors has yet to be elucidated (de Arruda et al. 1986, de Oliveira-Ferreira et al. 1990, Klein et al. 1991b, Tadei & Dutary 2000, da Silva-Vasconcelos et al. 2002, Póvoa et al. 2003, 2006, dos Santos et al. 2005, Galardo et al. 2007, da Rocha et al. 2008, Santos et al. 2009).

Recently, Foley et al. (2014) developed a study considering the percentage of the area predicted to be suitable for mosquito habitation based on ecological niche models of Amazon vectors. They found that *An. albitarsis* I, *Anopheles janconnae* and *Anopheles marajoara* had the highest percentage of their predicted suitable habitats overlapping the distribution models of *P. falciparum* and *P. vivax* [see details in Foley et al. (2014)]. They also concluded that phylogenetic proximity might be related to malaria vectorial importance within the *Albitarsis* group. The authors recognised that these findings would encourage additional studies of the transmission potential of these Amazonian *Anopheles* species.

An. aquasalis is distributed predominantly along the Atlantic Coast because of its tolerance to saltwater environments, including in Venezuela, where it is considered to be the primary coastal malaria vector of *P. vivax* (Galvão et al. 1942, Laubach et al. 2001, Póvoa et al. 2003, da Silva et al. 2006a).

Amazonian *Anopheles* species such as, *Anopheles deaneorum, An. marajoara, Anopheles mattogrossensis, An. nuneztovari, Anopheles oswaldoi, Anopheles rondoni* and *An. triannulatus* have been considered "naturally infected" with *Plasmodium* since they were captured with parasites in their blood meal (Galvão et al. 1942, Deane et al. 1948, de Arruda et al. 1986, Klein et al. 1991b, Branquinho et al. 1993, Tadei & Dutary 2000, Póvoa et al. 2001, 2003, 2006, da Silva-Vasconcelos et al. 2002, da Silva et al. 2006a, Galardo et al. 2007, da Rocha et al. 2008, Santos et al. 2009). However, their role as malaria vectors is not well defined.

Two crucial factors needed to label a mosquito a vector are the demonstration that the species is anthropophilic and identification of the same *Plasmodium* species or strain in patients from the same geographic region. In the field, the presence of *Plasmodium* oocysts in the mosquito midgut indicates parasite establishment in a susceptible vector. However, the discovery of only sporozoites in the dissected mosquito salivary gland can confirm that the life cycle is complete and consequently that the *Plasmodium* parasite can be transmitted by a bite to human hosts. Moreover, recognition of the infection rate (i.e., the percentage of individuals in a mosquito population that carry *Plasmodium*) is an important parameter for defining vector competence and thus a key indicator in the description of malaria dynamics and transmission biology in a given geographic region. In contrast, the sole presence of an apparent abundance of a species along with parasites in the ingested blood meal is not sufficient to implicate a mosquito as a vector (Smith et al. 2014).

Colonisation of American anophelines

Considering *An. darlingi*, *An. albitarsis s.l.* and *An. aquasalis is* as main vectors, only the latter species has been colonised for several years under laboratory conditions (Lima et al. 2004). The maintenance of mosquito vectors in a laboratory facilitates studies on their biology and behaviour and experimental studies to characterise details of their susceptibility to *Plasmodium* species, thus providing a greater understanding of malaria disease dynamics. Mosquito vectors of malaria from Africa and Asia have been well established in colonies and can be maintained in insectaries of several laboratories in different countries. Consequently, *Anopheles gambiae*,

the major vector in several African countries, is the most well studied mosquito, including its interaction with human and murine *Plasmodium* species that are considered causative agents of malaria (Moores 1953). Distinctly, the colonisation of *An. darlingi*, the major Amazon vector, has proven to be difficult, as has that of other New World anopheline species.

Several attempts to colonise American species of *Anopheles* under laboratory conditions have been conducted either unsuccessfully or with short-lived success. When describing the rationale for establishing a colony of *Anopheles quadrimaculatus,* Boyd et al. (1935) highlighted two key starting points: (i) an abundant supply of food for the larvae and (ii) a stable and optimal temperature. Galvão et al. (1944) used Boyd's technique with specifically sized cages (40 x 40 x 47 cm). They loaded approximately two thousand mosquitoes into each cage and the females started to lay eggs after seven days. Reproduction led to *An. albitarsis domesticus* (*An. marajoara*) mosquitoes reaching the seventh generation. Egg production in *Anopheles tarsimaculatus* (*An. aquasalis*), however, was low and was maintained by only a few dozen couples up to the fifth generation. The authors attributed the colonisation problems to a lack of mating due to the space and type of food offered to the males. To begin a mosquito colony there are numerous factors that need to be controlled for, including the fact that several species do not undergo free copulation under laboratory conditions (Martinez-Palacios & Davidson 1967). Thus, for the establishment of the colony, the induced copulation approach is often necessary. This method was developed by McDaniel and Horsfall (1957) for the *Aedes* spp and was later adapted by Baker et al. (1962) for *Anopheles*.

There are descriptions in the literature of various American *Anopheles* species that have been maintained in insectaries for short periods of time, including *Anopheles punctipennis*, *Anopheles maculatus*, *An. aquasalis, An. albitarsis*, *An. deaneorum* and *An. marajoara* (Baker et al. 1962, Ow-Yang & Maria 1963, Baker 1964, Arruda et al. 1982, Klein et al. 1990, Horosko III et al. 1997). In the 2000s, the colonisation of *Anopheles pseudopunctipennis*, which is considered an important vector of human *Plasmodium* spp along the Andes in several countries, was noted to have occurred by means of free intercourse (Lardeux et al. 2007). The adult mosquitoes were exposed to a blue strobe light for 20 min for several nights, encouraging them to copulate naturally under laboratory conditions. After a few generations, the researchers obtained a stable colony that reproduced by free mating. Corrêa et al. (1970) described some success in colonising and maintaining *An. darlingi* mosquitoes for about two years. Subsequently, however, Buralli and Bergo (1988) failed to achieve successful results from the same laboratory and using the same methodology. More recently, Moreno et al. (2014) described a method for *An. darlingi* colonisation that also used the strobe light approach. They reported that *An. darlingi* mosquitoes obtained after five generations were successfully infected with *P. vivax* by artificial membrane feeding similar to the previous work of Ríos-Velasquez et al. (2013) with field-captured mosquitoes.

One of the authors of this paper established colonies of two species of Neotropical anophelines 20 years ago. *An. albitarsis s.l.* was colonised in 1993 by induced copulation. After about two years of colony maintenance with induced copulation, we noticed the successful occurrence of free copulation; we used large cages with a thousand adults and a sex ratio of approximately 1:1 (Horosko III et al. 1997). *An. aquasalis* was settled in 1995 from the beginning by the free coupling method. In 1998, a second American malaria vector was colonised, *Anopheles albimanus*, which is one of the main vectors of malaria in Central America and in the south of Mexico (Zerpa et al. 1998). The authors used a simple and efficient maintenance method for mosquito mating and laying eggs.

Today, to the best of our knowledge and according to the specialised literature related to *Anopheles* species, only two long-term colonised American malaria vectors, *An. aquasalis* and *An. albimanus*, are maintained in laboratories and have been used for experimental studies, demonstrating that they are good models for studying the interaction of malaria vectors with *Plasmodium* species. As examples of these types of studies in *An. albimanus*, there are reports showing the susceptibility of the vector to *P. vivax* (Herrera et al. 2011, Solarte et al. 2011) and to the murine *P. berghei* (Serrano-Pinto et al. 2010, Herrera-Ortiz et al. 2011). For *An. aquasalis*, there have been studies developed by our group related to their susceptibility to *P. vivax* infection, including those related to gene expression during parasitic infection (Bahia et al. 2010, 2011, 2013, Ríos-Velasquez et al. 2013).

Searching for a model to study the *Plasmodium* interaction with an American mosquito vector

An. aquasalis in nature: distribution, habitat and population variability - *An. aquasalis* lives in sunny habitats with vegetation in fresh brackish water. It is believed that the mosquito prefers clean water such as that in stream pools, mangroves, ponds and ditches (Manguin et al. 1993, Grillet 2000). The demarcation of the *An. aquasalis* territory to coastal regions and its tolerance to salt water could be evolutionary adaptations that have been selected to avoid competition for food with other *Anopheles* mosquitoes (particularly during the larval phases), inserting the mosquito into the large and varied marine trophic chain (Sinka et al. 2010). The geographic distribution of *An. aquasalis* covers the southern coastal region of Central America, the Caribbean Islands and South America, but this species can penetrate eight-10 miles inland from the coast because it has a flight capacity of up to 8 km. Its presence at the Atlantic Coast has been reported from SP to Nicaragua and at the Pacific Coast from Costa Rica to Ecuador, as well as in the Antilles and Trinidad and Tobago (Faran 1980, Chadee et al. 1992, Zimmerman 1992, Consoli & Lourenço-de-Oliveira 1994).

