562ª ATA DA COMISSÃO EXAMINADORA DA TESE DE DOUTORADO EM QUÍMICA BIOLÓGICA DO PROGRAMA DE PÓS-GRADUAÇÃO EM QUÍMICA BIOLÓGICA APRESENTADA E DEFENDIDA POR **OCTAVIO AUGUSTO TALYULI DA CUNHA.**

No sexto dia do mês de julho de dois mil e vinte e dois, às 08h00min, realizou-se de forma totalmente remota e em concordância com o disposto na Resolução CEPG nº 01, de 16 de março de 2020, tendo sido gravada através do aplicativo Zoom®, a defesa da Tese de Doutorado em Química Biológica apresentada e defendida por **Octavio Augusto Talyuli da Cunha**, intitulada: **"Reflexos da fisiologia intestinal e imunometabolismo na competência vetorial do mosquito *Aedes aegypti*"**. A Comissão foi organizada obedecendo ao disposto nas Resoluções do Conselho de Ensino para Graduados da UFRJ e no Regulamento do Programa de Pós-graduação em Química Biológica, área de concentração em Química Biológica, estando constituída pelos Professores: (aprovado) **Anderson de Sá Nunes** (Professor Associado III da Universidade de São Paulo), (aprovado) **Luciana Jesus da Costa** (Professor Associado do Instituto de Microbiologia Professor Paulo de Góes, UFRJ), (aprovado) **João Trindade Marques** (Professor Adjunto da Universidade Federal de Minas Gerais), (aprovado) **Isabela Barbosa Ramos** (Professor Adjunto do Instituto de Bioquímica Médica Leopoldo de Meis,UFRJ), (aprovado) **Mauricio Roberto Viana Sant'anna** (Professor Adjunto da Universidade Federal de Minas Gerais), além de seu(ua) orientador(a), (aprovado) **Pedro Lagerblad de Oliveira** (Professor Titular do Instituto de Bioquímica Médica Leopoldo de Meis, UFRJ), sendo designado(a) Presidente da Banca e sem direito a voto. Após haver o(a) candidato(a) apresentado os resultados de sua tese, obedecendo ao prazo regimental, foi dada a palavra aos examinadores para arguição, na seguinte ordem: Anderson de Sá Nunes, Luciana Jesus da Costa e João Trindade Marques, tendo o(a) candidato(a) respondido às perguntas formuladas. Assim sendo, a Comissão Examinadora decidiu recomendar a outorga ao(à) candidato(a) do Grau de Doutor em Ciências (Química Biológica). Nada mais havendo a tratar, eu, Pedro Lagerblad de Oliveira, lavrei a presente Ata, que assino em meu nome e dos demais membros da Banca Examinadora.

Rio de Janeiro, 6 de julho de 2022.

_____

Pedro Lagerblad de Oliveira

(Presidente da Banca)

Universidade Federal do Rio de Janeiro
Instituto de Bioquímica Médica Leopoldo de Meis
Programa de Pós-Graduação em Química Biológica

Reflexos da fisiologia intestinal e imunometabolismo na competência vetorial do mosquito *Aedes aegypti*.

Octávio Augusto Talyuli da Cunha

Rio de Janeiro
Julho/2022

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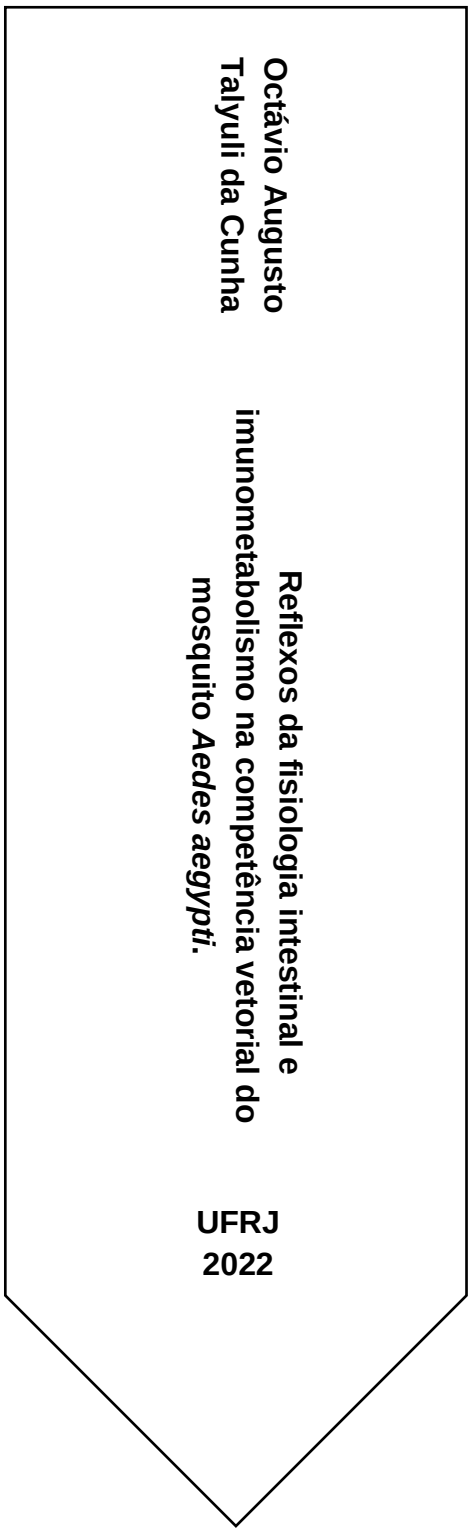
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Octávio Augusto Talyuli da Cunha

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Química Biológica, do Instituto de Bioquímica Médica Leopoldo de Meis, Universidade Federal do Rio de Janeiro, como parte dos requisitos necessários à obtenção do título de Doutor em Química Biológica.

Orientador:
Pedro Lagerblad de Oliveira
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Bioquímica Médica Leopoldo de Meis
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Rio de Janeiro
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FICHA DE APROVAÇÃO

Reflexos da fisiologia intestinal e imunometabolismo na competência vetorial do mosquito *Aedes aegypti*.

Octávio Augusto Talyuli da Cunha

Tese submetida ao Programa de Pós-graduação em Química Biológica, do Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro – UFRJ, como parte dos requisitos necessários à obtenção do título de Doutor em Química Biológica.

Aprovado por:

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Pedro Lagerblad de Oliveira

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Orientador

O presente trabalho foi realizado no Laboratório de Bioquímica de Artrópodes Hematófagos da Universidade Federal do Rio de Janeiro sob a orientação do Professor Pedro Lagerblad de Oliveira, na vigência de auxílios concedidos pelo CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), pela FAPERJ (Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro), PRONEX (Programa de Apoio a Núcleos de Excelência), FINEP (Financiadora de Estudos e Projetos), INCT-EM (Instituto Nacional de Ciência e Tecnologia – Entomologia Molecular). Parte dos dados obtidos foram realizados em período sanduíche no laboratório *Mosquito Immunity & Vector Competence Section* (MIVCS) do *National Institutes of Health* sob a orientacao da Doutora Carolina Barillas-Mury.

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Resumo

A hematofagia proporcionou as fêmeas do mosquito *Aedes aegypti* uma fonte rica em proteínas e lipídeos essenciais para a progressão da ovogênese. Entretanto, o sangue ingerido também se tornou um veículo para diversos patógenos, como arbovírus, que se replicam no organismo do inseto e são transmitidos durante uma segunda alimentação sanguínea. Sendo assim, o epitélio intestinal é considerado como a primeira barreira para os parasitas adquiridos no repasto sanguíneo. Interessantemente, a invasão do epitélio intestinal se dá concomitante a digestão do sangue, processo este que leva o inseto a uma intensa reprogramação fisiológica. Durante muito tempo se estudou como o sistema imune dos mosquitos reconheciam os patógenos e respondiam ao desafio. Entretanto, esses estudos se davam de forma destacada e pouco integrada com a fisiologia geral do inseto. Considerando que a invasão da barreira do epitélio intestinal pelos arbovírus e a digestão do sangue ocorrem no mesmo momento, nós propusemos nesta tese que há uma relação intrínseca entre os dois processos. Em outras palavras, a digestão do sangue e suas adaptações fisiológicas impactam diretamente a maneira a qual o inseto montará sua resposta imunológica a invasão viral. Em um primeiro momento, demonstramos como TOR (Target of Rapamycin), uma via canônica de controle metabólico, é induzida no intestino do mosquito *Aedes aegypti* frente aos aminoácidos obtidos pelo repasto sanguíneo e induz a proteólise do sangue, além de controlar a transcrição de genes chave para o metabolismo de carboidratos e lipídeos. TOR também possui ampla atuação na regulação da imunidade intestinal, impactando diretamente as vias de Toll, IMD e Jak/STAT, bem como o controle da proliferação da microbiota intestinal. A inibição de TOR causa um cenário de imunossupressão, o que favorece diretamente a infecção intestinal por Zika e Chikungunya. Nós demonstramos também que TOR regula a síntese da matriz peritrófica (PM), camada extracelular de quitina que reveste o bolo alimentar. Em uma segunda abordagem, mostramos o papel fisiológico de uma heme peroxidase (HPx1) essencial para a manutenção da função de barreira da PM na contenção e isolamento da microbiota. Uma vez que a HPx1 esteja depletada, o intestino responde aumentando a produção de ROS, o que reduz os níveis de microbiota e gera um dano tecidual. A quebra da homeostasia do intestino através do silenciamento de HPx1 leva a uma redução de cerca de 100x da carga de Dengue e Zika no epitélio intestinal. Nossos resultados demonstram que imunidade e alterações metabólicas induzidas pelo repasto sanguíneo estão intimamente relacionadas a maneira que o inseto controlará a infecção viral. A integração do imunometabolismo com a capacidade vetorial do mosquito *Aedes aegypti* abre novos caminhos de entendimento da dinâmica da infecção viral no inseto, bem como dos processos de resistência e tolerância frente ao desafio imune.

Abstract

The hematophagy provided *Aedes aegypti* female mosquitoes with a source of proteins and lipids essential for oogenesis. However, blood-feeding has also become a vehicle for several pathogens, such as arboviruses, which replicate in the insect's body and are transmitted during a second blood meal. Therefore, the intestinal epithelium is considered the first barrier for parasites acquired in the blood meal. Interestingly, the gut epithelium invasion occurs concomitantly with the blood digestion, a process that leads the insect to extensive physiological reprogramming. For a long time, it was studied how the immune system of mosquitoes recognized pathogens and responded to the challenge. However, these studies were poorly integrated with the general physiology of the insect. Considering that the gut invasion by arboviruses and the blood digestion occur at the same time, we proposed in this thesis that there is an intrinsic relationship between these two processes. In other words, blood digestion and its physiological adaptations directly impact the way in which the insect will mount its immune response to viral invasion. At first, we demonstrate how TOR (Target of Rapamycin), a canonical metabolic-controlling pathway, is induced in the *Aedes aegypti* mosquito gut by the amino acids obtained from the blood meal. This pathway induces blood proteolysis, in addition to controlling gene transcription of key enzymes for carbohydrate and lipid metabolism. TOR also has a broad role in regulating intestinal immunity, which directly impacts the Toll, IMD, and Jak/STAT pathways, as well as the midgut microbiota proliferation. TOR inhibition causes an immunosuppression scenario that allows intestinal infection by Zika and Chikungunya viruses. We also demonstrated that TOR regulates the peritrophic matrix (PM) synthesis, an extracellular layer of chitin that surrounds the food bolus. Furthermore, we showed the physiological role of a heme peroxidase (HPx1) essential for the maintenance of PM barrier function in the containment and isolation of microbiota. Once HPx1 is depleted, the gut responds by increasing ROS generation, which reduces microbiota levels and causes tissue damage. The breakdown of intestinal homeostasis through HPx1 silencing leads to a reduction of about 100x in the Dengue and Zika burden on the intestinal epithelium. Our results demonstrate that immunity and metabolic alterations triggered by the blood meal are closely related to the way in which the insect controls the viral infection. The integration of immunometabolism with the vectorial capacity of the *Aedes aegypti* mosquito opens new avenues for understanding the dynamics of viral infection in the insect, as well as the processes of resistance and tolerance upon immune challenges.

Sumário

Introdução

Os insetos representam a maior parte da biomassa terrestre. Muitos destes desenvolveram relações extremamente próximas com os serem humanos e são capazes de transmitir patógenos. Mosquitos do gênero *Aedes* (*Aedes aegypti* e *Aedes albopictus*) são os mais importantes vetores de arboviroses de relevância médica, como a Dengue, Zika, Chikungunya, Febre Amarela, dentre outros. Somente no Brasil, entre 2008 a 2019, dados do Ministério da Saúde apontam 10,6 milhões de casos confirmados de Dengue, com 6.429 óbitos (MINISTERIO DA SAUDE, 2020). Terapias eficazes antivirais ainda não estão disponíveis para a população e, desta forma, o controle da população de mosquitos, bem como medidas socioeducativas, são ainda as principais maneiras de redução dos casos de arboviroses.

Os mosquitos *Aedes aegypti* se alimentam naturalmente de seiva e fluidos vegetais, ricos em carboidratos, que sustentam suas atividades metabólicas basais. Entretanto, as fêmeas encontraram na hematofagia a fonte de nutrientes como aminoácidos e lipídios, para sustentarem as demandas fisiológicas relacionadas ao desenvolvimento de seus ovários e, posteriormente, dos ovos (HANSEN *et al.*, 2014).

Ao se alimentarem do sangue do hospedeiro vertebrado infectado por tais arbovírus, as fêmeas ingerem juntamente com sangue partículas virais que irão se estabelecer no inseto. O sangue infectado é direcionado para o intestino médio, onde será digerido. Neste mesmo momento, as partículas infecciosas virais são endocitadas e infectam o epitélio intestinal. Este, por sua vez, configura-se como a primeira barreira de infecção imposta pelo mosquito, compreendida por muitos como a mais importante e determinante para o sucesso da infecção (FRANZ *et al.*, 2015). A atuação do intestino como barreira envolve fatores genéticos e metabólicos do mosquito que irão impactar diretamente a infecção (BLACK IV *et al.*, 2002; FRANZ *et al.*, 2015). Mais recentemente, tem sido mostrado que a microbiota intestinal, que se encontra em franca expansão durante a digestão do sangue produz estímulos regulatórios que influenciam de forma decisiva o progresso da infecção (JUPATANAKUL; SIM; DIMOPOULOS, 2014; RAMIREZ *et al.*, 2012; XI; RAMIREZ; DIMOPOULOS, 2008). Após o estabelecimento da infecção no intestino, observa-se o escape de partículas virais para a hemocele do inseto,

culminando na disseminação da infecção e consequente invasão das glândulas salivares. Uma vez nestas glândulas, as partículas virais serão inoculadas, juntamente com a saliva, através da picada, no corpo do próximo hospedeiro em que o inseto se alimentará de sangue, completando assim o ciclo da doença.

Os insetos possuem um sistema imunológico de caráter inato coordenado por vias intracelulares de reconhecimento de patógenos ou de padrões associados a estes (RODGERS; GENDRIN; CHRISTOPHIDES, 2017). Tais vias são altamente responsivas a infecções bacterianas bem como a microbiota residente na luz do intestino. Além de reconhecimento bacteriano, as vias Toll, IMD e Jak/Stat já foram reportadas na literatura como importante moduladoras da resposta antiviral (ANGLERÓ-RODRÍGUEZ *et al.*, 2017; SIM *et al.*, 2013; SIM; JUPATANAKUL; DIMOPOULOS, 2014). De maneira geral, acreditava-se que Toll e Jak/Stat eram essenciais para a regulação da infecção viral (SOUZA-NETO; SIM; DIMOPOULOS, 2009; XI; RAMIREZ; DIMOPOULOS, 2008), entretanto, hoje já se demonstrou que a regulação da via de IMD também é importante para a infecção pelo controle que esta via exerce sob a microbiota intestinal (BARLETTA *et al.*, 2017). Além disto, linhagens de mosquitos com diferentes níveis de susceptibilidade ao vírus dengue mostram diferentes relações com as vias imunes e o controle que estas exercem na infecção viral (SIM *et al.*, 2013). No entanto, ainda não se sabe exatamente qual componente deste repertório molecular é capaz de limitar a infecção. Por exemplo, observa-se claramente a regulação transcricional dos peptídeos microbianos no intestino frente a infecção viral, mas a função destes na resposta antiviral ainda não foi elucidado. Especula-se que eles são capazes de atuar em partículas virais da mesma maneira que atuam na parede celular bacteriana, ou ainda interferirem na adsorção e no tráfico intracelular das partículas virais (AHMED *et al.*, 2019). Apesar de observado impacto na infecção viral, estas vias não são específicas no reconhecimento de estruturas virais, podendo, portanto, ser ativadas de maneira cruzada, através da interação com a microbiota ou outro fator. Já foi descrita, entretanto, uma via que reconhece especificamente estruturas virais, denominada via de RNA de interferência (RNAi), a qual dispara uma resposta imune mais eficiente (BONNING; SALEH, 2021). Uma vez ativada, a maquinaria molecular da via de RNAi mantem consigo um fragmento de cerca de 21 nucleotídeos do genoma viral, o qual guiará o reconhecimento de genomas virais que

estejam presentes no citoplasma da célula. Através do pareamento destes fragmentos com o genoma viral em replicação, ocorre a clivagem deste e consequente controle da carga viral.

Juntamente com a ativação das vias imunes, que culminarão na expressão de genes efetores como os peptídeos antimicrobianos, a geração de espécies reativas de oxigênio e nitrogênio também vem sendo reportada como importante componente das respostas antibacterianas e antivirais (BOTTINO-ROJAS *et al.*, 2018; LIU *et al.*, 2016; OLIVEIRA *et al.*, 2011; RAMOS-CASTAÑEDA *et al.*, 2008). De modo geral, o aumento de espécies oxidantes contribui para o controle da infecção, por meio de mecanismos moleculares ainda não totalmente elucidados, porém discute-se que isto ocorre tanto por oxidação direta da partícula ou de moléculas virais, bem como pela regulação de vias intracelulares que conduzem a ativação de efetores com atividade antiviral. Em contrapartida, a potencialização das respostas antioxidantes permite uma maior replicação viral (LIU et al., 2016; OLIVEIRA et al., 2017).

Apesar do entendimento da regulação das vias de imunidade inata durante uma infecção viral ter progredido consideravelmente nas últimas décadas (ROSENDO MACHADO; VAN DER MOST; MIESEN, 2021), resultados de análises transcriptômicas da infecção de tecidos digestivos mostram impacto da infecção na produção de enzimas digestivas e do metabolismo tanto glicídico quanto lipídico, em componentes do sistema de controle da proteostase como chaperonas, entre outros (ANGLERÓ-RODRÍGUEZ *et al.*, 2017; CHOTIWAN *et al.*, 2018; DONG; BEHURA; FRANZ, 2017). Assim, a literatura ainda precisa avançar em como os processos fisiológicos básicos da digestão do sangue se correlacionam com as vias de imunidade inata e impactam na infecção do inseto vetor. As partículas virais entram em contato com o organismo do inseto vetor no mesmo momento em que o sangue é digerido, mas não se conhece como os mecanismos relacionados ao processo digestivo são capazes de influenciar a infecção viral do epitélio intestinal.

Frente a uma alimentação de sangue, o organismo do mosquito passa por um amplo remodelamento fisiológico, observando-se síntese e secreção de diversas enzimas que sustentarão a digestão do sangue, a absorção de tais nutrientes e remodelamento do metabolismo, bem como modulações nas respostas imune

principalmente em resposta a proliferação da microbiota intestinal. Todos esses fatores são finamente regulados e trabalham de maneira dinâmica para manutenção da homeostasia. A interseção entre as regulações metabólicas que células e/ou um tecido apresentam durante um desafio imunológico e como, a partir do status energético, se dispara uma resposta imune dá-se o nome de imunometabolismo (MAN; KUTYAVIN; CHAWLA, 2017; O'NEILL; KISHTON; RATHMELL, 2016). Ou seja, um termo amplo que tenta integrar o metabolismo e a imunidade que durante anos foram estudados de maneira isolada.

Diante disto, esta tese foi centrada na relação entre os arbovírus e o epitélio intestinal de fêmeas de *Aedes aegypti*, orientada pela hipótese de trabalho de que a digestão do sangue regula a fisiologia – e o imunometabolismo intestinal, o que essencialmente determina o curso da infecção viral.

O primeiro capítulo desta tese apresenta uma revisão onde se faz uma discussão crítica da literatura acerca de como aspectos da fisiologia do inseto, em particular, a digestão do sangue pode impactar a competência vetorial de insetos e assim desenvolve uma reflexão acerca dos conceitos e vacâncias da literatura em torno da proposta central desta tese. Os dois capítulos seguintes abordarão experimentalmente tópicos discutidos no primeiro capítulo/revisão bibliográfica. Primeiro, mostraremos como a quinase TOR (Target of Rapamycin) controla de maneira ampla e eficiente o imunometabolismo intestinal e a capacidade vetorial do mosquito *Aedes aegypti*. Em um segundo momento discutiremos como a matriz peritrófica secretada pelo epitélio intestinal em resposta a alimentação com sangue é regulada pela atividade de uma heme peroxidase e como isto impacta fortemente a infecção viral.

Capítulo I

Revisão Bibliográfica

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Non-immune Traits Triggered by Blood Intake Impact Vectorial Competence

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Blood-feeding arthropods are considered an enormous public health threat. They are vectors of a plethora of infectious agents that cause potentially fatal diseases like Malaria, Dengue fever, Leishmaniasis, and Lyme disease. These vectors shine due to their own physiological idiosyncrasies, but one biological aspect brings them all together: the requirement of blood intake for development and reproduction. It is through blood-feeding that they acquire pathogens and during blood digestion that they summon a collection of multisystemic events critical for vector competence. The literature is focused on how classical immune pathways (Toll, IMD, and JAK/Stat) are elicited throughout the course of vector infection. Still, they are not the sole determinants of host permissiveness. The dramatic changes that are the hallmark of the insect physiology after a blood meal intake are the landscape where a successful infection takes place. Dominant processes that occur in response to a blood meal are not canonical immunological traits yet are critical in establishing vector competence. These include hormonal circuitries and reproductive physiology, midgut permeability barriers, midgut homeostasis, energy metabolism, and proteolytic activity. On the other hand, the parasites themselves have a role in the outcome of these blood triggered physiological events, consistently using them in their favor. Here, to enlighten the knowledge on vector–pathogen interaction beyond the immune pathways, we will explore different aspects of the vector physiology, discussing how they give support to these long-dated host–parasite relationships.

Keywords: tolerance, vector competence, blood-feeding, immunity, pathogens, parasite–vector interaction, insect physiology, midgut homeostasis

INTRODUCTION

For a long time, the traditional thinking on the insect vector–pathogen interaction described this relationship as impinging a low or even no fitness cost to the insect host (Sisterson, 2009; Powell, 2019). However, the discovery of the insect immune system brought to the scene a plethora of immune genes that were frequently modulated by the infection and, in several cases, the upregulation of these genes promptly reduced pathogen burden (Saraiva et al., 2016; Shaw et al., 2018; Telleria et al., 2018; Matetovici et al., 2019; Salcedo-Porras and Lowenberger, 2019). These findings led to a large number of studies on the immune signaling pathways and immune effector

genes capable of decreasing or blocking pathogen burden and vector competence, which could lead to the possibility of limiting the disease prevalence under field conditions.

The immunology conceptual framework that has dominated the last century is the idea that the capacity to eliminate the infectious agent is the hallmark of the organism's reaction against the infection. This so-called pathogen resistance and the path that eventually leads to health are considered synonymous with the elimination of the infectious agent. However, in the last decade, several studies have directed attention to the ability of organisms to reduce deleterious effects of infections without relying on the elimination of the parasite, but rather acting by alternative mechanisms that could restore tissue homeostasis, reducing self-inflicted damage caused by the host immune reaction or bythe pathogen directly (Chovatiya and Medzhitov, 2014). Some early studies have termed this disease endurance (Casadevalland Pirofski, 1999; Graham et al., 2005; Ayres et al., 2008). Later these processes directed to reduce fitness loss due to infection were grouped under the concept of disease tolerance (Medzhitov et al., 2012). The tolerance to disease is highlighted here as a way to fight infection. Mechanistic studies show thata wide variety of processes are implicated in disease tolerance, such as stress response, tissue homeostasis, wound repair, and energy metabolism (Shaw et al., 2018). Essentially, this fresh outlook in immunology changes the microbe-killing focus of theimmune response to the promotion of host health and survival through a global regulation of physiological responses (Lissner and Schneider, 2018). In insects, these studies were mainly performed with model insects, especially *Drosophila* (Lissner andSchneider, 2018) and the concept of disease tolerance has not yet been explored in the context of the interaction between insect vectors and the pathogens they transmit, as recently pointed(Oliveira et al., 2020).

Blood-feeding is a central event in the life cycle of both the insect vector and the pathogens they transmit. Typically, few parasites or viruses are taken up by the insect along with a blood meal. The number of pathogens in the blood meal is known as the critical bottleneck that will define the success of the infection. After a meal, the midgut becomes an aggressive environment, quickly populated by digestive enzymes that can potentially attack the pathogen. Moreover,it is colonized by an exuberant indigenous microbiota that may be a competitor for the incoming invader, at a clear numerical disadvantage. In addition, like all epithelial tissues, one of the main functions of the gut epithelium is to be a barrier of protection from the external world and to select what should enter into the organism. For most hematophagous insects, blood is the essential source of amino acids used tomake yolk proteins. Therefore, hormonal control of reproductionis usually triggered by blood intake, which is tightly linked tothe pace of blood digestion (Hagedorn, 2004). Several reports have shown hormonal effects on parasite infectivity, which, in some cases, have been attributed to a crosstalk between hormone signaling cascades and immune pathways (Nunes et al., 2020). Finally, for most blood-sucking insects, vertebrate hosts might be available more than once in a lifetime, and the effect of multiple blood meals have been shown to affect the life of pathogens by mechanisms that are not fully understood (Serafim et al., 2018). To summarize, the physiology of blood digestion of the hematophagous insect is the actual landscape where pathogens transmitted by them will thrive or not, a concept that was also revised by Nouzova et al. (2019). More importantly, the functioning of these processes ultimately defines the fitness cost of the infection to the insect vector, a major variable that affects vectorial capacity. Here we have addressed just some topics of vector physiology to illustrate the general concept that the performance of host organism integrated basic functionsis critical to the success of the vector/pathogen association. As these are multiple and comprehensive topics, our intentionis not to exhaust the analysis of the literature related to them. More than that, our goal is to highlight the need of examining the role of these and other non-immune basic aspects of insect physiology, not reviewed here, in determining the vector competence.

BLOOD DIGESTION AND METABOLIC SIGNALING

Some hematophagous arthropods feed on blood for their whole life cycle such as ticks and triatomine bugs, while in others, like mosquitoes and sandflies, only female adults feed on blood. In the adult stages, blood meals are strictly essential for oogenesis for most species. However, even for those few autogenous insects that use their teneral reserves to make the first batch of eggs, the following cycles of ovarian growth rely on vertebrate blood, and therefore, reproductive success depends on blood-feeding. The ingestion of a blood meal elicits a response in the gut-brain axis known to involve the central nervous system, the enteric nervous system, and the gastrointestinal tract (Hagedorn, 2004). The gut-brain crosstalk not only ensures the proper maintenance of gastrointestinal homeostasis but has multiple effects on insect physiology through neural, endocrine, immune, and humoral links (Gonzalez et al., 1999; Brown et al., 2008; Castillo et al., 2011; Gulia-Nuss et al., 2011). The nutritional intake connects intermediary metabolism to sexual maturation, oogenesis, microbiota colonization, and immune response, the latter being triggered by the encounter of the vector with pathogens.

Strictly speaking, digestion starts with hydrolysis of foodby a vast array of digestive enzymes secreted after a meal (proteinases, carbohydrases, and lipases) that, together with nutrient transporting proteins, are needed to process nutrients in the gut (Santiago et al., 2017). However, in the context of hematophagous insects, proteases have received far more attention because blood is composed mainly of proteins (90% of dry weight), leading those insects to translate an arsenal of proteases to support protein digestion (Lehane, 2005; Brackney et al., 2010; Henriques et al., 2017; Sterkel et al., 2017). In most insects, proteolysis is based on trypsin and other serine

Abbreviations: TG, triacylglycerol; TGFβ, transforming growth factor-beta; ILP, insulin-like peptides; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; NRF-2, nuclear factor erythroid 2–related factor 2; OXR1, oxidation resistance protein 1; cGMP, cyclic guanosine monophosphate.

proteases, in contrast to triatomines and ticks, where aspartic and cysteine proteases drive protein digestion (Sojka et al., 2008; Santiago et al., 2017; Henriques et al., 2021). Initially, protein degradation was described as accomplished by a few enzymes of each type, but genome sequencing in mosquitoes, triatomines, sandflies and ticks showed the existence of multiple copies of enzymes in all classes, revealing extensive gene duplications that probably occurred after the acquisition of the blood-feeding habit, suggesting the need for redundancy or some type of functional differentiation among enzymes of the same family (Sojka et al., 2008; Henriques et al., 2017; Santiago et al., 2017).

The possibility of pathogens being targeted by digestive enzymes and/or the manipulation of the host's digestion process by the pathogens has been addressed in several studies, butmost of them were performed before the genomic expansionof digestive proteases was acknowledged. Insect digestive tracts vary extensively in morphology and physicochemical properties, factors that greatly influence the potential interaction between their also diverse set of transmissible pathogens and digestive milieu. The activity of proteolytic enzymes in the gut of *Anopheles* mosquitoes does not appear to be affected by *Plasmodium* infection. In addition, differences in the activity of digestive proteases in general are not observed among mosquitoes with different levels of susceptibility to infection (Feldman et al., 1990; Chege et al., 1996; Jahan et al., 1999). However, proteolytic activities present in the midgut lumen are required to activate a chitinase secreted by these parasites that is essential in the initial event of midgut infection (Shahabuddin et al., 1993). In the *Leishmania mexicana-Lutzomyia*'s pair, the pathogen promotes a decrease of trypsin activity in the vector's gut, increasing parasite burden. Trypsin knockdown exerts the same effect (Sant'Anna et al., 2009), probably making the parasite more resistant to the highly degradative habitat of midgut lumen. In contrast, in the triatomine bug *Rhodnius prolixus*, the activity of cathepsin D-like enzymes increases upon infection by *Trypanosoma cruzi* (Borges et al., 2006). Still, its inhibition did not affect parasite development in the conditions tested (Garcia and Gilliam, 1980). As we can see, the existing examples concerning proteases in the vector-parasite interaction were based on the analysis of total enzymatic activities, which resulted from the action of multiple enzymes belonging to the same class. These facts might be the answer to the diversity of responses observed among the different groups of insects. It would be interesting to know what are the specific proteases involved in the different events and if they can have the same role in the different vector-parasite pairs.

For enveloped viruses, it is known that the establishment of a successful infection is highly dependent on the fusion of the viral envelope with host cell membranes, where the envelope proteins need to be activated by proteolytic processing by host cell proteases (Klenk and Garten, 1994). As for *Aedes aegypti* infection by dengue virus, treating the mosquito with a trypsin inhibitor before exposure to the virus decreases the midgut infection, which can be partially rescued when the virus is previously incubated with bovine trypsin (Molina-Cruz et al., 2005). In this case, the authors discuss the possibility of the virus be pre-processed by trypsin before gut epithelia invasion, enhancing its virulence. In contrast, another work published in 2008 showed that silencing the late trypsin 5G1 or the addition of soybean trypsin inhibitor to the infectious blood meals increased midgut infection rates by DENV-2 (Brackneyet al., 2008). The latter work was confirmed by a study showing that prior colonization of *Ae. aegypti* with the fungus *Talaromyces*sp. induced the downregulation of many digestive enzymes, including several trypsins, resulting in higher susceptibilityto dengue infection. Moreover, knockdown of these trypsin genes was able to recapitulate the fungus-induced decrease in viral infection (Angleró-Rodríguez et al., 2017b). Although controversial, the studies made with dengue and *Ae. aegypti* suggest that blood digestion mediated by trypsins may influence the rate of DENV-2 infection. Although the literature on the subject is scarce, proteases may influence vector infection by viruses. The determination of the precise time course of viral invasion and protease expression together with the repertoire of proteases in the mosquito midgut would allow for a more comprehensive approach of the contribution of each digestive enzyme for mosquito vectorial competence.

There is now a general agreement that the successful infection of an insect vector involves a tripartite interaction between the insect, the pathogens and the intestinal microbiota (Cirimotich et al., 2011; Ramirez et al., 2012), a concept that has been verified for different host/pathogens associations (Castro et al., 2012; Narasimhan and Fikrig, 2015; Romoli and Gendrin, 2018; Telleria et al., 2018). Interestingly, studies performed with mammalian models revealed that proteases both from the host or from the microbiota are important modulators of intestinal homeostasis and are involved in the interaction with pathogens (Buzza et al., 2010; Motta et al., 2019; Edogawa et al., 2020; Kriaa et al., 2020). Therefore, it is tempting to speculate if the influence of proteases on vector infection by pathogens is not at least partially mediated by a role of these enzymes on intestinal microbiota.

In addition to proteins, vertebrate blood is also enriched with lipids. Host lipid usage by pathogens and regulatory changes of lipid metabolism triggered by infection appear as keyplayers in several host-pathogen relationships, being essential determinants for vector competence, as recently revised by O'nealet al. (2020). Digestive lipases, such as TG lipases, are flux generating enzymes for lipid metabolism pathways. The essential process of absorption of digested lipids from the blood mealby midgut cells is followed by an increased lipid transport from the midgut to other tissues, to support development and oogenesis. This lipid transfer is promoted by lipophorin, the main hemolymph lipoprotein, and results in lipid accumulationin the fat body (revised by Gondim et al., 2018). Interestingly, *Plasmodium* oocysts in the *Aedes* gut basal lamina hijack mosquito lipophorin to support the parasite development (Atella et al., 2009) and knockdown of lipophorin by RNA interference (RNAi) strongly restricted development of *Plasmodium* oocysts, reducing their number by 90% (Cheon et al., 2006; Rono et al., 2010). Similarly, induction of lipophorin synthesis is observed in *C. quinquefasciatus* with the filaria *Wuchereria bancrofti* (Kumar and Paily, 2011).

Host lipid remodeling is also observed in vertebrate infection by arboviruses to support their replication (Ng et al., 2008; Fernández de Castro et al., 2016; Leier et al., 2020). Similar

reprogramming events have already been shown in vector cells (Perera et al., 2012; O'neal et al., 2020). An increase in the number of lipid droplets has been observed in *Aedes albopictus* C6/36 cells infected with dengue virus (Samsa et al., 2009). Furthermore, lipidomic analyses of the same DENV-infectedcells revealed a large number of differentially expressed genes of diverse classes of lipids such as phospholipids and sphingolipids (Perera et al., 2012). On the same way, *Ae. aegypti* Aag2 cell line showed a regulation in the expression of lipid-related genes upon dengue 2 infection (Barletta et al., 2016). Besides,*in vivo* lipidomics showed that dengue infection in *Ae. aegypti* mosquitoes changed the lipid profile, mainly based on the inhibition of acylglycerolphosphate acyltransferase (AGAPT1), leading to an accumulation of phospholipids that support the viral replication (Chotiwan et al., 2018; Vial et al., 2019).

The blood-fed *Ae. aegypti* gut seems to increase the expression of many lipid-related genes, such as fatty acid synthase and perilipin-like proteins, which boost lipid droplet formation after blood meal (Barletta et al., 2016). Lipid droplets wereshown in mammals to serve as a signaling platform involved with the synthesis of bioactive lipids (eicosanoids) (Vallochiet al., 2018). In *Aedes* mosquitoes, it has been shown that the midgut epithelia synthesize prostaglandins in response tothe microbiota expansion in a phospholipase A-dependent manner that tunes the innate immune system against viral infection (Barletta et al., 2020). Similarly, *Anopheles gambiae* midgut produces prostaglandins in response to microbiota elicitors upon *Plasmodium* invasion, which triggers a cellular immune response (Barletta et al., 2019). The bug *R. prolixus* humoral and cellular responses seem to be modulated by eicosanoids as well (Azambuja et al., 2017). Using *T. rangeli* infection model, it was demonstrated that the insect reduces the arachidonic acid (the eicosanoid precursor) circulating in the hemolymph, leading to an inhibition of hemocytes phagocytic activity in the hemolymph (Garcia et al., 2004b; Figueiredoet al., 2008). Although eicosanoids/prostaglandins are part ofthe immune molecular arsenal in insects, these arthropods lack the canonical cyclooxygenase (COX), a key enzyme to convert the arachidonic acid into eicosanoids (Varvas et al., 2009). This is intriguing because those insects respond to the treatment with pharmacological COX inhibitors, such as indomethacin and acetylsalicylic acid, impairing the eicosanoid synthesis and modulating the immune response (Garcia et al., 2004a; Barletta et al., 2020). A question that remains open is what are theenzymes that play the role of canonical vertebrate COX in insects.In this sense, a specific peroxinectin named Pxt, that catalyzes the formation of the prostaglandin H2 (PGH_2), was identifiedin the follicle cells of *Drosophila* (Tootle et al., 2011). Later the same COX-Like activity was identified in the moth *Spodoptera exigua* (Park et al., 2014). Additionally, Barletta et al. (2019) showed that *An. gambiae* heme peroxidases 7 and 8 are importantenzymes to synthesize the prostaglandin by the mosquito gut epithelia, suggesting possible candidates for alternative enzymes with COX-like activities.

The interplay between the lipid metabolism pathways and the autophagy-related molecular machinery, named as lipophagy, and its role in physiological and pathological processes in mammals has received increased attention in the last few years (Schulze et al., 2017; Kounakis et al., 2019). Recently, it was demonstrated that the Chagas' disease vector *R. prolixus* can use lipophagy during starvation to increase life span and locomotor activity (Santos-Araujo et al., 2020). It would be interesting to verify how this lipophagic machinery works under infection by either *T. cruzi*, a parasite limited to the intestinal environment, or *T. rangeli*, which is capable to invade the hemocoel and colonize salivary glands. Moreover, in enteric-infected *Drosophila*, an immune response is assembled by a lipophagy-dependent activation of DUOX (Lee et al., 2018). Thus, the contribution of lipophagy to the success of vectors' infection by their respective pathogens, remains obscure and deserve to be investigated.

Insects do not synthesize cholesterol *de novo*, meaning that this lipid has to be absorbed from the diet over their lifetime (Clark and Block, 1959; Zande, 1967). It has been shown in mammalian models that different immune challenges entail cholesterol mobilization (Tall and Yvan-Charvet, 2015).In the case of viruses, the infection interferes in several aspects of cholesterol metabolism, needed for the formation of cell membranes and intimately related to both the entry of viral particles in the cell and their exportation (Osuna-Ramoset al., 2018). Unfortunately, literature on vector biology only tangentially looked at this particular aspect, pointing out some genes involved in cholesterol metabolism and cellular traffic, such as the Niemann Pick 1 protein and the Sterol Carrier Protein 2, as host factors that allow the viral multiplication inthe mosquito (Junjhon et al., 2014; Jupatanakul et al., 2014, 2017; Fu et al., 2015; Chotiwan et al., 2018). Dengue infection blocking by *Wolbachia* in mosquitoes also correlates with changes in cholesterol metabolism, trafficking, and accumulation (Geoghegan et al., 2017). Moreover, even though cholesterol is known as a precursor for the hormone ecdysone (Canavoso et al., 2001), the association between viral infection and hormonal signaling is largely unknown. Nevertheless, definitive evidence showing the contribution of dietary cholesterol to the viral replication in mosquito is still lacking. Additionally,it is also unexplored how serum cholesterol fluctuation in populations from endemic areas could correlate to the viral transmission by mosquitoes.

Some medical relevant parasites such as Apicomplexan and Trypanosomatids, similarly to insects, lack the capacity of *de novo* cholesterol synthesis (Coppens, 2013; Pereira et al., 2015). In order to differentiate and proliferate in the insect gut, they obtain cholesterol from the vertebrate's plasma low-density lipoprotein (LDL) (Labaied et al., 2011; De Cicco et al., 2012; Petersen et al., 2017). Lipophorin, mentioned previously to be hijacked from mosquitoes by parasites, might be the lipoprotein responsible for cholesterol import during the parasite insect stage. However, this hypothesis remains to be tested.

Most articles that compare carbohydrate metabolism of vectors-fed in sugar-rich diets with those fed on blood have focused on the fat body and physiological homeostasis. Sugar metabolism in these animals is controlled at the hormonal level by ILP and juvenile hormone (JH) (Clifton and Noriega, 2011; Hansen et al., 2014; Hou et al., 2015; Roy et al., 2015). It is increasingly clear that parasites can dramatically change the cellular energy metabolism of their arthropod vectors, as recently

revised by Samaddar et al. (2020). Still, for mosquito-arbovirus interactions, the knowledge on such metabolic alterations is limited. An *in vitro* study showed that Zika virus infection in *Ae. albopictus* drives the glucose metabolism toward the pentose phosphate pathway, differently from human cells that increase flux to the tricarboxylic acid cycle (Thaker et al., 2019). Activation of the pentose pathway provides NADPH for antioxidant pathways, which control the intracellular redox state. Maintaining the redox balance would be beneficial for viruses as it would protect them from oxidative damage. However, the relevance of these changes to the course of viral infection has not been experimentally addressed in the literature yet, despite alterations in expression of metabolic enzymes being regularly observed in transcriptomic analyses of infected vector digestive apparatus (Padrón et al., 2014; Angleró-Rodríguez et al., 2017a; Etebari et al., 2017; Narasimhan et al., 2017; Coutinho-Abreu et al., 2020). A large amount of gene expression data on vector infection has now accumulated, and it could be used to direct studies focusing on the crosstalk between canonical immunity pathways and carbohydrate/energy metabolism, the so-called immunometabolism, and the relevance of them to vector biology (Samaddar et al., 2020).

As in other organisms, in addition to its digestive role, the digestive tract functions as a nutrient sensor. Intestinal signaling is involved in integrative processes that link nutritional availability with behavior and metabolism and the microbial intestinal world, including eventual pathogens that come along with the food. One of the few reports on this subject showed that in *Ae. aegypti* blood intake triggers nutrient-sensing signaling, such as the Target of Rapamycin (TOR), responsible for translation of early trypsin (Brandon et al., 2008). This is extremely important for the course of digestion, as this initial event coordinates the late digestive phase (Barillas- Mury et al., 1995; Brackney et al., 2010). A broad spectrumof cellular mechanisms depends on a signaling pathway. The routes taken will rest on the pairs of signaling molecules and receptors that trigger the process. In addition to nutrients such as amino acids and heme, which act also as signalingmolecules (Hansen et al., 2004; Oliveira et al., 2011a; Bottino- Rojas et al., 2015; Short et al., 2017), a blood meal brings also regulatory peptides that act as neurochemicals and hormones, like vertebrate insulin, insulin like growth factor (IGF1), TGF-β and other cytokines. Furthermore, the presence of parasites in the blood meal can antagonize or potentiate the effects of these vertebrate-borne signaling molecules in the vector organism (Pakpour et al., 2013a). The resulting cellular responses can be beneficial or detrimental to pathogen development. Most of the studies on signaling pathways and vector susceptibility/resistance to infection have focused on their role in the activation of immune pathways. These studies have been extensively discussed previously by others (Pakpour et al., 2013a, 2014; Urbanskiand Rosinski, 2018; Sharma et al., 2019; Nunes et al., 2020). Among the best-known signaling pathways in insect vector species is the insulin/insulin-like growth factor signaling pathway(IIS), which is involved in the regulation of growth, longevity, reproduction and immunity. *An. stephensi* stimulated with human ILPs induces ROS-mediated signaling, without oxidative damage that culminates with NFκB inhibition, allowing the *Plasmodium falciparum* oocyst development (Surachetpong et al., 2011; Pakpour et al., 2012). On the other hand, dietary insulin showed a negative impact on flavivirus replication in *Ae. aegypti* and *Ae. albopictus* cells, and *Culex quinquefasciatus* adult mosquitoes, in a mechanism dependent on JAK/Stat activation (Ahlers et al., 2019). Furthermore, it was shown that insulin receptor knockdown in *C. quinquefasciatus* blocks filarial parasite development (Nuss et al., 2018).

The IIS pathway comprehends two branches: the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3K)/Akt. A series of studies have shown that both branches are modulated by host blood components. Host growth factors/cytokines affect the mosquito-malaria parasite interaction by modulating the MAPK signaling pathway. Ingested human IGF1 reduces phosphorylation of the MAPK ERK signaling protein in *An. stephensi* midgut and decrease the intensity and prevalence of *P. falciparum* infection (Drexler et al., 2013). Accordingly, the mammalian host TGF-β-1 induces the expression of nitric oxide synthase and reduces the prevalence of *Plasmodium* infection in *An. stephensi*. This effect is inhibited by the activation of ERK (Surachetpong et al., 2009). Interestingly, TGF-β also appears to be critical for the survival of parasites such as *T. cruzi* and *Leishmania amazonensis* in mammalian hosts (Barral-Netto et al., 1992; Ming et al., 1995; Omeret al., 2000). However, the impact of host TGF-β on the interaction of these parasites with their vectors has not yet been investigated.

Regarding the PI3K/AKT, Corby-Harris et al. (2010) showed that the overexpression of an activated form of Akt in *An. stephensi*, a regulator of IIS, shortened the mosquito lifespan and increased resistance to *P. falciparum*. Lately, the same group showed that the sustained Akt activation in the mosquito midgut resulted in mitochondrial dysfunction coupled to Akt- mediated repression of autophagy and compromised midgut epithelial structure. The perturbation of midgut homeostasis enhanced parasite resistance and decreased mosquito lifespan (Luckhart et al., 2013).

Insulin-like peptides induced by blood-feeding trigger vitellogenesis in the fat body of *Ae. aegypti* and act as regulators of the blood digestion in the gut (Gulia-Nuss et al., 2011; Roy and Raikhel, 2012). In *An. stephensi*, ILPs interfere inthe mosquitoes intermediary metabolism and nutrient intake (Pietri et al., 2016). Interestingly, *P. falciparum* soluble productsinduce the expression of ILPs in *An. stephensi*, through boththe MEK/ERK and PI3K/Akt branches of IIS and inhibiting *P. falciparum* development *in vivo* by affecting mosquito immune effector genes (Pietri et al., 2015). This ILP-mediated inhibition of parasite development is somehow contradictory with the previous findings that human insulin could favor the parasite growth, raising the possibility that even being structurally similar, vector and host insulins can elicit distinct gut responses to *Plasmodium* infection. Moreover, Castillo et al. (2011) showed that the insulin signaling pathway can directly regulate hemocyte proliferation in *Ae. aegypti*. The same study also highlights an interesting observation regarding blood-feeding: resistance and tolerance to the same bacterial pathogen dramatically change

due to blood meal digestion and/or mobilization of resources for reproduction (Castillo et al., 2011).

The PKC pathway is part of this complex signaling network that responds to infection in different vectors. Inhibition of PKC blocks West Nile virus entry in C6/36 cells by inhibiting endosomal sorting (Chu et al., 2006). In *Ae. aegypti*, activation of PKC by the heme released during the digestion of blood decreases ROS production and allows an increased proliferation of indigenous microbiota (Oliveira et al., 2011a). However, the effect of PKC pathway on viral infection has not been investigated until now. PKCs have also been shown to be expressed in the midgut epithelia of *An. gambiae* and *An. stephensi* after a blood meal. As in *Ae. aegypti*, the *An. stephensi* PKC activation was also linked to the decrease of midgut epithelial barrier, resulting in the greater development of *P. falciparum* oocysts (Pakpour et al., 2013b), without modulation of NF-κB-dependent immune factors, thus indicating that the regeneration of the midgut epithelium is essential for infection control, as also suggested by Taracena et al. (2018).

So far, in spite of the advances discussed here, it remains widely unclear how this network of nutritional signaling pathways reflects on the parasite/host interaction. This reinforces the need to build a more holistic view of the interplay between metabolic changes and the canonical immune responses to vector transmitted pathogens that occur after a blood meal, using the integrative conceptual framework of immunometabolism.

PERITROPHIC MATRIX

The mammalian gastrointestinal epithelium is protected by the secretion of a mucus layer, mostly composed of highly glycosylated proteins (mucins) (Johansson et al., 2013; Sicard et al., 2017). The hydrophilic O-linked oligosaccharides that coat these proteins give them the physical and chemical properties that support the mucus protective role. Mucins are secreted by specialized cells, such as goblet cells in the intestine, and have a short half-life, which ensures the constant renewal of the mucus barrier (Deplancke and Gaskins, 2001). This barrier is essential to the digestive tract as it protects the tissue from mechanical damage by food particles, chemical aggression (pH and action of digestive enzymes) and limits direct contact with the microbiota. Although frequently neglected or ignored, insect gut also presents a bonafide mucous layer, with transcriptomic data revealing abundant expression of mucins in the midgut (Terra et al., 2018). The peritrophic matrix (PM) is an extracellular structure found in the intestinal lumen of insects that is ascribed a major role as a protective layer in the midgut, usually described as analogous to the vertebrate mucus. The PM was first described by Lyonet in 1762 and is composed of glycosylated proteins embedded in chitin fibers (for reviews see Lehane, 1997; Terra, 2001, Terra et al., 2018). PMs can be classified into two different types: Type 1 is secreted by all epithelial cells as a continuous gel-like structure that completely packages the food bolus and is formed in response to feeding. The type 2 PM is a membranous structure characterized by its constitutive secretion by a midgut region called cardia and delimits an area between the epithelium and the PM, the ectoperitrophic space. It has a tubular morphology and lines the whole gut epithelium (Shao et al., 2001). Adult mosquitoes such as *Ae. aegypti* and *Anopheles* sp. secrete type 1 PM after a blood meal but present the type 2 PM at larval stages. The same is observed in the sandfly *L. longipalpis* (Secundino et al., 2005). *Glossina* spp. and *Drosophila* present the type 2 PM also in the adult stage. Hemipteran insects like the Chagas' disease vectors are an exception in that they lack the classical PM and the lipidic perimicrovillar membranes are responsiblefor the PM functions (Terra, 2001; Hegedus et al., 2019). In the case of ticks, the PM is a chitin-containing extracellular layer that covers the digestive cells, displaying a very distinct morphology to that of a typical insect PM (Matsuo et al., 2003; Grigoryeva, 2010; Kotsyfakis et al., 2015), probably reflecting their peculiar tick digestive physiology, based on intracellular digestion of host blood proteins (Lara et al., 2005).

In most insects, the PM functions as a molecular sieve, controlling the traffic of molecules between the intestinal epithelium to the lumen, compartmentalizing the digestion and protecting the epithelium from potentially cytotoxic molecules (Billingsley and Rudin, 1992). However, several of these roles have been scarcely investigated in blood-feedinginsects such as mosquitoes. For example, it has been shownthat disruption of PM by the action of exogenous chitinasesincreases the blood digestion rate, an unexpected effect thatwas attributed to augmented access of intestinal proteases tothe blood bolus (Villalon et al., 2003). Also, the *Ae. aegypti*PM binds heme released during the digestion of hemoglobin, which was hypothesized to reduce its oxidative potential (Pascoa et al., 2002).

In contrast, the PM acting as a barrier that limits exposure to the microbial world has been more thoroughly investigated. In mammals, the relative lack of an exuberant immune response against the intestinal microbiota has been attributed to the mucus acting as a barrier that avoids direct contact betweenthe microbiota and the epithelium and not to the microbiota subverting the host immune response, a phenomenon named as "immunological ignorance" by Hooper (2009). Thus, immune homeostasis is attained largely by the mucus layer limiting the exposure of enterocytes to the microbiota (Li et al., 2018). Similarly, in insects, the secretion of the PM approaches this pivotal immune barrier function, as it compartmentalizes the microbiota and its immune elicitors, avoiding the overexposure of the gut epithelium (Buchon et al., 2009; Kuraishi et al., 2011, 2013; Weiss et al., 2014). In this way, it was demonstrated that *An. gambiae* mosquitoes express a heme peroxidase, which is essential to crosslink the PM proteins/mucins, supporting the correct assembly of this barrier. Once this peroxidase is knocked down, the PM barrier is compromised and the gut epithelia is exposed to microbial elicitors that over activate the intestinal immune system (Kumar et al., 2010).

When the microbiota and the PM layer barrier function become unbalanced, the intestinal cell homeostasis is rapidly affected. Both in mosquito and *Drosophila* the gut stem cell populations respond to biotic and abiotic injury and their activation is prevented by the PM presence (Micchelli and Perrimon, 2006; Buchon et al., 2013; Janeh et al., 2017;

Taracena et al., 2018). In *Ae. aegypti*, when the PM structure is compromised by inhibition of chitin synthesis, the epithelial midgut is exposed to the microbiota, which in turn activates the generation of ROS, causing tissue damage and leading to a regenerative response based on mitotic proliferation of progenitor cells (Taracena et al., 2018). Complementary to this mechanism, it was shown in *Anopheles* mosquitoes that genes related to the synthesis of chitin and peritrophins – that together form the structural backbone of the PM – have their expression stimulated by proliferation of the intestinal microbiota (Rodgers et al., 2017). This effect closely recapitulates the mammalian response of goblet cells that prompt the secretion of stored mucus after exposition to native and pathogenic bacteria (Coconnier et al., 1998; Deplancke and Gaskins, 2001).

In mosquitoes, the intestinal microbiota experiences an explosive expansion after a blood meal. Of course, this microbial blooming is fueled by the sudden increase in the availability of nutrients, jumping from a few thousand bacterial cells in sugar-fed *Ae. aegypti* to a plateau of a few million cells in a single midgut by 12 h after blood-feeding (Oliveira et al., 2011a). However, this is probably well below the microbial population that could be supported by an unrestrained growth of bacteria in about 2 ml of blood, posing a question that has not yet been properly addressed: how fine-tuning regulation of the microbial growth is attained? At least one of such mechanisms is the production of ROS by the gut epithelium, as already mentioned above (Ha et al., 2005a; Oliveira et al., 2011a). Consistently, it was shown that gut ROS production is also attenuated by the barrier function of the PM (Taracena et al., 2018), highlighting the existence of mechanisms that balance the microbe-killing mucosal response in a way that is neither too detrimental to the host nor to the microbiota.Of interest, this mode of operation can also be relevant during infection by pathogenic microorganisms.

The first encounter between the insect host and a vectored parasite coincides with the blood digestion in the midgut, exactly when the PM is formed, making it plausible that this immune barrier can regulate the infection's success. Anopheline mosquitoes ingest *Plasmodium* gametocytes that will fertilize and generate the ookinetes, which will invade the intestinal epithelium by secreting chitinases activated by mosquito digestive enzymes, allowing the parasite to traverse the PM. The invasion will happen around 24 h after feeding, concomitantly with PM formation peak (Huber et al., 1991; Shahabuddin et al.,1993). Moreover, it has been shown that proteins present in the matrix can function as anchors for the parasite, promoting its penetration process (Zhang et al., 2015). Although one would expect the PM might impose a barrier to the parasite infectionin the gut, several reports showed instead that the absence of this structure decreases the gut parasitemia (Billingsley and Rudin, 1992; Shahabuddin et al., 1995; Baia-da-Silva et al., 2018). The PM regulation is also essential for Leishmanial infectionof sandflies (Coutinho-Abreu et al., 2010). *Leishmania* parasites do not invade the epithelium but hide in the ectoperitrophicspace (between the epithelium and the PM) anchored to epithelialcells, which likely protect them from the action of digestive proteases (Pimenta et al., 1997; Ramalho-Ortigao, 2010). For the*Glossina* – *T. brucei* pair, for a long time it was not understood how the parasites crossed the flies' PM. However, recent evidence was provided that the expression of genes related to the PM formation was influenced by infection. In these studies, the authors show that *T. brucei* targets the cardia, causing the discontinuation of PM type I secretion and allowing them to invade the ectoperitrophic space (Aksoy, 2019; Rose et al., 2020).

In the Chagas disease parasite replicative stage, the *T. cruzi* epimastigotes, adhesion to the kissing bug perimicrovillar membranes seems important for their division (Gonzalez et al., 1999). Treatment of the intestinal tissue with antiserum against the perimicrovillar membrane reduces the trypanosomatid development in the vector (Gonzalez et al., 2006). As mentioned above, kissing bugs don't have a PM but perimicrovillar membranes, which are phospholipid membranes secreted by the gut and that, analogous to the PM, define a perimicrovillar space (Terra, 1988). The *T. cruzi* epimastigotes are attached to these perimicrovillar membranes through their flagella and membrane glycoinositolphospholipids. Hydrophobic proteins located in their surface and sugar residues present in perimicrovillar membrane glycoproteins appear to be necessary for this interaction (Zingales et al., 1982; Golgher et al., 1993; Pereira-Chioccola et al., 2000; Alves et al., 2007; Nogueira et al., 2007). In *R. prolixus*, it has been shown that another function of the perimicrovillar membranes is the promotion of the aggregation of heme molecules, forming nucleation sites that convert heme into hemozoin crystals and hence preventing heme toxicity toward both the host and the parasite, that consequently creates a favorable environment for pathogen growth (Oliveira et al., 2000; Stiebler et al., 2014; Ferreira et al., 2018).

The role of the *Ae. aegypti* matrix in controlling viral infections has not been investigated so far. It is not clear whether the viral particle can get through the pores of the matrix or if the invasion of the epithelium occurs in the first hours after the meal, when the matrix has not yet been completely modeled. The latter hypothesis is the most accepted by the community, although there is a knowledge gap behind this topic (Franz et al., 2015).

In non-hematophagous insects, it is suggested that the peritrophic matrix secretion integrates the hormonal signaling, mediated by ecdysone, to the pathways downstream to nutritional sensors (Merzendorfer and Zimoch, 2003). Feeding mosquitoes with an artificial diet of low nutritional value that promotes distension of the epithelium stimulates the synthesisof a fragile and short-lived matrix, different from the robust structure observed upon blood-feeding (Dinglasan et al., 2009; Whiten et al., 2018). Thus, it is worth assessing whether the matrix synthesis is controlled by nutritional/metabolic sensors (such as the target of rapamycin, TOR and AMP-activated proteinkinase, AMPK) regulated upon blood arrival, in addition to the molecules released during digestion, such as heme, hormonal signaling induced by digestion (e.g., ecdysone and ILP) or microbial community expansion.

The understanding of the PM as a barrier that coordinates the insect intestinal immune activation beyond its digestive aspect, leads to new perceptions and insights. For example, maintenance of cellular homeostasis in addition to tissue damage repair may be central to disease tolerance (Oliveira et al., 2020), while current state of literature focuses on infection resistance. Future studies

are needed that address how the microbe-associated patterns are presented to the gut epithelia, and consequently how the classical immune system will be tuned and shape the parasite life history.

INTESTINAL REDOX HOMEOSTASIS

Historically, research on reactive oxygen species (ROS) was pushed to a central position in biology after the discovery of superoxide dismutase (McCord and Fridovich, 1969). The scene was dominated for about 30 years by the study of the role of ROS in pathologic conditions (Lambeth, 2007; Liu et al., 2018), where oxidative stress was defined as an imbalance between antioxidant mechanisms and production of ROS by several sources, including microbe-killing NADPH oxidases of immunecells. However, the discovery of the signaling role of nitric oxide (NO) in the regulation of diverse aspects of cell physiology, followed by several reports on hydrogen peroxide acting as a second messenger of several hormones and growth factors, led toa change in paradigm, with the introduction of redox signaling and redox homeostasis as novel steering concepts (Reth, 2002; Jones, 2006; Veal et al., 2007; Jones and Sies, 2015).

As already mentioned above, the digestive apparatus of most animals is also home to an abundant and specific microbial community that is now recognized as having a major and pleiotropic impact on the physiology of the metazoan host (Douglas, 2019). However, the mechanisms that control the growth rate of the intestinal microbiota are still not fully understood. Seminal studies in *Drosophila* revealed that an intestinal Dual oxidase (DUOX) produced H_2O_2 in response to the presence of microorganisms in the gut lumen (Ha et al., 2005a,b). Importantly, they also showed that the gut epithelium was protected from H_2O_2 by its dismutation to H_2O by a heme-peroxidase that at the time was mistakenly called "Immune regulated catalase." A DUOX enzyme found in *Ae. aegypti* mosquitoes has its activities decreased upon ingestion of a blood meal, leading to a marked reduction of ROS levels in the midgut (Oliveira et al., 2011a). Inhibition of the mosquito DUOX caused an increase in the size of the indigenous intestinal microbiota (Oliveira et al., 2011a), revealing a role of ROS metabolism in fine-tuning the symbiotic relationship between the commensal microbial community and the mosquito, in addition to its canonical immune action in the defense against pathogens.

Several other reports addressed the relation between ROS and pathogens in insect vectors. In an elegant work, Liuet al. (2016) showed that RNAi silencing of mosquito DUOX increased dengue virus replication in the midgut. Viral NS1 protein present in the host plasma enhanced susceptibility of the mosquito to DENV infection by reducing expression of DUOX, as well as of NoxM/Nox4-art (a NOX-4 homolog specific of the arthropod lineage, Gandara et al., 2017). Moreover, the iron concentration in the host's blood was inversely correlatedto the prevalence and viral load of mosquito infection by dengue virus (Zhu et al., 2019). The catalase knockdown in*Ae. aegypti* changed the mosquito's susceptibility to DENV but had no impact on the Zika virus (ZKV) establishment. On the other hand, the ZKV viral load in the mosquito midgut was decreased by the redox imbalance promoted by down-regulation of NRF2 antioxidant transcription factor, suggesting that other components of the redox homeostasis downstream of NRF2 are involved in the control of ZKV infection (Bottino-Rojaset al., 2018). Among those, there are canonical NRF2 targets, such glutathione S-transferase and cytochrome P450, suggested to be active in maintaining tissue homeostasis during blood digestion in the mosquito (Bottino-Rojas et al., 2018, 2019). The reduction of viral load by ROS has been explained in most cases by assuming it is inflicting direct damage to theviral particle. However, experimental proof for this hypothesisis still lacking. Interestingly, an alternative mechanism emerged from the demonstration that redox imbalance and its associated cellular damage in *Ae. aegypti* and *Ae. albopictus* midgut led to increased programmed cell death, triggering a homeostatic response based on tissue-repairing mitotic activity of intestinal stem cells (Janeh et al., 2017; Taracena et al., 2018). The same report showed that this increased cellular turnover in the gut epithelia negatively impacted vector susceptibility toarbovirus infection (Taracena et al., 2018). Notwithstanding, in mosquito strains naturally refractory to viral infection, resistance was dependent on the proper recruitment of stem cells via Delta/Notch signaling pathway (Taracena et al., 2018).

In a way similar to what happens with the mosquito down-regulation of ROS production after a blood meal (Oliveiraet al., 2011a), the kissing bug *R. prolixus* also decreases the intestinal ROS generation after blood-feeding (Gandara et al., 2016). However, this is not triggered by the dietary heme, as it was shown for *Ae. aegypti* (Oliveira et al., 2011a), but by the nutritional intake, which increases amino acid levels and activates the TOR pathway, impacting negatively the production of mitochondrial ROS by an yet uncharacterized mechanism (Gandara et al., 2016). Nogueira et al. (2015) showed that while high levels of H_2O_2 reduced growth of the *T. cruzi* epimastigote stages, low levels increased proliferation. In contrast, antioxidant molecules reduced the proliferation of epimastigotes but increased conversion to the infective trypomastigote form, revealing that ROS levels in the bug digestive apparatus have a complex regulatory role on the life cycle of the parasite.

The phlebotomine *L. longipalpis* presents an interesting illustration of how redox homeostasis in the normal gut physiology is relevant for the parasite. In the sandfly, there is a decrease in the intestinal ROS generation upon feeding (Diaz-Albiter et al., 2012), similar to *Ae. aegypti* (Oliveira et al., 2011a) and *R. prolixus* (Gandara et al., 2016). ROS levels are increased by infection with a *Serratia marcescens* strain pathogenic for the sandfly, and uric acid (an antioxidant) administration to the sugar meal increased virulence of this bacteria, revealing that the ROS-producing pathways in the gut can be controlled by immune signaling (Diaz-Albiter et al., 2012). In contrast, *Leishmania mexicana* infection does not increase ROS levels after a blood meal (Diaz-Albiter et al., 2012), but silencing the sandfly catalase or maintaining them fed in sugar meal supplemented with H_2O_2 decreased the intestinal parasite load. This result revealed that not only is the parasite capable of evading immune activationof ROS, but it is additionally benefited by the down-regulation

of ROS levels that is part of the normal physiology of the host. However, this goes beyond simple immune evasion by the parasite, as another report, also from Dillon's group, showed that *Leishmania* infection indeed protected flies from death by *Serratia* co-infection, but without reducing *Serratia* levels (Sant'Anna et al., 2014), strongly suggesting that the *Leishmania* is acting by triggering tolerance to disease mechanisms, thereby reducing damage and preventing fitness loss, without killing of the pathogen (in this case, the *Serratia* bacterium).

A *Plasmodium* refractory *An. gambiae* strain was appointed to have intrinsic higher levels of H_2O_2, which increases even more after an infectious blood meal as part of its antiparasitic defense mediated mainly by hemocytes (Kumar et al., 2003; Molina-Cruz et al., 2008) and this could be attributed to higher mitochondrial ROS production in the refractory mosquitoes (Oliveira et al., 2011b; Gonçalves et al., 2012). The antioxidant defenses induced by blood-feeding in this model seem to be in part under the control of a redox sensor, called OXR1, that regulates both the expression of antioxidant enzymes and the success of parasite infection (Jaramillo-Gutierrez et al., 2010).

Anopheles mosquitoes have a unique redox metabolism upon feeding and infection, described as part of the so-called "time bomb model" where the midgut invasion by the parasites triggers an epithelial response based on protein nitration, activation of peroxidases, and, consequently, apoptosis of those invaded cells (Han et al., 2001; Kumar et al., 2004). This complex intestinal response to the ookinete invasion was molecularly dissected, revealing the role of NOX5, a NADPH oxidase member, as the source of ROS. It works with a heme peroxidase to mediate the parasite nitration (Oliveira et al., 2012). While these studies point to a conventional "immune" function for NOX5, recently, the NOX5 enzymes were shown to regulate muscular function, both in mammalian blood vessel smooth muscle contraction and in the intestinal peristalsis in *R. prolixus* (Montezano et al., 2018), adding some more complexity to this scenario and bringingabout the possibility of the existence of a crosstalk between physiological mechanisms and the microbiota.

The role of reactive nitrogen species (RNS) in the metabolism of hematophagous vectors was initially revealed by the demonstration that increases in NO levels limited the development of *P. falciparum* in *An. stephensi* (Luckhartet al., 1998). Subsequent works performed by the same groupled to the molecular characterization of nitric oxide synthase (NOS) (Luckhart and Rosenberg, 1999) and the modulation of NOS expression during infection by factors such as the parasite's hemozoin pigment (Akman-Anderson et al., 2007) and glycosylphosphatidylinositols (Lim et al., 2005). It was also shown that the *An. gambiae* NOS gene is controlled by Jak/Stat pathway upon infection with *P. berghei* (Gupta et al., 2009). Nitrogen reactive species have a complex chemistry, acting not only through the canonical effect of NO on cGMP formation, but also via its reaction with superoxide forming peroxynitrite. This highly reactive intermediate modifies amino acid side chains and generates derivatives such as nitrotyrosine or nitrosothiols, which are formed in the mosquito gut and are modulated by infection, having profound effects on cell signaling (Kumar et al., 2004; Gupta et al., 2005; Peterson and Luckhart, 2006; Peterson et al., 2007; Oliveira et al., 2012). While protein nitration was shown to be relevant in triggering an anti-plasmodium response (Oliveira et al., 2012), global and mechanistic analyses of nitrosative signaling on insect physiology are still scarce. More recently, it has been reported that the kissing bug *R. prolixus* produces NO in response to *T. rangeli* (Whitten et al., 2007), and NOS inhibition allowed the proliferation of *T. cruzi* parasites in the insect gut (Batista et al., 2020). Nonetheless, the role of RNS-involved pathways and their oxidative implications to parasites/virus infection of mosquitoes or other vectors is still largely unknown and exposes an important avenue for future investigation.

Another way to positively modulate intestinal ROS in mosquitoes is through native microbiota elicitors (Oliveira et al., 2011a; Xiao et al., 2017), and this can have consequences for pathogens. An *Enterobacter* strain isolated from the midgut of wild-caught mosquito was shown to decrease *P. falciparum* infection by inducing ROS generation in the gut. The infection load was restored in the presence of vitamin C (Cirimotich et al., 2011). Accordingly, in *An. gambiae*, blood digestion increases catalase expression and activity in the midgut epithelium and catalase knockdown turns the mosquito more resistant to *P. falciparum* infection (Molina-Cruz et al., 2008). In contrast, in *An. aquasalis*, infection with *Plasmodium vivax* is increased upon catalase silencing (Bahia et al., 2013). Along with thepositive effect of ROS on *T. cruzi* development in triatomine bugs discussed above (Nogueira et al., 2015), this report on *P. vivax* and *An. aquasalis* highlight the complexity of the links between redox homeostasis and parasite/host relationship, which is not explained by a simplistic microbe-killing role of ROS. Even in the several reports mentioned above where ROS levels are inversely correlated with pathogen infection (such as in the *Aedes*/arbovirus), it is not completely clear how much these ROS are produced under the control of canonical immune signaling pathways and how much is derived from the "regular" physiology, such as handling of heme and iron intake, control of microbiota, muscular activity or reticulum stress.

REPRODUCTIVE PHYSIOLOGY AND HORMONAL REGULATION

Vertebrate blood-feeding is a decisive evolutionary trait needed to obtain nutrients for egg development. Different species vary dramatically in their reproductive output. Some insects, like mosquitoes, are able to lay hundreds of eggs each time they take a blood meal and this feature impacts deeply their density in endemic areas (Shaw and Catteruccia, 2019). The reproductive fitness of vectors represents a promising target to prevent disease transmission because it interferes directly with the burden caused by large populations. Nonetheless, there is evidence that these organisms balance their energy investment into different life processes, often leading to fitness trade-offs between survival, immunity, and reproduction (Schwenke et al., 2016). Therefore, biological pathways essential for reproductive fitness directly or indirectly influence elements that govern vectorial capacity.