An. aquasalis is an important *P. vivax* vector that is present at the Atlantic and Pacific coasts from Central America to southern Brazil. In situations in which the mosquito density increases, females can be the vectors of human malaria, especially in the absence of domestic animals, which are their usual food source. For exam-

ple, Giglioli (1963) reported the effect of mechanisation on a rice farm in Guyana, which led to the disappearance of buffalo in the region. This resulted in a change in the behaviour of *An. aquasalis* that had man as its main blood source. Nevertheless, this mosquito species has been associated with several outbreaks of malaria in several countries (Deane 1986, Berti et al. 1993, Laubach et al. 2001, Mouchet et al. 2008). In most of the territory it inhabits, this species is exophilic, zoophilic and crepuscular, but in the drier northeast area it is frequently endophilic and bites human hosts. The females are opportunists, feeding in both intra and peridomiciliary areas of animals and humans. They begin to bite at sunset, reaching maximum activity in the early evening before decreasing later at night (Flores-Mendoza et al. 1996). Usually the mosquitoes rest in their peridomestic habitats before and after the blood meal.

Due to the importance of *An. aquasalis* as a vector of human malaria, it is necessary to perform studies to evaluate the genetic structure of diverse populations. In general, many *Anopheles* species are formed by complexes of cryptic species. The taxonomic elucidation of these complexes could reflect on the epidemiology and even on the control of malaria (Rosa-Freitas et al. 1998). To elucidate the dilemma of whether a given species is highly polymorphic or a complex of related species, an integrated approach of performing several studies is necessary. These studies comprise taxonomic investigations applying morphological, behavioural and molecular tools.

In its previous description, *An. aquasalis* was divided into two varieties: *An. tarsimaculatus* var. *aquacaelestis*, presenting the second hind tarsus with less than 1/6 of the length being black and *An. tarsimaculatus* var. *aquasalis*, with nearly 1/2 of its length being black (Curry 1932). Based on the morphological characters, many synonymous examples were proposed for this species. In 1941, Komp changed the name of the species known as *An. tarsimaculatus* var. *aquacaelestis* to *Anopheles (Nyssorhynchus) emilianus* by analysing egg characteristics. By studying the morphological characteristics of the eggs, larvae and adults, da Ramos (1942) renamed the same species *An. (N.) oswaldoi guarujaensis*. While working in Venezuela in 1948, Anduze (1948) found two different tonalities of mosquitoes and changed the name of the so-called *An. aquacaelestis* and *An. aquasalis* to var. *guarauno* and var. *delta,* respectively. Garcia et al. (1977) were working in Venezuela and studying several morphological characteristics in 1977 when they described *An. aquasalis* as a new species called *Anopheles (Nyssorhynchus) deltaorinoquensis*. While still working on Venezuelan mosquito populations in 1997, Maldonado et al. (1997) showed that the egg morphology of *An. aquasalis* varies within the species. More recently, a systematic study based on the morphological characteristics supported the single species status for *An. aquasalis* (Sallum et al. 2000). However, as a result of these data using morphological tools, the species complex dilemma has yet to be resolved.

To elucidate the taxonomic relationships among *An. aquasalis* and *An. emilianus* in Venezuela, Perez and Conn (1992) conducted a chromosomal banding pattern study on polytene chromosomes of different mosquito populations from endemic and non-endemic areas in that country. They observed that the banding patterns of the populations were identical to the standard chromosome map of *An. aquasalis* from Brazil. In 1993, Conn et al. analysed populations of *An. aquasalis* from Venezuela, Trinidad and Brazil using restriction enzyme digestion of mitochondrial DNA (mtDNA). The five enzymes surveyed yielded 12 mtDNA haplotypes. Estimates of mtDNA sequence divergence between all the populations were within the range of interspecific distances calculated for members of the anopheline species complexes. These results suggest a possible interspecific division in *An. aquasalis* populations north and south of the Amazon River delta (Conn et al. 1993, Linley et al. 1993). In 2002, examining variations in a fragment of the mitochondrial cytochrome oxidase I gene from five *An. aquasalis* Brazilian populations from PA and AP, Fairley et al. (2002) tested the hypothesis that the freshwater Amazon River acts as a barrier to gene flow in northeastern Brazil. Analytical results suggested that the localities within this region of northeastern Brazil constitute a single large population of *An. aquasalis* that spans the Amazon River delta.

To test the populations on either side of the Orinoco River (which is another potential freshwater barrier to gene flow for *An. aquasalis*), intragenomic heterogeneity of the internal transcribed spacer (ITS)1 and ITS2 arrays were investigated by Fairley et al. (2005) in mosquito populations from two geographic locations each in Brazil and in Venezuela and in a single location in Suriname. No sequences from either ITS had a diagnostic distribution or were informative for distinguishing between these populations, providing additional support for the status of *An. aquasalis* as a single species. In this same year, the relationship between *An. aquasalis* and other Amazonian malaria vectors was tested using the rDNA sequence ITS2. The results showed that this marker is compatible with the morphological taxonomic key established for Amazonian mosquitoes and that ITS2 sequence data has proven to be useful in species identification and potentially to solve taxonomic problems (Marrelli et al. 2005). The same results were obtained in Colombia (Cienfuegos et al. 2011). Specifically, there were only five point mutations reported for ITS2 (Fairley et al. 2005). Two interesting questions that remain are how great is the morphological and genetic variability of *An. aquasalis* in endemic areas and are these factors related to vector competence for malarial parasites.

Experimental Plasmodium infection of mosquito vectors - One keystone step to understanding the *Plasmodium* life cycle is the development of infectious mosquito vectors. Experimental infection models are used to understand the biology of the interaction between *Plasmodium* parasites and *Anopheles* mosquitoes. Most research projects have used laboratory models consisting of the human parasite *P. falciparum*, murine parasites *P. berghei* and *Plasmodium yoelii* and the avian parasite *P. gallinaceum* interacting with *An. gambiae*, *An. stephensi, An. albimanus* and *Ae. aegypti* mosquitoes. These mosquito species show different susceptibilities to infection

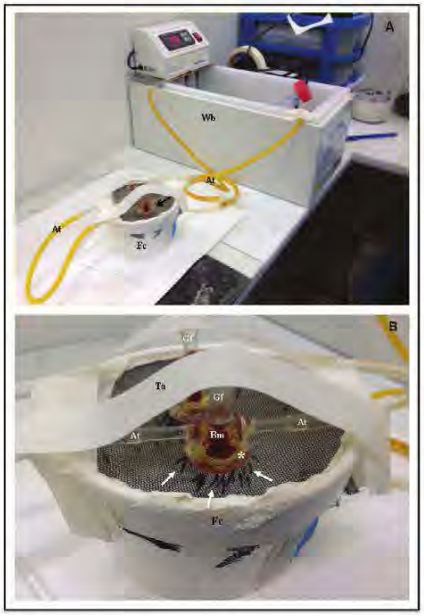

Fig. 4: photographs of the apparatus for developing experimental infection of *Anopheles aquasalis*. A: a small feeding cage (Fc) for containing the mosquito is seen connected to yellow aquarium tubings (At) that are linked to a water thermal bath (Wb) with 37-39°C circulating warm water; B: large magnification image of A in Figure showing details of the Fc. Note the glass feeder (Gf) device placed over a black mesh clothing fabric (asterisk) that is covering the Fc. The Gf is filled with an infective blood meal (Bm), linked to the At and covered by a chicken skin membrane (asterisk). Note several mosquitoes (arrows) in the feeding activity (arrows); Ta: tape for holding the Gf.