In general, it is considered that activation of immune responses decreases reproductive output in a diverse array

of insects. Amongst blood-feeding vectors, parasite-induced fecundity reduction is a strategy that is evident in many vector/parasite associations (Hurd, 2003). In malaria-mosquito systems, a challenge with bacterial components or *Plasmodium* infection promotes apoptosis of follicle cells and reduces the accumulation of protein in the ovaries, as well as the number of eggs laid (Hogg and Hurd, 1995; Ahmed et al., 2002; Ahmed and Hurd, 2006; Pigeault and Villa, 2018). An immune-mediated arrest of oogenesis was also reported in other disease vectors such as the triatomine bug *R. prolixus* and tsetse flies (Hu et al., 2008; de Medeiros et al., 2009), suggesting resource allocation toward immunity to achieve recovery from infection. Besides, it is also true that reproductively active insects have reduced resistance to infection (Schwenke et al., 2016). In mosquitoes, it was shown that the same molecular processes involved in delivering blood-acquired nutrients to maturing eggs also favor the development of *Plasmodium* oocysts in the midgut and diminish the efficiencyof parasite killing by the mosquito immune system (Ronoet al., 2010). Recently, it was shown that transgenic *An. gambiae* mosquitoes with reduced reproductive capacity have a significantly higher malaria transmission potential, due to an increase in parasite growth rates (Shaw et al., 2020). Due to the direct implications in currently proposed control strategies (e.g., eggless mosquitoes for population suppression) and the vacancy of descriptions of resource reallocation mechanisms in other insect vector species, this subject deserves a greater deal of attention in the field.

Hormonal control is a critical mechanism for the physiological trade-off between reproduction and immunity. JH and 20-Hydroxyecdysone (20E) are key regulators of metamorphosis and reproduction in all holometabolous insects (as reviewedby Roy et al., 2018). Specifically, the balance between JH and 20E is essential for egg maturation. In most insects, increased JH levels promote egg production and provisioning and, in contrast, high 20E titers result in the resorption of immature vitellogenic eggs (Gruntenko and Rauschenbach, 2008). However, in female mosquitoes, digestion and ovarian development are physiologically integrated through a cascadeof ecdysteroid signaling initiated after a blood meal (Hansenet al., 2014). Beyond the induction of synthesis and secretion of yolk protein precursors in the fat body, 20E is shownto regulate a number of additional genes that could impact parasite development in different species. In *D. melanogaster*, ecdysone triggers a precise signaling pathway shown to modulate expression levels of antimicrobial peptides and interfere with resistance mechanisms in the context of bacterial infections (Flatt et al., 2008; Rus et al., 2013). The chemical inhibition of ecdysone signaling in the blood-feeding triatomine *R. prolixus*is able to suppress cellular and humoral immune responses, disrupting gut microbial homeostasis (de Azambuja et al., 1991; Vieira et al., 2021).

Anopheline mosquitoes are a unique model for ecdysone studies due to their strict anautogeny and male transfer of 20E to females during a monandrous copulation. The mating- induced increase in oogenesis is mediated by vitellogenic lipid transporters that also facilitate *Plasmodium* developmentby reducing the parasite-killing ability (Rono et al., 2010). Additionally, mating affects longevity and induces changes in the midgut that can increase susceptibility to the parasite (Dao et al., 2010; Dahalan et al., 2019). In contrast, the topical application of a 20E agonist shortens lifespan, prevents mating and egg production, and significantly blocks *P. falciparum* development (Childs et al., 2016). Therefore, 20E exerts a long-range regulation of multiple physiological processes that are highly relevant to the mosquito's competence to transmit malaria: reproductive success, parasite development, and longevity. Recently, a direct influence of 20E on cellular immune function and antipathogen immunity in mosquitoes was demonstrated. Blood-feeding of *An. gambiae* females or direct 20E injections increase phagocytic activity and this ecdysone-mediated immune priming reduces bacteria and *Plasmodium berghei* survival (Reynolds et al., 2020). However, some argue that in natural settings, the coevolution of parasite and vector has led to a less conflicting relationship, in which the immune response is toned down, and the potential cost of infection for invertebrate hosts is minimized (Mitchell and Catteruccia, 2017). Werling et al. (2019) provided evidence of a positive correlation between mosquito and parasite fitness dependent on 20E signaling. By genetic ablation of ovary development and impairment of 20E endogenous production, it was determined that ecdysone signaling is required for *P. falciparum* development via the production of mosquito host-derived lipids (Werling et al., 2019). This supports a model where the provision of lipid molecules during vitellogenesis is used by the parasite to increase survival and optimize its transmission (Costa et al., 2018). Therefore, the intricate interplay mediated by 20E between insect reproductive physiology and parasite development remains partially unresolved. It is possible that ecdysone signaling has tissue and/or threshold-specific actions, enabling the establishment of infection while boosting anti-parasite responses through distinct mechanisms. To warrant a proper impact in pathogen transmission of future discoveries, further research should ideally consider field/natural conditions and be focused on parasite-vector based combinations that occur in the wild.

Juvenile hormones control almost every aspect of insect's life. The seminal studies of Wigglesworth, started in the early 1930s, established the existence and major roles of JHin insects, regulating tissue morphogenesis, vitellogenesis and immune response, acting primarily as an 'inhibitory hormone' (Wigglesworth, 1965). Additionally, strong evidence across a range of insect taxa endorse the model in which mating increases JH titers and suppresses 20E, promoting egg development and inhibiting immune capability (Schwenke et al., 2016). Much of what we know of the molecular regulation of JH in blood-feeding species comes from studies in *Ae. aegypti*. Here, therate of JH synthesis in young stages of mosquitoes is in close correlation with their nutritional status (Noriega, 2004). JH also has essential functions in adult females where it controls post-eclosion maturation, leads to reproductive competency and ability to feed on blood, and regulates gene expression after blood-feeding (Clifton and Noriega, 2011; Roy et al., 2015). Aside from morphological alterations in the ovary, transcriptional JH-induced changes in the fat body render this tissue competent to respond to ecdysone produced by the ovary after blood-feeding

(Zou et al., 2013). Moreover, JH is delivered by *Ae. aegypti* males during mating, which increases egg development by directing nutritional resources toward reproduction (Klowden and Chambers, 1991; Clifton et al., 2014). In *Drosophila*, a similar mating-induced expression of JH results in a remodeling of the female midgut, leading to cell division, increased organ size and ultimately a higher food intake (Reiff et al., 2015). However, in mosquitoes, a midgut-remodeling process in mated females has not been addressed yet. This event is relevant in disease vectors because it could be aimed to favor nutrient absorption toward egg provisioning, but also benefit pathogen developmentby deviating resources from immunity and supporting a higher parasite count due to an expanded gut area. JH is transportedto target tissues by the hemolymph carrier juvenile hormone- binding protein (JHBP) where it binds to the methoprene- tolerant (Met) receptor and exerts its pleiotropic effects. One of the overall processes affected by Met depletion in mosquitoes is lipid metabolism (Wang et al., 2017), which could have a deep impact on the mounting of immune responses to pathogens and parasite maturation (Cheon et al., 2006). Recently, Kim et al. (2020) described a specific role for JH, through JHBP mutation, in regulating innate immune responses and the developmentof hemocytes in *Ae. aegypti*. JHBP-deficient mosquitoes are immunosuppressed at the humoral and cellular levels, and present a severe susceptibility to bacterial infection (Kim et al., 2020). These results call for more detailed studies exploring the role of JH signaling on vector infection by disease pathogens.

Blood-feeding initiates a complex series of physiological events in the gut, fat body and ovary that are integrated by the actions of JH, 20E and peptide hormones. Among the topics that could earn further exploration, the modulating roleof sex hormones and their effects on non-reproductive organs are poorly understood in blood-feeding insects. Recently, in *Drosophila*, it was proven that an ecdysone-dependent signaling from the ovaries to the gut promotes growth of the intestine (Ahmed et al., 2020). In addition, the insect midgut produces certain hormones when it recognizes harmful components or pathogenic bacteria in an ingested meal; concurrently, these hormones regulate other tissues and organs (as reviewed in Wu et al., 2020). Given that blood digestion, parasite development and vitellogenesis require the coordination of molecular events inthree different abdominal tissues, these inter-organ relationships have relevance in the context of vector–parasite interactions and deserve further attention. Furthermore, between insects of the different taxa, the diversity in life history traits may lead to distinctive adaptations of these systems (Schwenke et al., 2016) and the study of reproductive processes with respect to species-specific features can help the identification of novel targets for vector control.

MULTIPLE BLOOD-FEEDINGS

The competence to support pathogen development variesbetween vectors due to many biological aspects, which include not only immunity but also feeding behavior and nutritionalstatus (Lefèvre et al., 2013). Frequency of feeding is undisputedly an important factor in relation to human infections with insect-transmitted diseases (Kramer and Ebel, 2003; Das et al., 2017). Multiple blood meals can increase vectorial capacity by promoting the contact of the disease-carrying insect with susceptible hosts. In the study of vector biology, it is widely accepted for blood-feeding dipterans that host seeking is halted by a full blood meal (Klowden, 1990). However, a numberof observations where gravid females still display host-seeking behavior motivated a reconsideration of this assumption (Scott et al., 1993; Guzman et al., 1994; Beier, 1996; Matthews et al., 2016). As most vectors of diseases need to take additional blood meals after becoming infected to complete the transmission cycle, pathogens may have evolved mechanisms to promote their success, redefining the role of uninfected blood-feedings in the epidemiology of these diseases.

One of the classic entomological parameters used in malaria transmission models rely on the proportion of bites experienced per person and the number of total bites taken by a mosquito per gonotrophic cycle (Tedrow et al., 2019). Due to their low reserves, anophelines frequently seek more than one blood meal at each oviposition cycle (Beier, 1996). This behavioral aspect of *Anopheles* females appears to increase not only fecundity, but also longevity and resistance to insecticides (Oliver and Brooke, 2017). Interestingly, the development of the malaria parasite seems to equally affect and be affected by frequentblood meals, which accelerate oocyst maturation and sporozoite development (Beier et al., 1989; Ponnudurai et al., 1989), and are induced by pathogen–vector manipulation to further enhance transmission (Cator et al., 2012). Shaw et al. (2020) proved that previously infected *An. gambiae* females, when provided a second uninfected blood meal, present an increase in oocyst growth rates and faster accumulation of sporozoites in the salivary glands, which can indeed amplify local malaria transmission potential (Shaw et al., 2020). Therefore, this previously overlooked multiple feeding behavior is a justifiable current trend of investigation in vector-borne pathogen transmission.

In the phlebotomine sand fly species *Lutzomyia*, a higher proportion of insects heavily infected can be found afterthe second blood meal (Moraes et al., 2018). Also, the subsequential feeding induces a faster proliferation of *Leishmania* parasite infective forms and its rapid migration to the vector proboscis, increasing vectorial competence during the second gonotrophic cycle (Elnaiem et al., 1994; Vivenes et al., 2001). Furthermore, it has been recently proven that the taking of a second uninfected blood meal by *Leishmania*-infected sand flies triggers a specialized developmental stage of the parasites, the retroleptomonad promastigotes, which is a replicative form and amplifies both the host infection and the infectiveness of the bite (Serafim et al., 2018).

As previously mentioned, blood-feeding triggers intense physiological changes to the gut tissue – including mechanical distention of the midgut, altered cell homeostasis, and changed permeability of the basal lamina – that could aid pathogen dissemination out of the midgut (Okuda et al., 2007; Dong et al., 2017; Taracena et al., 2018). Indeed, in the mosquito *Ae. aegypti*, stretching of the gut tissue over consecutive blood-feedings seems to be a critical factor causing the midgut basal lamina to become

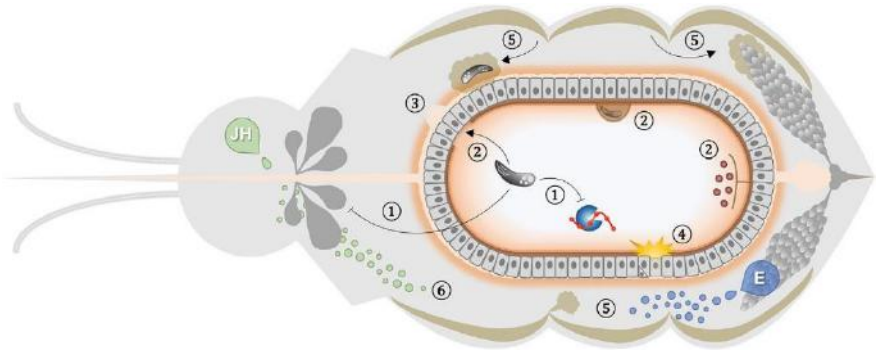

FIGURE 1 | Generic representation of non-immune elements that affect vector-pathogen interaction. The scheme depicts a frontal plane of a blood-feeding insect showing the relative position and interactions of the different non-immune players involved in diverse parasite-vector associations. Organ and cell sizes are not up to scale. (1) Parasites can manipulate feeding rate and digestion activity of the host (Stierhof et al., 1999; Sant'Anna et al., 2009). (2) Peritrophic matrix regulates intestinal infection, requiring parasite escape to establish invasion (Huber et al., 1991; Rose et al., 2020); allowing parasite anchoring to ectoperitrophic space (Pimenta et al., 1997; Zhang et al., 2015); and compartmentalizing microbiota to ensure immune ignorance of the epithelium (Weiss et al., 2014). (3) Blood-feeding-induced microperforations in the basal lamina support pathogen dissemination (Kantor et al., 2018; Armstrong et al., 2020). (4) Rupture of the peritrophic matrix barrier activates ROS generation that triggers an epithelial response to infection (Oliveira et al., 2011a; Taracena et al., 2018). Juvenile Hormone (JH) and Ecdysone (E) are key regulators of the physiological trade-off between reproduction and immunity. (5) Ovary ecdysone production exerts paramount effects such as fat body-induced provision of lipid molecules during vitellogenesis that can reduce the parasite-killing ability and support its development (Rono et al., 2010; Werling et al., 2019). (6) Juvenile hormone influence diverse physiological processes in the insect that can impact pathogen success such as ability to feed on blood, midgut remodeling, reproductive competency, gene expression regulation, lipid metabolism and immune response mounting (Clifton and Noriega, 2011; Zou et al., 2013; Roy et al., 2015; Kim et al., 2020).

permissive for viral escape (Kantor et al., 2018; Cui et al., 2019). Due to blood-feeding-induced microperforations in the basal lamina, virus-infected individuals fed an additional non-infectious blood meal disseminate and transmit viruses more efficiently than single-fed mosquitoes (Armstrong et al., 2020). It is interesting to note that the previous discussed transfer of male reproductive gland substances during mating in *Ae. aegypti* can increase blood-feeding frequency, potentially affecting pathogen transmission by female mosquitoes (Villarreal et al., 2018). One could simply postulate that the enhanced or accelerated parasite/pathogen development upon a sequential feeding is due to the higher availability of nutrients. However, other hypotheses can explain this phenomenon, like the aforementioned diversion of energy from the immune response to support oogenesis, and further evolutionary adaptations exploited by the parasite that favor their own development and transmission.

One of the most intriguing propositions in this field is that vector-borne parasites directly manipulate phenotypictraits of their vectors and hosts in ways that increase the contact between them, hence favoring transmission. Major observational examples include *Plasmodium*, *Leishmania,* and *Trypanosoma* spp. manipulating the behavior of mosquitoes, sand flies and kissing bugs, respectively (Hurd, 2003). Frequently studied changes include alterations of biting rates in vectors and increased attractiveness of vertebrate hosts (Lefèvre andThomas, 2008). Moreover, interesting direct evidence suggests that parasite infection reduces the insect feeding efficiency, prolonging probing time, thus enhancing the likelihood of infecting multiple hosts during a single feeding cycle (Stierhof et al., 1999; van den Abbeele et al., 2010). According to this hypothesis, in malaria-mosquito systems, vector manipulation by the parasites decreases vertebrate host seeking during the pre-infectious phase, lowering the risk of mosquito death during early parasite development. Once the vectors have become infectious, these proceedings are again increased (Schwartz and Koella, 2001). Hence, mosquitoes harboring transmissible sporozoites would be more likely to bite several people pernight (Koella et al., 1998). However, most of the evidence of manipulation comes from avian or rodent model systemsand is focused on isolated components of mosquito host- feeding process (e.g., host detection, probing, piercing, blood ingestion and terminating the feed) (Friend and Smith, 1977). Therefore, the complexity of human malaria models makesit difficult to characterize how infection affects this multiple set of behaviors (Cator et al., 2012). Furthermore, when long evolutionary association between specific *Plasmodium* and *Anopheles* species combinations are tested, distinct or null alterations are shown (Nguyen et al., 2017; Stanczyk et al.,2019). Overall, behavioral manipulations stand as a complex phenomenon that continues to require careful observations with the use of different methods and multidisciplinary approaches (Lefèvre et al., 2006).

Regardless of the mechanism, vector-borne disease transmission depends on the frequency at which the insect vector bites humans. Therefore, fundamental investigations on the impact of complex vector behaviors, and of the ecology and evolution of vector–pathogen interactions, remain key aspects needed to generate better predictions of disease transmission and of the efficacy of control interventions. The varied transmission strategies evolved by pathogens and the vectors' behavioral changes induced by them can affect how current control tools work. Moreover, biting frequency and how bites are distributed among different people also can have significant epidemiologic effects (Woolhouse et al., 1997; Cooper et al., 2019).

High multiple-feeding rates can explain why reducing vector populations alone is difficult for prevention and support the argument for additional studies on feeding behavior (Harrington et al., 2014). Given that multiple blood-feedings directly increase the number of potentially infective encounters, this impact should be considered on model predictions and, accordingly, shape specific vector control strategies. This could mitigate the possibility of underestimating transmission intensity, which could lead to a misunderstanding of the impact of vector control (Tedrow et al., 2019).

CONCLUDING REMARKS

The growing impact of vector-borne diseases urgently calls for the development of new entomological interventions.Increasing knowledge on insect biology and insect–pathogen interactions that help unravel the processes that determine vectorial capacity will fuel innovative approaches to stop transmission (Shaw and Catteruccia, 2019). Insect hosts can resist infection or limit/tolerate the deleterious effects caused by the pathogen. Tolerance refers to all host defense mechanisms that limit 'damage to functions and structures' during infection, without interfering with pathogen load, as defined more than 60 years ago by plant pathologists (Caldwell et al., 1958). Therefore, resistance has a negativeeffect on pathogen fitness, whereas tolerance does not. The genetic trade-off between resistance and tolerance can shape the successful evolutionary interactions in a vector-pathogen system (Lambrechts and Saleh, 2019; Oliveira et al., 2020).

Here, we strengthen the argument that the insect vector response to infection does not merely activate immune pathways as a mechanism of resistance. It also encompasses a broad range of adaptive consequences, including metabolic alterations,stress responses, and tissue repair. Many of these are related to blood-feeding and reproduction (**Figure 1**). These events can lead to improved survival of the insect despite active pathogen replication. The impact of infection on the vector can thus be tuned by the parasite to favor both physiological host homeostasis and completion of the transmission cycle. Moreover, the target for natural selection is seldom one isolated organ or a discrete event, but rather are the multisystemic processes that involve pathogen acquisition and development within the vector. Despite recent advances in the knowledge of physiological mechanisms that can work as non-canonical immunological traits, several elements have yet to be unfolded. Future novel vector control strategies may arise rooted in integrated system biology research to target physiological aspects that act as protective mechanisms and contribute to tolerance to infection. In this way, innovative and effective tools can be used, in an evolutionary considerate manner, to mitigate the great burden imposed on societies by vector-borne diseases.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Capítulo II

Artigo – em preparação

TOR-mediated immunometabolism drives *Aedes aegypti* midgut physiology and viral infection susceptibility

TOR-mediated immunometabolism drives *Aedes aegypti* midgut physiology and viral infection susceptibility

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Abstract

Hematophagous insects face drastic metabolic reprogramming once they feed on blood. Moreover, blood-feeding exposes them to parasites and viruses. *Aedes aegypti* mosquitoes are the major vectors for medical-relevant arboviruses. Here we show that the blood digestion triggers signaling through the Target of Rapamycin (TOR) pathway in the mosquito gut, which drives a metabolic shift in carbohydrate and lipid metabolism. This pathway plays a role in coordinating the intestinal innate immune responses that take place after a blood meal. TOR inhibition by rapamycin led the mosquitoes to an immunosuppressed state, favoring the microbiota proliferation, and Zika and Chikungunya virus infection of the gut epithelia, increasing the infection intensity and prevalence. Rapamycin also blocks the peritrophic matrix (PM) synthesis, a barrier that compartmentalizes the gut microbiota and blood digestion, however, without triggering a strong antibacterial immune response. TOR inhibition shut down most of the known microbial sensing in the gut, with no antimicrobial peptide synthesis and ROS generation, even with no PM barrier. Taken all together, our data show that the nutritional signaling and TOR activation is essential to regulating the intestinal immunometabolism that reflects on the mosquito vector competence for arboviruses.

Introduction

All living cells share the ability to use energy conserved in macromolecules or their constituent building blocks. Oppositely, cells can synthesize new complex molecules, depending on their energetic requirements. Most recently, the literature endeavors the understand the cross-talk between the metabolic pathways and the immune pathways, an approach that has been disciplinary referred to as immunometabolism. This holistic view integrates the metabolic needs of immune-compromised cells to license them to play their biological role. Two kinases are well-known to drive the metabolism, the AMP-kinase (AMPK) and the Target of Rapamycin (TOR), and both seem to be differentially activated in pro and anti-inflammatory cells [1,2].

The kinase *Target of Rapamycin* (TOR) at first was correlated with fungicidal immune response. However, promptly was characterized as a pleiotropic regulator of cell growth, immune responses, and anabolic processes [3–5]. This kinase nucleates distinct proteins forming two complexes (TORC1 and TORC2) that promote cell anabolism, growth, and proliferation. Classically, the TORC1 is allosterically inhibited by rapamycin, and it has been shown that amino acids, growth factors, and cytokines are positive physiological modulators [4]. The TORC1 complex allows cell growth, once it affects protein synthesis (through S6 Kinase, 4EBP, and eIF2-alpha phosphorylation), lipid storage, and mitochondrial metabolism. On the other hand, the TORC2 is inhibited by rapamycin only in chronic exposures and acts through the cytoskeleton and metabolism [4].

The *Aedes aegypti* mosquitoes represent a public health threat since they transmit the most relevant arboviruses, such as dengue, zika, and chikungunya. The midgut epithelium is considered the first and most important immune barrier to viral development in the mosquito organism [8]. In their natural environment, mosquitoes change from starvation or a nectar-feeding (carbohydrate-rich) to a blood meal two to three times their weight before feeding. In contrast, vertebrate blood is a rich source of amino acids, as proteins account for more than 85% of its dry weight. Therefore, hematophagy pushes the mosquito cell physiology through a full metabolic reprogramming, a scenario where the TOR signaling is likely to have a central role. The role of TOR in mosquito physiology after a blood meal has been studied and linked to the participation of the fat body and ovary in insect reproduction, but, surprisingly, little work has addressed the implications of TOR in the immunometabolism juncture in the midgut of blood-fed insects.

During the blood digestion, the midgut epithelium goes through a deep transcriptional change to support the hydrolysis of blood components, nutrient absorption, the peritrophic matrix synthesis, and the innate immune response to the native microbiota expansion [7,9,10]. Moreover, this is the landscape in the mosquito gut where the viral particles must thrive to establish a successful infection in the organism. Here we show that the TOR has a pleiotropic effect upon blood-feeding, coordinating key functions of

the midgut physiology after a blood meal. We also show that this pathway acts in the Zika and Chikungunya intestinal infection. Therefore, our data suggest that TOR couples the nutritional status sensing to the *A. aegypti* immune response in the gut.

Methodology

Ethics statement

All the animal care and experimental protocols were conducted following the guidelines of the institutional care and use committee (Comissão de Ética no Uso de Animais, CEUA-UFRJ) and the NIH Guide for the Care and Use of Laboratory Animals. The protocols were approved under the registry # CEUA-UFRJ 149/19. The animal facility technicians at the Instituto de Bioquímica Médica Leopoldo de Meis (UFRJ) carried out all aspects related to rabbit husbandry under strict guidelines to ensure careful animal handling.

Aedes aegypti mosquitos

A. aegypti (Red Eye strain) mosquitoes were raised at the Universidade Federal do Rio de Janeiro, Brazil, under a 12-h light/dark cycle at 28°C and 70% relative humidity. The larvae were fed with dog chow and adults were maintained in a cage and given a solution of 10% sucrose *ad libitum*. Three- to seven-day-old females were used in the experiments.

Mosquito meals

Aedes aegypti females were artificially fed with heparinized rabbit blood supplemented with (1) 20μM of rapamycin (Sigma) diluted in DMSO, (2) DMSO, (3) 2mg/ml soybean trypsin inhibitor. Three hours before feeding, the sugar solution was removed from the mosquito cage to encourage the artificial feeding, which was performed through glass–feeders sealed with *Parafilm*. Mosquitoes were fed also with the Substitute of Blood-meal (SBM) supplemented with 50μM of heme or the heme vehicle, as described elsewhere [11,12]. For antibiotic treatment, an autoclaved 5% sucrose solution was supplemented with penicillin (200U/mL) and streptomycin (200μg/mL) (Invitrogen) and offered to the mosquitos for 3 days before blood-feeding. Three pools of at least seven midguts were dissected 6 or 24 hours after feeding and kept in TRIZOL reagent for RNA extraction. All the experiments were performed at least three times.

Western blotting

Mosquitoes were starved for at least 6 hours and then fed in a Schneider cell culture media supplemented or not with 40 μl per ml of 100× non-essential amino acids (Invitrogen, 11140050) and 80 μl per ml 50x MEM amino acid solution (Invitrogen, 11130051). The midgut epithelia of sugar-fed, amino acid-fed, or amino acid + rapamycin-fed mosquitoes were dissected 1.5 hours after feeding and incubated in a protease and phosphatase inhibitor cocktail (Invitrogen). The samples were homogenized, and the total protein content was determined by Pierce 660nm Protein Assay Kit method. Protein extracts (20μg) from the midgut epithelia were boiled in LDS buffer and DTT for 10 min

and then resolved in 4-12% Bis-Tris SDS-page using MES buffer. The proteins were dry transferred to nitrocellulose membrane through iBlot System (P0 program – 7 min at 20V). The membranes were blocked in a blocking solution containing 3% albumin and 1% non-fat milk in TBS-T for at least 1 hour. The membrane was incubated with Drosophila pS6Kinase (Cell signaling, #9209) primary antibody diluted 1:2500 in blocking solution ON, at 4ºC, in a rocker machine. The secondary antibody (HRP) was diluted at 1:7500 in a blocking solution and incubated for 2 hours at room temperature. The membranes were developed using SuperSignal West Dura Extended (Thermo).

Digestion and ovary development

The females were artificially fed with blood supplemented with rapamycin or SBTI and their midguts were dissected. Pools of five midguts were collected immediately after feeding (0 hour) and 18-, 24-, and 48-hours post-feeding. The midguts were placed in a tube containing a protease inhibitor cocktail (50 µg/mL SBTI, 1mM benzamidine, 1mM PMSF), homogenized and the protein content was measured by the Lowry method [13]. Ovaries from 24 hours post-feeding with rapamycin were dissected, placed in a protease inhibitor cocktail, and protein was measured. After the feeding, each individual female was transferred to a cage containing wet filter paper to allow oviposition. The eggs laid were counted 7 days post-feeding.

Peritrophic matrix staining and permeability

Midguts from mosquitoes 18 hours post-feeding were dissected and fixed in a 10% neutral buffered formalin solution for ON. The fixed samples were embedded in paraffin and microtome sections were performed and placed in glass slides. The slides were incubated with 100µg/ml of wheat-germ agglutinin conjugated with fluorescein isothiocyanate (WGA-FITC) in PBS for 40 minutes at room temperature [11]. The WGA-FITC was washed out and the slices were incubated with 1µg/mL of DAPI in PBS for 5 minutes, for nuclei staining, and washed out. A drop of Prolong Gold (Invitrogen, P36934) was applied, and a cover slide was laid. To access the peritrophic matrix permeability, insects were artificially fed with blood containing or not rapamycin and supplemented with 1mg/ml of 4KDa Dextran-FITC (Sigma). The midguts were dissected 18 hours after feeding, fixed, and processed as mentioned above. The slides were observed on a Leica Epifluorescence inverted microscope (Leica Microsystems, Germany).

RNA extraction, cDNA synthesis and qPCR

Total RNA was extracted by TRIZOL reagents following the manufactor's protocol of pools of 10 mosquito guts in each. The RNA (1ug) was treated for 30 min at 37ºC with DNAse I and the cDNA were synthesized with the High-Capacity cDNA Reverse transcription kit (Applied Biosystems) using Random Primers. Quantitative PCR (qPCR) was performed in a StepOnePlus Real-Time PCR System (Applied Biosystems) using

Power SYBR-green PCR master MIX (Applied Biosystems). The primers used in the qPCR analyses are listed in supplemental table 1.

Mosquito survival curve

Females were fed with blood supplemented or not with rapamycin and with *Serratia marcescens* (1 OD_{600nm}/ml). The bacteria were grown in Luria-Bertani media shaking at 37ºC, 200rpm, ON. The culture was centrifuged, the pellet was resuspended in PBS, and the optical density was read at 600nm. The insect mortality was observed daily for 4 days after bacterial feeding.

Viral infection

Zika (PE243/201, GenBank accession number KX197192.1) and Chikungunya (BHI3745/H804709) were propagated in *Aedes albopictus* C6/36 cell line for 7 days in Leibovitz-15 media (Gibco #41300–039, supplemented with 5% fetal bovine serum, tryptose 2.9g/L, 10 mL of 7.5% sodium bicarbonate/L; 10 mL of 2% L-glutamine, 1% of non-essential amino acids (Gibco #11140050) and 1% penicillin/streptomycin, pH 7.4) and the aliquots of the culture supernatant were clarified and storage at -70ºC. The viral titers were determined by plaque assay of 10-fold serial dilutions and inoculated in Vero cell line (harvest in DMEM media, 2% FBS, 1% pen/strep, 0.8% methylcellulose), 4 days of incubation at 37ºC and 5% CO_2, as previously described. The cell fixing and staining were performed by adding a solution of 1% crystal violet in methanol: acetone solution (1:1 v/v) for 1 hour at room temperature.

Mosquitoes were orally infected by mixing 40% rabbit red blood cells washed twice with PBS, 50% of the viral stock, and 10% of decomplemented rabbit serum. The insects were fed with 10^5 CFU/ml of each virus and infected midguts were dissected 4 days post-ZKV infection and 3 days post-CHKV infection.

ROS generation

Midguts were dissected 18 hours after feeding and incubated with 50µM of dihydroethidium (hydroethidine; DHE; Invitrogen/Thermo Fisher Scientific, MA - USA) in Leibovitz 15 (L-15) medium for 20 min at room temperature in the dark. The dye medium was removed, and the midguts were gently washed in PBS. The fluorescence of the oxidized DHE was acquired using a Zeiss Observer.Z1 with a Zeiss Axio Cam MrM Zeiss, and the data were analyzed using AxioVision software in a Zeiss-15 filter set (excitation BP 546/12 nm; beam splitter FT 580 nm; emission LP 590 nm)

Statistical Analyses

All the graphs and statistical analyses were performed with the GraphPad 6 software. For qPCR experiments, the relative gene expression was calculated by the Comparative Ct Method [14] and expressed as the mean of ΔΔCt values.

Results and discussion

The blood-feeding induces TOR activation in the midgut

The blood digestion in the mosquito gut exposes the epithelia to an amino acid overload and the TOR pathway is modulated by cellular levels of amino acid. Amino acid feeding triggered the TOR pathway in the gut epithelia, as observed in figure 1A by the phosphorylation of TOR – target p70S6 kinase, and the addition of 20μM of rapamycin in the amino acid-supplemented meal abolished the target phosphorylation. This confirmed that the rapamycin treatment efficiently inhibited the TOR kinase activity. The same result was previously reported by Brandon et al. [15] who showed that 20μM of rapamycin was enough to abolish the TOR phosphorylation of p70S6 Kinase and 4-EBP. The authors also showed that TOR regulates the translation of early trypsin, a key protease that begins blood digestion. To validate the experimental design of delivering rapamycin through blood-feeding, the digestion rate was measured in blood-fed mosquitoes (control) or supplemented with rapamycin or soybean Trypsin Inhibitor (SBTI, as a positive control). Figure 1B shows that rapamycin decreased the blood digestion rate. Dong et al., 2017, also showed that *A. aegypti* fed on a protein-rich buffer increased the expression of several digestive proteases [16]. It has been shown that TOR activation is essential to vitellogenesis, pointing to crosstalk of nutritional and hormonal signaling in the regulation of the development of ovaries and fat body after a blood meal [17,18]. Inhibition of TOR also impacted the embryogenesis, leading to delayed oviposition in *Riphicephalus microplus* tick [19]. Figure 1C shows that the rapamycin-fed mosquitoes delayed the ovary development, and it impacts the number of eggs laid (Figure 1C).

Carbohydrate and lipid metabolism is modulated by the TOR pathway

The metabolic reprogramming does not exclusively affect the protein synthesis and export pathways, but also the most basic energy metabolism pathways. After a blood meal, the expression of key glucose metabolism enzymes is strongly up-regulated in the midgut, such as hexokinase and lactate dehydrogenase (LDH), whereas a trehalose transporter (TRET) is downregulated (Figure Sup 1). In contrast, rapamycin-fed mosquitoes showed an opposite profile, once the hexokinase and LDH were downregulated and the TRET was increased (Figure 1D), disclosing that TOR modulates the carbohydrate catabolism and absorption. The glycolytic status of the organism is emphasized upon a blood-feeding in mosquitoes and the heme release from the hemoglobin digestion supports this metabolic shift [12,20,21]. The hypoxia-induced transcription factor HIF1 has been associated with the TOR activation in mammals and controls the glycolytic fate [22–24]. Also, it has been reported that this transcription factor is downstream of TOR in *A. aegypti* reared in a food-restricted condition, in a microbiota–mediated hypoxia manner [25]. Here we show that the HIF1 expression in the gut is induced after a blood-feeding and TOR inhibition by rapamycin downregulates the HIF1

gene (Figure 1D), suggesting a transcriptional axis of TOR x HIF1 and glycolytic metabolism.

TOR is an anabolic regulator and favors lipogenesis and lipid droplet formation in mammals [26], in crosstalk with leptin signaling [27]. Barletta et al. (2015), showed that the blood meal up-regulates genes involved in lipid synthesis and increases lipid droplet numbers [28]. The hypothesis here is that TOR is involved in the lipogenic signaling network during blood digestion. After 24 hours of rapamycin-feeding, the expression of genes committed with the lipid metabolism and lipid droplet assembly was decreased (Figure 1D). Barletta et al., 2015, discussed that the microbiota proliferation followed by a blood - meal triggers lipid droplet remodeling. Here we suggest that the nutrient signaling mediated by TOR is also important to sustain the lipid biosynthetic metabolism. Moreover, the transcription factor PGC1 alpha is known to stimulate lipolytic pathways [29]. We showed that PGC1 gene expression is downregulated in the gut of blood-fed mosquitoes when TOR is active, and, conversely, that it is activated by rapamycin treatment. Overall, our data suggest that TOR drives the gut epithelia towards an anabolic status after blood–feeding.

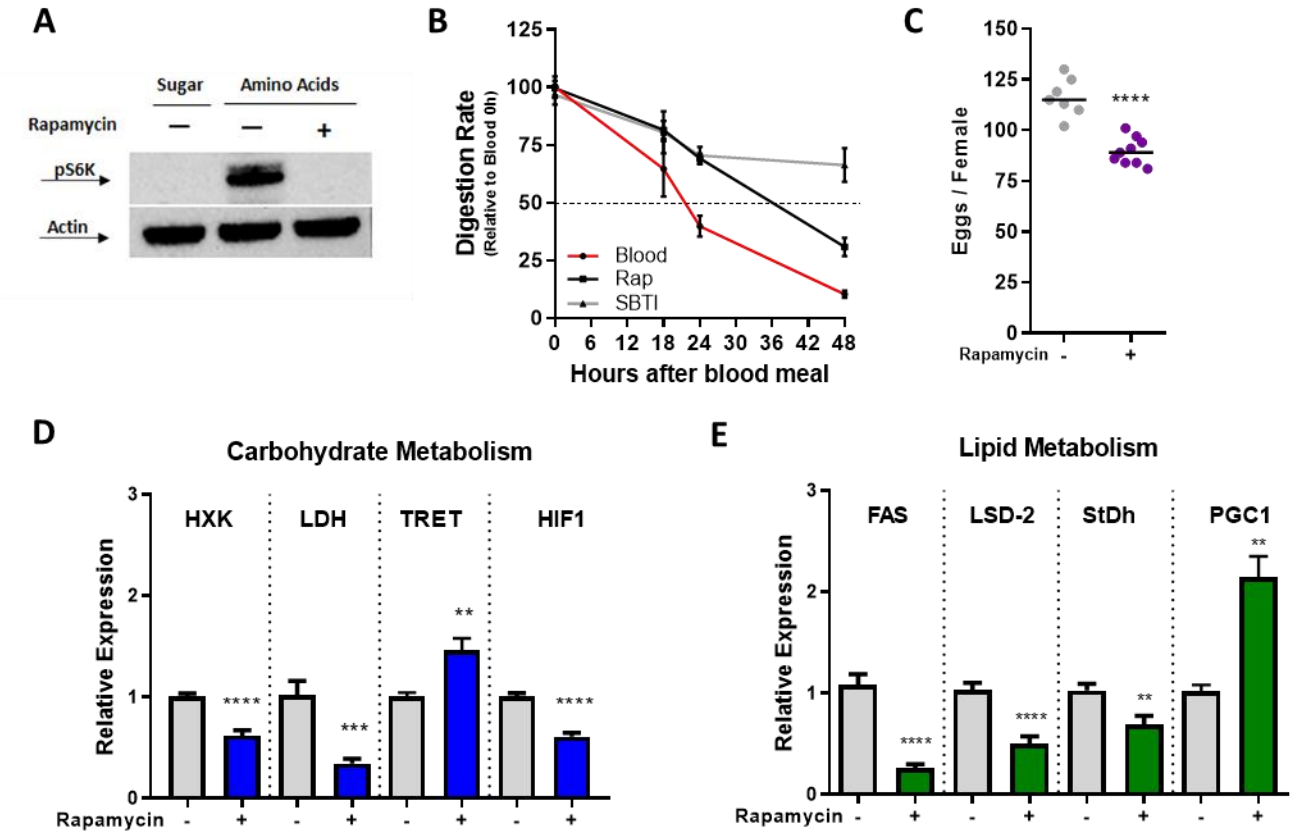


Figure 1. Metabolic shift mediated by TOR pathway. A) Western blotting of gut epithelia extracts of sugar-fed or 90 min post amino acids-feeding. Rapamycin (20µM) was added to the meal. B) Digestion rate measured by total protein amounts in the gut after feeding with blood (control), blood + 20µM of Rapamycin or blood + 2mg/ml of SBTI

(n=5 guts per condition). C) Number of eggs laid per female 7 days after feeding (Control n=7, Rap n=9). ****p<0.0001. Data are median, Mann-Whitney test. Gene expression in the midgut of carbohydrate (D) or lipid (E) metabolism genes by qPCR, using RP49 gene as endogenous control, 24 h after blood-feeding (at least 6 pools for each condition were analyzed). **p<0.005, ***p<0.001, ****p<0.0001. Data are mean +/- SEM, T-test.

Rapamycin acts as an immune suppressor in *Aedes* midgut

The TOR activation is crucial to support the pro-inflammatory polarity of macrophages and lymphocytes in mammals in acute infectious challenges or in chronic immune pathologies [1,30,31]. After the blood meal, the mosquito midgut microbiota population scales up to levels 100 times higher and, therefore, the tissue faces a challenge [32], which triggers signaling by innate immunity pathways. Then, we hypothesized that after the blood meal, the intestinal immune response is also orchestrated by TOR activation. Supplemental figure 2 shows that after the blood meal the gut immunity is enhanced as shown by the up-regulation in gene expression of immune-related genes, as previously observed [33]. Figure 2 shows that the rapamycin treatment led the midgut to an extensive immune suppression, affecting gene expression of the transcriptional factors of the three innate immune pathways Toll, IMD, and Jak/Stat pathways (Figure 2A), bacterial recognition proteins (2B), and antimicrobial peptides (2C). Indeed, Dong et al., 2017, showed that a protein-rich buffer diet enhances intestinal immunity in a virus-independent fashion [16]. The repressive scenario promoted by rapamycin feeding allows the microbiota to overgrow beyond the blood-fed control levels (Figure 2D). The immune suppression caused by TOR inhibition made the mosquitoes more susceptible to a bacterial infection (Figure 2E). It has been reported that protein–fed mosquitos increased the expression of immune-related genes compared to the saline–fed insects [16], consistent with the hypothesis proposed here that TOR, via amino acid sensing, modulates the *A. aegypti* intestinal immune system. Despite the presence of an increased bacterial population in the gut, ROS production by the intestinal epithelia does not show alterations when insects are fed with rapamycin-supplemented blood (Figure 2F), a lack of response that reveals a TOR role also in the function of this essential arm of gut immunity. Gandara et al., 2017, showed that TOR pathway signaling after blood intake decreased ROS levels in the gut of *Rhodnius prolixus*, a Chagas disease vector [34]. However, the authors did not look whether this axis of amino acids x TOR x ROS affected microbiota proliferation and insect immune responses. The immunosuppressant effect of rapamycin is largely known for mammals [31,35], similarly to data presented here showing decreased *A. aegypti* gut innate immunity. However, Feng et al., 2021, reached an opposite result [36] working with *Anopheles stephensi, showing that mosquitoes injected with rapamycin or* fed on rapamycin-treated mice displayed increased immunity activation, through the IMD pathway, which eventually led to resistance to *Plasmodium berghei* infection. In that report, the authors found that the

RNA seq of *A. stephensi* carcass showed an immune enhancement upon rapamycin treatment. These discrepant outcomes need further investigation, but it is possible that different mosquito species or even different mosquito tissues can distinctly respond to the TOR activation upon immune challenge.

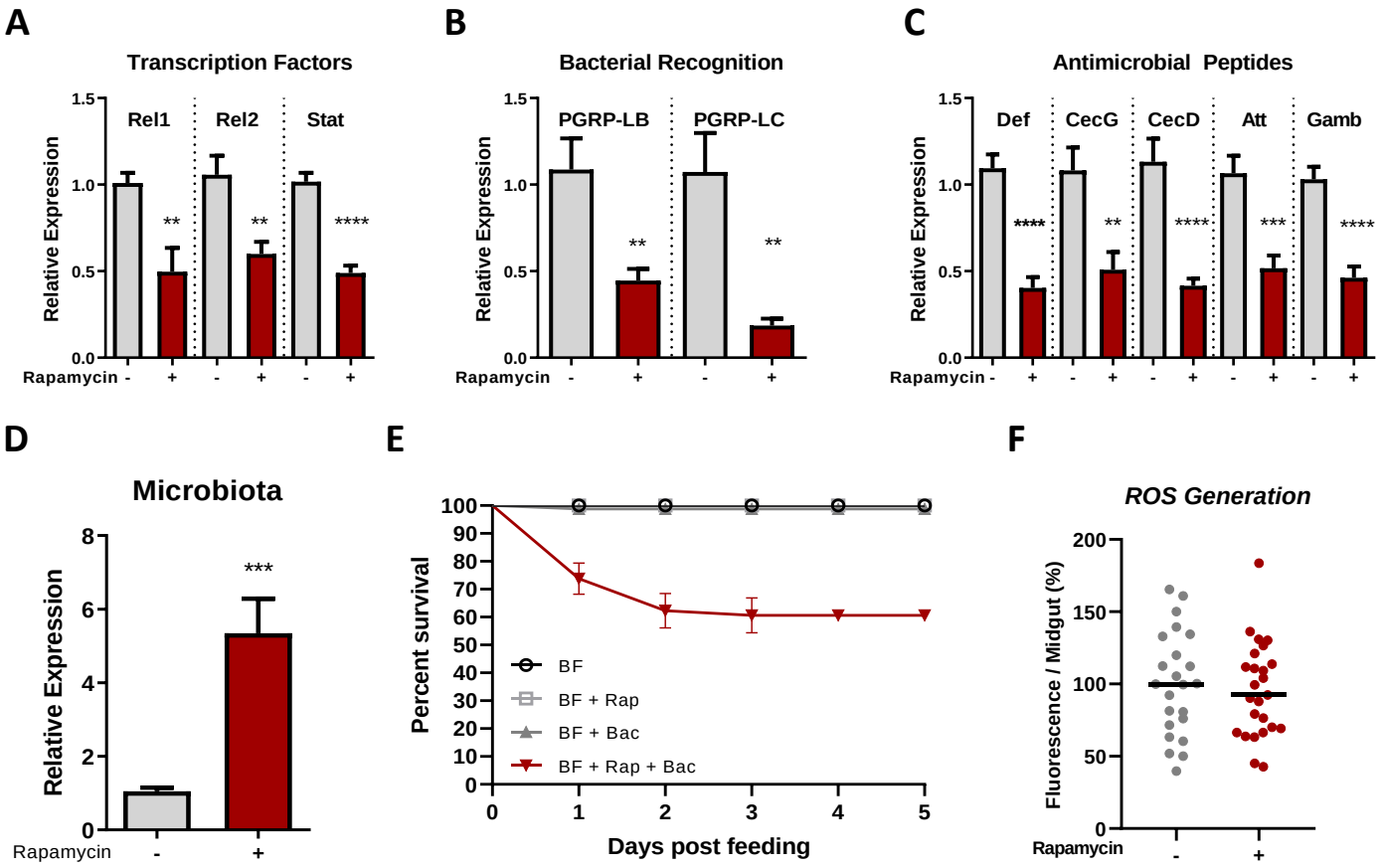


Figure 2. Intestinal immunosuppression. A-D) Expression of immune-related genes and eubacterial 16S gene in the midgut by qPCR 24 h after blood-feeding (at least 6 pools for each condition were analyzed). **p<0.005, ***p<0.001, ****p<0.0001. Data are mean +/- SEM, T-test. E) Survival curve of mosquitoes fed with blood +/- rapamycin and challenged with *Serratia marcescens* (BF n=117, BF+Rap n=115, BF+Bac n=190, BF+Rap+Bac n=121). The number of dead insects was counted daily for 5 days after feeding. F) Quantitative analysis of the fluorescence intensity of oxidized DHE from individual midguts at 18 h ABM (- Rap, n=23; + Rap, n=25).

Inhibition of TOR allows arbovirus to infect the midgut

Since the virus must infect the gut epithelial cells when TOR is active, and this kinase controls the intestinal immunity and microbiota (Figure 2), the impact of TOR on arboviral infection was evaluated. The mosquitoes were fed with infected blood in the presence of rapamycin and the viral titers were measured in the midgut by plaque assay. In these experiments, infections were performed with either Zika virus (a Flavivirus) or Chikungunya(an Alphavirus). Figure 3 shows that rapamycin significantly increased the number of viral particles in the midgut tissue (infection intensity), however, the effect was even more pronounced in the proportion of mosquitoes displaying infection (prevalence), independently of the virus used. This result showed that immunosuppression due to TOR inhibition stands not only for bacteria but also for viruses' infections, and highlights that the blood digestion triggers immune mechanisms that have antiviral effects. As Rel 1 and STAT transcription factors were downregulated by the rapamycin (fig 2A), these pathways appear as likely candidates part of the immune signaling network under the control of TOR, in line with previous reports that showed that TOLL and Jak/STAT pathways play antiviral roles in *A. aegypti* [37–39].

Interestingly, once a eukaryotic cell gets infected by a virus, a frequent remarkable event is the host cell translation shut-off, in a TOR-repressive manner in some cases [40–42]. This mechanism favors the viral genome translation, by alternative pathways, to the detriment of host RNA translation. Also, it prevents the interferon-mediated immunity triggered by TOR activation [43,44]. As the capped RNA translation is one canonical role of the TORC1 in mammals, it has been used as an explanation for the interference of this pathway on viral replication in insects *in vitro.* Rapamycin treatment of *Aedes albopictus* C6/36 cell line reflected lower Sindbis titers [45], an opposite scenario that was observed in *A. aegypti* Aag2 cell line [46] and here in the *A. aegypti* gut. Perhaps, the C6/36 *in vitro* model used to molecularly understand mosquito–virus interaction should not be the best choice once this cell line lacks a major antiviral pathway, RNAi, that may affect other outcomes of the viral replication [47]. Moreover, Zika NS4A and NS4B proteins blocks TOR activity in fetal neural stem cells, triggering autophagy and enhancing its replication [48], and rapamycin induces CHKV replication in the HEK-293 cell line [49]. In the sandfly *Lutzomyia* downregulating the TOR pathway with galactosamine, led to impaired blood digestion, as observed here in *A. aegypti*, and also significantly enhances the Leishmania infection intensity, prevalence, and differentiation in the fly gut [50].

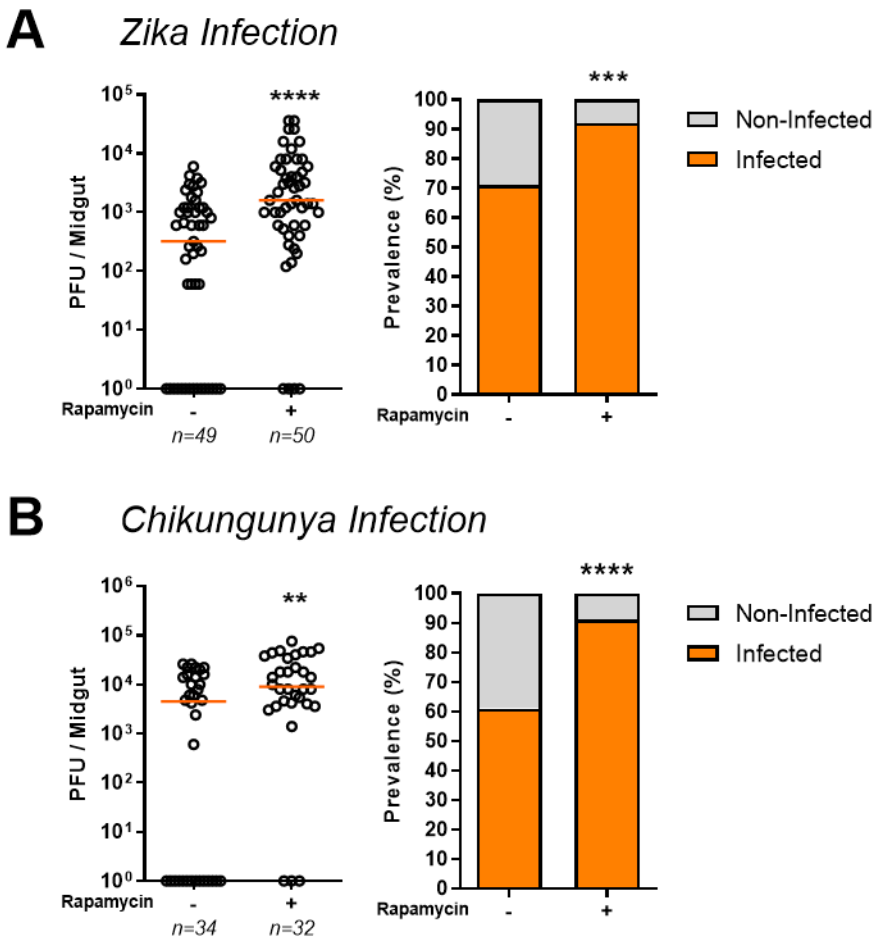


Figure 3. Viral infection is enhanced upon TOR inhibition. Infection intensity and prevalence of mosquito gut infected with Zika (A) or Chikungunya (B) virus, assessed by plaque assays. Each dot represents an individual gut and red bars represent median values. Mann-Whitney test. For infection prevalence, a chi-square test was performed.

Peritrophic Matrix synthesis is TOR-dependent

The Peritrophic Matrix (PM) secretion is a hallmark of the blood digestion in *A. aegypti* females. This acellular layer is composed of chitin fibrils and high glycosylated proteins that protect the midgut epithelia from direct contact with the blood bolus [51]. PM has been mentioned as an insect analog of mammalian intestinal mucus layer once it filters many elicitors from the microbiota, playing a central role in the local immunity [52]. The molecular mechanism behind the control of PM synthesis is lacking in many insects, including *A. aegypti*. Figure Sup 3A shows that the blood meal induces the expression in the mosquito midgut of both chitin synthase genes and one peritrophin (Aper50, a chitin-

binding protein that is a structural component of the PM) [53]. Rapamycin decreased the expression of both chitin synthase genes but not the Aper50 gene (figure 4A), suggesting that TOR, together with other mechanisms, contributes to PM synthesis. Blood-feeding induces microbiota proliferation in the midgut lumen [32], which in *Anopheles coluzzii* triggers PM synthesis [54]. However, our results showed that the microbiota growth does not seem to count for the *A. aegypti* PM synthesis (Figure 4B and Sup 3B). PM staining and assessment of its barrier function by evaluating penetration of labeled dextran in mosquitoes fed on blood supplemented with rapamycin showed compromised function of the PM by TOR inhibition (figure 4C-D), as the gut epithelia is infiltrated by the dextran present in the blood bolus due to the lack of a proper PM barrier. Protein absence in the meal has been reported to fail in triggering PM synthesis or resulting in secretion of a fragile structure [16,55]. The PM is an important site to nucleate heme detoxification, suggesting an extracellular antioxidant function [56]. We showed previously that heme triggers the expression of Heme Peroxidase 1 in the gut that controls the PM formation and is necessary to the acquisition of the PM barrier function using the dextran permeability assay [57]. However, we could not observe any influence of dietary heme on the chitin synthase gene expression (Figure Sup 3C), which indicates that PM formation is a complex process that receives inputs from multiple signaling pathways.

Previous reports showed that compromising PM formation either by inhibiting chitin synthesis [58] or through HPx1 silencing [57] leads the gut epithelia to respond to increased contact with the microbiota through increasing ROS generation [57]. In contrast, although rapamycin blocked PM formation (fig X), the ROS levels in the gut were not increased (fig x) and the microbiota overgrew (fig x). It suggests that the immunosuppression caused by TOR inhibition acts mechanistically upstream to the immune transcriptional regulation, being involved also to the tissue microbial sensing. The oxidative burst caused by the microbiota interaction with the epithelia is meanly driven by DUOX activation [32, 59-61], which mechanism is not transcriptionally-regulated [62]. These data reinforce our hypothesis that TOR activation during blood digestion is decisive for the intestinal immune responses.

The PM synthesis is controlled in some ways by TOR activation. However, some peritrophins genes, from *A. aegypti* and *A. coluzii*, have putative STAT binding motifs in their promoter region [39,54]. As we showed in figures 2 and 3, TOR regulates the expression of both, APER50 (peritrophin) and STAT and it's known that those pathways interact in mammals [63]. To know if TOR can modulate the expression of PM components via STAT transcription factor, we searched an available *A. aegypti* PM proteomic database to search for genes that potentially have STAT motifs in their promoters [64]. As result, out of 155 putative secreted proteins, 55 had at least one STAT bind motif. Figure X shows that STAT–silenced mosquitoes decreased the expression of one of the chitin synthase genes and the peritrophin genes 24 hours after blood–feeding. This is important molecular evidence of how the *A. aegypti* PM synthesis is controlled

upon feeding and it directly suggests that STAT is a downstream factor of TOR. However, we cannot exclude that more than one transcription factor regulates the PM-associated genes.

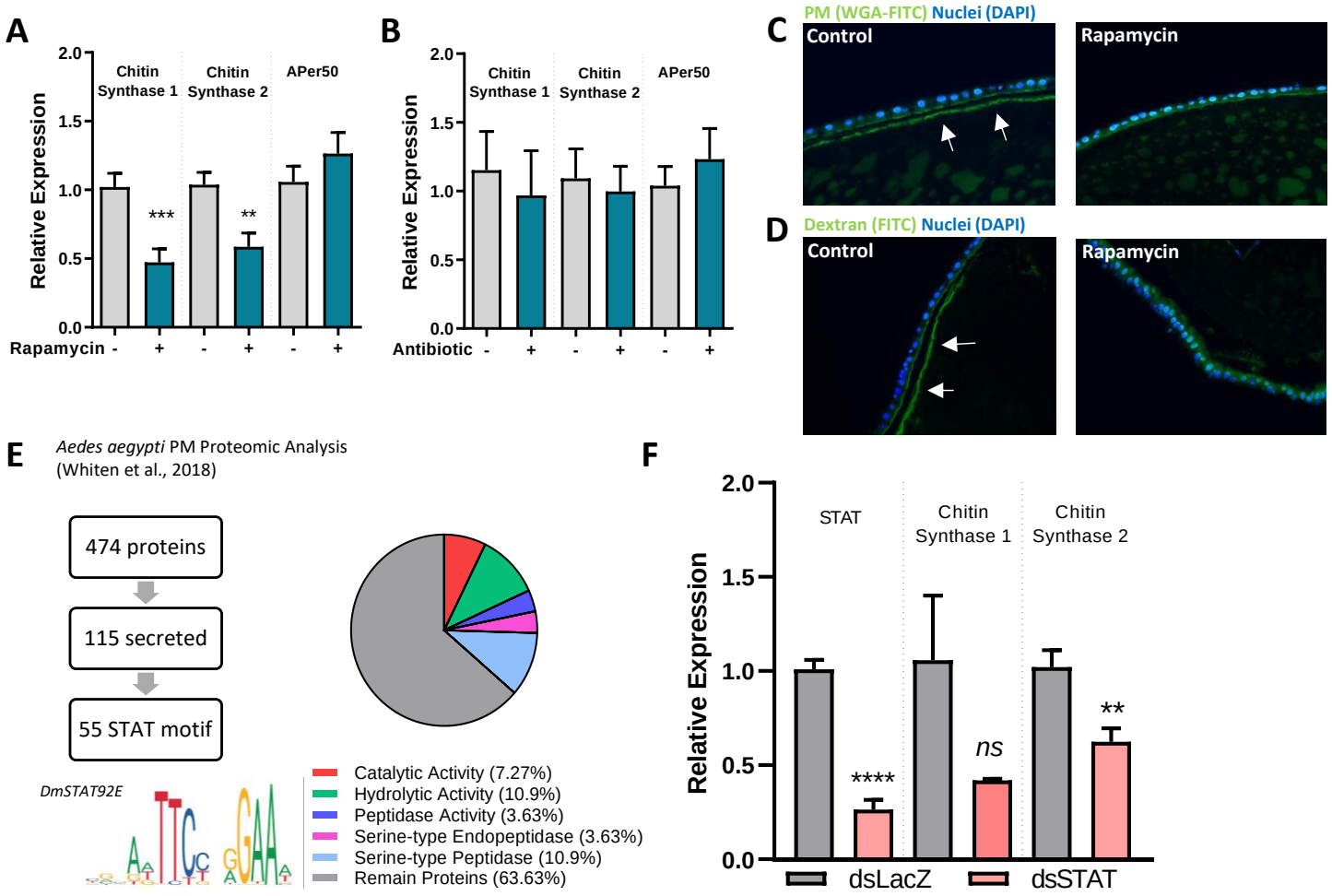


Figure 4. TOR triggers PM synthesis. A-B) Expression of two different chitin synthase genes evaluated in the mosquito gut 24 h after feeding, by qPCR. *p<0.05, **p<0.005. Data are mean +/- SEM, T-test. C) Peritrophic matrix staining in histological sections of mosquito midgut 18 h after feeding (representative pictures). D) Histological sections of mosquito midgut 18 h after blood feeding containing Dextran-FITC (representative pictures). E) Schematic work-flow of STAT motif binding sites in 5' upstream gene region of PM-associated genes. F) Expression of PM-associated genes in mosquitos' control (dsLacZ) or knockdown for STAT transcription factor (dsSTAT) in the mosquito gut 24 h after feeding, by qPCR (at least 6 pools for each condition were analyzed). **p<0.005, ***p<0.001. Data are mean +/- SEM, T-test.

Conclusions

The blood-feeding imposed physiological adaptions to the hematophagous organisms. The blood digestion triggers several signaling pathways in the *A. aegypti* gut that can impact the insect vector competence. Here we show that activation of the TOR pathway upon feeding not only orchestrated a large metabolic shift but also, is necessary for PM synthesis, a for rendering the intestinal epithelia capable to mobilize its immune pathways to respond to microbiota growth and limit viral infection and replication.

Supplementary data

Fig Sup 1

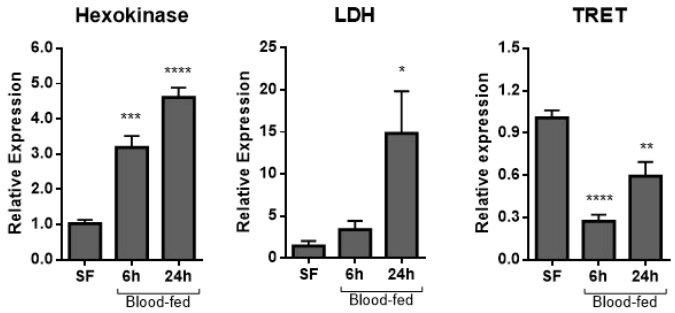


Fig Sup 2

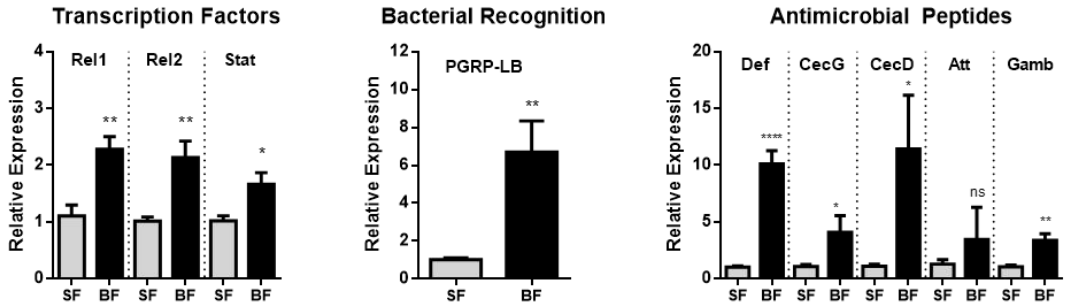
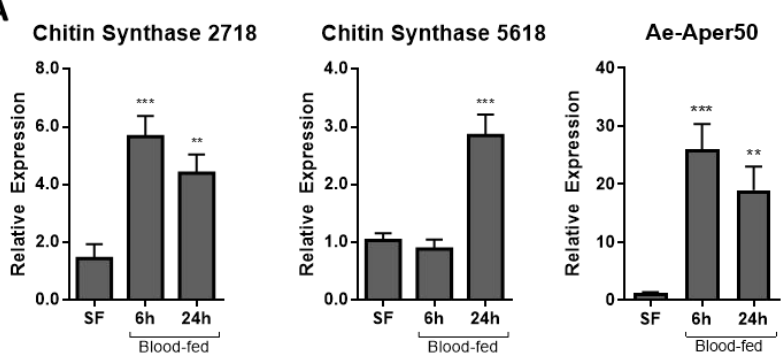


Fig Sup 3

A



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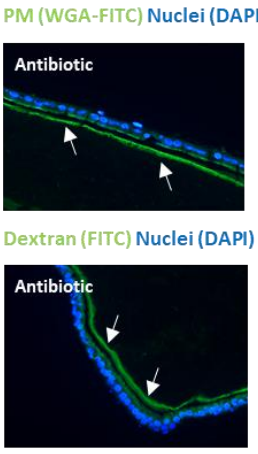

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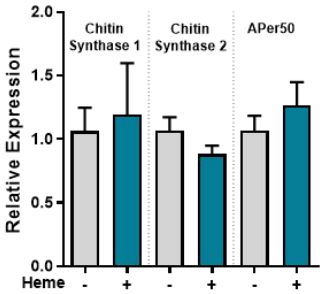

Legend

Figure Sup 1. Relative expression of genes involved in carbohydrate metabolism in the mosquito gut after blood feeding. Data are normalized by the sugar-fed control group (at least 6 pools for each condition were analyzed). *p<0.05, **p<0.005, ***p<0.001, ****p<0.0001. One-way Anova, Dunnett's comparison.

Figure Sup 2. Relative expression of genes involved in immune responses in the mosquito gut 24 h after blood-feeding. Data are normalized by the sugar-fed control group (at least 6 pools for each condition were analyzed). *p<0.05, **p<0.005, ***p<0.001, ****p<0.0001. T test.

Figure Sup 3. A) Relative expression of genes involved in PM synthesis in the mosquito gut after blood feeding. Data are normalized by the sugar-fed control group (at least 6 pools for each condition were analyzed). **p<0.005, ***p<0.001. One-way Anova, Dunnett's comparison. B) Peritrophic matrix histological sections of mosquito midgut 18 h after feeding (representative pictures). C) Relative expression of genes involved in PM synthesis in the mosquito gut 24 h after feeding on substitute of blood meal (SBM) in the presence or absence of heme. Data are normalized by the SBM - Heme control group (at least 6 pools for each condition were analyzed). **p<0.005, ***p<0.001. T test.

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Capítulo III

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The *Aedes aegypti* peritrophic matrix controls arbovirus vector competence through HPx1, a heme–induced peroxidase.

The *Aedes aegypti* peritrophic matrix controls arbovirus vector competence through HPx1, a heme–induced peroxidase.

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Abstract

Aedes aegypti mosquitoes are the main vectors of arboviruses. The peritrophic matrix (PM) is an extracellular layer that surrounds the blood bolus and acts as an immune barrier that prevents direct contact of bacteria with midgut epithelial cells during blood digestion. Here, we describe a heme-dependent peroxidase, hereafter referred to as heme peroxidase 1 (HPx1). HPx1 promotes PM assembly and antioxidant ability, modulating vector competence. Mechanistically, the heme presence in a blood meal induces HPx1 transcriptional activation mediated by the E75 transcription factor. HPx1 knockdown increases midgut reactive oxygen species (ROS) production by the DUOX NADPH oxidase. Elevated ROS levels reduce microbiota growth while enhancing epithelial mitosis, a response to tissue damage. However, simultaneous HPx1 and DUOX silencing was not able to rescue bacterial population growth, as explained by increased expression of antimicrobial peptides (AMPs), which occurred only after double knockdown. This result revealed hierarchical activation of ROS and AMPs to control microbiota. HPx1 knockdown produced a 100-fold decrease in Zika and Dengue 2 midgut infection, demonstrating the essential role of the mosquito PM in the modulation of arbovirus vector competence. Our data show that the PM connects blood digestion to midgut immunological sensing of the microbiota and viral infections.

Introduction

Mosquito-borne viruses are emerging as global threats to public health. Female mosquitos ingest infected blood from a host and transmit the virus to another host during the next blood-feeding. As the first insect tissue infected by the virus, the midgut is the initial barrier that the virus must overcome to establish itself in the mosquito (Black IV et al., 2002). Because blood digestion occurs in the midgut concomitantly with viral infection of epithelial cells, digestion-triggered physiological events have a major influence on the course of intestinal infection (Talyuli et al., 2021).

The peritrophic matrix (PM) in mosquitoes is a semi-permeable chitinous acellular layer secreted by intestinal cells after blood feeding. The PM completely envelopes the blood bolus, and its structure avoids direct contact of the digestive bolus with the midgut epithelia (Lehane, 1997; Shao et al., 2001). The PM is the site of deposition of most of the heme produced from blood hemoglobin hydrolysis, thus limiting exposure of the midgut cells to harmful concentrations of heme, a pro-oxidant molecule (Pascoa et al., 2002). Extensive gut microbiota proliferation occurs in most hematophagous insects after a blood meal. Therefore, the PM is a barrier that limits interaction of the tissue with the intestinal microbiota (Kuraishi et al., 2013; Oliveira et al., 2011; Terra et al., 2018), playing a role analogous to the mammalian intestinal mucous layer (Terra et al., 2018). The PM is mainly composed of chitin and proteins, and correct assembly of this structure is crucial to its barrier function. Additionally, the PM is a barrier for parasites such as *Plasmodium*, *Trypanosoma brucei,* and *Leishmania major*, which must attach to or traverse the PM to complete their development in an insect vector (Coutinho-Abreu et al., 2010; Rose et al., 2020; Shahabuddin et al., 1995; Weiss et al., 2014).

There are several studies on the role of reactive oxygen species (ROS) and redox metabolism on the gut immune response to pathogens. In *Drosophila melanogaster,* ROS production by a dual oxidase enzyme (DUOX, an NADPH oxidase family member) is triggered by pathogenic bacteria. The self-inflicted oxidative damage arising from DUOX activation is prevented by hydrogen peroxide scavenging via an immune-regulated catalase (IRC) (Ha, Oh, Bae, et al., 2005; Ha, Oh, Ryu, et al., 2005). In *Anopheles gambiae*, *Plasmodium* ookinete midgut invasion triggers a complex epithelial response mediated by nitric oxide and hydrogen peroxide that is crucial to mount an effective mosquito antiplasmodial response (Kumar & Barillas-Mury, 2005). Furthermore, an *Anopheles gambiae* strain genetically selected to be refractory to *Plasmodium* infection exhibits enhanced activation of JNK-mediated oxidative stress responses (Garver et al., 2013; Jaramillo-Gutierrez et al., 2010). In *Aedes aegypti*, it has been proposed that the Dengue NS1 viral protein decreases hydrogen peroxide levels, preventing an oxidative intestinal environment, which is an adverse condition for both Dengue and Zika viral infection (Bottino-Rojas et al., 2018; Liu et al., 2016). Catalase silencing in the *Aedes aegypti* gut reduces the dengue infection prevalence rate (Oliveira et al., 2017). The ROS generation by DUOX plays a key role in modulating proliferation of the indigenous microbiota, growth of opportunistic pathogenic bacteria, and Dengue virus infection (Ha et al., 2009; Liu et al., 2016; Oliveira et al., 2011).

Kumar et al. (2010) showed that heme peroxidase 15 (HPx15), also referred to as immunomodulatory peroxidase (IMPer), is expressed in the *A. gambiae* midgut and uses the hydrogen peroxide generated by DUOX as a substrate to crosslink proteins of the mucous layer in the ectoperitrophic space, limiting diffusion of immune elicitors from the gut microbiota and thus preventing activation of midgut antimicrobial responses to commensal bacteria. IMPer silencing results in constant activation of epithelial immune responses against both bacteria and *Plasmodium* parasites (Kumar et al., 2010). A similar immune barrier role for the PM against parasite infection has also been shown in tsetse flies infected with *T. brucei* and sandflies infected with Leishmania (Ramalho-Ortigao, 2010; Rose et al., 2020). Therefore, most of the studies on the PM of insect disease vectors have focused on its role as a barrier for parasites, but much less is known about the influence of PM on viral infections or its contribution to gut homeostasis and immune responses in *A. aegypti*.

Here, we show that HPx1, a heme peroxidase associated with the *A. aegypti* PM, has a dual role, acting in the PM assembly crucial for its barrier function and as an antioxidant hydrogen peroxide-detoxifying enzyme. This role of HPx1 in midgut physiology and immunity highlights that dietary heme is a signal that by triggering HPx1 expression and PM function, produces a homeostatic response that controls ROS and AMP immune effectors, microbiota expansion, and viral infection.