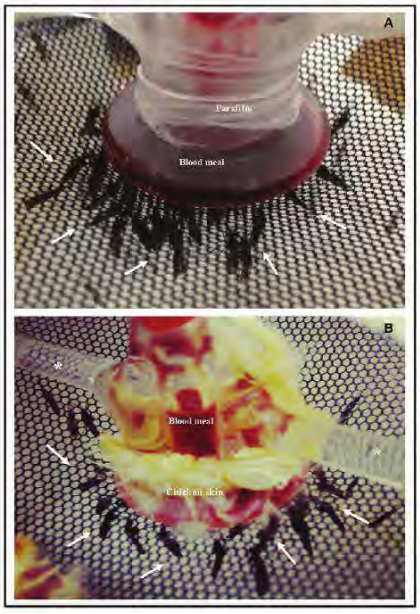

Fig. 5: photographs showing details of the glass feeders for developing experimental infection of *Anopheles aquasalis*. A, B: images of the glass feeders filled with infected blood meals over black mesh clothing for retaining the mosquitoes inside the feeding cages; A: the glass feeder is covered with an artificial membrane and piece of parafilm; B: a glass feeder covered with a natural membrane, dissected chicken skin. The lateral side of the glass feeders (asterisks) are linked to aquarium tubings (not showing) for maintaining the circulating warm water. Inside the feeding cages, several mosquitoes are seen in the feeding activity (arrows in A and B).

by the *Plasmodium* spp. All of these parasite species are cultured in the laboratory or maintained in experimental animals, making it easy to develop experimental research, but some combinations of parasite-mosquito do not occur in nature and might not resemble the real interactions seen between parasites and their vectors (Boete 2005).

In the past, experimental infection of mosquito vectors was initiated by direct placement of the mosquitoes on the skin of malarial patients to encourage feeding (Klein et al. 1991a, c, da Silva et al. 2006b). Due to ethical issues, these types of studies are currently leaning towards the use of membrane-feeding assays instead in order to minimise the human interaction factor. Several studies have confirmed that offering a blood meal through a membrane-feeding device is as efficient as direct feeding on human skin for the study of *Plasmodium* infection of mosquito vectors. A comparative study developed by Gouagna et al. (2013) compared the field-based xenodiagnoses and direct membrane feeding assays evaluating the infectiousness to *An. gambiae* and concluded that the infection rates were similar with both methods. The membrane assay to infect mosquitoes is a simple method and can easily be applied in a laboratory without any sophisticated or complex devices.

From P. vivax infected patients to Amazon mosquito vectors - Today, it is possible to infect Amazon vectors in laboratories located in Manaus, the capital city of AM. The collaboration between three institutions, namely National Institute for Amazonian Research, Amazonian Oswaldo Cruz Foundation and Doctor Heitor Vieira Dourado Foundation for Tropical Medicine (FMT-HVD), has provided good conditions for developing important studies related to *Plasmodium* interaction with mosquito vectors. *P.* vivax is one of the most important causative agents of malaria in humans and is the most widespread and present parasite in America (Cruz et al. 2013); therefore, we decided to focus on its interaction with mosquito vectors. We used blood samples from adult vol-

unteers (ages >18 years) infected with *P. vivax* for our experiments and diagnosed malaria using thick blood smears stained with Giemsa stain. Approximately 3 mL of blood were collected from volunteers by venipuncture. After blood collection, all the patients were treated at the FMT-HVD or in the health posts where they were diagnosed, following ethical procedures determined by the Brazilian Health Ministry.

A simple experimental protocol was used to infect the mosquito vectors (Figs 4, 5). Briefly, adult mosquitoes were sugar-starved overnight prior to infection. Blood samples infected with *P. vivax* were offered to the mosquitoes for a period of 45-90 min *via* a membrane-feeding assay through a glass feeder device (Figs 4B, 5A, B). A Parafilm® membrane was used to cover the glass device (Fig. 5A). Other natural membranes that can also be used for the experiments include the skin from two-three day-old chicks (Figs 4B, 5B) or from young mice or hamsters. During the experimental infection, blood was held at 37-39ºC through a hose system connected to a thermal bath (Fig. 4A). Engorged mosquitoes were separated in rearing boxes. Five-eight days after ingesting infective blood meals, the midguts from the experimentally infected mosquitoes were dissected in phosphate buffered saline (PBS), stained with 2% commercial mercurochrome (Merbromin), placed under a cover glass and examined for the presence of oocysts. Additionally, 12-14 days after infection, the mosquito salivary glands were dissected in PBS in order to observe the sporozoites.

Improving the knowledge of the vectorial competence of Amazonian anopheline populations to *Plasmodium* is necessary to better understand the transmission of malaria in the region. At the end of 2013, our group published an article showing the characteristic aspects of the experimental *P. vivax* infection of key *Anopheles* species from the Brazilian Amazon and other surrounding South American countries (Ríos-Velasquez et al. 2013). This study compared the infection of four field-captured anophelines with the colonised *An. aquasalis*. The following mosquito species were studied: (i) *An. darlingi*, the major malaria vector in all countries located in the Amazon Region, (ii) *An. aquasalis* and *An. albitarsis s.l.*, also proven vectors, and (iii) *An. nuneztovari s.l.* and *An. triannulatus s.l.*, which have been found to be infected, but their status as vectors is not yet well defined. Larvae from the anophelines were collected in the field and reared until the adult stages, except for *An. aquasalis,* which was obtained from a well-established colony. All *Anopheles* species tested were susceptible to experimental *P. vivax* infection with the patient isolates. However, the proportion of infected mosquitoes and the infection intensity measured by oocyst number varied significantly among the species. Colonised *An. aquasalis* mosquitoes showed the highest infection intensity. It was also observed that the components of the serum (by way of inactivation) could modify the infection rates, increasing the infection in *An. darlingi* and *An. triannulatus s.l.*, but diminishing infection in *An. albitarsis s.l.* and *An. aquasalis*. The gametocyte density in the infected blood meal varied among the mosquito species. *An. albitarsis s.l.*, *An. aquasalis* and *An. nuneztovari s.l.* had higher infection rates than *An. darlingi*. This study was the first to characterise the experimental development of *P. vivax* in *Anopheles* vectors from the Amazon. The data found enabled us to infer that the *P. vivax*-vector interaction presents variations depending on the species analysed (Ríos-Velasquez et al. 2013). This fact could have a direct impact on the vector competence of the anopheline species. Moreover, this comparative study demonstrated and endorsed *An. aquasalis*, the main vector in coastal South and Central America, as a feasible laboratory model. Both *An. aquasalis*, from an established colony, and *P. vivax,* from malarial patients, are now being used by our group as a model of human malaria transmission (Bahia et al. 2010, 2011, 2013, Ríos-Velasquez et al. 2013).

The cultivated P. falciparum parasite and mosquito vector interaction - P. falciparum is the human malaria parasite with the most devastating clinical consequences. In laboratories located close to the endemic regions, it is possible to study the interaction of *P. falciparum* with mosquito vectors by feeding the mosquito with collected infected blood from local patients (Harris et al. 2012). However, with the introduction of the continuous culture of *P. falciparum,* it is now possible to study the factors involved in parasite-vector interactions in the laboratory far from the endemic areas. The first successful continuous culture was established and described by Trager and Jensen (1976).

The adaptation of several lines of *P. falciparum*-producing gametocytes in laboratories allowed the infection of colonised mosquito vectors (Trager & Jensen 1976, Carter & Miller 1979). Several studies have been performed by distinct research groups allowing the characteristics of *P. falciparum* inside some important vectors from Africa and Asia, including the molecular aspects of the interaction and the immune response to the parasite infection to be understood (Rodrigues et al. 2012, Ramirez et al. 2014). Additionally, studies have shown that mosquito species exhibit a wide range of susceptibility to infection with a given *P. falciparum* line (Collins et al. 1986, Lambrechts et al. 2005) and different *Plasmodium* isolates also vary in their ability to infect a given mosquito strain (Niare et al. 2002, Lambrechts et al. 2005, Riehle et al. 2006).

A degree of adaptation was suggested between geographically isolated populations of *An. gambiae* and *P. falciparum* when an *An. gambiae* colony was successfully selected for resistance to New World *P. falciparum* isolates, but remained susceptible to those of African origin (Collins et al. 1986). Different vector-parasite interactions may have evolved through adaptation in the African *An. gambiae* and *P. falciparum,* allowing this parasite population to evade the mosquito's immune response (Lambrechts et al. 2007). African and New World *P. falciparum* populations show moderate genetic divergence (Volkman et al. 2007, Jambou et al. 2010) that could drive the differences in their infectivity. It appears that genetic differences in both the mosquito and the parasite affect the efficiency of mosquito infection and disease transmission (Molina-Cruz et al. 2012). Recent studies show that Brazilian and African lines (7G8 and NF54, respectively) infecting *An. gambiae* (African vector) differ in their ability to evade the mosquito's im-

mune system and thioester-containing protein 1 (TEP1) (a complement like system) is correlated with parasite invasion (Molina-Cruz et al. 2012). Also of interest is an article demonstrating that *P. falciparum* development in a non-malaria vector, *Culex quinquefasciatus*, is blocked by the mosquito immune response after ookinetes have crossed the midgut epithelium and come in contact with the mosquito haemolymph (Molina-Cruz et al. 2013).