Materials and Methods

Ethics Statement

All the animal care and experimental protocols were conducted following the guidelines of the institutional care and use committee (Comissão de Ética no Uso de Animais, CEUA-UFRJ) and the NIH Guide for the Care and Use of Laboratory Animals. The protocols were approved under the registry #CEUA-UFRJ 149/19 for rabbit use and 075/18 for mice immunization and antiserum production. The animal facility technicians at the Instituto de Bioquímica Médica Leopoldo de Meis (UFRJ) carried out all aspects related to rabbit and mice husbandry under strict guidelines to ensure humane animal handling.

Mosquitos

The *Aedes aegypti* females (Red-Eye strain) used in this study were raised in an insectary of Universidade Federal do Rio de Janeiro. Approximately 200 larvae were reared in water-containing trays and fed dog chow. Pupae were transferred to plastic cages, and adults were fed *ad libitum* with 10% sucrose solution in cotton pads. The insects were kept in a 12 h dark/light period-controlled room at 28 °C and 80% humidity. Blood feeding was performed using rabbit ears or artificially through glass feeders sealed with Parafilm and connected in a circulated water bath at 37 °C. Substitute of blood meal (SBM) is a previously described artificial diet with a chemically defined composition (Talyuli et al., 2015) and was used in experiments in which the presence of heme was modulated. For this study, only bovine albumin and gamma-globulin were used as protein sources, and no hemoglobin was added. Hemin was solubilized in 0.1 M NaOH and neutralized with 0.01 M sodium phosphate buffer (pH 7.4). Antibiotics (penicillin 200 U/ml and streptomycin 200 µg/mL) in autoclaved 5% sucrose solution were supplied for 3 days before feeding with blood or SBM.

Catalase activity

Midguts were dissected in cold 50% ethanol, and epithelia were separated from the blood bolus surrounded by the PM. The samples were immediately transferred to tubes with a protease inhibitor cocktail (50 µg/mL SBTI, 1 mM benzamidine, 1 mM PMSF). The midgut epithelial samples were directly homogenized, but the PM-enriched fraction samples were centrifuged 3x at 10000 × g for 5 min at 4 °C to remove as much of the blood bolus as possible. Hydrogen peroxide detoxification activity was measured based on peroxide absorbance (240 nm for 1 min) in the presence of mosquito homogenates (Aebi, 1984), and the protein concentration was determined according to Lowry (LOWRY et al., 1951). For *in vitro* inhibition experiments, samples were incubated with different concentrations of 3-amino- 1,2,4-triazole for 30 min at 4 ˚C before enzymatic activity assays (Oliveira et al., 2017).

Double-strand RNA synthesis and injections

To synthesize dsRNA, a first PCR was performed using mosquito whole-body cDNA as a template. The product was diluted 100x and used in a second reaction with T7 primers. dsLacZ was used as a control and amplified from a cloned plasmid containing the LacZ gene. Double-strand RNA synthesis was performed using a MEGAscript T7 transcription kit (Ambion/Thermo

Fisher Scientific, MA - USA). The reaction was performed overnight at 37 °C. Each product was precipitated with 1 volume of isopropanol and 1:10 volume of 3 M sodium acetate (pH 3). Four-day-old females were cold-anesthetized, and a double shot of 69 nl of 3 µg/µL dsRNA was injected into the mosquito thorax using a Nanoject II (Drummond Scientific, PA - USA). For double-silencing experiments, the dsRNA mixture containing both dsHPx1 and dsDUOX was lyophilized and then resuspended to half of the original volume. One day after injection, the females were blood-fed.

RNA extraction, cDNA synthesis, and qPCR

Total RNA was extracted from midgut samples using TRIzol reagent following the manufacturer's protocol. The RNA (1 µg) was treated for 30 min at 37 °C with DNAse, and cDNA was synthesized with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems/Thermo Fisher Scientific, MA - USA) using random primers. Quantitative PCR (qPCR) was performed with a StepOnePlus Real-Time PCR System (Applied Biosystems/Thermo Fisher Scientific, MA - USA) using Power SYBR-green PCR master MIX (Applied Biosystems/Thermo Fisher Scientific, MA - USA). The RP49 gene was used as an endogenous control, and the primers used in the qPCR analyses are listed in Supplemental Table 1.

HPx1 antiserum

Aedes aegypti HPx1 (AAEL006014) was cloned into pET15b at the NdeI (5') and BamHI (3') restriction sites. The export signal predicted by SignalP software (Petersen et al., 2011) was removed from the purchased codon-optimized sequence (GenScript, NJ - USA). Plasmid pET15b containing the HPx1 protein-coding sequence was transformed into the *Escherichia coli* BL21(DE3) strain. Cells were grown at 37 °C in 2xYT medium containing 100 µg/L ampicillin. After reaching OD_{600} 0.4, the cells were cooled and supplemented with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and the cells were incubated at 25 °C overnight. The cells were then harvested and resuspended in buffer A (200 mM Tris pH 8.0, 500 mM NaCl, 5 mM imidazole, 1% Triton X-100, 10% glycerol, 10 mM β-mercaptoethanol) supplemented with 2 mg/mL lysozyme. After breaking the cells by ultrasonic treatment, the insoluble fraction was collected by centrifugation. Because the recombinant HPx1 obtained by this protocol was not soluble, the corresponding protein band was cut from the SDS–PAGE gel, and the protein was extracted from the gel (Retamal et al., 1999). Immunization of BalB/C mice was performed by injecting two shots of 50 and 25 µg of antigen intraperitoneally, spaced by 21 days, using Freund's complete and incomplete adjuvant, respectively, in a 1:1 ratio (1 antigen:1 adjuvant). Two weeks after the second shot, blood was extracted by cardiac puncture, and the serum was isolated and frozen for further use.

HPx1 Western Blotting

Pools of midgut epithelia and PMs were dissected and immediately placed in tubes containing a protease inhibitor cocktail. The samples were denatured at 95 °C in the presence of SDS sample buffer, and a volume equivalent to 1 midgut/slot was resolved by SDS–PAGE. The gel was blotted onto PVDF membranes (Bjerrum Schafer-Nielsen buffer - 48 mM Tris, 39 mM glycine, 0.037% SDS - pH 9.2, 20% methanol) for 1 h at 100 V and blocked with 5% albumin in TBS-T (Tris 50

mM, pH 7.2, NaCl 150 mM, 0.1% Tween 20) overnight (ON) at 4 °C. The membranes were incubated with 1:5000 anti-HPx1 primary antiserum diluted in blocking solution for 5 h at room temperature. The primary antibody solution was removed, and the membrane was washed with TBS-T (3X) before incubation with alkaline phosphatase-conjugated anti-mouse secondary antibody (1:7500 in blocking solution) for 1 h at room temperature (RT). The membrane was washed and developed using NBT/BCIP alkaline phosphatase substrates.

Putative HPx1 gene promoter *in silico* analysis

The MEME motif-based algorithm (Bailey et al., 2009) was used to analyze a 2500 bp sequence upstream of the 5' transcription start site of the HPx1 gene (*Aedes aegypti* genome, version AaegL3.4). The Fimo tool (Grant et al., 2011) was used to specifically search for cis-regulatory elements associated with ecdysone molecular signaling, as previously described (Kokoza et al., 2001).

PM permeability

HPx1-silenced mosquitoes were artificially fed rabbit blood containing 1 mg/ml dextran-FITC (Sigma, FD4), and the midguts were dissected 18 h after feeding. The midguts were fixed in 4% paraformaldehyde solution in 0.1 M cacodylate buffer for 2 h and placed ON in a 15% sucrose solution in phosphate-buffered saline (PBS; 10 mM Na-phosphate buffer, pH 7.2, 0.15 M NaCl) at RT. Then, they were incubated in a 30% sucrose solution for 30 h. The following day, the midguts were infiltrated with 50% Optimal Cutting Temperature/OCT (Tissue-TEK, Sakura Finetek, CA - USA) in 30% sucrose solution for 24 h and then ON in 100% OCT. The samples were frozen at -70°C until use, and serial 14-µm-thick transverse sections were obtained using an MEV Floor Standing Cryostat (SLEE Medical, Nieder-Olm - Germany). The slices were placed on glass slides and mounted with Vectashield containing DAPI mounting medium (Vector laboratories, CA - USA). The sections were examined in a Zeiss Observer.Z1 with a Zeiss Axio Cam MrM Zeiss through excitation BP 546/12 nm, beam splitter FT 580 nm, and emission LP 590 nm.

Reactive oxygen species measurement

Midguts were dissected at 18 h after blood feeding and incubated with 50 µM dihydroethidium (hydroethidine; DHE; Invitrogen/Thermo Fisher Scientific, MA - USA) diluted in 5% fetal bovine serum-supplemented Leibovitz 15 medium for 20 min at room temperature in the dark. The dye medium was removed, and the midguts were gently washed in dye-free medium. The fluorescence of the oxidized DHE was acquired using a Zeiss Observer.Z1 with a Zeiss Axio Cam MrM Zeiss, and the data were analyzed using AxioVision software in a Zeiss-15 filter set (excitation BP 546/12 nm; beam splitter FT 580 nm; emission LP 590 nm) (Oliveira et al., 2011).

Mitosis labeling

HPx1-silenced mosquitoes were blood-fed, and the midguts were dissected 18 h after feeding. The midguts were fixed in 4% paraformaldehyde solution for 30 min, permeabilized with 0.1% Triton X-100 for 15 min at RT, and blocked ON at room temperature in a solution containing PBS, 0.1% Tween 20, 2.5% BSA, and 10% normal goat serum. All samples were incubated overnight

with a mouse anti-PH3 primary antibody (1:500, Merck Millipore, Darmstadt - Germany) diluted in blocking solution at 4 °C and then washed 3x for 20 min each in washing solution (PBS, 0.1% Tween 20, 0.25% BSA). The midguts were incubated with a secondary goat anti-mouse antibody conjugated with Alexa Fluor 546 (Thermo Fisher Scientific, MA - USA) for 1 h at room temperature at a dilution of 1:2000, and nucleic acids were stained with DAPI (1 mg/ml, Sigma) diluted 1:1000. PH3-positive cells were visualized and counted using the Zeiss fluorescence microscope mentioned above (Taracena et al., 2018).

Virus infection and titration

Zika virus (ZKV; Gen Bank KX197192) was propagated in the *Aedes albopictus* C6/36 cell line for 7 days in Leibovitz-15 medium (Gibco #41300–039, Thermo Fisher Scientific, MA - USA; pH 7.4) supplemented with 5% fetal bovine serum, tryptose 2.9 g/L, 10 mL of 7.5% sodium bicarbonate/L; 10 mL of 2% L-glutamine/L, 1% nonessential amino acids (Gibco #11140050, Thermo Fisher Scientific, MA - USA) and 1% penicillin/streptomycin (Oliveira et al., 2017) at 30 °C. Dengue 2 virus (DENV, New Guinea C strain) was propagated in C6/36 in MEM media (GIBCO #11095080, Thermo Fisher Scientific, MA - USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin for 6 days (Jupatanakul et al., 2017). The cell supernatants were collected, centrifuged at 2,500 × g for 5 min, and stored at -70 °C until use. Mosquitoes were infected with 10^5 PFU/ml ZKV or $2x10^7$ PFU/ml DENV in a reconstituted blood meal prepared using 45% red blood cells, 45% of each virus supernatant, and 10% rabbit serum (preheated at 55 °C for 45 min). Four days after Zika infection or seven days after Dengue infection, the midguts were dissected and stored at -70 °C in 1.5 ml polypropylene tubes containing glass beads and DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The samples were thawed and homogenized, serially diluted in DMEM, and incubated in 24-well plates with a semiconfluent culture of Vero cells (for ZKV samples) or BHK21 cells (for DENV) for 1 h at 37 °C and then incubated with DMEM 2% fetal bovine serum + 0.8% methylcellulose (Sigma, M0512) overlay for 4 days at 37 °C and 5% CO_2 in an incubator. The plates were fixed and stained for 45 min with 1% crystal violet in ethanol/acetone 1:1 (v:v).

Statistical analyses and experimental design

All experiments were performed in at least three biological replicates, and samples correspond to pools of 5–10 insects. The graphs and statistical analyses were performed using GraphPad 8 software. For qPCR experiments, relative gene expression was calculated by the Comparative Ct Method (Pfaffl, 2001), and the result is expressed as the mean of ΔΔCt values compared to a housekeeping gene (RP-49, AAEL003396) (Gentile et al., 2005).

Results

Aedes aegypti PM detoxifies hydrogen peroxide

After a blood meal, the antioxidant capacity of the *Aedes aegypti* midgut is increased by expressing enzymes and low molecular weight radical scavengers (Sterkel et al., 2017). These protective mechanisms are complemented by the capacity of the PM to sequester most of the heme produced during blood digestion, which has been proposed to be a preventive antioxidant defense, as heme is a pro-oxidant molecule (Devenport et al., 2006; Pascoa et al., 2002). Figure 1A shows that *A. aegypti* PM exhibited hydrogen peroxide detoxifying activity up to 24 h ABM followed by a sharp decrease at 36 h, close to the end of blood digestion. The specific activity of the PM hydrogen peroxide scavenging activity was comparable to the activity found in the midgut epithelia 24 h after blood-feeding, which is attributed to a canonical intracellular catalase (SUP1A). However, silencing of the “canonical” intracellular catalase (AAEL013407-RB) did not alter the PM’s ability to detoxify hydrogen peroxide at 24 h after feeding (Fig 1B), suggesting that this activity in the PM is not due to the midgut intracellular catalase (AAEL013407-RB) (Oliveira et al., 2017). This hypothesis received support from the observation that the hydrogen peroxide decomposing activity of the PM and midgut epithelia showed distinct *in vitro* sensitivity to the classical catalase inhibitor amino triazole (SUP1B). Moreover, neither depletion of the native microbiota by antibiotic treatment (SUP1C) nor feeding the insect an artificial diet (cell-free meal) devoid of catalase (SUP1D) altered PM hydrogen peroxide detoxification, additionally excluding the hypothesis of an enzyme originating from the microbiota or host red blood cells.

HPx1-dependent hydrogen peroxide scavenging by the PM

We hypothesized that the observed PM hydrogen peroxide detoxifying activity should be attributed to another enzyme encoded by the mosquito genome. Peroxidases are a multigene family of enzymes and the genome of *A. aegypti*, as with most other organisms, has many peroxidases. Peroxidases are grouped in three large families: glutathione, heme, and thioredoxin peroxidases. As the PM is an extracellular structure, we initially searched for peroxidases with a predicted signal peptide. Interestingly, this search identified ten peroxidases, all of them belonging to the heme peroxidase family, which also includes the secreted peroxidases of *Anopheles gambiae* (Kumar et al., 2010) and *Drosophila melanogaster* (Ha, Oh, Ryu, et al., 2005). Phylogenetic analysis showed that *A. aegypti* heme peroxidase 1 (HPx1) is a close homolog of the peroxidase HPx15/IMPer that promotes the crosslink of extracellular proteins in the gut lumen of *A. gambiae* (FIG 1C). As the HPx15/IMPer from *A. gambiae* was shown to be secreted by the midgut epithelia, we used the presence of a secretion signal peptide as an additional feature to indicate HPx1 as the *A. aegypti* PM enzyme responsible for decomposing hydrogen peroxide. Fig. 1D shows that the blood meal induced HPx1 gene expression in the gut. Moreover, western blotting showed that most of the HPx1 in the midgut was bound to the PM, with a minor fraction being associated with the epithelia (Fig. 1 E), and RNAi silencing of HPx1 expression significantly

decreased hydrogen peroxide detoxification by the PM (Fig. 1 E; HPx1 silencing efficiency: SUP1F).

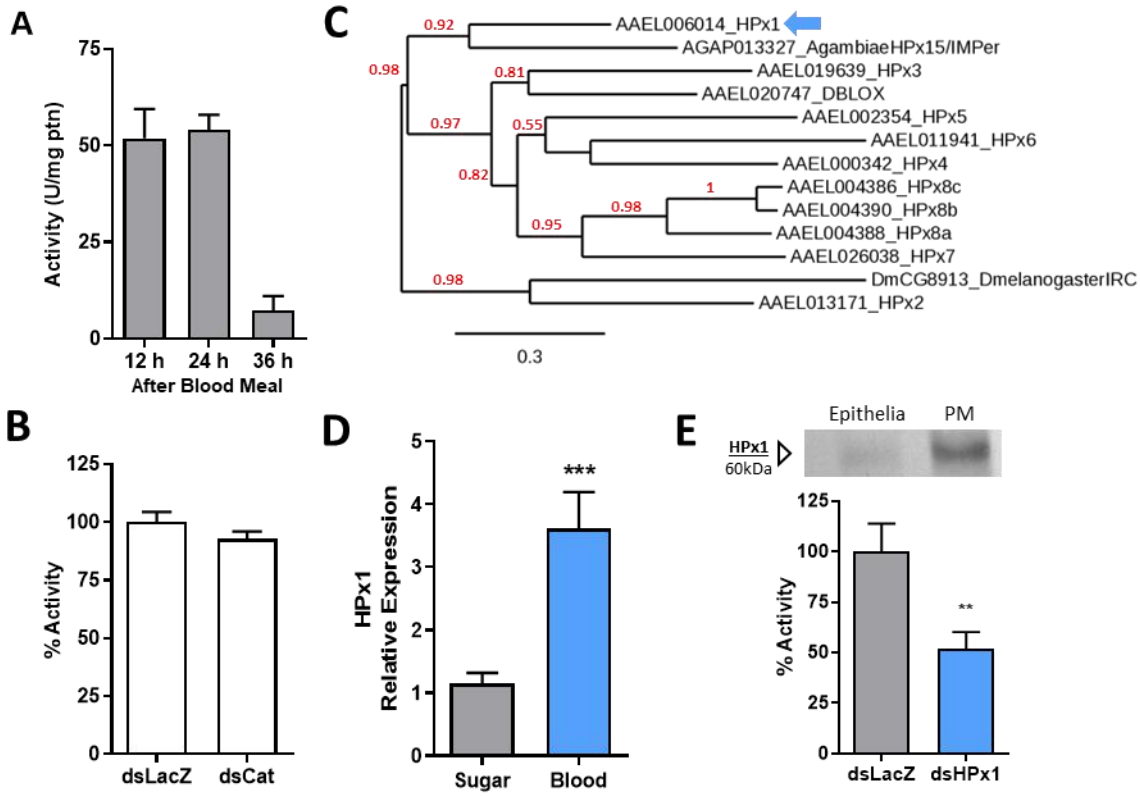


Figure 1. Hydrogen peroxide scavenging by the *Aedes aegypti* peritrophic matrix. A) PMs were dissected at different times after a blood meal (ABM), and their catalase specific activity was measured (12 h n=3, 24 h n=3; 36 h n=2). B) Catalase activity of PMs dissected from control (dsLacZ-injected) and catalase (AAEL013407-RB)-knockdown insects at 24 h AMB (dsLacZ n=9; dsCat n=9). C) Phylogenetic tree of heme peroxidases from *Aedes aegypti* (AAEL), *Anopheles gambiae,* and *Drosophila melanogaster*. Maximum likelihood analysis was performed, and the numbers in each branch represent bootstraps. (D) HPx1 expression in midguts at 24 h ABM relative to the sugar-fed control (Sugar n=11; Blood n=11). (E) Western blot of the HPx1 protein in 20 µg of gut epithelia and PM extracts at 18 h ABM and catalase activity of PMs dissected from control and HPx1-knockdown insects (dsLacZ n=8; dsHPx1 n=13). The full western blot membrane is shown in Supplementary Figure 1E. Data are the mean +/- SEM. **p> 0.005, ***p<0.001 for the T test for D and E.

The E75 transcription factor mediates heme-induced HPx1 midgut expression

A blood meal triggers large changes in the gene expression pattern of *A. aegypti* and, among several factors, the heme released upon hemoglobin proteolysis acts as a pleiotropic modulator

of transcription (Bottino-Rojas et al., 2015). Feeding the insects with SBM with or without heme revealed that heme significantly regulated HPx1 gene expression (Fig. 2A). Accordingly, lower hydrogen peroxide decomposition activity was observed in the PM secreted by females fed SBM without heme compared to the blood-fed insects, a phenotype rescued by heme supplementation (Fig. 2B). *In silico* analysis of the HPx1 promoter gene region revealed putative binding sites for E75, a hormone-responsive transcription factor that functions as a heme and redox sensor (SUP2A) (Cruz et al., 2012). E75 knockdown significantly reduced HPx1 gene expression in the midgut after blood feeding, revealing a molecular mechanism for triggering HPx1 by heme (Fig. 2C; E75 silencing efficiency: SUP2B). Proliferation of the gut microbiota in response to blood feeding is known to induce expression of several genes in the midgut. However, neither microbiota depletion by oral administration of antibiotics (aseptic) nor reintroduction of a bacterial species (*Enterobacter cloacae*) into antibiotic-treated mosquitoes affected HPx1 gene expression (SUP2C). Thus, we propose the molecular signaling model shown in Fig. 2D.

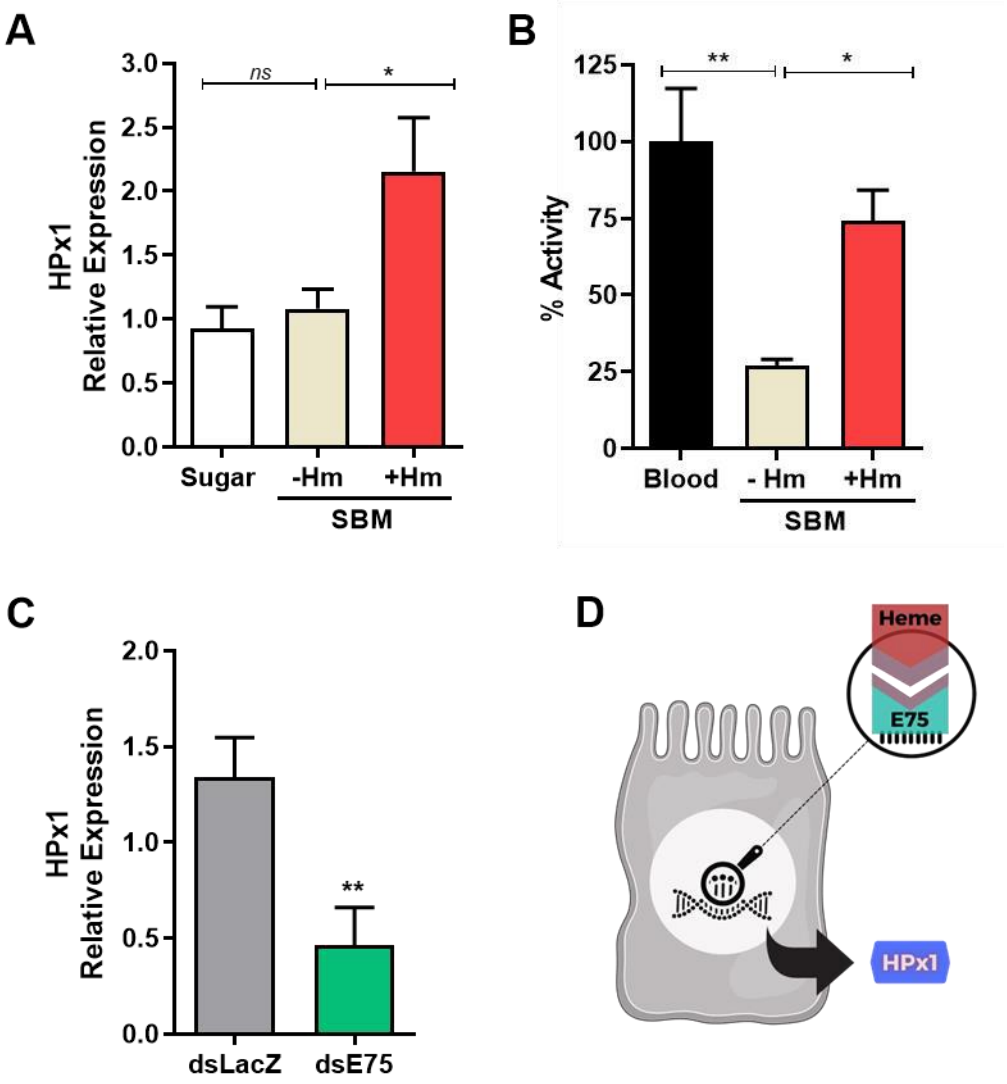


Figure 2. HPx1 expression is controlled by dietary heme and the E75 transcription factor. A) HPx1 expression in the midgut of sugar-fed or at 24 h after SBM feeding (without heme or supplemented with 50 µM of heme) (Sugar n=5; -Hm n=14; +Hm n=14). B) Catalytic activity of PMs from mosquitoes fed different diets at 24 h postfeeding (Blood n=3 pools of 10 PM each; -Hm n=4 pools of 10 PM each; +Hm n=4 pools of 10 PM each). C) HPx1 expression in midguts from control (dsLacZ) and dsE75-injected mosquitoes at 24 h ABM (dsLacZ n=3; dsE75 n=4). D) Schematic model of molecular signaling for HPx1 expression in the mosquito midgut. *p<0.05, **p<0.005, ns = not significant. Data are the mean +/- SEM. One-way ANOVA with Dunnett's post-test for A and B and the T test for C.

HPx1 contributes to PM assembly and regulation of the gut bacterial population

The PM is a semipermeable matrix that controls traffic of molecules between the intestinal lumen and the epithelia, and its correct assembly is essential to fulfilling its barrier function. Fig. 3A shows that control mosquitoes fed fluorescent dextran retained the polymer on the gut luminal side, a proxy of the barrier function of the PM. In contrast, HPx1-silenced mosquitoes presented strong fluorescence in the epithelial layer, suggesting a role for HPx1 in the proper assembly of the PM, as its permeability barrier function was compromised by HPx1 silencing. As this alteration in permeability might expose the epithelium to bacterial elicitors from the proliferative microbiota, we evaluated ROS production, known as an antimicrobial defense. Figs. 3B and 3B' show that HPx1 silencing increased ROS levels in the midgut. The increase in ROS in HPx1-silenced mosquitoes was due to increased contact of the gut epithelia with the microbiota because oral administration of the antibiotics prevented the increase in the ROS levels, as observed in HPx1-silenced mosquitos (Fig. 3C). Exposure to damage signals and elevated ROS has been shown to activate intestinal stem cell mitosis (30). Indeed, HPx1 silencing increased phosphorylated H3-histone levels (Fig. 3D) in midgut epithelial cells, indicative of mitotic activity and suggestive of epithelial remodeling in response to oxidative imbalance. This highlights the key role of HPx1 in tissue homeostasis. The native midgut bacterial load was significantly reduced after HPx1 silencing (Fig. 3E). However, this was not due to canonical immune signaling pathways, as neither expression of two antimicrobial peptides, Attacin and Cecropin G, nor the bacterial sensor PGRP-LB by midgut cells was significantly different from that of dsLacZ controls (Fig. 3F), suggesting that ROS levels control microbiota proliferation.

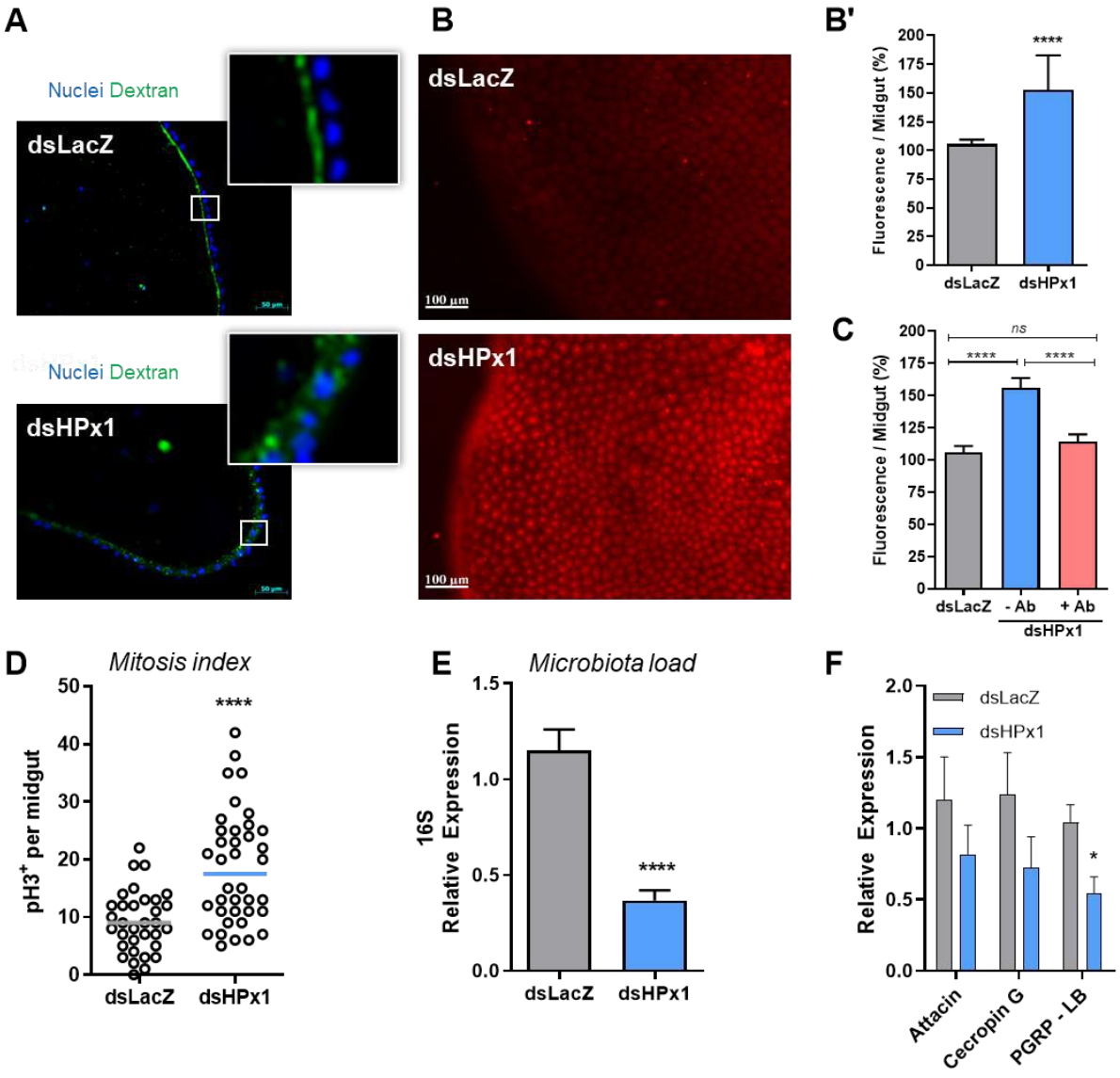


Figure 3. The role of HPx1 in PM assembly. A) Midgut transverse slices at 18 h ABM supplemented with dextran. Green: dextran – FITC. Blue: DAPI nuclear staining. Insets highlight dextran localization. B) Representative images of ROS levels measured by DHE oxidation in individual midguts at 18 h ABM. B') Quantitative analysis of the fluorescence intensity of oxidized DHE (dsLacZ, n=35; dsHPx1, n=39). C) Quantitative analysis of the fluorescence intensity of oxidized DHE from individual midguts at 18 h ABM (dsLacZ, n=32; dsHPx1 - Ab, n=34; dsHPx1 + Ab, n=27). D) Mitosis index in the mosquito midgut at 18 h ABM (phospho-histone H3) (dsLacZ n=33; dsHPx1 n=38). E) The intestinal microbiota load analyzed through eubacterial ribosomal 16S gene expression by qPCR at 24 h ABM (dsLacZ n=6; dsHPx1 n=7). F) Immune-related gene expression upon HPx1 silencing at 24 h ABM by qPCR (dsLacZ n=8; dsHPx1 n=7). *p<0.05, ****p<0.0001, ns = not significant. Data are the mean +/- SEM. The T test for B', D, E and F, and one-way ANOVA with Tukey's posttest for C.

HPx1 and DUOX coordinate intestinal immunity

NADPH-oxidases are a family of ROS-producing enzymes related to the immune system. Members of the dual oxidase (DUOX) group have been shown to play an essential role against bacterial challenge in the insect intestinal environment (Ha, Oh, Bae, et al., 2005; Kumar et al., 2010; Oliveira et al., 2011). Silencing HPx1 alone significantly increased ROS levels (Fig. 4A). In contrast, ROS levels were similar to those of dsLacZ controls in females in which HPx1 and DUOX were co-silenced (Fig. 4A and 4B), indicating that DUOX activity is the source of ROS when HPx1 is silenced. Unexpectedly, despite lowered ROS levels in double-silenced mosquitoes, bacterial levels remained reduced (Fig. 4C). This antibacterial response appears to be mediated by activation of canonical immune signaling pathways, as evidenced by increased expression of antimicrobial peptides and PGRP-LB in HPx1/DUOX co-silenced females (Fig. 4D-F).

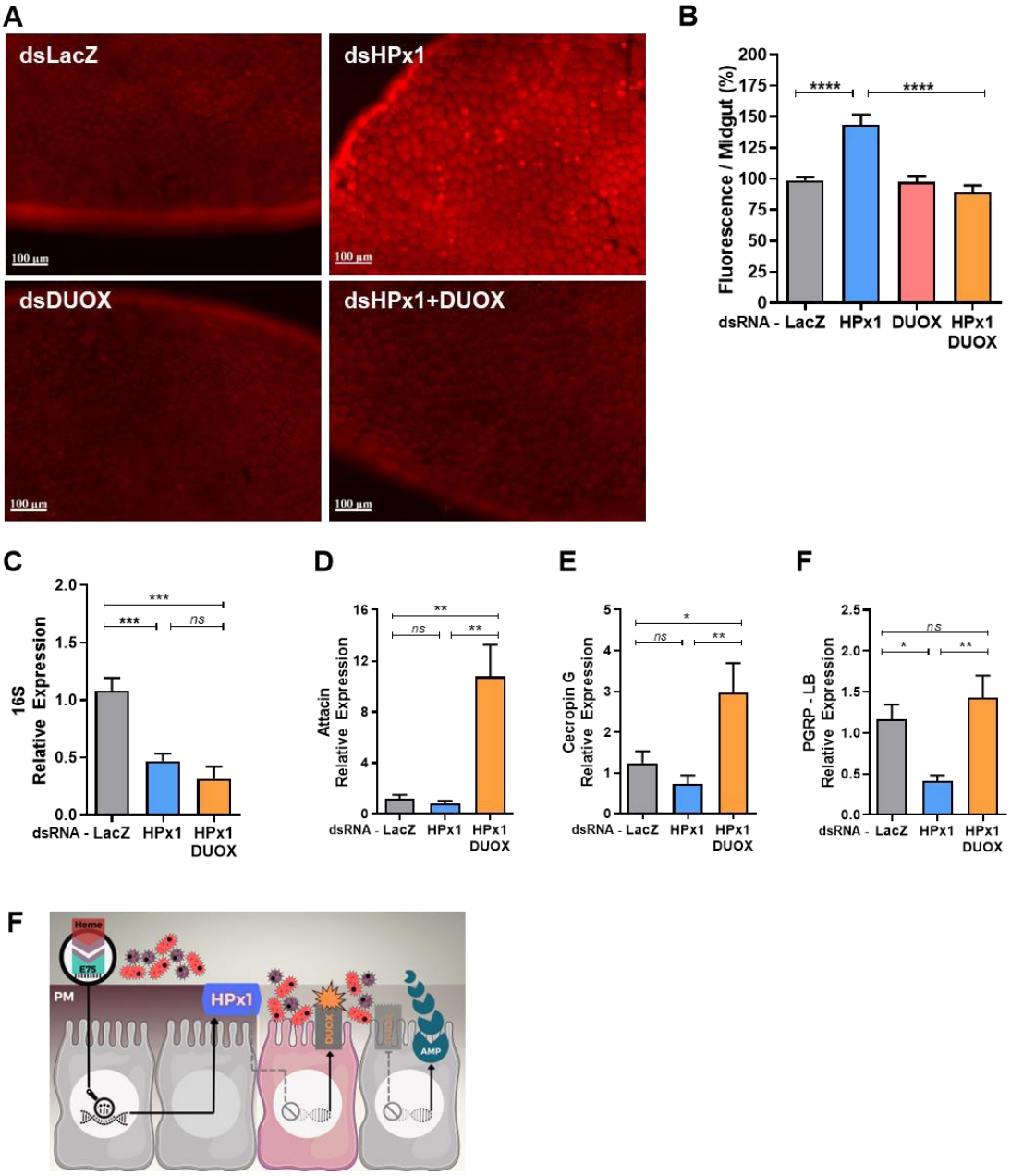
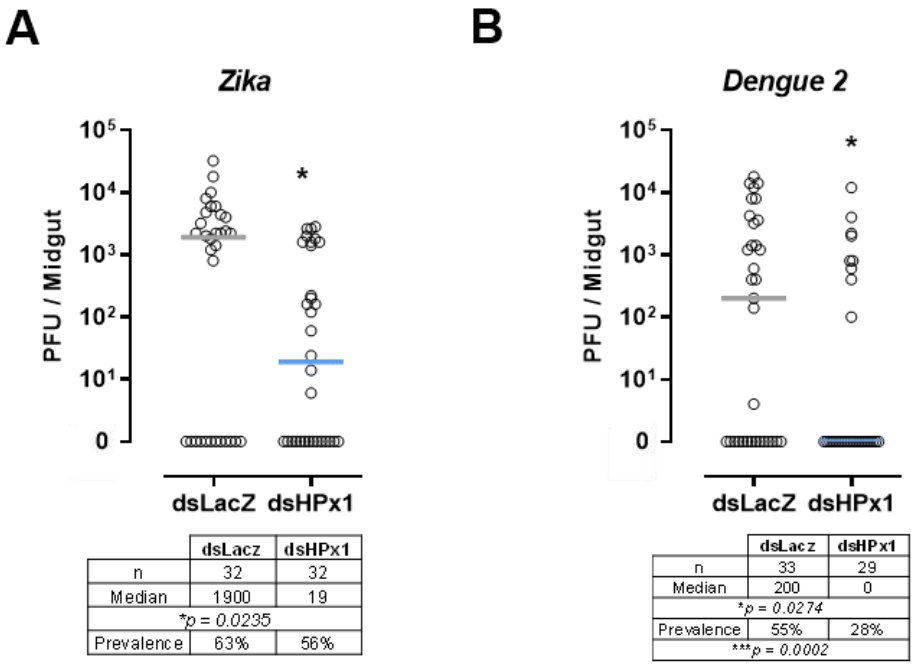


Figure 4. DUOX activation upon HPx1 silencing and hierarchical mode of activation of innate immunity in the mosquito midgut. A) Representative images of ROS levels measured by DHE oxidation in individual midguts at 18 h ABM. B) Quantitative analysis of the fluorescence intensity of oxidized DHE (dsLacZ, n=38; dsHPx1, n=43; dsDUOX, n=21; dsHPx1+DUOX, n=31). C) The intestinal microbiota load analyzed through eubacterial ribosomal 16S gene expression by qPCR at 24 h ABM (dsLacZ n=6; dsHPx1 n=6; dsHPx1+DUOX n=4). D-F) Immune-related gene expression upon HPx1 silencing at 24 h ABM by qPCR (at least n=5 for each condition). F) Schematic panel of intestinal immune activation showing that the PM integrity mediated by HPx1 activity isolates the gut microbiota, and once this integrity is lost, DUOX and antimicrobial peptides

(AMPs) are activated. **p<0.005, ***p<0.001, ****p<0.0001, ns = not significant. Data are the mean +/- SEM. One-way ANOVA with Tukey's post-test for A', B, and C.

Intestinal homeostasis impacts Zika and Dengue virus infection

The midgut epithelium is the first tissue that a virus must invade to establish a successful infection. By modulating diffusion of immune elicitors, HPx1 is crucial to maintaining gut homeostasis, allowing the proliferation of bacteria from the gut microbiota without triggering an immune response. Investigation of the effect of HPx1 silencing on midgut infection with Zika (ZKV) and Dengue virus (DENV) showed that silencing HPx1 expression dramatically reduced ZKV and DENV midgut infection at 4 and 7 days postfeeding, respectively (Fig. 5A and B). Titers of ZKV and DENV decreased approximately 100-fold in the gut of HPx1-silenced mosquitoes, along with a reduction in infection prevalence.



	dsLacz	dsHPx1
n	32	32
Median	1900	19
*p = 0.0235		
Prevalence	63%	56%

	dsLacz	dsHPx1
n	33	29
Median	200	0
*p = 0.0274		
Prevalence	55%	28%
***p = 0.0002		

Figure 5. Viral infection in HPx1-silenced insects. A) Zika titers in midguts at 4 days postinfection. B) Dengue 2 titers in midguts at 7 days postinfection. Viral titers were assessed by the plaque assay. Each dot represents an individual mosquito gut, and bars indicate the median. * p< 0.05; Mann–Whitney test for A and B. The prevalence statistical analysis was performed by the Chi-square test followed by Fisher's exact test.

Discussion

The *Aedes aegypti* PM is an acellular layer that surrounds the blood bolus throughout the digestion process and limits direct contact of the epithelium with the midgut content and the intestinal microbiota, which undergoes massive proliferation upon blood feeding. In female mosquitoes, PM formation occurs in response to ingestion of a blood meal, following a time course that is finely coordinated with the pace of blood digestion. However, the signaling pathways that trigger PM secretion in adult mosquito females have not been elucidated nor has the impact of this structure on viral infection. Here, we characterize an intestinal secreted peroxidase (HPx1) that functions in PM assembly, contributing to its barrier function and promoting microbiota growth by preventing an antimicrobial response. This PM function has a permissive role for viral replication in the mosquito gut, thus constituting a novel determinant of vector competence. Importantly, dietary heme triggers HPx1 gene expression using the heme-dependent transcription factor E75, allowing synchronization of PM maturation with blood digestion by sensing the free heme released as hemoglobin is digested.

Similar to all blood-feeding organisms, mosquitoes face an oxidative challenge due to large amounts of heme – a pro-oxidant molecule – released by hemoglobin degradation. Therefore, preventing oxidative damage through ROS detoxification is a hallmark of their physiology (Sterkel et al., 2017). HPx1 mediates a novel mechanism to promote redox balance in the *Aedes* midgut through its hydrogen peroxide scavenging activity and by modulating PM barrier function. We also show that HPx1 allows proliferation of the gut microbiota without activating a DUOX-mediated oxidative burst by limiting exposure of gut epithelial cells to microbial immune elicitors. We have previously shown that *Aedes aegypti* catalase (AAEL013407-RB), the main intracellular hydrogen peroxide scavenger, is induced in the blood-fed midgut of females (Oliveira et al., 2017). Here, we demonstrate that HPx1 contributes to the overall peroxide scavenging capacity of the gut in a way that is independent of epithelial intracellular catalase but at comparable activity levels (Fig 1). Although peroxidases are less efficient in decomposing hydrogen peroxide than catalases, it is known that some heme peroxidases also have high catalase activity (Vidossich et al., 2012; Vlasits et al., 2010).

A. gambiae IMPer/HPx15 belongs to anopheline-specific expansion of the heme peroxidase family (Kajla et al., 2016) nested in the same branch of the peroxidase family of *A. aegypti* HPx1 and immune-regulated catalase (IRC; CG8913) of *D. melanogaster* (Ha, Oh, Ryu, et al., 2005; Konstandi et al., 2005; Waterhouse et al., 2007). However, *Drosophila* IRC has a conserved heme peroxidase domain structure (Pfam PF03098) but lacks a catalase domain (Pfam PF00199), despite having a high hydrogen peroxide dismutation activity (Ha, Oh, Ryu, et al., 2005), which is a feature of catalases but uncommon among peroxidases. Interestingly, IMPer/HPx15 of *Anopheles* is also expressed in the female reproductive tract, induced by the ecdysone transferred along with the sperm during insemination, due to ecdysone-responsive elements present in the promoter region (Shaw et al., 2014). Ecdysone-responsive element sequences close to the *Aedes* HPx1 gene have been identified *in silico* (Zhao et al., 2001). Therefore, *Aedes* HPx1, IRC, and AgIMPer are secreted enzymes that modulate interactions of the midgut with commensal and pathogenic bacteria. Nevertheless, their role in the biology of the reproductive organs has not been well established. Although our data reveal that HPx1, IRC, and

IMPer share sequence and functional homology, it is not possible at present to speculate which roles are ancestral and which were acquired secondarily during the evolution of dipterans.

A. gambiae IMPer has been proposed to crosslink external matrix proteins by forming di-tyrosine bridges, reducing the accessibility of microbial elicitors to the intestinal cells (Kumar et al., 2010). In *Drosophila*, however, a transglutaminase enzyme crosslinks PM proteins, also protecting the midgut epithelia from damage (Kuraishi et al., 2011; Shibata et al., 2015). In this study, we show that HPx1 associates with the PM and modulates gut permeability in *A. aegypti* (Fig. 4). When the PM structure was compromised by HPx1 silencing, we observed immediate responses from the epithelium that increased ROS levels, which was attributed mainly to DUOX activation by microbial elicitors. The role of PM in preventing elevated ROS production in the gut epithelium was also observed when the PM was compromised by the chitin inhibitor diflubenzuron administered in a blood meal for *A. aegypti* females (Taracena et al., 2018). A simple hypothesis to explain how elevated ROS might lower virus infection is that they directly attack the virus. However, arbovirus infection of midgut cells is thought to occur early during digestion, several hours before proliferation of microbiota occurs and before the PM is secreted (Franz et al., 2015). Therefore, it is unlikely that extracellular ROS produced in response to bacterial elicitors in HPx1-silenced insects would directly attack virus particles that will be localized intracellularly when these molecules start to increase in the lumen. In general, cellular antiviral mechanisms are most plausibly responsible for hampering viral infection upon HPx1 silencing. Among these possible mechanisms, the elevated extracellular ROS levels derived from DUOX activation are indeed sensed by the gut as a danger signal, evoking a tissue-repairing response the hallmark of which is stem cell proliferation (Fig. 4), a homeostatic response coupled to cell death in response to insult and damage. Interestingly, Taracena et al. proposed that different degrees of resistance to infection among mosquito strains are related to different capacities to promote a rapid increase in stem cell proliferation; hence, faster cell death, followed by cell renewal from stem cell activation, is a process that reduces viral infection (Taracena et al., 2018). One could hypothesize that this mechanism is responsible for the decrease in ZKV and DENV infection promoted by HPx1 silencing.

In mammals, the mucus layer allows commensal bacteria from the gut microbiota to thrive without eliciting microbicidal immune responses by the intestinal mucosa. In other words, the mammalian mucus layer acts as a physical barrier that leads to “immunological ignorance” by preventing a state of constant immune activation and chronic inflammation of the intestine in response to immune elicitors from the normal microbiota (Chassaing et al., 2015; Hooper, 2009; Macpherson et al., 2005). In mosquitos, the PM is secreted when the microbiota peaks in number to approximately 100-1000 times the population found before a blood meal (Oliveira et al., 2011), representing a potential massive immune challenge to this tissue. Hixson et al. suggested that immune tolerance to the indigenous microbiota might be mediated by high expression of caudal and PGRP genes, leading to low expression of antimicrobial peptides in epithelial cells from the posterior gut (Hixson et al., n.d.). Here, we highlight the role of PM-associated HPx1 in limiting exposure of the epithelium to immune elicitors from the expanded microbiota observed postfeeding. Before blood feeding, the PM is absent, and bacteria interact with the epithelium, leading to ROS generation and damage-induced repair (Oliveira et al., 2011; Taracena et al., 2018). When the first line of immediate response to immune elicitors (redox mediated) is further

prevented by simultaneous DUOX silencing, a reaction is activated (antibacterial peptides expression) to limit microbial growth. These data suggest hierarchal immune activation in the *A. aegypti* midgut fundamentally orchestrated by the PM integrity. This is similar to what was reported for *A. gambiae*, whereby bacterial elicitors lead to DUOX-mediated activation of IMPer, which promotes cross-linking of extracellular proteins (Kumar et al., 2010). However, in this report, an epithelial cell mucous layer (and not the PM) was indicated as the site of action of the peroxidase. Regarding the mode of activation of this pathway, the findings shown herein are also fairly similar to those for *Drosophila*, with ROS produced by DUOX being primarily triggered by bacterial pathogens, and the IMD pathway induces antimicrobial peptide production upon activation failure (Buchon et al., 2009; Ha et al., 2009; Hixson et al., n.d.; Ryu et al., 2006).

Traditionally, immunology has focused on how hosts eliminate pathogens while fighting infections, but in the last decade, there has been a growing interest in how hosts endure infection by utilizing disease tolerance, including diminishing both the direct damage caused by the pathogen and the self-inflicted damage due to the host immune reaction directed at the elimination of the pathogen. In this study, we showed that PM-associated HPx1 is pivotal to maintaining gut immune homeostasis, acting as a tolerance mechanism that prevents responses to the microbiota. Additionally, the fact that HPx1/PM disruption causes a drastic reduction in the viral load within the gut epithelia suggests that this mechanism is essential to the tolerogenic status of the gut to viral replication and directly contributes to vector competence.

Together, our results indicate that the *A. aegypti* PM supports midgut homeostasis during blood digestion. Heme derived from blood hemoglobin digestion regulates expression of HPx1, an enzyme that has a central role in the assembly of a fully functional PM, through the heme-sensitive E75 transcription factor. Thus, HPx1 maintains immunological ignorance of the midgut epithelia toward the microbiota, allowing a state of microbiota and viral tolerance and preventing tissue damage.

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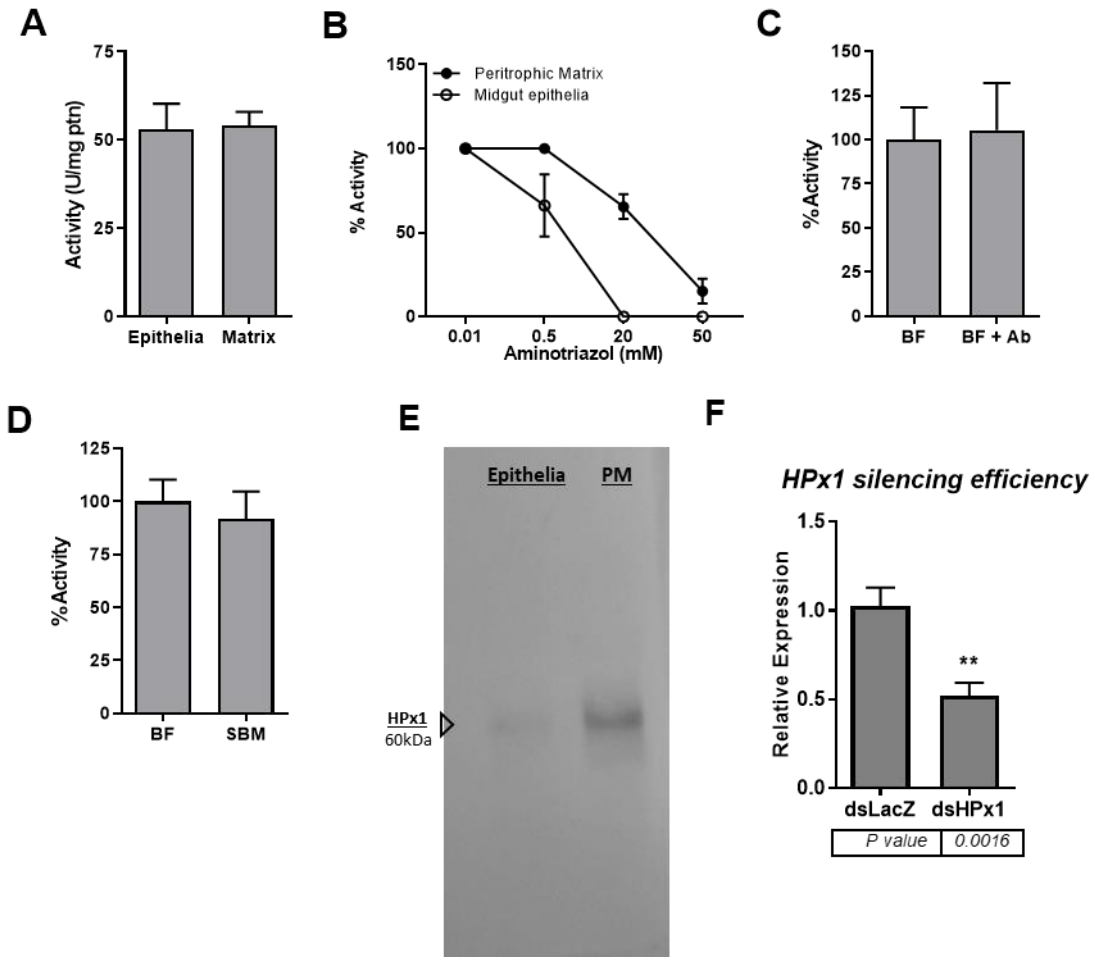
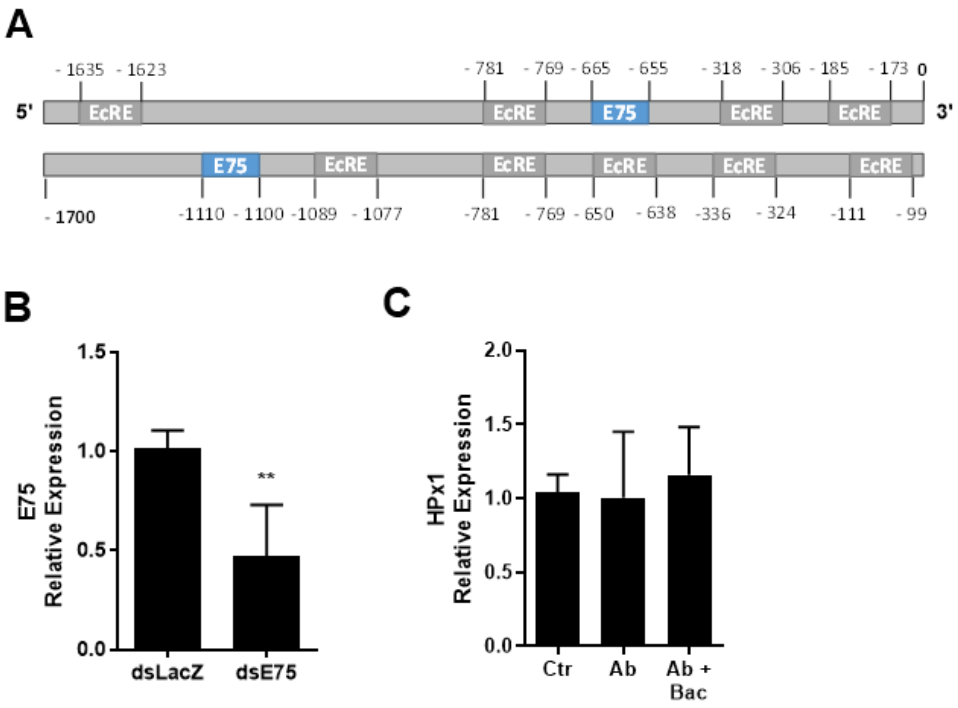
Supplementary figures

Supplementary Figure 1



SUP1: A) Catalase-specific activity comparison of the intestinal epithelia and PM at 24 h ABM. B) *In vitro* sensitivity of midgut epithelia and PM samples to aminotriazole, a catalase/peroxidase inhibitor. C) Mosquitos were treated with (or without) an antibiotic cocktail in a sugar meal, and PM catalase activity was assayed at 24 h ABM (BF n=10; +AB n=11). (D) Catalase activity comparison of the PM at 24 h ABM for mosquitoes fed blood or SBM, a chemically defined artificial diet (BF n=21; +AB n=17). E) HPx1 western blot of midgut epithelia and PM protein extracts, referring to Fig. 1E.

Supplementary Figure 2



SUP2: A) Schematic illustration of the E75 and ecdysone receptor-binding motifs in the promoter region of HPx1. Numbers refer to nucleotide positions relative to the transcription start site. B) E75 silencing efficiency in midguts at 24 h ABM. C) HPx1 expression in midguts at 24 h ABM. Control mosquitoes were fed a regular sucrose solution before blood feeding. Ab mosquitoes were pretreated with antibiotics before blood feeding. Ab + Bac mosquitoes were pretreated with antibiotics and fed blood containing *Enterobacter cloacae at* 1 OD/ml (Ctr n=6; Ab n=6; Ab+Bac n=5). Data are the mean +/- SEM.

Supplementary Table 1

Primer list

RP-49 AAEL003396-RA	For	GCTATGACAAGCTTGCCCCCA
	Rev	TCATCAGCACCTCCAGCT
dsCatalase AAEL013407 –RB	For	taatacgactcactatagggACTCCACTTGCTGTGCGTTT
	Rev	taatacgactcactatagggTCTCCCTTAGCAATAGCGTTG
HPx1 AAEL006014-RA	For	TCCTGTGCATCCTGACTGAG
	Rev	CGTTGTCGCAGAAGATACGA
dsHPx1 AAEL006014-RA	For	taatacgactcactatagggAGATGTTGTACCGACGATGG
	Rev	taatacgactcactatagggATTTGGTGGCCACTCGTATC
16S	For	TCCTACGGGAGGCAGCAGT
	Rev	GGACTACCAGGGTATCTAATCCTGTT
dsE75 AAEL007397	For	taatacgactcactatagggGCTACCCTGTCCGGTCAAT
	Rev	taatacgactcactatagggCTCGGCTTCACCTTTCCTGT
E75 AAEL007397	For	TGTCTGCAGTCGATCGTTTC
	Rev	TGCTGCCGTAGGAGTTCTT
Attacin AAEL003389-RA	For	TTGGCAGGCACGGAATGTCTTG
	Rev	TGTTGTCGGGACCGGGAAGTG
Cecropin G AAEL015515-RA	For	TCACAAAGTTATTTCTCCTGATCG
	Rev	CGATGTAGCATTCGGTGATG
PGRP-LB AAEL010171-RA	For	ATTTAACGTCGTGGGAGCAC
	Rev	TCACTTGGCGATGACCAATA
DUOX AAEL007563-RA	For	GGATTTGTGCCCATCCTATG
	Rev	AACCGTGTAGATCGCTGCTT
dsDUOX AAEL007563-RA	For	taatacgactcactatagggATAATGTGGTCGCCAAGAGG
	Rev	taatacgactcactatagggTGGGACCGAACAGTTTATCC

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Discussão

Apesar de conceitualmente simples, a ideia de que a digestão do sangue, e todo o remodelamento fisiológico que ela implica ao inseto vetor, está diretamente relacionada com a habilidade deste em lidar com infecções parasitarias é muito pouco explorada na literatura, especialmente no contexto de infecções virais (TALYULI *et al.*, 2021). A literatura assume que as partículas virais entram em contato com o epitélio intestinal momentos após a ingestão do sangue infectado (FRANZ *et al.*, 2015), entretanto a replicação viral se dá ao passo que a digestão também progride. Sendo assim, os mecanismos fisiológicos disparados pela ingestão de sangue e os desdobramentos da digestão podem influenciar diretamente a capacidade vetorial e como o epitélio intestinal lida com a infecção viral.

Como apresentado no primeiro capítulo desta tese, a ingestão de grandes volumes de sangue em resposta a pressão seletiva para minimização do número de visitas ao hospedeiro leva os insetos vetores a profundas alterações metabólicas, que suportam a digestão e a absorção e uso dos nutrientes, especialmente no esforço reprodutivo (STERKEL *et al.*, 2017). A hematofagia também impõe a estes insetos adaptações aos potenciais danos oxidativos promovidos pela liberação de altas concentrações de heme e ferro, elementos que propagam espécies reativas levando ao desequilíbrio redox (OLIVEIRA *et al.*, 2011). Tais mecanismos disparados pela digestão incluem a secreção de barreiras físicas e regulação da transcrição gênica que contribuem para a manutenção da homeostasia frente a variação dos fluxos metabólicos decorrentes da ingestão de um enorme volume de nutrientes. Além disso, um traço marcante da biologia destes organismos é o fato de a população microbiana residente na luz do trato digestivo sofrer uma drástica expansão em poucas horas após a alimentação com sangue, com variações de até três ordens de grandeza (OLIVEIRA, JOSE HENRIQUE M *et al.*, 2011). Embora seja frequente na literatura a observação de que existe um aumento da expressão de gene imunes após um repasto sanguíneo (BARLETTA *et al.*, 2020; DONG; BEHURA; FRANZ, 2017; PRICE *et al.*, 2015), a amplitude desta resposta contrasta com a variação observada do tamanho da microbiota, sugerindo a existência de mecanismos de tolerância ou atenuação do desafio imunológico que contribuam para

a aceitação da microbiota (HIXSON *et al.*, 2022; STERKEL *et al.*, 2017). Além disso, fêmeas adultas de insetos vetores, como *Aedes aegypti, Lutzomyia longipalpis, Anopheles aquasalis e Rhodnius prolixus*, apresentam uma maior carga de microbiota intestinal quando comparadas aos machos das respectivas espécies (BARLETTA FERREIRA *et al.*, 2022), dados estes que corroboram o argumento de que fêmeas apresentam um estado imune mais tolerante ao estabelecimento da microbiota. Este complexo panorama fisiológico é o que patógenos humanos encontram no organismo do vetor. Alguns destes eventos favorecem a infecção dos insetos, outros, por sua vez, criam barreiras que precisam ser ultrapassadas, determinando soluções de compromisso construídas no curso de um processo de coevolução de vetores e patógenos que foi essencial para o estabelecimento de relações parasitárias bem sucedidas (TALYULI *et al.*, 2021). Consequentemente, o desbalanço dessa relação, ou seja, a perda desta homeostasia impacta diretamente na infecção e desenvolvimento dos parasitas.

O capítulo II desta tese evidenciou como as alterações metabólicas e as regulações imunes estão intimamente ligadas e coordenadas no epitélio intestinal, após a alimentação, pela atividade da quinase TOR. Apesar do entendimento de que esta quinase seja ativada por aminoácidos, em modelos como mamíferos e leveduras, e que 90% do peso seco do sangue é composto por proteínas, poucas vezes a literatura de artrópodes hematófagos se debruçou no entendimento da importância de TOR para a fisiologia intestinal após o repasto sanguíneo. Menos ainda, na relação desta via com a capacidade vetorial. Incialmente foi discutido que algum sinal nutricional fosse importante para a regulação das tripsinas intestinais após a alimentação e que os aminoácidos poderiam mediar tal sinalização (BARILLAS-MURY; NORIEGA; WELLS, 1995; NORIEGA *et al.*, 1996). Anos depois, foi atribuída a ativação de TOR, o controle da tradução e atividade da tripsina de fase inicial da digestão (BRANDON *et al.*, 2008; GULIA-NUSS *et al.*, 2011). Foi demonstrado também que a presença de aminoácidos e ativação de TOR é importante para a regulação do equilíbrio redox intestinal frente a ingestão de sangue no barbeiro *Rhodnius prolixus* (GANDARA *et al.*, 2016). Nesta tese, observamos que a inibição de TOR promoveu um efeito altamente pleiotrópico, regulando múltiplas enzimas-chave da digestão de carboidratos e lipídios, além de

impactar diretamente a digestão como já apontado anteriormente pela literatura. Nossas observações foram feitas exclusivamente no epitélio intestinal em um momento no qual ocorre o pico da digestão do sangue. Entretanto, sabemos que há uma coordenação metabólica entre o intestino e diversos outros tecidos do mosquito, como o corpo gorduroso (local de armazenamento de lipídeos e síntese de vitelo) e o músculo de voo. A função de TOR no corpo gorduroso direcionando a síntese de vitelo é algo bastante estudado em mosquitos, mas a integração metabólica entre os tecidos ainda não foi explorada na literatura (HANSEN *et al.*, 2004, 2005; PARK *et al.*, 2006). A alimentação induz uma cascata de sinalização celular promovida pelo hormônio ecdisona (HANSEN *et al.*, 2014). A ação sinérgica de TOR e ecdisona já foi observada relacionada à síntese de vitelogenina, entretanto esta correlação não foi feita no epitélio intestinal, nem tampouco um estudo mais aprofundado de quais fatores de transcrição responsivos a ecdisona também estariam sendo ativados por TOR. A coordenação entre TOR e a atividade hormonal promovida pelos peptídeos similares a insulina (*insulin-like peptides,* ILPs) já foi brevemente reportada (GULIA-NUSS *et al.*, 2011), mas nada se sabe desta rede de sinalização no contexto da infecção viral.

Nossos dados concordam com o conceito de que a droga rapamicina (via inibição de TOR) possui um efeito imunossupressor, tal como descrito de forma bastante consistente em modelos vertebrados. Em mamíferos, sabe-se que a atividade de TOR está direcionada com a polarização da resposta inflamatória, bem como com a diferenciação e ativação de células imunes (DUMONT; SU, 1995; O'NEILL; KISHTON; RATHMELL, 2016; POWELL *et al.*, 2012). No entanto, observações recentes em *Anopheles stephensi* demonstraram um papel imunossupressor mediado pela via de TOR, onde o uso de rapamicina potencializou as respostas imunes anti-*Plasmodium berghei* mediadas pela via de IMD e pelo sistema análogo ao complemento (FENG *et al.*, 2021). Diferentemente, no nosso estudo o uso de rapamicina levou a uma regulação negativa pronunciada dos peptídeos antimicrobianos e de proteínas que reconhecem os padrões associados a patógenos, sugerindo um efeito de imunossupressão e consequente permissividade a patógenos tais como vírus e bactérias. Isso nos permite explorar mais a fundo os conceitos de imunometabolismo, ou seja, de como as alterações metabólicas (e suas respectivas vias celulares de sinalização) são capazes

de sustentar a resposta imunológica a nível celular e tecidual (MAN; KUTYAVIN; CHAWLA, 2017; SAMADDAR *et al.*, 2020) em diferentes insetos vetores.

O tratamento dos insetos com rapamicina reduziu a expressão de genes imunes, como demonstrado no Capítulo II, porém ainda não fomos capazes de entender molecularmente em que momento essa regulação acontece. A ideia de que a ativação de TOR seja necessária para a síntese de um possível mensageiro que ative a imunidade intestinal não foi explorada nesta tese. Uma hipótese é que o monitoramento nutricional feito por TOR pode inativar fatores de transcrição repressores da imunidade, que por sua vez, ainda estariam atuantes no epitélio intestinal quando a rapamicina seja administrada aos insetos. Ainda que de forma especulativa, um candidato a esta função seria o fator de transcrição responsivo a AMP cíclico (CREB). CREB é um repressor da vitelogênese no corpo gorduroso de mosquitos que não se alimentaram de sangue (DITTMER *et al.*, 2003). Apesar dos autores não discutirem este ponto, pode ser que haja um *looping* de regulação entre a ativação de TOR e a repressão de CREB, para a consequente síntese de proteínas de vitelo. Ou ainda que haja mecanismos de *feedback* negativo entre os sinais fisiológicos que mantêm CREB ativo pré-alimentação e a condição fisiológica observada durante a digestão do sangue. Além disso, CREB pode estar relacionado a um panorama de imunossupressão em mamíferos, uma vez que compete com NF-kB pela proteína co-ativadora p300, essencial para a atividade de ambos fatores de transcrição (WEN; SAKAMOTO; MILLER, 2010). Interessantemente, a proteína p300 (também chamada de *CREB-binding protein*, CBP) foi alvo de estudo por Amarante e colaboradores (ver anexo 10 desta tese) no contexto de infecção de mosquitos por vírus Zika. Os dados mostram que o silenciamento de p300/CBP leva a uma redução da expressão de genes imunes e aumento da viremia no mosquito, confirmando o papel imune deste fator também em mosquitos. Experimentos adicionais que aderecem esta hipótese ainda precisam ser realizados.

Outra possível explicação para o efeito na imunidade intestinal mediado por TOR, seria que esta quinase regule algum fator de transcrição da família GATA. Já foi demonstrado que um dos fatores GATA é alvo da fosforilação de TOR no corpo gorduroso de *Aedes aegypti,* favorecendo assim a vitelogênese (ATTARDO *et al.*, 2003; PARK *et al.*, 2006). Tanto em drosófila quanto em *Anopheles aquasalis*, membros desta

família já foram descritos com funções imunes (BAHIA *et al.*, 2018; SENGER; HARRIS; LEVINE, 2006; TINGVALL; ROOS; ENGSTRÖM, 2001), entretanto não se conhece esta função em *Aedes aegypti*.

A diferenciação e atividade de células pro-inflamatórias como macrófagos M1 e linfócitos TH1 e TH17 parece ser dependente da ativação de TOR, seja pela regulação metabólica, seja pela interação com fatores de transcrição como STAT (BYLES *et al.*, 2013; DELGOFFE *et al.*, 2011; WEICHHART; HENGSTSCHLÄGER; LINKE, 2015). Além disso, sabe-se que a ativação desta quinase nas células imunes também está relacionada no controle da expressão de genes dependentes de NF-kB. Deste modo, torna-se importante compreender o papel desta via de sinalização na ativação e proliferação de hemócitos, agentes da imunidade celular nos insetos. Sabe-se que a alimentação com sangue é capaz de induzir a proliferação dos hemócitos de *Aedes aegypti* e *Anopheles gambiae* (CASTILLO, J.C.; ROBERTSON; STRAND, 2006), e ao menos em *Aedes aegypti,* num mecanismo aparentemente dependente da ativação de membros da famílias de ILPs (CASTILLO, JULIO; BROWN; STRAND, 2011). ILPs e TOR são sinérgicos na sinalização para a síntese de vitelogenina no corpo gorduroso e na digestão do sangue no intestino de *Aedes aegypti* (GULIA-NUSS *et al.*, 2011; ROY; HANSEN; RAIKHEL, 2007). Sendo assim, a hipótese de que TOR também estaria atuando junto com este hormônio na proliferação de hemócitos e em suas atividades imunológicas é bastante promissora. O estudo dos hemócitos em *Aedes aegypti* por si já é um tópico bastante escasso na literatura, sendo a correlação destas populações celulares com TOR e a infecção viral totalmente desconhecida.

TOR por usa vez também exerceu impacto importante no curso da infecção intestinal dos mosquitos por Zika e Chikungunya. A supressão das vias de imunidade inata causada pela rapamicina é o caminho mais obvio de explicação para o aumento da intensidade e prevalência das infecções. No entanto, podemos ainda discutir o fato de que os vírus manipulam diferencialmente a maquinaria de tradução proteica celular, e essa regulação está claramente conectada com a atividade de TOR (ROTH *et al.*, 2017; WALSH; MATHEWS; MOHR, 2013). Apesar de nossos resutlados apontarem para regulação da transcrição, a função de TOR durante a infecção viral em insetos

possivelmente possa ser explicada também pela modulação das vias de tradução proteica.