The identification of Brazilian *P. falciparum* lines that produce infective gametocytes will provide important information that will elucidate the parasite/vector interaction that is indispensable for future studies aimed at developing new strategies for blocking malaria transmission. The susceptibility of *An. aquasalis* and *An. darling*i to this parasite under laboratory conditions needs to be further investigated.

Non-human Plasmodium species as a model for studying the interaction with mosquito vectors - P. berghei, *P. yoelii* and *Plasmodium chabaudi* are murine parasites that have been adapted in the laboratory and are considered good models to investigate malaria in mammals and also to study parasite-mosquito interactions. These *Plasmodium* species have been used in different laboratories for several years to infect *An. gambiae, Anopheles funestus*, *An. quadrimaculatus* and *An. stephensi*, all of which are malaria vectors in Africa and Asia, mainly due to the vectors' high susceptibility to infection with various malaria parasite species and strains (Yoeli et al. 1964, Vaughan et al. 1991, Sinden et al. 2002, Alavi et al. 2003, Akaki & Dvorak 2005, Frischknecht et al. 2006, Hume et al. 2007, Lo & Coetzee 2013, Xu et al. 2013).

There are several advantages of using an animal model of malaria and many research groups worldwide have begun using murine *Plasmodium*-based experimental models to better understand the interaction between malaria parasites and vectors. Essentially, these models have been helpful in the evaluation of potential interventions for malaria control and to generate and test hypotheses about the biology of human malaria and drug tests (Killick-Kendrick 1978, Jaramillo-Gutierrez et al. 2009, Xu et al. 2013).

P. berghei was first found in the gut and salivary glands of *Anopheles dureni* (its natural invertebrate host) in Central Africa. Later, the parasite was isolated from the vertebrate host, the tree rat, *Grammomys surdaster,* before being was passed on to white rats and resulting eventually in the K173 strain (Vincke 1954, Yoeli 1965, Sinden et al. 2002). *P. berghei* has largely been used as a reliable experimental model for malaria studies because of its relatively simple requirements for laboratory maintenance and the availability of permanent green fluorescent-labelled strains (Franke-Fayard et al. 2004). Consequently, *P. berghei* is one of the most commonly studied *Plasmodium* species, particularly for elucidating the interactions between the parasites and their hosts (Anderson et al. 2004, Baldacci & Menard 2004, Ishino et al. 2004, Levashina 2004, Siden-Kiamos & Louis 2004). *P. yoelii* was originally found and isolated from rats in Central Africa. Three subspecies are recognised, namely *P. yoelii yoelii, P. yoelii nigeriensis* and *P. yoelii killicki,* and they are widely used to study host immune responses and the genetic basis of parasite phenotypes. *P. chabaudi* is a parasite of the African thicket rat, *Thamnomys rutilans*; it has been adapted to develop in the laboratory mouse and is one of the best laboratory models for the study of malaria. The species is one of the most common murine models that have been utilised within vaccine research. *P. berghei* and *P. yoelii* transgenic lines that constitutively express green fluorescent protein (GFP) can develop throughout the entire life cycle in the vertebrate host and these mosquito vectors have been very useful in laboratorial experiments.

P. gallinaceum is an avian malaria parasite that is phylogenetically closer to *P. falciparum* than it is to many other malaria species (McCutchan et al. 1996, Roy & Irimia 2008) and has intriguingly become very useful in laboratories because it can be infected and complete its entire cycle in *Ae. aegypti* mosquitoes and in *Aedes fluviatilis* (Tason & Krettli 1978, de Camargo et al. 1983, Pimenta et al. 1994, Gupta et al. 2005). This model is now widely used for understanding the cell biology of parasitic infection and the routine chemotherapy test in chicks (Carvalho et al. 1992, Rocha et al. 1993a, b, Ramirez et al. 1995, Krettli et al. 2001, da Rocha et al. 2004, Maciel et al. 2008, Rodrigues et al. 2008).

Few studies regarding New World vectors have been developed to date. *An. albimanus,* a Central America malaria vector, can be infected by *P. yoelii,* but cannot be effectively infected by *P. berghei* (Vaughan et al. 1994, Noden et al. 1995, Brucker & Bordenstein 2013). However, Frischknecht et al. (2006) demonstrated that a transformed GFP-*P. berghei* line can complete its life cycle in this North American vector. However, the susceptibility of two important human malaria vectors of this parasite in South America, *An. aquasalis* and *An. darlingi,* requires further investigation under laboratory conditions. It was recently shown that *An. funestus,* an important vector in Sub-Saharan Africa, is permissive for *P. berghei* development, which is in contrast with previous reports (Xu et al. 2013). This kind of work highlights the importance of fully testing New World anopheline species for *P. berghei* experimental infections using different parasite strains and mosquito populations.

The establishment of experimental infections using *An. aquasalis* mosquitoes from colonies and *P. yoelii* and *P. berghei* parasites could provide an interesting model for studying malaria in the Amazonian scenario. It could definitely be the first step in finally understanding the biology underlying *P. vivax* and/or *P. falciparum* infection of Brazilian vectors.

The immune response of the mosquito vector to *Plasmodium* infection

Understanding the molecular mechanisms involved in the development of the parasites in the vectors is an important step in determining the interaction process and vectorial competence. Mosquitoes, like other organisms, produce humoral and cellular immune responses. A large range of molecules can be produced against pathogens such as bacteria, fungi, viruses and *Plasmodium* spp and can be secreted by mosquito or-

gans and tissues as fat bodies, haemocytes and midgut cells (Yagi et al. 2004, Cirimotich et al. 2010). Recent studies using microarrays and transcriptome techniques have described how *Plasmodium* parasites can modulate the expression of immune genes in *An. gambiae* and *An. stephensi* (Dimopoulos et al. 2002, Xu et al. 2005, Dong et al. 2006, Baton et al. 2009). Actually, many studies have produced evidence supporting the fact that the vectorial competence of a determined vector depends on the action of the mosquito immune system during the infection process with *Plasmodium* species.

During several steps of the life cycle, mosquito immune defences can kill parasites, thereby controlling or eliminating the infection. Once *Plasmodium* parasites are ingested by female mosquitoes during blood feeding, they face the harsh environment of the digestive tract. It has been previously observed that these parasites can negatively or positively modulate the gene expression and activity of many of the mosquito's digestive enzymes (Gass & Yeates 1979, Jahan et al. 1999, Somboon & Prapanthadara 2002). There are several phenomena related to the mosquito vector's defences that can occur. For example, the production of nitric oxide synthase (NOS) by the vector occurs from the period before the invasion of the intestinal epithelium to the time when the parasite crosses the epithelial cells. NOS is responsible for activation of the production of the antimicrobial peptides that are responsible for the death of a large number of ookinetes in the insect gut (Luckhart et al. 1998, Dimopoulos et al. 2001, Olayan et al. 2002, Herrera-Ortiz et al. 2011). Moreover, NOS is also an important component of the nitration process in *Plasmodium*-invaded midgut cells and targets parasites for complement activation through TEP1 protein (Oliveira et al. 2011). Additionally, due to this immune response (at least for the human *Plasmodium*), less than 10 ookinetes can successfully cross the intestinal epithelium and form viable oocysts (Ghosh et al. 2000). This means that only a small proportion of the ingested parasites will be able to successfully escape the interior of the intestine, cross over the PM and invade the epithelial cells of the intestine. Activation of the melanisation cascade may also occur during the crossing of the intestinal epithelium. A cascade of serine proteases which activates PPOs through a second cascade leads to the deposition of melanin and free radicals that are involved in the death of ookinetes (Luckhart et al. 1998, Hoffmann et al. 1999, Ghosh et al. 2000, Ligoxygakis et al. 2002, Cirimotich et al. 2010). The ookinetes that survive the onslaught of the immune system will release the sporozoites. In the haemolymph, the phagocytosis of sporozoites by mosquito haemocytes has been described in *Ae. aegypti* and *An. gambiae* (Hillyer et al. 2003, 2007). In addition to their phagocytic activity, these haemocytes are able to secrete substances that assist in promoting the death of the parasite (Blandin & Levashina 2007). Antimicrobial peptides that are rapidly produced by the fat body of the insect also represent an important step in fighting the infection. Actually, there is an intensive role that the mosquito's immune system has to constantly undergo in order to fight back the infection.

The insect's defense mechanisms are activated by intracellular immune signalling pathways. Toll, immunodeficiency (IMD) and JAK/STAT are the three major immune pathways, first described in *Drosophila* and then in *Anopheles* (Cirimotich et al. 2010). The Toll pathway activation by *P. berghei* is able to restrain parasite survival in *An. gambiae* (Frolet et al. 2006). Over-activation of this pathway by silencing the negative regulator cactus dramatically reduced *P. berghei* loads in *An. gambiae*, *An. stephensi* and *An. albimanus*, but not *P. falciparum* numbers in these same mosquito species (Garver et al. 2009). Interestingly, the IMD pathway plays an important role in limiting *P. falciparum* infection. Depletion of caspar, the negative regulator of the IMD pathway, promotes a *P. falciparum*-refractoriness phenotype in *An. gambiae* mosquitoes. However, the same phenotype is not achieved when *P. berghei* is used (Garver et al. 2009).