Outras frentes que podem representar novas relações de TOR com a competência vetorial seria existência de possíveis modulações que os arbovírus possam exercer nos mecanismos de tradução proteica em mosquitos. Contudo, infelizmente, a literatura de insetos vetores negligencia esta área do conhecimento. Para citarmos alguns exemplos, a maneira como o inseto lida com 1) o reconhecimento de estruturas genômicas virais como IRES (*Internal Ribosome Entry Site*), 2) os mecanismos alternativos a TOR de tradução cap-dependente via ativação da quinase MNK, 3) a regulação da fosforilação de eIF2-alfa no controle do fluxo de tradução e geração de grânulos de estresse impactando a replicação viral.

Outra vertente interessante da competência vetorial, é a capacidade do tecido infectado se remodelar a partir das injúrias que sofre, uma preocupação central da proposta do papel da tolerância a patógenos. A atividade mitótica de células tronco residentes do intestino parecem ser um marco importante do curso da infecção viral, além de apresentar diferentes perfil de regulação em linhagens de mosquitos com diferentes graus de susceptibilidade a arbovírus (Taracena et al., 2017). A função de TOR exercida no controle do ciclo celular é algo bastante discutido em drosófila, mamíferos e leveduras (SCHMELZLE; HALL, 2000; ZHANG *et al.*, 2000), entretanto, não sabemos como esta via pode regular a proliferação e diferenciação de células progenitoras intestinais durante a infecção viral. Mecanisticamente, processo de endoreplicacao do material genético em células poliploides intestinais vem sendo apontado como outro mecanismo importante regulado pela infecção viral em *Aedes aegypti* (SERRATO-SALAS *et al.*, 2018). Inibição de vias celulares que afetem a progressão do ciclo celular são tidas como reguladoras da endoreplicacao (ZIELKE; EDGAR; DEPAMPHILIS, 2013) e o papel de TOR neste contexto em mosquitos ainda não foi explorado.

Nossas discussões foram construídas a partir de fenótipos observados após a administração da rapamicina junto com o sangue. Essa droga está fortemente associada a inibição do complexo 1 de TOR (TORC1) quando administrada de maneira aguda, como foi feito nos experimentos desta tese. Apesar disto, experimentos adicionais são

necessários para se entender diferencialmente as funções fisiológicas dos complexos 1 e 2 de TOR no mosquito *Aedes aegypti*, bem como os impactos na competência vetorial. O silenciamento gênico de componentes específicos de cada complexo como Raptor e Rictor, podem nos ajudar a entender melhor tais funções individuais até então desconhecidas na literatura. Entretanto, nossos resultados indicam para uma direção a se seguir, com diversas novas possibilidades a se explorar da função de TOR na fisiologia dos insetos vetores.

Conhecida desde 1762, a matriz peritrófica é classicamente descrita como uma barreira para que o bolo alimentar não entrasse em contato diretamente com o epitélio intestinal, limitando a exposição do tecido a agentes abrasivos ou componentes químicos agressivos como algumas enzimas digestivas ou o próprio pH (LEHANE, 1997). Apesar de conhecido o fato de que o epitélio intestinal dos mosquitos secreta a matriz frente ao repasto sanguíneo, os mecanismos moleculares responsáveis pela regulação da formação da matriz eram desconhecidos. Os resultados do segundo capítulo revelaram um papel essencial da via de sinalização de TOR na síntese da matriz, ao mesmo tempo em que o terceiro capítulo mostra um papel para o heme da dieta através da regulação da peroxidase HPx1 na manutenção da estrutura e da função de barreira da PM, revelando uma complexa rede de sinalização envolvida na formação da PM. Diversas enzimas que contribuem para a formação da matriz peritrófica estão diretamente relacionadas com a atividade de TOR e outros sinais fisiológicos como a presença de heme e a microbiota não são necessários para a ativação transcricional destas enzimas. A proposta de que a função de TOR na formação da matriz se dê pelo fator de transcrição STAT, mostra uma outra via de interação entre vias de sinalização imunes com a sinalização nutricional promovida pela digestão.

Durante certo tampo, discutiu-se na literatura também que a barreira física da matriz peritrófica seria importante na contenção de parasitas, como o *Plasmodium*, ao limitar a invasão do epitélio intestinal de mosquitos. Alguns experimentos, no entanto, foram realizados na tentativa de desfazer essa barreira, sob a premissa de que isso levaria ao aumento da infecção dos mosquitos. Curiosamente, o que acontecia era o contrário. Mosquitos com a matriz peritrófica comprometida possuíam menos oocistos

em desenvolvimento, ou não apresentavam diferenças significativas (BAIA-DA-SILVA *et al.*, 2018; BILLINGSLEY; RUDIN, 1992; SHAHABUDDIN *et al.*, 1993). Entretanto, em 2010, foi proposto que o epitélio intestinal é isolado da microbiota proliferativa após a alimentação por uma camada de mucinas (provavelmente associada a matriz peritrófica) altamente interligadas. A construção desta barreira era mediada por uma heme peroxidase (IMPER/HPx15) que, quando ausente, expunha o epitélio as bactérias e isso promovia uma ativação imune no tecido que determinava uma redução pronunciada da infecção pelo plasmódio (KUMAR et al., 2010).

Aqui estudamos a função de barreira imune da PM em *Aedes aegypti*. Nossos dados revelam que ela também atua isolando a microbiota intestinal, num mecanismo dependente de uma peroxidase (HPx1) filogeneticamente próxima a HPx15 de anofelinos e a uma peroxidase intestinal de Drosophila, também implicada na modulação da relação com a microbiota (embora nesse caso, a sua ação tenha sido atribuída a capacidade de detoxificação de peróxido de hidrogênio) (HA *et al.*, 2005). Adicionalmente, observamos que a HPx1 é regulada transcricionalmente por um fator de transcrição dependente de heme e de ecdisona, o E75. O papel sinalizador do heme durante a ingestão de sangue pelo *Aedes* já havia sido abordado antes em um trabalho do grupo (OLIVEIRA, JOSE HENRIQUE M *et al.*, 2011), onde foi mostrado que essa molécula determinava a redução da produção de espécies reativas de oxigênio pelo epitélio através da inibição de uma DUOX mediada por proteína quinase do tipo C. A indução da expressão de HPx1 é independente de PKC (dado não mostrado), e a ação aqui descrita, mediada pelo E75 revela o caráter sinalizador altamente pleiotrópico do heme da dieta sobre a fisiologia do inseto. De outro lado, o disparo da produção de ecdisona pela alimentação através do eixo de sinalização neuroendócrino é conhecido como um aspecto central da fisiologia da hematofagia, ao acoplar ingestão de sangue e reprodução (BROWN *et al.*, 1998). O mecanismo aqui proposto descortina um *cross-talk* entre a via de sinalização de OEH/ecdisona e o metabolismo de heme do epitélio intestinal com prováveis impactos sistêmicos que merecem ser explorados em estudos futuros. O fator de transcrição E75, juntamente com heme, também já foi demonstrado em *Aedes aegypti* como importante regulador da vitelogênese no corpo gorduroso (CRUZ *et al.*, 2012). Nesta tese demonstramos a importância dele no intestino médio

para a síntese da matriz peritrófica. Entretanto não conseguimos descrever ainda a relação entre a sinalização de ecdisona via heme e E75 com as regulações promovidas pela ativação de TOR.

Esses resultados revelam o mecanismo molecular que controla a formação desta barreira e mostram que a sua ruptura compromete a homeostasia do epitélio intestinal. A homeostasia promovida pela compartimentalização da microbiota é alcançada pela "ignorância imunológica" do tecido intestinal orquestrada pela matriz. De forma relevante, havia um consenso tácito na área de que a PM não seria relevante para a infecção por arbovírus, uma vez que esta aconteceria nas primeiras horas após a infecção, portanto antes da formação da matriz, que era entendida como uma barreira meramente física a infecção (FRANZ *et al.*, 2015). Os nossos resultados mostram um papel regulatório da PM sobre a fisiologia do tecido digestivo e do organismo como um todo, que desta forma, também é crucial para o estabelecimento de infecções virais no intestino do mosquito. A relação entre a PM e a infecção viral também era desconhecida na literatura e os resultados apresentados no terceiro capítulo desta tese apontam nesta direção.

Outro ponto relevante é o estado tolerogênico que o intestino médio apresenta tanto permitindo a proliferação da microbiota, quanto de certa forma, a infecção viral, como revisado anteriormente (OLIVEIRA, JOSÉ HENRIQUE; BAHIA; VALE, 2020). Nossos resultados apontam para um papel primordial da matriz peritrófica na manutenção deste estado. Outro fato curioso é o de que a porção anterior do intestino médio, onde teoricamente não há a formação da matriz peritrófica, apresenta uma população microbiana reduzida por alta ativação imune. Ao passo que a porção posterior, a qual há a secreção da matriz, e há alta proliferação microbiana, não se observa a mesma amplitude de resposta imune, estando vários genes de resposta tolerogênica sendo regulados (HIXSON *et al.*, 2022). O entendimento da tolerância imunológica ainda é bastante superficial em insetos vetores, mas a matriz peritrófica se apresenta como um componente central deste perfil imune.

Sendo assim, o conjunto dos nossos resultados reforçam o conceito de imunometabolismo, apontando para a necessidade de uma visão abrangente e

unificadora sobre a fisiologia e a imunologia, como um caminho promissor para compreendermos as relações parasitárias.

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Anexos

Artigos publicados durante o doutorado, em colaboração com grupos internos e externos a UFRJ.

1. Angleró-Rodríguez et al., 2017. An aedes aegypti-associated fungus increases susceptibility to dengue virus by modulating gut trypsin activity. *Elife*. 10.7554/eLife.28844

2. Taracena et al., 2018. Regulation of midgut cell proliferation impacts Aedes aegypti susceptibility to dengue virus. *PLoS Negl. Trop. Dis.* 10.1371/journal.pntd.0006498

3. Bottino-Rojas et al., 2018. The redox-sensing gene Nrf2 affects intestinal homeostasis, insecticide resistance, and Zika virus susceptibility in the mosquito Aedes aegypti. *J. Biol. Chem.* 10.1074/jbc.RA117.001589

4. Padilha et al., 2018. Zika infection decreases Aedes aegypti locomotor activity but does not influence egg production or viability. *Mem. Inst. Oswaldo Cruz*. 10.1590/0074-02760180290

5. Bottino-Rojas et al., 2019. Non-canonical transcriptional regulation of heme oxygenase in Aedes aegypti. *Scientific Reports*, *9*(1), 1–12. 10.1038/s41598-019-49396-3

6. Padilha, K. P et al., 2020. Chikungunya infection modulates the locomotor/flight activity of Aedes aegypti. *Sleep Science*. https://doi.org/10.5935/1984-0063.20200018

7. Vasconcellos et al., 2019. Natural infection by the protozoan Leptomonas wallacei impacts the morphology, physiology, reproduction, and lifespan of the insect Oncopeltus fasciatus. *Scientific Reports*, *9*(1), 17468. 10.1038/s41598-019-53678-1

8. Resck et al., 2020. Unlike Zika, Chikungunya virus interferes in the viability of Aedes aegypti eggs, regardless of females' age. *Sci Rep*. doi: 10.1038/s41598-020-70367-6.

9. Barletta et al., 2020. Prostaglandins regulate humoral immune responses in Aedes aegypti. *PLoS Negl Trop Dis*. doi: 10.1371/journal.pntd.0008706.

10. Amarante et al., 2021. Zika virus infection drives epigenetic modulation of immunity by the histone acetyltransferase CBP of Aedes aegypti. Aceito para publicacao Plos Neglected Tropical Diseases.

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RESEARCH ARTICLE

An *Aedes aegypti*-associated fungus increases susceptibility to dengue virus by modulating gut trypsin activity

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Abstract Transmission of dengue virus (DENV) requires successful completion of the infection cycle in the *Aedes aegypti* vector, which is initiated in the midgut tissue after ingestion of an infectious blood meal. While certain *Ae. aegypti* midgut-associated bacteria influence virus infection, little is known about the midgut-associated fungi (mycobiota), and how its members might influence susceptibility to DENV infection. We show that a *Talaromyces* (*Tsp_PR*) fungus, isolated from field-caught *Ae. aegypti,* render the mosquito more permissive to DENV infection. This modulation is attributed to a profound down-regulation of digestive enzyme genes and trypsin activity, upon exposure to *Tsp_PR*-secreted factors. In conclusion, we show for the first time that a natural mosquito gut-associated fungus can alter *Ae. aegypti* physiology in a way that facilitates pathogen infection.

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Introduction

Arthropod-borne viral diseases have an enormous global public health impact. While dengue virus (DENV) remains the most important arbovirus worldwide, the recent emergence of Chikungunya and Zika virus have exacerbated the impact of *Aedes*-transmitted diseases. The mosquito *Aedes aegypti* is the principal vector of DENV. Successful transmission of the virus requires completion of its infection cycle in the mosquito, beginning in the midgut tissue and eventually ending up in the saliva, through which it can be introduced into a new host.

The mosquito acquires the virus through a blood meal from an infected vertebrate host. The blood is digested in the insect's midgut through the enzymatic action of a variety of proteolytic enzymes and provides the necessary nutrients for egg production (***Noriega and Wells, 1999***). The proteolytic activity of the midgut impairs DENV infection, and specific trypsins are associated with this modulation of susceptibility (***Brackney et al., 2008***; ***Molina-Cruz et al., 2005***). In the midgut lumen, the virus also encounters a variety of microorganism, which constitutes the vector midgut microbiota. Studies have shown that some bacterial species can inhibit DENV infection and other human pathogens (***Bahia et al., 2014***; ***Dennison et al., 2014***; ***Ramirez et al., 2012***, ***2014***). However, the midgut microbiota also comprises fungi, but little is yet known about the mosquito's mycobiome (***Angleró-Rodríguez et al., 2016***; ***da Costa and de Oliveira, 1998***; ***da S Pereira et al., 2009***). Most studies have focused on entomopathogenic fungi for use in mosquito control (***Dong et al., 2012***; ***Scholte et al., 2007***). Fungi are ubiquitous in the environment, especially in tropical regions, and studies have shown the association of filamentous fungi, including *Talaromyces* species with various vectors of human pathogens (***Akhoundi et al., 2012***; ***da Costa and de Oliveira, 1998***; ***da S Pereira et al., 2009***; ***Jaber et al., 2016***; ***Marti et al., 2007***).

In the present study, we have identified a *Talaromyces* species fungus from Puerto Rico (*Tsp_PR*) from the midgut of field-caught *Ae. aegypti* that renders the mosquitoes more susceptible to DENV infection, through transcriptional and enzymatic inhibition of trypsin enzymes.

Results

An *Ae. aegypti* gut-associated *Tsp_PR* fungus augments DENV infection of the mosquito midgut

Adult female *Aedes sp.* mosquitoes were collected in the dengue endemic Maunabo region of southeastern Puerto Rico. After surface sterilization of mosquitoes, the midguts were dissected and plated on agar for fungal growth. Among the isolated fungi, we identified a *Talaromyces* species fungus and characterized at the microscopic (***Figure 1***) and molecular levels. Sequence analysis using the rRNA internal transcribed spacer (ITS) showed a 100% similarity to the fungus *Talaromyces* species and 99% *Penicillium* species. *Talaromyces* is often classified as a *Penicillium* species classification, since in 2011, species in the *Penicillium* subgenus *Biverticillium* were reclassified as *Talaromyces* (***Pitt, 2014***). To address the ability of *Tsp_PR* to colonize the mosquito midgut, we fed mosquitoes on fungus spore-laced sugar solution for 2 days, and then monitored the total fungus colony-forming units (CFUs) in the whole mosquito and midguts for 25 days (***Figure 2A,B***, ***Figure 2—source data 1***). *Tsp_PR* was detected in the whole fungus-exposed mosquitoes and midguts during the entire time course. The assay also identified a small number of fungi in the control mosquito cohort but not *Tsp_PR*. We also investigated whether the presence of the fungus in the mosquito gut had any effect on mosquito longevity. Introduction of the live *Tsp_PR* spores through sugar feeding for 2 days did not affect mosquito survival up to 38 days (p=0.3073) (***Figure 2C***, ***Figure 2—source data 1***).

To investigate whether the presence of *Tsp_PR* in the mosquito midgut can modulate susceptibility to DENV infection, we introduced whole fungus spores to *Ae. aegypti* mosquitoes via feeding on a spore-containing sugar solution prior to a DENV-infected blood meal (***Figure 2C,D***, ***Figure 2—source data 1***). Two mosquito strains were used: the DENV-susceptible Rockefeller strain and the partly resistant Orlando strain (***Sim et al., 2013***). Seven days after DENV infection, midguts were dissected, and DENV titers were enumerated. Spore ingestion by Rockefeller and Orlando strains females resulted in a significant (p<0.001) increased DENV infection. Upon spore ingestion, both mosquito strains showed an increased DENV infection prevalence, as a measure of the proportion of mosquitoes that were infected, however it was only significantly higher in the Orlando strain. Further studies were performed in the Orlando strain.

Talaromyces sp. and related fungi are known to produce a variety of secondary metabolites and proteins (***Bara et al., 2013***; ***Klitgaard et al., 2014***). It was unclear whether the influence of *Tsp_PR* on *Ae. aegypti's* susceptibility to DENV required the presence of whole live fungi or was mediated by a secreted factor. To distinguish between these two possibilities, we prepared a sucrose suspension of the *Tsp_PR* secretome containing a filtered fungus culture without spores and mycelia. Mosquitoes were fed on this suspension prior to the ingestion of DENV-infected blood (***Figure 3A***, ***Figure 3—source data 1***). In a separate experiment, mosquitoes were fed on a sugar solution containing a heat-treated fungus secretome preparation prior to ingestion of DENV-infected blood, in order to address the nature of the factor(s) that influence DENV infection (***Figure 3B***, ***Figure 3—source data 1***). Mosquitoes that had ingested the fungus secretome-containing solution showed a significantly increased virus infection (p=0.002). In contrast, the heat-treated solution did not enhance DENV infection of the mosquito midgut (p=0.548). Taken together, these findings indicate that *Tsp_PR* secretes one or more heat-sensitive molecules that enhance DENV infection in *Ae. aegypti* midguts.

Mosquito are exposed to a variety of fungi in nature. In order to test whether the *Tsp_PR*-mediated increase of DENV infection is fungus-specific, we performed fungus-exposure and DENV infection assays with a *Penicillium chrysogenum* that had also been isolated from field-caught mosquitoes. Feeding mosquitoes on whole *P. chrysogenum* conidia, or culture filtrates, did not affect DENV infection (p=0.829, p=0.867, respectively) (***Figure 4A,B***, ***Figure 4—source data 1***). We have previously associated *P. chrysogenum* with an enhancement of *Plasmodium* infection in *Anopheles gambiae* (***Angleró-Rodríguez et al., 2016***). To test whether *Tsp_PR* could influence malaria parasite infection in its vector we provided *Tsp_PR* to *An. gambiae* mosquitoes prior to infection with

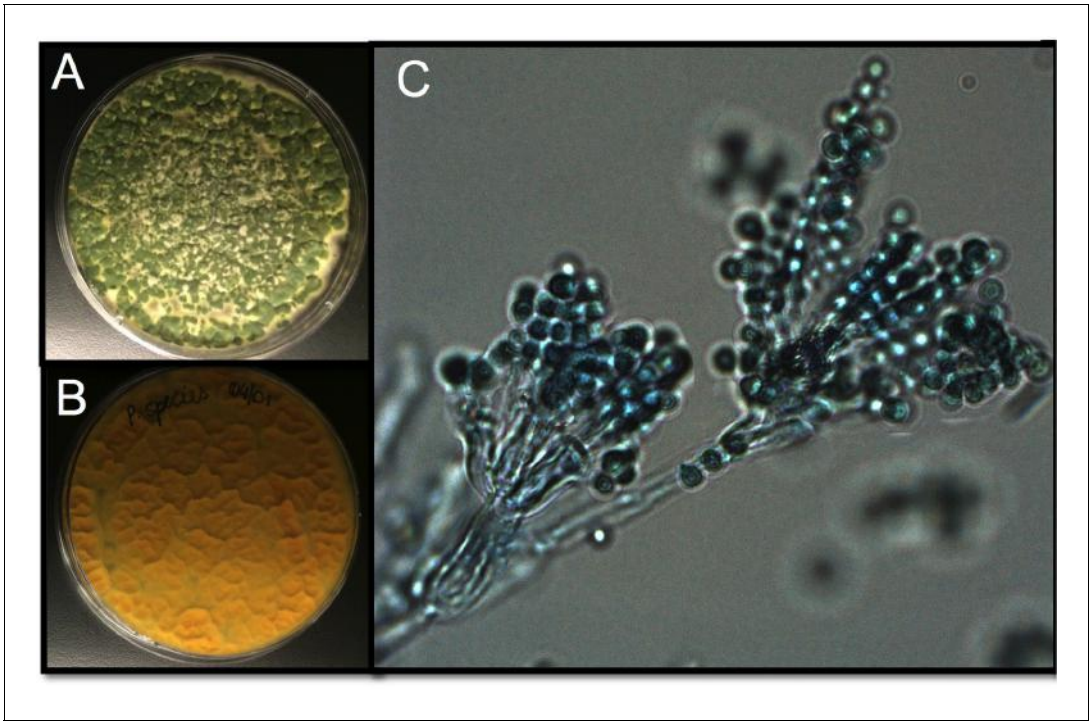

Figure 1. *Tsp_PR* morphology. After isolation, the fungus was grown on Sabouraud agar and characterized macroscopically and microscopically. (**A**) Top and (**B**) bottom view of the fungus on Sabouraud agar. (**C**) Microscopic view of the typical brush-like biverticillated conidiophore of *Talaromyces sp.* fungi.

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Plasmodium falciparum, and this resulted in increases parasite oocysts numbers on the mosquito gut (p=0.015) (***Figure 4C***, ***Figure 4—source data 1***). These results show that *Tsp_PR* can enhance infection of different human pathogens in different mosquito vectors.

Tsp_PR-secreted factor(s) do not affect the mosquito midgut microbiota or DENV infection of aseptic mosquitoes

Some species of *Tsp_PR* and the closely related *Penicillium* are known to produce bioactive compounds with anti-bacterial activity (***Bara et al., 2013***; ***Klitgaard et al., 2014***). To investigate whether *Tsp_PR* produces antibacterial factors, we performed a bacterial growth inhibition assay by a disc diffusion antibiotic sensitivity test (***Figure 5A***). We examined possible *Tsp_PR*-mediated growth inhibition of the following bacteria isolated from field-caught mosquito midguts (***Ramirez et al., 2012***): the three Gram-negative bacteria *Serratia marcescens, Chromobacterium haemolyticum*, and *Enterobacter hormaechei*; and the three Gram-positive bacteria *Bacillus subtilis*, *Staphylococcus capprae*, and *Lactococcus lactis*. The disk soaked in the *Tsp_PR* secretome solution did not show any growth inhibitory activity for any of the six tested bacteria, when compared to an antibiotic control.

To investigate whether *Tsp_PR*-secreted molecules can influence the mosquito midgut bacterial load, we quantified the total cultivable bacteria of the midguts of fungus secretome-exposed and non-exposed mosquitoes using CFU assays (***Figure 5B***, ***Figure 5—source data 1***). Exposure to the *Tsp_PR* secretome did not affect the total bacterial midgut load (p=0.147). Previous studies showed that reduction or elimination of the mosquito midgut microbiota, through antibiotic-treatment, (aseptic mosquitoes) significantly increases susceptibility to DENV in *Ae. aegypti* (***Xi et al., 2008***). Next, we tested whether the fungus-secretome would influence DENV infection of aseptic mosquitoes (***Figure 5C***, ***Figure 5—source data 1***). *Tsp_PR* secretome-fed aseptic mosquitoes did not differ from the aseptic control cohort with regards to DENV infection intensity (p=0.867), while it showed a slightly higher but non-significant, infection prevalence. These data suggest that a bacteria-related factor may in some way influence the fungus-mediated effect on DENV infection.

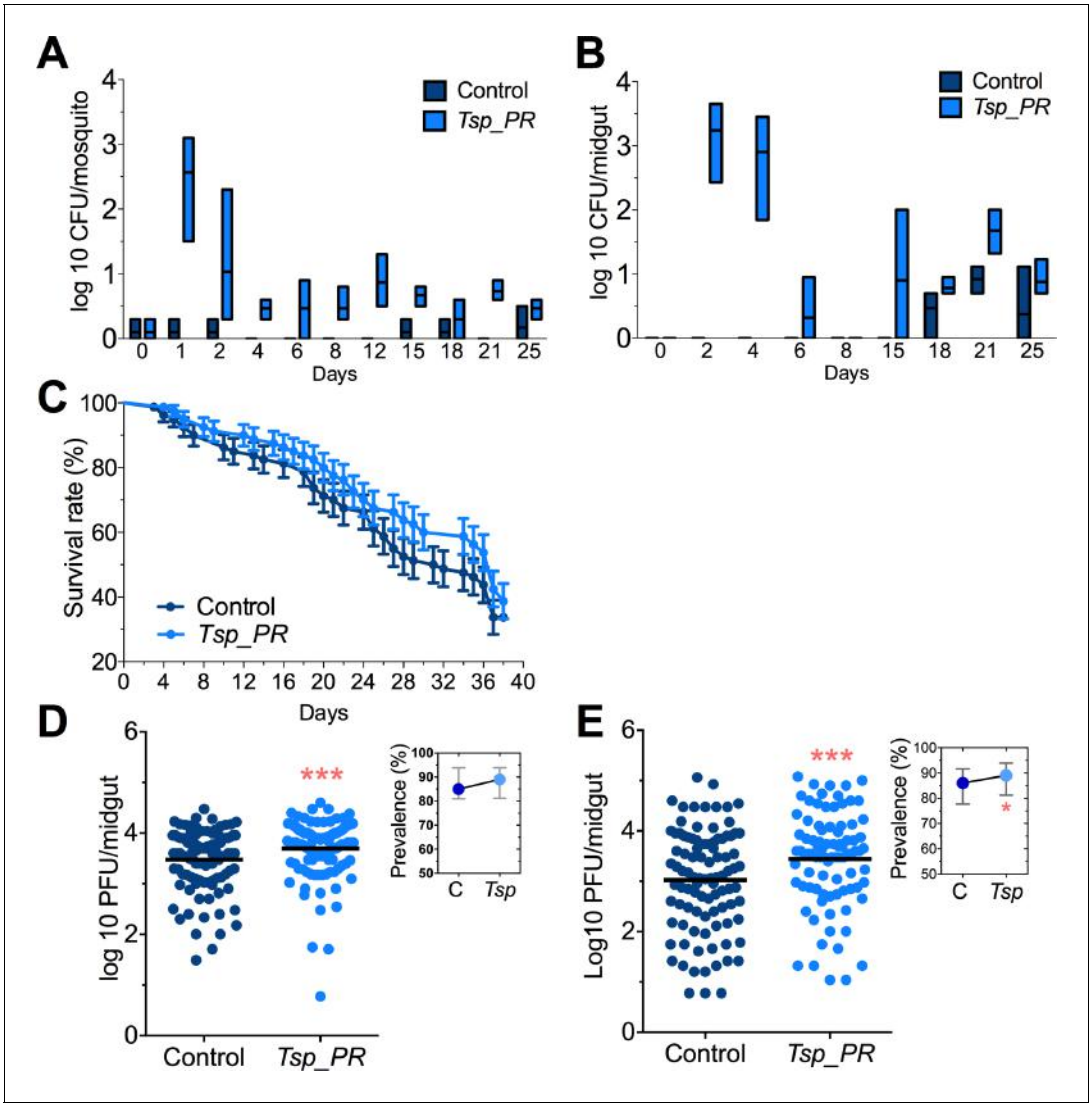

Figure 2. *Tsp_PR* fungus significantly increases DENV infection in *Aedes* mosquito midguts. *Aedes* mosquitoes were mock-fed or fed for 48 hr with 10% sucrose solution containing 1×10^9 *Tsp_PR* spores. After spore feeding, (**A**) Fungus colonization in whole mosquitoes or (**B**) midguts. The presence of *Tsp_PR* in the mosquito was monitored for 25 days after introduction by enumerating fungal CFUs on Sabouraud agar with antibiotics cocktail from three independent experiments, the line indicates the mean and bars the maximum and minimum ranges. (**C**) Survival assays. Female mosquitoes fed with *Tsp_PR* spores or unfed were monitored in a daily basis for 38 days in three independent experiments (N = 80, p=0.3073). Error bars represent ± SE. (**D**) Rockefeller strain mosquitoes, (Control, N = 123; *Tsp_PR*, N = 120) or (**E**) Orlando strain mosquitoes (Control, N = 113; *Tsp_PR*, N = 99) were infected with a blood meal containing DENV; at 7 days post-infection (dpi), the midguts were dissected. Each dot represents a plaque-forming unit (PFU) transformed to $\log_{10}$ in individual midguts from three independent experiments. The line indicates the mean. Upper right boxes show the prevalence of infected mosquitoes, error bars represent the 95% confidence interval. *p<0.05, ***p<0.001,.

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Tsp_PR-secreted factors stimulate down-regulation of genes encoding blood-digesting enzymes

Next we explored the influence of the *Tsp_PR* secreted factors on the mosquito transcriptome, as a measure of its molecular physiology, in order to provide clues to the mechanism that could be responsible for the influence on DENV infection. A genome-wide microarray-based transcriptome comparison between the midguts of fungus-secretome solution-exposed and non-exposed

mosquitoes reveled regulation (23 up-regulated and 22 down-regulated) of a variety of genes belonging to different functional classes (***Table 1***) (***Figure 6A***). Forty-eight percent (11 genes) displaying increased transcript abundance after exposure to the *Tsp_PR* secretome belonged to the redox class and are putatively involved in various oxidoreductive processes, including detoxification. As many as 82% (9 genes) of this redox class encoded cytochrome P450 proteins, suggesting that the fungus activates the detoxification machinery in the mosquito midgut. A significant proportion (55%, 12 genes) of the 22 down-regulated genes were functionally related to blood digestion, and predominantly proteolysis (41%) (***Table 1***) (***Figure 6B***). Twenty-two percent of the total down-regulated genes encoded trypsins. These results suggest that the *Tsp_PR* secretome causes an impairment of the mosquito's ability to digest the blood meal.

Fungus-secreted molecules impair trypsin activity of the *Ae. aegypti* midgut

Earlier studies have shown that mosquito ovary development is correlated with the amount of digested blood and reabsorption of nutrients for egg production (***Bryant et al., 2010***; ***Lea et al., 1978***). Poor digestion of the blood meal therefore results in decreased ovary development. Hence, ovary development after blood feeding can serve as a proxy assay for blood-digestion efficiency. We performed an assay in which *Tsp_PR* secretome-exposed and subsequently blood-fed mosquitoes were microscopically evaluated for ovary follicle developmental stage after a non-infectious blood meal. Mosquitoes exposed for 48 hr to the fungus secretome prior to blood feeding showed a significantly decreased in ovary development (p=0.025). Only 9% of the mosquitoes had fully developed ovary follicles (***Figure 7A***, ***Figure 7—source data 1***); in contrast, untreated mosquitoes had a higher level (29%) of fully ovary follicle development. We also, examined the mosquito body weight at 1 hr and 4 hr after a blood meal. Mosquitoes treated with *Tsp_PR* secretome were heavier than non-treated mosquitoes at 48 hr post-blood meal, indicating impairment of blood digestion (p=0.001) (***Figure 7B***, ***Figure 7—source data 1***).

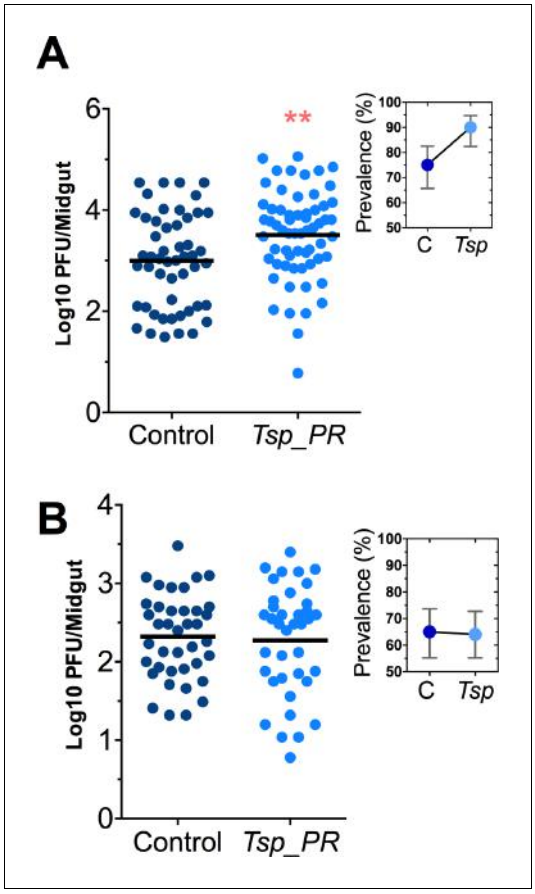

Figure 3. Heat-sensitive *Tsp_PR* secreted molecule(s) render mosquitoes more susceptible to DENV infection. DENV titers by plaque assay. Orlando strain mosquitoes were mock-fed or fed for 48 hr with a 10% sucrose solution a *Tsp_PR* filtered solution, which contained only (**A**) the fungus-secreted molecules (Control, N = 68; *Tsp_PR*, N = 68), or (**B**) a heat-treated *Tsp_PR* fungus-secreted molecules (Control, N = 60; *Tsp_PR*, N = 61). Mosquitoes were infected with a blood meal containing DENV, and midguts were dissected at 7 dpi. Each dot represents a log $_{10}$ PFU in individual midguts from three independent experiments. The line indicates the mean. Upper right boxes show the prevalence of infected mosquitoes, error bars represent the 95% confidence interval. **p<0.01.

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Following blood ingestion, gut epithelial cells synthesize and secrete diverse enzymes to digest the protein-rich meal (***Borovsky, 2003***). Trypsin activity accounts for most of the proteolytic activity during the mosquito's digestion of blood (***Barillas-Mury et al., 1995***; ***Noriega and Wells, 1999***). Our transcriptomic analysis showed a down-regulation of several trypsin genes in fungus-treated mosquitoes in the absence of a blood meal.