In *An. gambiae*, the JAK/STAT pathway mediates the killing of *P. falciparum* and *P. berghei* in the late infection phases after midgut invasion. Disruption of this pathway by silencing the transcription activator, STAT-A, promotes *P. berghei* oocyst development. Meanwhile, the over-activation of the JAK/STAT pathway by depletion of the suppressors of cytokine signalling triggers NOS expression and decreases the infection levels (Gupta et al. 2009).

Reactive oxygen species (ROS) are generated by mitochondrial activity and/or activation of the immune system in mosquitoes (Kumar et al. 2003, Molina-Cruz et al. 2008, Gonçalves et al. 2012). In *An. gambiae*, the ROS-producing dual oxidase protein and an haemeperoxidase (HPX2) are able to secrete a dityrosine network. This network prevents strong immune activation of the midgut by commensal gut bacteria. When *Plasmodium* ookinetes invade epithelial cells, the dityrosine network is disrupted and a high level of NO, which has a strong negative effect on parasite survival, is produced (Kumar et al. 2010). In addition, the invasion of the *An. gambiae* midgut epithelium by the *P. berghei* ookinetes induces the expression of a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, NADPH oxidase 5 and HPX2, which catalyses protein nitration leading to parasite opsonisation and killing through complement action in the mosquito's haemolymph (Oliveira et al. 2011). Although ROS can promote parasite killing, they can also be hazardous to mosquito cells. Therefore, ROS production should be compartmentalised and their life-span must undergo fine regulation by the activation of detoxifying enzymes such as catalase and superoxide dismutase (SOD). In *An. gambiae*, catalase expression and activity is inhibited by *P. berghei* infection. The silencing of this enzyme decreases *P. berghei* survival (Molina-Cruz et al. 2008), emphasising that ROS are important immune effectors against *Plasmodium* parasites.

Another major process in insect defense is the melanisation immune response that is present in the major classes of arthropods. Factors present in the haemolymph mediate melanin synthesis when the recognition of non-self is activated and a CLIP cascade culminates in the limited proteolysis and conversion of inactive prophenoloxidase proenzyme (PPO) into active phenoloxi-

dase (PO). Subsequent oxidation of phenols by PO leads to the production of quinones that polymerise to form melanin. Several serine proteases have been identified and characterised in the haemolymph of *Anopheles* in the presence of *Plasmodium*. Changes in the conformation of some membrane receptors activate a serine protease, which in turn triggers the activation of the PPO cascade that activates the melanisation immune response. PO is a very active enzyme and its activation intermediates are toxic both to invading microorganisms and for the insect itself. Therefore, its activation is limited to the site of infection and if not, it could lead to widespread and lethal melanisation for insects. In the plasma and haemocytes, inhibitory proteins such as serpins (SRPNs) can be found that regulate the activity of serine proteases (Volz et al. 2006). In mosquitoes, SRPNs regulate the cascade of PPO and determine whether or not malaria parasites are lysed, mainly *via* the activation of the Toll and IMD pathways (Gulley et al. 2013).

Many functional genetic studies have demonstrated in the *An. gambiae*/*P. berghei* system that melanisation can eliminate dead ookinetes (Blandin et al. 2004) or directly mediate ookinete killing, based on the mosquito's genetic background (Volz et al. 2006). The melanisation response of *Plasmodium* has been particularly followed in refractory mosquitoes such as the *An. gambiae* strain (L35), which melanises most *Plasmodium* species including the Brazilian *P. falciparum* 7G8 line; it is highly susceptible to some African *P. falciparum* strains such as LE5 and NF54 (Collins et al. 1986). Recently, Molina-Cruz et al. (2013) investigated whether these parasite lines differed in their ability to evade the mosquito's immune system. Silencing key components of the mosquito's complement system (TEP1, LRIM1 or APL1) prevented melanisation of 7G8 parasites, reverting to the refractory phenotype. In contrast, it had no effect on the intensity of the infection with NF54, indicating that this line is able to evade the mosquito's complement system. Furthermore, when L35 females were co-infected with a line that is melanised (7G8) and one that survives (3D7), this resulted in mixed infections with both live and encapsulated parasites in individual midguts. The African 3D7 parasites were able to evade the mosquito complement system even when 7G8 parasites were being melanised, indicating that immune evasion is parasite-specific and not systemic in nature. These findings suggest that evasion of the *An. gambiae* immune system by *P. falciparum* may be a result of parasite adaptation to sympatric mosquito vectors and may be an important factor driving malaria transmission (Molina-Cruz et al. 2012).

In the interaction studies of *Plasmodium* with their vector, more attention has been paid to the TEP1 that has a similar structure to that of vertebrate C3. Mosquito haemocytes synthesise and release TEP1 in the haemocoel. TEP1 acts as an opsonin, promoting the phagocytosis of Gram-negative and Gram-positive bacteria in a thioester-dependent manner (Levashina et al. 2001). It was also observed that TEP1 can bind and mediate the killing of the midgut stages of *P. berghei* parasites (Blandin et al. 2004) and efficient binding of TEP1 to the ookinetes requires previous parasite targeting by midgut protein nitration (Oliveira et al. 2011). Specifically, TEP1 binds to the surface of the *P. berghei* ookinetes escaping from the basal side of the mosquito midgut epithelium, mediating the death of the parasite (Blandin et al. 2004). Moreover, TEP1-depleted susceptible and refractory (L35) *An. gambiae* mosquitoes showed enhanced development of *Plasmodium* oocysts, clearly demonstrating its anti-parasitic effect (Blandin et al. 2004) for *P. berghei* (Molina-Cruz et al. 2012) and for *P. falciparum*. Considering the LRIM1, LRR and APL1C cited in the above paragraph that also displayed a similar knock-down phenotype to that of TEP1 and increased *P. berghei* oocyst numbers in susceptible and L35 refractory mosquitoes, as well as inhibiting ookinete melanisation (Osta et al. 2004, Riehle et al. 2008, Povelones et al. 2009), there is a functional collaboration between these three proteins in mosquito anti-parasitic defence. Further studies of these complex molecules are necessary for a complete understanding of the innate immunity of these malarial vectors.

Haemocytes are the main players of the insect cellular response. The haemocyte types can vary greatly from flies to mosquitoes (Blandin & Levashina 2007). In *An. gambiae*, the main haemocyte populations are prohaemocytes, progenitor cells, granulocytes, phagocytic cells and oenocytoids (Rodrigues et al. 2010). They are responsible for the melanisation and encapsulation of pathogens in the haemolymph. In addition, haemocytes can also produce humoral effectors that target *Plasmodium* parasites (Pinto et al. 2009). Recent studies have demonstrated that different *Plasmodium* species can trigger haemocyte differentiation in *An. gambiae* (Ramirez et al. 2014) and an increase in the granulocyte population is associated with immune protection towards subsequent *P. berghei* infections (Rodrigues et al. 2010).

The *Plasmodium* life cycle is a complex process and one could argue that this complexity is due to the parasite's ability to alter itself on a cellular and molecular level. Recent studies have determined that the expression of *Plasmodium* surface proteins can control the vector infection. The *P. falciparum* gamete surface protein genes *Pfs48/45* and *Pfs47* have been shown to have highly polymorphic regions (Conway et al. 2001, Anthony et al. 2007). Population studies have demonstrated an extreme geographical divergence of allele frequencies for both the *Pfs48/45* and *Pfs47* genes. This strong population structure is not observed in other *P. falciparum* genes. The *Pfs48/45* and *Pfs47* genes have seven and 18 single nucleotide polymorphisms (SNPs), respectively, while other genes have fewer SNPs. The African lines had the most diverse combinations of these genes, whereas parasites from Brazil and Peru have the same SNP combination. Recently, Molina-Cruz et al. (2013) identified *Pfs47* as an essential survival factor for *P. falciparum* that allows the parasite to evade the immune system of *An. gambiae*. *Pfs47* suppresses midgut nitration responses that are critical in activating the complement-like system. Thus, the disruption of *Pfs47* reduced parasite survival in the mosquito. These authors also provide evidence that *Pfs47* population structure may be due to the adaptation of *P. falciparum* to different *Anopheles* vector species present

outside of Africa. Understanding the molecular mechanisms involved in this step is crucial to interfering with the development of *Plasmodium* in mosquitoes.

Immune response of *An. aquasalis* to *P. vivax* infection

Because the genome sequence of this mosquito is still not available, differential subtraction mRNA libraries were generated to investigate how *P. vivax* infection modulates *An. aquasalis* gene expression (Bahia et al. 2010). Infection down-regulated the expression of the genes related to mosquito embryogenesis and energy metabolism, which was consistent with the notion that the activation of the immune system towards *Plasmodium* has a negative impact on reproductive fitness (Hopwood et al. 2001, Ahmed & Hurd 2006). In contrast, only 3% of the obtained sequences were related to immunity. This weak immune activation could be associated with a high compatibility between *P. vivax* and *An. aquasalis,* as demonstrated for other parasite-vector combinations (Jaramillo-Gutierrez et al. 2009).

Regarding the harsh environment of blood digestion in the *P. vivax*-*An. aquasalis* model, the expression of a chymotrypsin-like protease was heavily inhibited by infection 24 h after this infection occurred, showing that the parasite can negatively modulate this gene expression. The same effect was not observed for a carboxypeptidase A-like protein also found in this anopheline (Bahia et al. 2010). However, *P. vivax* infection induced the expression of a member of the SRPN family. These are classical inhibitors of serine proteases that participate in blood digestion and the melanisation cascade (Dana et al. 2005, Michel et al. 2005). It is still unclear, however, whether these changes in digestive enzymes could have a protective effect on *P. vivax* development in *An. aquasalis* mosquitoes.

In *P. vivax*-infected *An. aquasalis*, catalase and SOD expression was induced 36 h post-infection (p.i.) in the whole mosquitoes. This induction was not observed in the infected midguts. However, midgut catalase and SOD activities were significantly lower 24 h after infection, indicating that *P. vivax* parasites can modulate the detoxifying response post-transcriptionally (Bahia et al. 2013). The silencing of catalase increased *P. vivax* infection and prevalence. These results are in contrast with previous reports for *An. gambiae* (Molina-Cruz et al. 2008) and suggest that ROS are necessary for *P. vivax* development in *An. aquasalis* mosquitoes, leading this parasite to manipulate the detoxification system accordingly.

The role of IMD and Toll pathways on the *P. vivax*-*An. aquasalis* interaction remains unclear. *P. vivax* can induce the expression of the antimicrobial peptide cecropin in *An. aquasalis* mosquitoes (Bahia et al. 2010) and cecropin production is under the control of IMD and Toll pathways in other mosquito species (Meister et al. 2005, Moon et al. 2011, Pan et al. 2012).

Bahia et al. (2011) showed that the JAK/STAT pathway is also activated in *P. vivax*-infected *An. aquasalis* mosquitoes, but at an earlier stage than previously reported for *An. gambiae* (Gupta et al. 2009). The expression of STAT, the negative regulator protein inhibitor of activated STAT1 and the immune effector NOS was induced by *Plasmodium* at 24 and 36 h p.i. NOS is an important component of the nitration process that targets parasites for complement activation (Gonçalves et al. 2012). Besides to silencing of STAT promoted *P. vivax* development in *An. aquasalis* mosquitoes. The effect of the STAT pathway on *P. vivax* infection at later stages is yet to be investigated.

Consideration of anopheline genomes and those of New World vectors

The 2002 publication of the *An. gambiae sensu stricto* (Holt et al. 2002) and the *P. falciparum* (Gardner et al. 2002) genomes marked a breaking point in the field of malaria vector biology research. The *Anopheles* project wrapped together decades of classic genetics knowledge, allowing us to better understand issues such as chromosome and gene architecture. It also allowed vector biologists to plunge into the area of comparative genomics through which the first comparisons made (Christophides et al. 2002, Zdobnov et al. 2002) addressed matters such as the composition of the immunity-related gene repertoire. In the post-genome era, several genetic engineering tools and strategies for vector control have arisen, have been implemented and have been assessed (Alphey et al. 2002, Lycett & Kafatos 2002, Scott et al. 2002, Benedict & Robinson 2003, Riehle et al. 2003, Tabachnick 2003, Toure et al. 2004, Sinkins & Gould 2006, Takken & Knols 2009, Isaacs et al. 2011, Sumitani et al. 2013). Nevertheless, the high diversity and plasticity that *Plasmodium* parasites have shown in vertebrate and invertebrate hosts have led to the assumption that the parasites evolve faster and adapt rapidly, more so than human and anopheline hosts (Carius et al. 2001, Cohuet et al. 2010). As a consequence of this phenomenon and with the experiences thus far accumulated, the vector biology community understood that sequencing the genomes of multiple mosquito and parasite species would be imperative to understanding and manipulating the vector-parasite interactions.

For this purpose, efforts were jointly channelled *via* the *Anopheles* Genomes Cluster (AGC), which in 2008 formed the basis of what would become the first anopheline comparative genomics consortium (Besansky 2014). The committee identified and selected 16 mosquito species whose genomes and transcriptomes were about to be published (Neafsey et al. 2013) and made available through the VectorBase (Megy et al. 2012). Unfortunately, *An. albimanus* is the only American vector listed in the project and no attention was paid to the Amazon mosquitoes that are the vectors of the majority of the human cases on the continent.

The evolutionary vector-parasite dynamics, vectorial competence traits and mosquito behaviour could have been shaped by multiple factors such as specific genotype combinations. Experimental evidence and theories explaining how the genomic composition of a mosquito species determines whether it is refractory or susceptible towards infection by a species (strains) of *Plasmodium* parasite have been published (Billingsley & Sinden 1997, Norris et al. 2001, Osta et al. 2004, Lambrechts et al. 2005, Riehle et al. 2007, Jaramillo-Gutierrez et al.

2009, Harris et al. 2010). There is also a great body of literature connecting vector biology with non-genetic components such as ecological factors (Schmid-Hempel & Ebert 2003, Lambrechts et al. 2005, Tripet et al. 2008, Tripet 2009, Wolinska & King 2009).

As stated by the AGC (Moreno et al. 2010, Besansky 2014), sequencing the genome of mosquito species that capture and represent the evolutionary and phenotypic divergence within the anopheline vectors distributed throughout the world is critical. It is the consensus among the community that envisioning a eukaryote genome project requires looking at it as a continuous process of innovation, re-sequencing and annotation (Li et al. 2006, 2010, Sharakhova et al. 2007, Moreno et al. 2010). Together with the *An. gambiae s.s.* genome, other annotated anopheline assemblies will provide a platform for gaining genome-wide evolutionary and population genetic insights into the mechanisms of speciation and the biological processes that influence the ability of the mosquitoes to transmit malaria parasites to humans.

It has also been brought to the attention of the vector community that the genomic aspects of vectorial capacity and competence have not been uniformly studied (Cohuet et al. 2006, 2010) and some have been largely overlooked, both in terms of the species analysed and the gene families addressed by experimental biology. For example, rapid progression has been made regarding mosquito immunity, insecticide resistance and olfaction genetics. However, the genetic determinants of parasite virulence, mosquito adaptation to human environments and the evolutionary forces exerted on vectors by the parasite and the microbiome associated with them, are still progressing slowly. The area of comparative genomics is rapidly evolving and developing tools. Therefore, the number of questions that vector biology can answer through sequenced and published genomes has expanded (Zdobnov et al. 2002, Reddy et al. 2012). Major analytical themes now include topics such as molecular evolution and speciation, chemoreception, circadian rhythm, development, repetitive and transposable elements, reproduction, secretomes, rearrangements of chromosomal architectures, neuropeptides and behaviour, blood/sugar metabolism and so on.

The Neotropical vectors represent an interesting target to understand how competent malaria transmission evolved in a different ecological setting and also followed different human settling conditions (Fagundes et al. 2008, Hubbe et al. 2010, O'Rourke & Raff 2010, Bodner et al. 2012, Yalcindag et al. 2012). It is believed that the interactions between the actors of the malaria transmission triad - humans, Neotropical vectors and *Plasmodium* parasites - are relatively recent on the American continent. For example, the main Neotropical malaria vector, *An. (Nyssorhynchus) darlingi*, which diverged from *An. (Cellia) gambiae* approximately 100 million years ago, could have evolved in a human and parasite-free environment for several million years (Moreno et al. 2010).

When we add up all of the biological evidence and take into account the fact that malaria is a malady that still imposes a high burden upon the people who live in the Amazon Basin (> 500 thousand cases are reported annually), sequencing the genome of a Neotropical vector seems important. Thus, in 2013, this became a reality with the publication and upload onto the VectorBase of the *An. darlingi* genome (Marinotti et al. 2013). This project was performed at the behest of the Brazilian National Council for Research and set a cornerstone for future basic and applied comparative genomics studies. Such research endeavours will be able to start answering long sought-after answers regarding the biology of malaria in an American context and will focus on generating genetic and chemical tools (e.g., insecticides, bacterial larvicides and paratransgenesis strategies) for vector control that better adjust to the ecological and public health conditions in Latin America.

The Brazilian malaria research network is aware of the pitfalls that were addressed and elegantly presented by the AGC regarding the ordeals of the *de novo* assembly of complex eukaryote genomes. Critical aspects of genome sequencing and assembly have been proposed for discussion in the vector biology community due to the open nature of the AGC work. Such topics include: the necessity of isogenic colonies, DNA template quality, genomic library building techniques and heterozygosity-solving algorithms, amongst others. It is the opinion of the Brazilian malaria research network that the time is right to embark on the establishment of a suitable model for research that benefits from the experience and data generated by the *An. darlingi* genome and together expands and enriches the depth of knowledge of American vector biology.

As proof of the steps being taken by research groups in Brazil towards the advancement of genomic sciences, we can also mention the ongoing *An. aquasalis* genome project. This will bridge the vacuum that currently exists between the *An. darlingi* model and its use in experimental biology research. The absence of colonies of this species in several laboratories and the highly heterozygous nature of its genome assembly still hinder its potential as a research model.

The *An. aquasalis* species has viable, operating colonies throughout Brazil. It is pertinent that *An. aquasalis* has been used in experimental infections and transmission assays with multiple *Plasmodium* species. Therefore, this species is positioned as a top model for the understanding of malaria transmission within the Brazilian context. The peculiar bionomics of the *An. aquasalis* mosquito (Sinka et al. 2010) has prompted us to expand and explore other "genomic" areas, in particular the reconstruction of the associated consortium of bacteria and viruses that could be predicted from the massive parallel sequencing process is of interest. Next Generation Sequencing (NGS) technology has evolved into an impressive tool that ranges from genome assembly to microbiome screening (Mardis 2011). When carefully implemented and combined with the experimental designs of genome sequencing projects, metagenomics could become a key element to deconvolute the complex inner insect ecosystem.

As a final thought, we believe that tailored measures of vector control that respond to local conditions and transmission patterns are sorely needed in our region. Targeted interventions based on the growing existence of genomic data pertaining to tandems of Neotropical

vectors and *Plasmodium* parasites could enhance the control strategies that already exist. Building the capacity to generate and use comparative genomics data from local anopheline species is therefore justified.

Modulation of *Plasmodium* infection by the mosquito vector microbiota

Amongst the metazoans, insects are by far the most diverse and abundant clade (Basset et al. 2012). Their success can be explained in part by the relationships they have established with beneficial members of their associated microbiome. The term microbiota defines the microbial communities that stably or transiently colonise insect epithelia as well as intracellular compartments and target organs. They may vary from bacteria to viruses, yeasts and protists. The bacterial component of this ecosystem is to date the most studied and characterised (Ng et al. 2011a, Gendrin & Christophides 2013, Minard et al. 2013). These symbiotic microbiomes or consortiums are beneficial for their insect hosts in many ways (Dillon & Dillon 2004, Azambuja et al. 2005, Thomas et al. 2012, Engel & Moran 2013), including the following: as dietary supplementation, for the enhancement of digestive mechanisms, to help tolerate environmental perturbations, for protection from parasites (Degnan & Moran 2008) and pathogens (Nartey et al. 2013) and for the maintenance and/or enhancement of host immune system homeostasis. Furthermore, the absence or elimination of the microbial fauna and even the modification of its composition can reduce the fitness of the harbouring insect (Thomas et al. 2012). This observed influence of the microbiome on its host has been referred to as the extended phenotype and can range from mutualism to parasitism, as well.

Recently, the study of microorganisms living in the insect gut has increased considerably. The last decade has seen the publication of multiple relevant studies ranging from diversity screening metagenomic surveys (Baumann 2005, Lindh et al. 2005, Carpi et al. 2011, Dinparast et al. 2011, Lindh & Lehane 2011, Ng et al. 2011a, b, Chavshin et al. 2012) to molecular studies on how the gut bacteria interact with the host's immune system and respond to infection (Azambuja et al. 2005, Chouaia et al. 2010, Boissiere et al. 2012).

It is not within the scope of this review to provide an exhaustive analysis on metagenomics or the architecture and dynamics of this micro-ecosystem within Culicine vectors. Recent revisions cover these topics substantially and creatively (Dillon & Dillon 2004, Engel & Moran 2013, Gendrin & Christophides 2013, Minard et al. 2013). Our aim is to briefly call attention to recent advancements that malaria vector control has generated regarding microbiota and its association with vector competence traits. Many of them have been greatly enhanced by the use of metagenomic tools that have allowed us to discover and explore how microbial species could be used in paratransgenesis and malaria transmission-blocking strategies.

Metagenomics emerged as a derivation of classic microbial genomics with the key difference being that it bypasses the requirement for obtaining pure cultures for sequencing (Glass et al. 2010, Huttenhower 2012, Kim et al. 2013). We now have the ability to obtain genomic information directly from microbial communities in their natural habitats and study them in a concerted manner, describing their species composition and even predicting the potential genomic functions and metabolic capabilities they possess (Wooley et al. 2010, Williamson & Yooseph 2012).

As NGS has skyrocketed, our potential to generate genomic data benchmarking (Ansorge 2009) has gained relevance, providing guidance to experimental biologists that encounter themselves with a myriad of available bioinformatics tools (Delcher et al. 2007, Huson et al. 2007, Meyer et al. 2008, Angly et al. 2009, Clemente et al. 2010, Glass et al. 2010, Gerlach & Stoye 2011, Jiang et al. 2012). As users of such technology, we would like to stress that when designing experiments that encompass metagenomic data generation, it is imperative to consider points such as: sampling techniques, DNA/RNA extraction protocols, sequencing platforms, assembly, taxonomic binning, gene annotation tools, statistical analysis and data/meta-data sharing formats (Wommack et al. 2008, Tanenbaum et al. 2010, Wooley et al. 2010, Thomas et al. 2012). The availability of standardised procedures (Field et al. 2008, Tanenbaum et al. 2010) and platforms for data storage and sharing are becoming increasingly important to ensure that the output of individual projects can be assessed and compared (Thomas et al. 2012).

Metagenomic screening assays are now being used to determine the diversity of microorganisms and viruses residing in arthropod vectors of medical importance. Such assays allow human health agencies and research groups to monitor endemic infections, perform real-time surveillance of newly emerging zoonotic pathogens, discover etiological agents and discover how they associate with and within their host (Bishop-Lilly et al. 2010, Carpi et al. 2011, Ng et al. 2011a, b, Mokili et al. 2012).

Due to their importance as vectors of malaria, anopheline mosquitoes have been the targets of multiple efforts to profile their microbiota (Gendrin & Christophides 2013). Behind this effort lies the knowledge that bacteria living in the midgut have been found to modulate the response of the mosquitoes towards *Plasmodium* infection (Pumpuni et al. 1993, Dong et al. 2009, Boissiere et al. 2012, Eappen et al. 2013), have the potential to block infections and can be used as genetic transformation vehicles (Pumpuni et al. 1993, Dong et al. 2009, Weiss & Aksoy 2011, Boissiere et al. 2012, Ricci et al. 2012, Eappen et al. 2013). Below, we summarise some of the key findings regarding the impact of microbiota on the *Plasmodium*-Culicidae interaction model.

Both laboratory and field mosquito strains have been found to be associated with microbial organisms that particularly colonise the gut. They consist primarily of Gram-negative bacteria of the Enterobacteriaceae family. Field populations of *An. gambiae* and *An. funestus* were found to contain 16 bacterial species spanning 14 genera (Lindh et al. 2005). The laboratory populations of *An. gambiae* and *An. stephensi* also presented a wide variety of bacteria, especially of the genus *Asaia*, *Enterobacter*, *Mycobacterium*, *Sphingomonas*, *Serratia* and *Chryseobacterium* (Favia et al. 2007, Dong et al. 2009). Bacteria of the *Asaia* genus were also found in *Ae. aegypti* mosquitoes (Pidiyar et al. 2004, Rani et al.

2009, Gaio et al. 2011). In addition, beyond the digestive tract, studies have shown that the species of this genus are also able to colonise the salivary gland and ovaries of mosquitoes and are usually acquired through vertical transmission (Favia et al. 2007).

It has been shown that gut bacteria may have an impact on vectorial competence by inhibiting the sporogonic development of malaria parasites within the mosquito vector (Pumpuni et al. 1993, 1996, González-Cerón et al. 2003, Dong et al. 2009, Cirimotich et al. 2011). Pumpuni et al. (1993, 1996) also showed, whilst manipulating the bacterial content, that Gram-negative bacteria inhibit oocyst formation in whole or in part and that the same action was not observed with Gram-positive bacteria.

Evidence of this influence of the intestinal microbiota on the life cycle of the parasites has been demonstrated for other insects such as sandflies and tsetse flies (Schlein et al. 1985, Welburn & Maudlin 1999).

Recent studies suggest that *Enterobacter* species in the gut of *Anopheles arabiensis* mosquitoes originating from Zambia act directly on *P. falciparum,* blocking the development of the parasite and making this population refractory to infection. This refractoriness was associated with the generation of the ROS that interfere with the development of the parasite and kills it before its invasion of the intestinal epithelium (Cirimotich et al. 2011).

Previous studies suggest that bacteria in the gut lumen modify the intestinal environment and inhibit the development of parasites by the actions of the immune system by overexpression of immunity genes, culminating in an increased rate of production of antimicrobial peptides (Pumpuni et al. 1996, Ratcliffe & Whitten 2004, Michel & Kafatos 2005). Such peptides are likely to play a key role not only in the control of pathogenic or symbiotic bacteria, but also in the development of infections by parasites (Beard et al. 2001, Boulanger et al. 2004). Interestingly, the mosquito immune system acts against bacterial growth and also eliminates a large number of parasites modulating the intensity of infection in mosquitoes infected with *P. berghei* or *P. falciparum* (Meister et al. 2009).

It has also been proposed that certain bacteria taxa can induce a reductive environment within the mosquito midgut, thus aiding in the detoxification of reactive oxygen and nitrogen species, a fact that would allow for an aggressive immune response of the mosquito when infected by the parasite (Wang et al. 2011).

In *Ae. aegypti,* antibiotic treatment affects the progression of *P. gallinaceum* infection. It was observed that mosquitoes treated with kanamycin partially inhibited the sporogonic development of *P. gallinaceum,* while carbenicillin-treated mosquitoes were significantly more susceptible to infection. Although both antibiotics are effective against Gran-negative bacteria, carbenicillin also affects Gram-positive bacteria (AS Orfano et al., unpublished observations).

Recent results obtained in our laboratory show that the expression of AMPs of *Ae. aegypti* mosquitoes is modified with antibiotic treatment and subsequent infection with *P. gallinaceum*. Insects treated with kanamycin had increased expression of defensin 24 h and 36 h after being fed an infective blood meal, in comparison with a group of mosquitoes not treated with antibiotics that were fed an infective blood meal. This period in particular occurs when the ookinete begins to invade the intestinal epithelium, reducing infection. Similar results were observed in similar experiments with *An. gambiae* mosquitoes upon infection with *P. berghei*; a peak of defensin expression was detected at 26 h after the antibiotic-treated mosquitoes were fed an infective blood meal (Richman et al. 1997). In our model, when the insects are treated with carbenicillin and infected, the expression levels of defensin were inferior to those of the control mosquitoes at 24 h and 36 h after blood feeding, revealing a less active immune system, which probably leads to a greater susceptibility to the avian malaria parasite.

In conclusion, we would like to highlight the fact that vector biology has made great advancements over the past years and many results have been attained by "synergic" approaches with computational science as a key element. Many interesting theories are now being discussed and explored regarding the hologenomic basis of speciation (Nikoh et al. 2008, Rosenberg & Zilber-Rosenberg 2011, Ni et al. 2012, Brucker & Bordenstein 2013) and how bacteria and viruses may be shaping the genomes and phenotypes of harbouring organisms (Gorski et al. 2003, Crochu et al. 2004, Degnan & Moran 2008, Keeling & Palmer 2008, Nikoh et al. 2008, Klasson et al. 2009, Rohwer et al. 2009, Holmes 2011, Rosario & Breitbart 2011, Ni et al. 2012, Reyes et al. 2012, Stern et al. 2012, Horie et al. 2013, Husnik et al. 2013, Ioannidis et al. 2013, Seed et al. 2013). When we take into consideration the fact that there are one million bacteria and 10 million viral particles per millilitre of surface seawater (Suttle 2005, Ng et al. 2011b, Rosario & Breitbart 2011), maximising the NGS sequencing data generated by the *An. aquasalis* genome project becomes an opportunity to explore many of these new avenues. These vast surroundings and potentially associated microcosms may have left their mark upon the coevolving larval stages of this species while developing in brackish waters.

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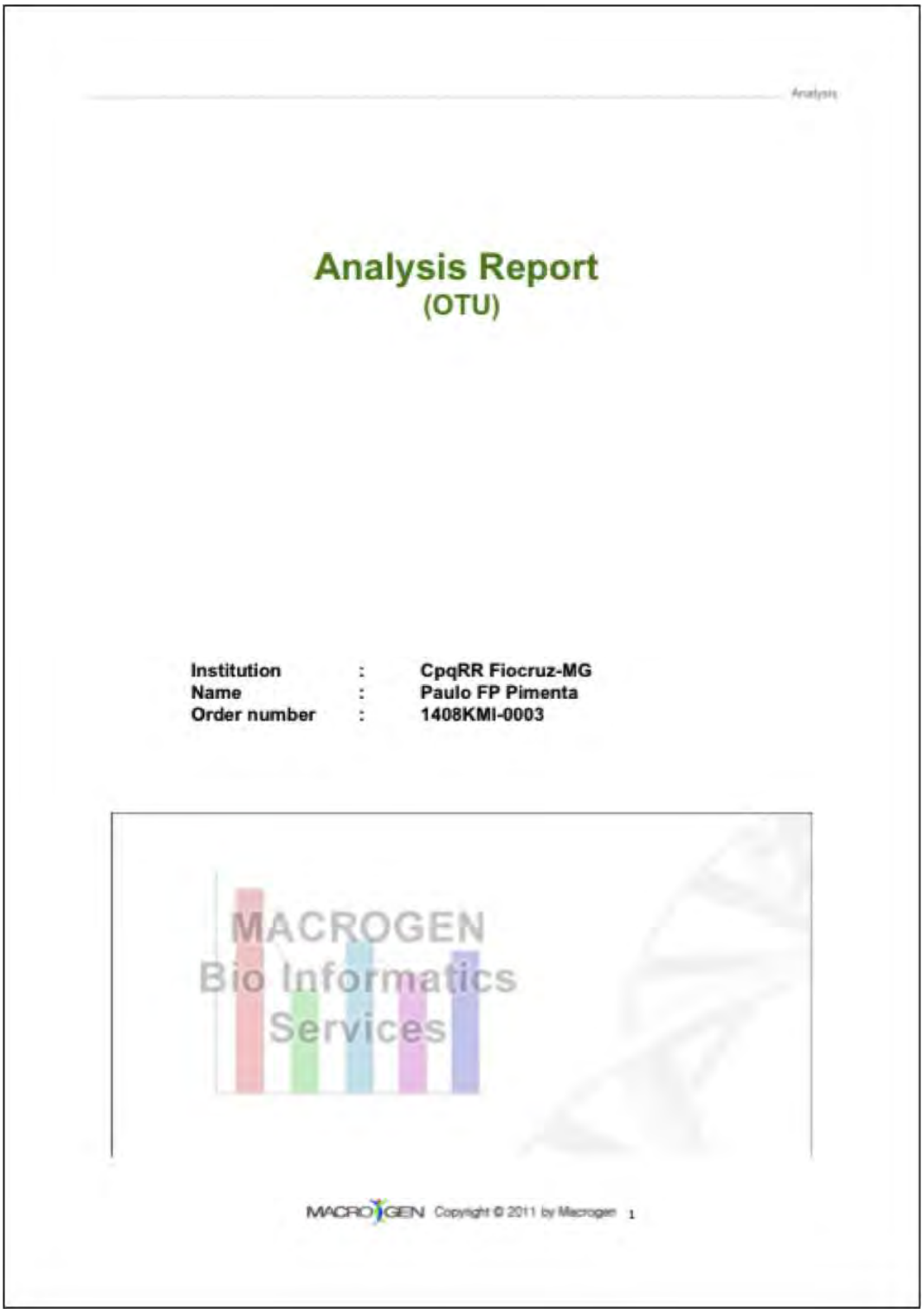
Anexos

Anexo 1: Relatório enviado pela Macrogen sobre a predição de fOTUs associados de *A. aquasalis*.



Analysis

Analysis Report
(OTU)

Institution	:	CpqRR Fiocruz-MG
Name	:	Paulo FP Pimenta
Order number	:	1408KMI-0003

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