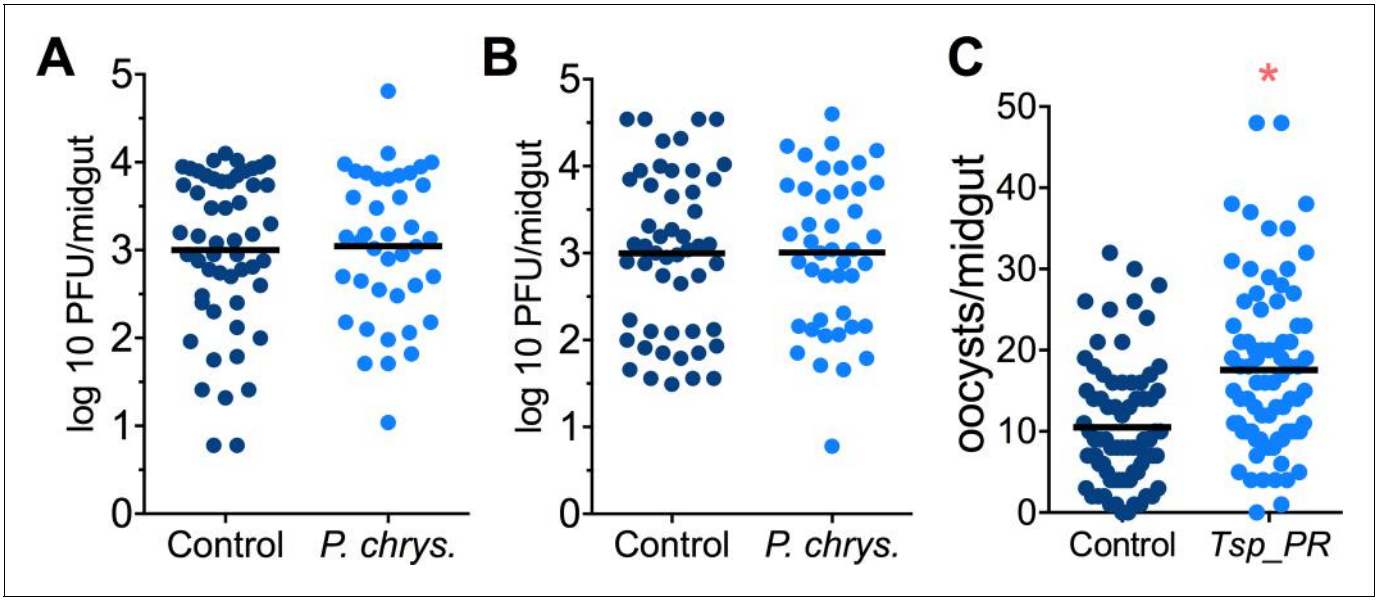

Figure 4. *Penicillium chrysogenum* does not modulate DENV infection in *Aedes* mosquito midguts, while *Tsp_PR* render *An. gambiae* more susceptible to *Plasmodium* infection. *Penicillium chrysogenum* was isolated from field-caught *Anopheles sp.* mosquitoes and re-introduced into *Aedes* mosquitoes to test the modulation of DENV infection. *Aedes* Orlando strain was mock-fed or fed for 48 hr with a 10% sucrose solution containing (**A**) 1×10^9 *P. chrysogenum* spores (Control, N = 61; *P. chrysogenum*, N = 47) or (**B**) fungus-secreted molecules (Control, N = 68; *P. chrysogenum*, N = 53). After fungus feeding, the mosquitoes were infected with a blood meal containing DENV; at 7 days post-infection (dpi), the midguts were dissected. Each dot represents a PFU value in individual midguts from three independent experiments. The line indicates the mean. (**C**) Influence of *Tsp_PR* on *P. falciparum* infection of *An. gambiae*, as a measured by oocyst numbers 7 days after feeding on a *P. falciparum* gametocyte culture (infection intensity). The mosquito cohort (N = 79) that had been exposed to a *Tsp_PR* -laced sucrose solution for 48 hr prior to parasite infection had a significantly higher *P. falciparum* infection than did the non-fungus-exposed control cohort (N = 76). Graphs show three independent experiments. Each dot represents a single midgut, and bars represent the mean. *p<0.05.

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Next, we performed assays to determine whether the *Tsp_PR* secretome down-regulates midgut trypsin enzymatic activity in vivo, by simply assaying the enzymatic activity of mosquito guts after exposure to the *Tsp_PR* secretome at 24 hr after a blood meal. The results showed a significantly lower trypsin enzymatic activity in the midgut of the experimental *Tsp_PR* secretome-exposed group (p<0.001) than in the non-treated control mosquitoes (***Figure 7C***, ***Figure 7—source data 1***).

The *Tsp_PR* secretome appeared to stimulate a reduction in trypsin enzymatic activity as a consequence of the transcriptional down-regulation of trypsin genes. However, we also proceeded to test whether the *Tsp_PR* secretome could directly influence trypsin enzymatic activity in vitro. This assay showed that the fungus secretome significantly decreased the in vitro activity of a commercial trypsin (p<0.01) in absence of mosquito's guts (***Figure 7D***, ***Figure 7—source data 1***). To provide functional confirmation that the *Tsp_PR* secretome-regulated trypsins could influence DENV infection, we silenced selected trypsin genes using RNA interference (RNAi)-mediated gene silencing and compared DENV titers to that of a control cohort that has been treated with a GFP dsRNA. Silencing efficiency was evaluated (***Figure 8A***, ***Figure 8—source data 1***) for AAEL010196, T196 (***Figure 8B***, ***Figure 8—source data 1***); AAEL013707, T707 (***Figure 8C***, ***Figure 8—source data 1***); AAEL013714, T714 (***Figure 8D***, ***Figure 8—source data 1***); AAEL013715, T715 (***Figure 8E***, ***Figure 8—source data 1***). Silencing of T714 and T715 resulted in a modest increased DENV infection intensity, while simultaneous silencing of all trypsin genes (Tmix) significantly increased DENV infection intensity (p<0.05). Silencing of T714 resulted in the greatest increase of DENV infection prevalence (p<0.001) (***Figure 8F***, ***Figure 8—source data 1***). These results indicate that a transcript reduction of multiple

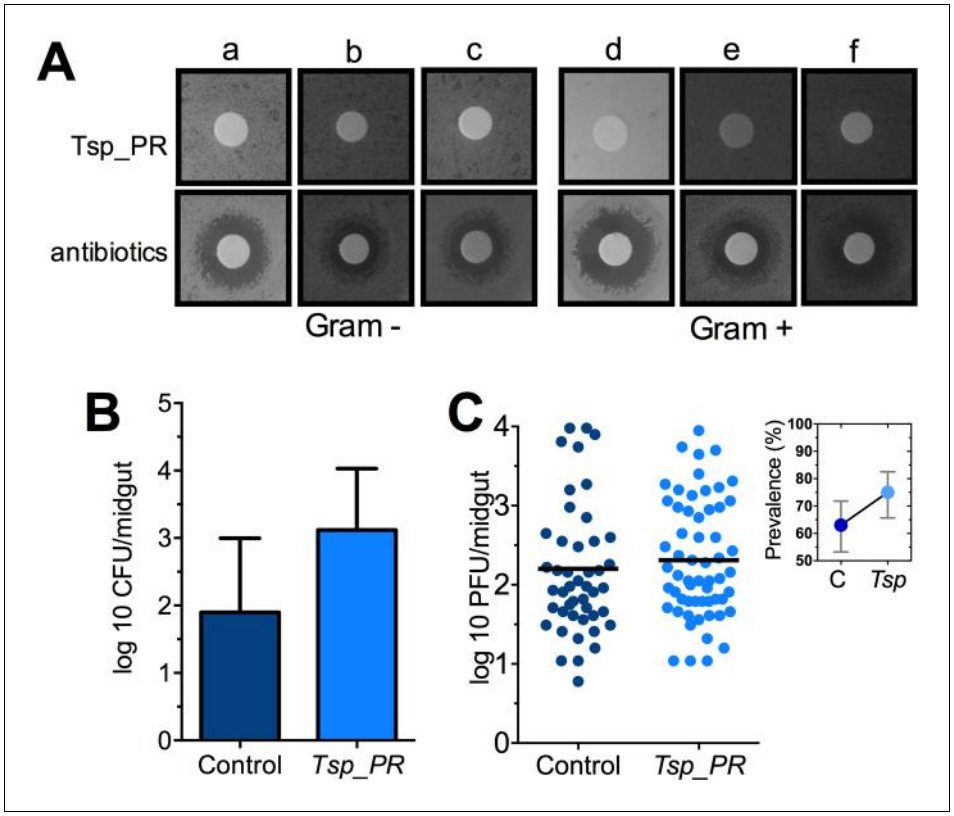

Figure 5. The *Tsp_PR* secreted molecule(s) do not affect bacterial load or DENV infection in aseptic mosquitoes. (**A**) Bacterial growth inhibition assay. Six bacterial isolates of field-caught mosquitoes (***Ramirez et al., 2012***) were independently plated on LB agar and covered with a disk soaked in a *Tsp_PR* secretome solution or antibiotic cocktail. Three isolates were Gram-negative bacteria: *Serratia marcescens* (**a**), *Chromobacterium haemolyticum* (**b**), and *Enterobacter hormaeche* (**c**). Three were Gram-positive bacteria: *Bacillus subtilis* (**d**), *Staphilococcus capprae* (**e**), and *Lactococcus lactis* (**f**). Bacterial inhibition was indicated by the presence of a bacterial inhibition zone around the disk. (**B**) Total bacterial loads. Midguts of secretome solution-exposed and unexposed mosquitoes were collected, homogenized, and plated on LB agar. Bacteria were counted as CFU. Error bars represent ± SD of three independent experiments p=0.202. (**C**) DENV titers in aseptic mosquitoes. Mosquitoes were treated with an antibiotic cocktail via a sugar meal 4 days before the fungal treatment and were mock-fed or fed for 48 hr on a *Tsp_PR* secretome solution prior to DENV infection. Each dot represents the PFU after 7 dpi in individual midguts from three independent experiments (Control, N = 75; *Tsp_PR*, N = 78). The line indicates the mean, p=0.867. Upper right box shows the prevalence of infected mosquitoes, error bars represent the 95% confidence interval.
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The following source data is available for figure 5:

Source data 1. Raw data and statistics summary for ***Figure 5B,C***.
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trypsins has a promoting effect on DENV infection, and trypsin T714 appears to exert the strongest contribution.

Taken together, these results show that one or more *Tsp_PR*-secreted factors influence blood digestion through the modulation of trypsin expression and activity, which in turn, affects susceptibility to DENV infection.

Discussion

Here we show that a fungus that is naturally associated with *Aedes* sp. in the field can enhance virus infection, and thereby potentially also enhance DENV transmission. Several studies have shown that the mosquito gut microbiota plays a critical role in determining the outcome of pathogen infection

Table 1. Significantly regulated genes in *Tsp_PR* secretome-exposed mosquitoes.
Log_2 values of differential mRNA abundances (*Tsp_PR* exposed/non-exposed) of genes.

Gene description	Gene ID	Log_2
trypsin	AAEL010196	−2.33
trypsin, putative	AAEL013714	−2.25
trypsin	AAEL010203	−2.07
Catalytic activity, serine-type endopeptidase activity, proteolysis	AAEL017520	−1.99
trypsin	AAEL013715	−1.95
serine-type enodopeptidase, putative	AAEL001690	−1.64
saccharopine dehydrogenase	AAEL014734	−1.35
Sialin, Sodium/sialic acid cotransporter, putative	AAEL004247	−1.25
hypothetical protein	AAEL013835	−1.22
alkaline phosphatase	AAEL000931	−1.19
trypsin	AAEL013707	−1.19
hypothetical protein	AAEL007591	−1.08
carboxypeptidase	AAEL010776	−1.07
triosephosphate isomerase	AAEL002542	−1.03
leucinech transmembrane proteins	AAEL005762	−0.90
serine-type enodopeptidase, putative	AAEL001701	−0.90
Conserved hypothetical protein (chitin-binding domain type 2)	AAEL017334	−0.89
sterol carrier protein-2, putative	AAEL012697	−0.82
hypothetical protein	AAEL002875	−0.82
lysosomal acid lipase, putative	AAEL004933	−0.81
hypothetical protein	AAEL002963	−0.78
lysosomal alpha-mannosidase (mannosidase alpha class 2b member)	AAEL005763	−0.76
ornithine decarboxylase	AAEL000044	1.70
glucosyl/glucuronosyl transferases	AAEL003099	1.23
conserved hypothetical protein(salivary protein [Culex])	AAEL009985	1.05
cytochrome P450	AAEL014607	1.01
cytochrome P450	AAEL014609	0.99
cytochrome P450	AAEL006811	0.97
cytochrome P450	AAEL014616	0.96
cytochrome P450	AAEL014608	0.95
hypothetical protein	AAEL004317	0.94
hypothetical protein	AAEL005669	0.92
hypothetical protein	AAEL002263	0.90
glucosyl/glucuronosyl transferases	AAEL010386	0.88
CRAL/TRIO domain-containing protein	AAEL003347	0.87
alpha-amylase	AAEL010537	0.85
hypothetical protein	AAEL011203	0.83
glucose dehydrogenase	AAEL004027	0.82
hypothetical protein	AAEL009198	0.81
glutamate decarboxylase	AAEL010951	0.80
cytochrome P450	AAEL008846	0.79
Vanin-like protein 1 precursor, putative	AAEL006023	0.78
cytochrome b5, putative	AAEL012636	0.77

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Table 1 continued

Gene description	Gene ID	Log_2
cytochrome P450	AAEL009131	0.76
cytochrome P450	AAEL014893	0.75

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and that different species of gut-associated bacteria can inhibit DENV and *Plasmodium* infection in *Aedes* and *Anopheles* mosquitoes, respectively (**Dong et al., 2009**; **Pumpuni et al., 1996**; **Ramirez et al., 2012**). Studies of the mosquito microbiota have mainly focused on bacteria, whereas the influence of fungi on mosquito physiology and pathogen infection remains largely understudied. Most studies concerning the mosquito mycobiota have explored the biology of entomopathogenic fungi, such as *Metarhizium anisopliae* and *Beauveria bassiana*, for use in mosquito control (**Darbro et al., 2011**; **Scholte et al., 2007**). The association of diverse fungi, with different developmental stages of mosquitoes in the field is reported (**da Costa and de Oliveira, 1998**; **da S Pereira et al., 2009**), and fungi, including *Talaromyces* sp., have also been isolated from the midgut of other arthropods such as ticks, sandflies, and kissing bugs (**Akhoundi et al., 2012**; **Marti et al., 2007**). Their presence is not surprising, since mosquitoes and many other insect vectors of disease are constantly exposed to a fungus-rich environment, including the larval breeding habitat, the plant nectars on which the adults feed, and the sites on which they rest.

We have shown that the presence of *Tsp_PR* spores or fungus secreted-molecules in the midgut tissue of the mosquito results in higher DENV infections in two different *Ae. aegypti* strains. However, modulation of infection does not seem to be a general property of a closely related *Penicillium* species, since exposure of *Ae. aegypti* to whole spores or the secretome of another mosquito-associated species, *Penicillium chrysogenum*, did not affect DENV infection (**Angleró-Rodríguez et al., 2016**). Our study suggests that *Tsp_PR* produces a factor(s) that is a likely a heat-sensitive protein or metabolite that alters the mosquito's susceptibility to DENV. Mosquitoes treated with the fungus secretome did not display differences in bacterial load in their midguts, indicating that the fungus-secreted factor(s) does not compromise the ability of *Ae. aegypti* to control its intestinal microbiota.

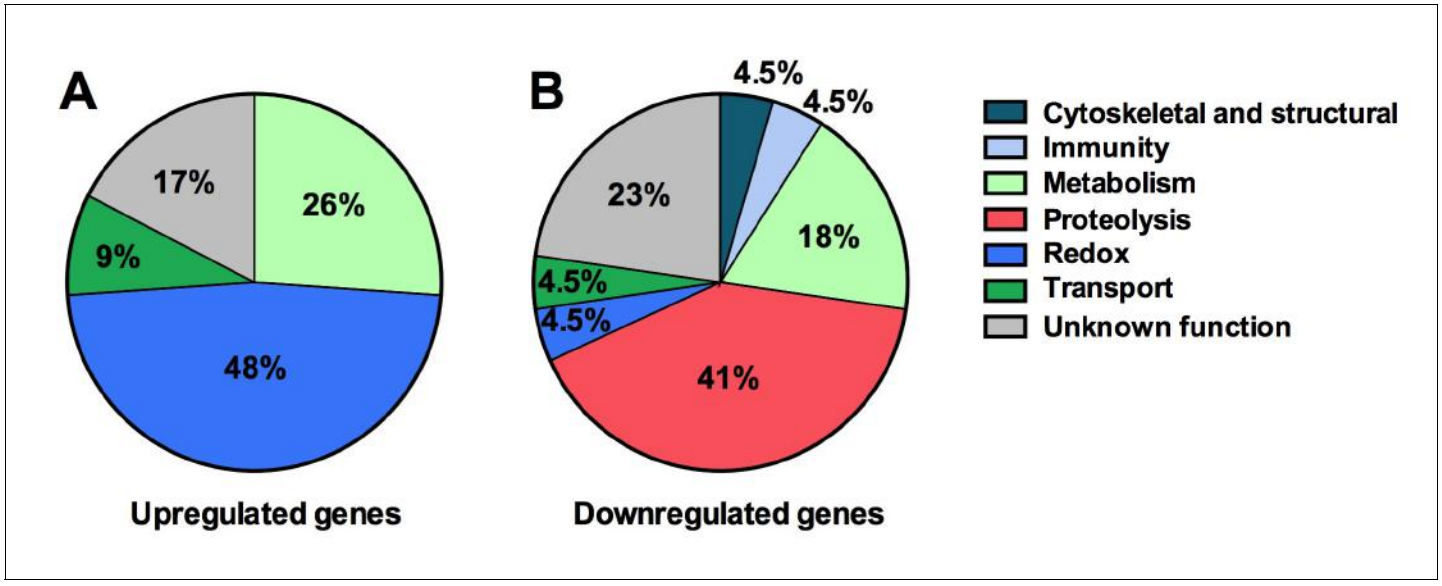

Figure 6. *Tsp_PR* secreted-molecule(s) –induced gene regulation. Functional classification in real numbers of the differentially expressed genes in mosquito midguts treated with *Tsp_PR* secretome for 48 hr, as compared to those of untreated mosquitoes. The fungus treatment-responsive genes are presented in ***Table 1*** and ***Supplementary file 1***.

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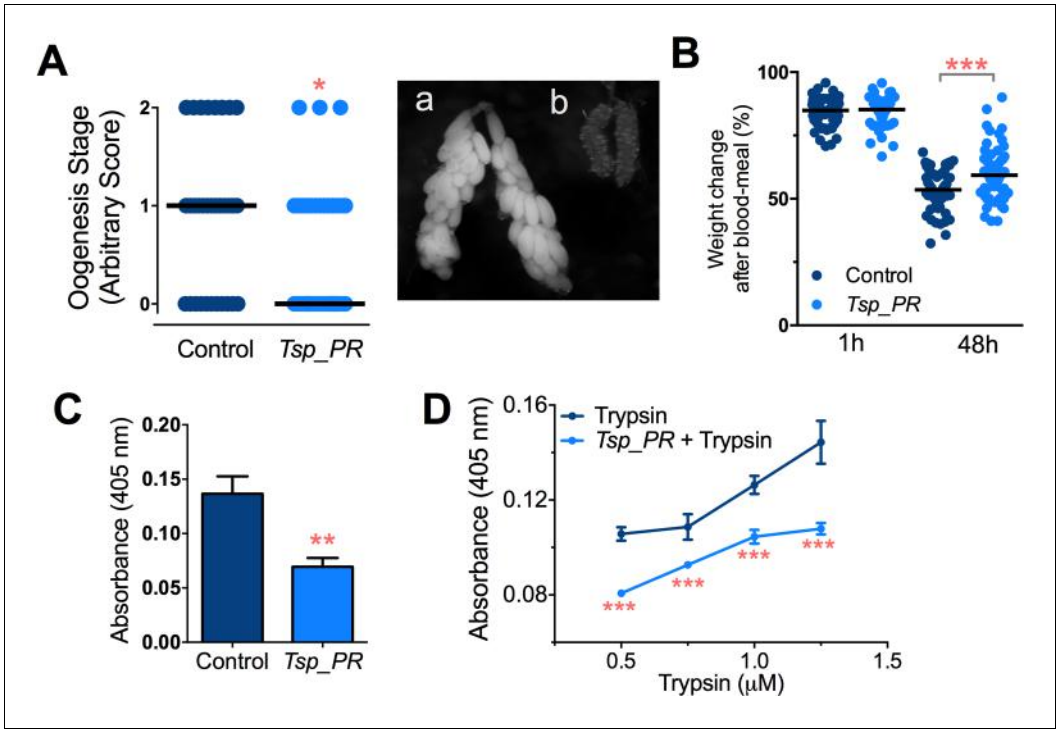

Figure 7. Inhibition of *Ae.aegypti* midgut trypsin activity by *Tsp_PR*-secreted molecule(s). (**A**) (*left*) Ovary development based on an arbitrary score of the ovary size at 6 days after a blood meal, 0 for small round follicles, 1 for intermediate size follicles, and 2 for fully developed follicles, with the elongated shape of normal mature eggs. Control, N = 34; *Tsp_PR*, N = 34, line represents the median, of three independent experiments. (*right*) Light microscopy picture of (**a**) a completely developed ovary follicle, which represents a score 2 (**b**) small round follicles, represent score 0. (**B**) Change in mosquito body weight after 1 hr (Control, N = 66; *Tsp_PR*, N = 74), (p=0.784) and 48 hr (Control, N = 58; *Tsp_PR*, N = 74) (p=0.001) of a non-infected blood meal (**C**) Trypsin in vivo enzymatic activity in midguts of mosquitoes treated or mock-treated with *Tsp_PR* secretome. Error bars represent ± SEM of three independent experiments. (**D**) Trypsin in vitro enzymatic assays of *Tsp_PR*'s ability to inhibit commercial trypsin activity. The activity was measured at various concentrations of trypsin. *Tsp_PR* represents the control group in which the fungus filtrate was added but no trypsin, and the absence of trypsin activity was experimentally confirmed (not shown). Error bars represent ± SEM of three independent experiments. *p<0.05, **p<0.01, ***p<0.01.

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The following source data is available for figure 7:

Source data 1. Raw data and statistics summary for ***Figure 7***.

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Our transcriptomic analysis of how the *Tsp_PR* influences mosquito physiology pointed to a profound regulation of genes principally related to metabolism and digestion. Among the top up-regulated genes were ornithine decarboxylase, which is associated with polyamine biosynthesis; glucosyl/glucuronosyl transferases, which play an important role in the detoxification of xenobiotics and the regulation of endobiotics (***Ahn et al., 2012***); and cytochrome P450, which is involved in detoxification (***Strode et al., 2008***). Interestingly, a considerable number of up-regulated genes were associated with detoxification, suggesting that the fungus imposes a certain level of toxicity. However, *Tsp_PR* exposure did not seem to affect mosquito longevity. Perhaps the up-regulation of the detoxification machinery effectively reverses a detrimental effect.

Among the up-regulated genes, only glucosyl/glucuronosyl transferase (AAEL003099) modulates DENV infection in *Ae. aegypti* (***Sim et al., 2013***). An earlier study showed that this gene was expressed at a lower level in *Ae. aegypti* strains that are refractory to DENV and at a higher level in DENV-susceptible strains, suggesting that the AAEL003099 gene acts as a DENV host factor. These findings are in agreement with our study, in which *Tsp_PR* secretome-exposed mosquitoes showed

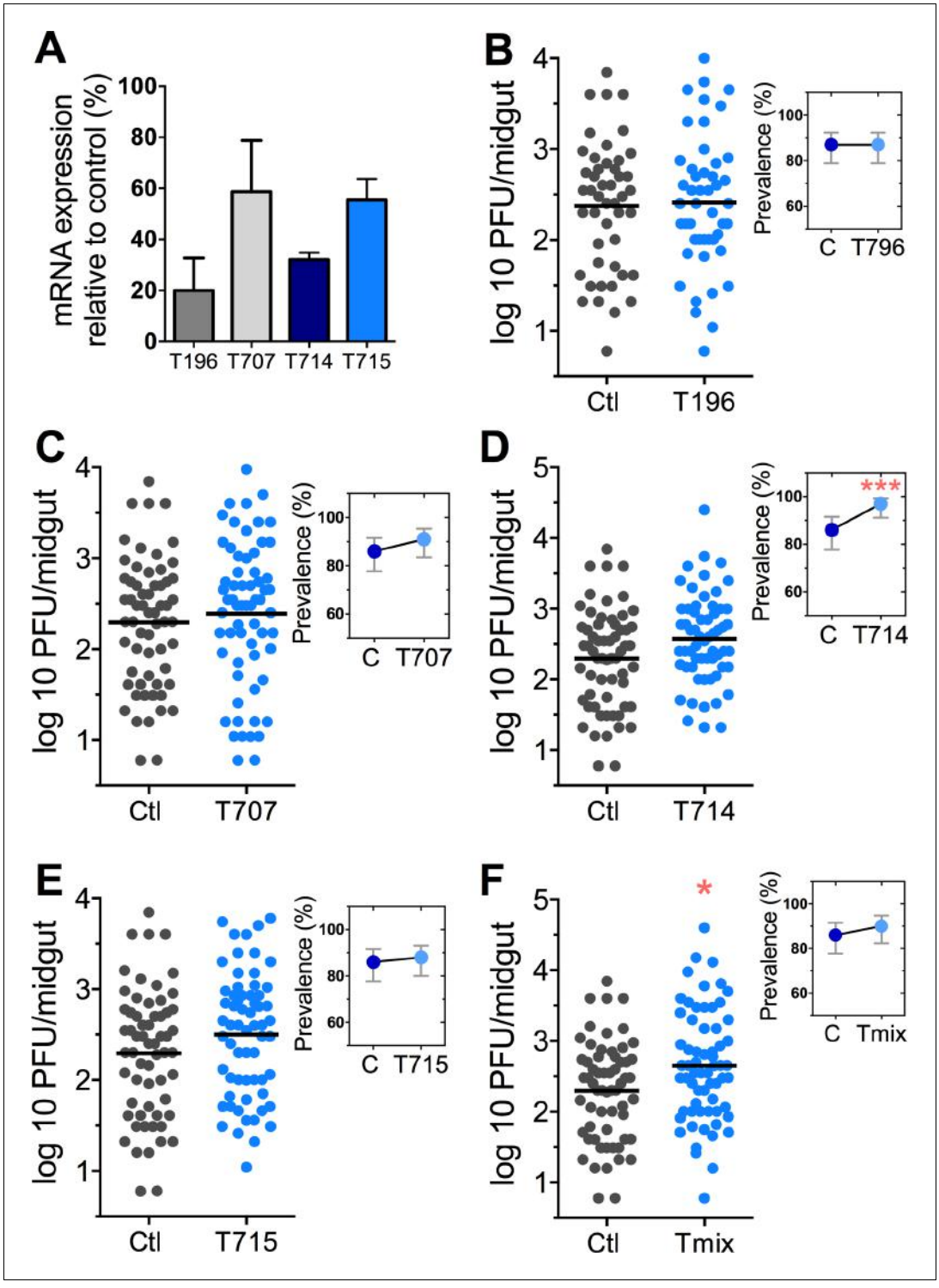

Figure 8. DENV infection after dsRNA-mediated silencing of trypsin genes. (**A**) Trypsin genes abundance after dsRNA-mediated gene silencing, (AAEL010196 (T196), AAEL013707 (T707), AAEL013714 (T714), AAEL013715 (T715). (**B–F**) DENV infection intensity of trypsin genes-silenced mosquitoes are compared to GFP dsRNA-treated control mosquitoes (**B**) T196 (Control, N = 54; T196, N = 54), (**C**) T707 (Control, N = 71; T707, N = 69), (**D**) T714 (Control, N = 71; T714, N = 62), (**E**) T715 (Control, N = 71; T715, N = 72), (**F**) Simultaneous silencing of all trypsins (Tmix) (Control, N = 71; Tmix, N = 71). The line indicates the mean, each dot represents the $\log_{10}$ PFU after 7 dpi in individual midguts from four independent biological experiments, except T196 which represents three

Figure 8 continued on next page

Figure 8 continued

independent experiments. Upper right boxes show the prevalence of infected mosquitoes, error bars represent the 95% confidence interval. *p<0.05, ***p<0.001.

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The following source data is available for figure 8:

Source data 1. Raw data and statistics summary for ***Figure 8***.

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an elevated expression of AAEL003099. It is therefore possible that a *Tsp_PR*-mediated up-regulation of AAEL003099 contributes to the elevated DENV infection levels. Another up-regulated gene associated with proviral effect is ornithine decarboxylase, a recent study showed that the polyamine biosynthesis is associated with an enhanced infection of Chikungunya and Zika viruses in humans (***Mounce et al., 2016***).

The most striking expression signature that resulted from *Tsp_PR* secretome exposure was a profound overrepresentation of down-regulated proteolysis genes. Trypsin genes were highly represented in this category, and they play a crucial role in the blood-digesting process in the mosquito midgut. Two groups of trypsins are produced after the blood meal: early trypsins during the first 6 hr post-blood meal, and the late trypsins between 8 and 36 hr post-blood meal (***Barillas-Mury et al., 1995***; ***Noriega and Wells, 1999***). However, the early trypsin mRNA is produced prior to feeding and is stored in the midgut epithelial cells, remaining untranslated until blood ingestion (***Noriega and Wells, 1999***). Our transcriptomic analysis revealed trypsin AAEL010196 to be the most down-regulated, followed by four other trypsin genes (AAEL013714, AAEL010203, AAEL013715, and AAEL013707). We confirmed the functional significance of trypsin transcript depletion on DENV infection using RNAi-mediated gene knock-down studies.

A phylogenetic analysis based on the trypsin gene nucleotide sequences revealed that the AAEL013715 and AAEL013707 cluster together and show a closer resemblance to the early trypsin EA1 gene, which has been associated with *Aedes* blood digestion (***Figure 9***). The trypsin genes AAEL013714, AAEL010203, and AAEL010196 have a close resemblance to two well-characterized late trypsins, 5G1 and LT (***Brackney et al., 2010***). Studies of trypsin 5G1 have established a link between trypsin activity and permissiveness to DENV infection, since RNAi-mediated repression of this gene results in greater susceptibility to the virus (***da Costa and de Oliveira, 1998***). This finding

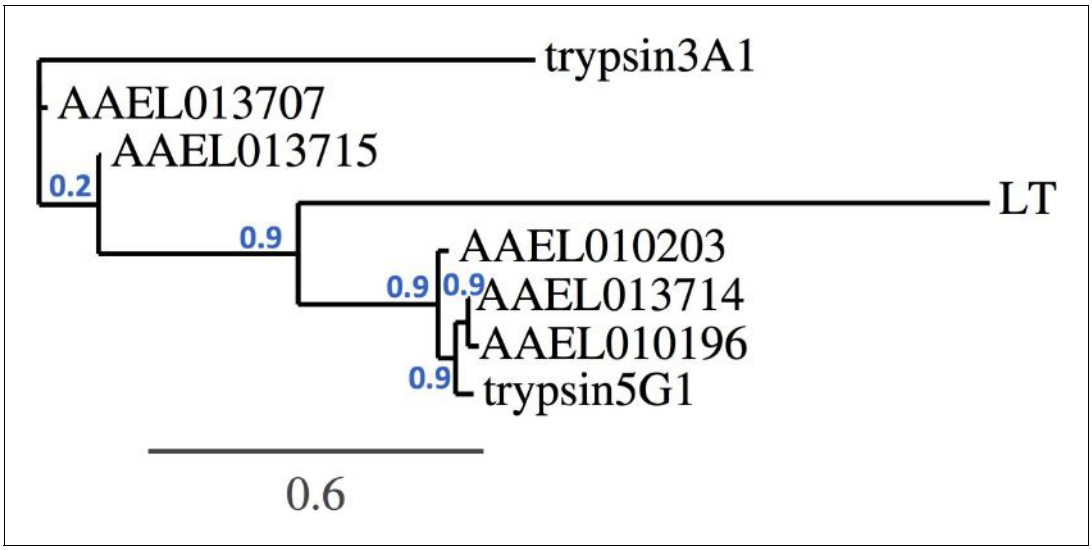

Figure 9. Trypsin phylogeny. Phylogenetic tree of the nucleotide alignment of trypsins regulated by *Tsp_PR* and others associated with the *Aedes* midgut. Branch support values represent approximate likelihood ratios, constructed using the program PhyML 3.0 approximate likelihood-ratio test (***Dereeper et al., 2008***).

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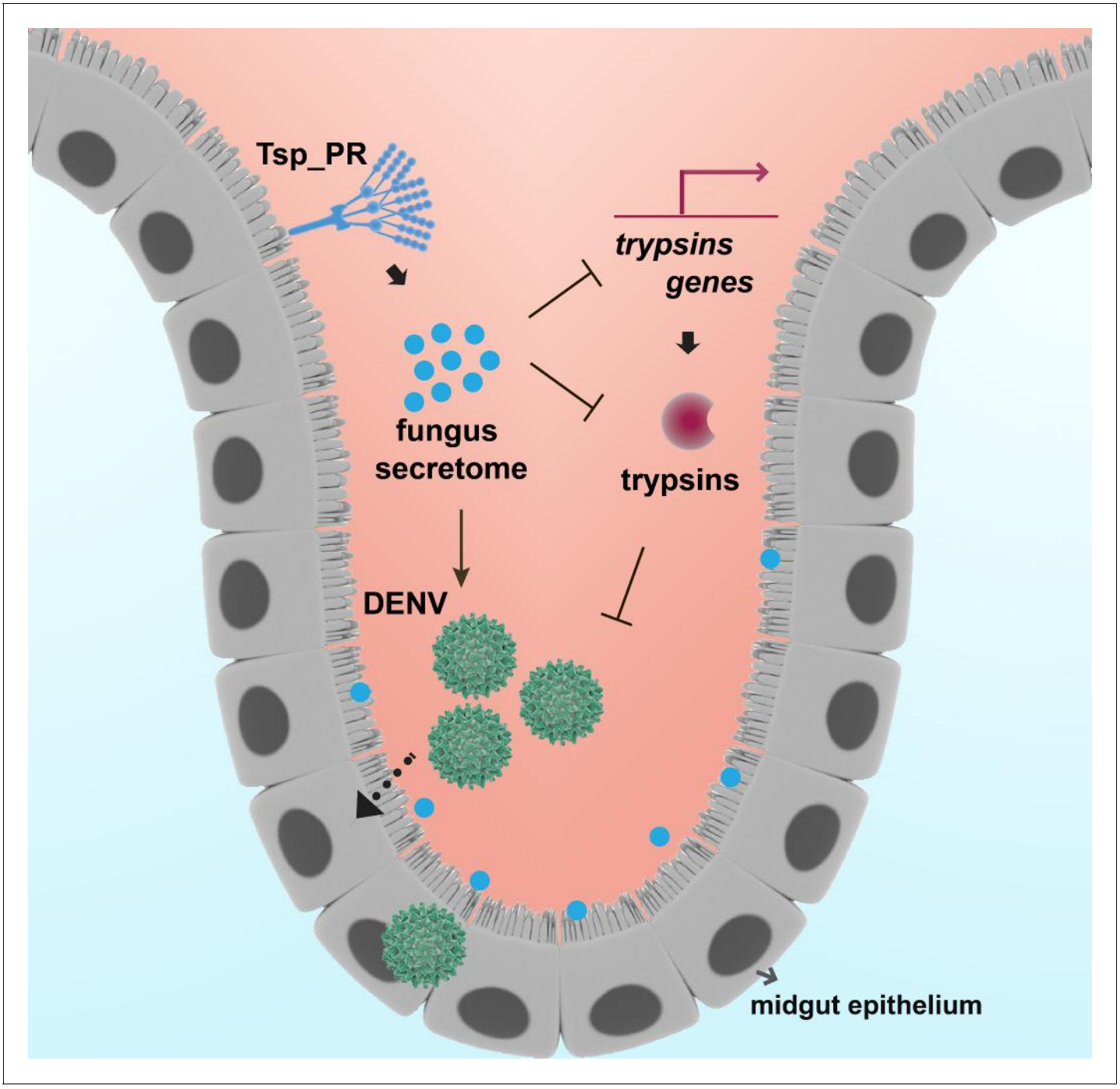

Figure 10. Model of *Tsp_PR*-mediated increased *Ae. aegypti* permissiveness to DENV. *Tsp_PR* secreted factors render *Ae. aegypti* more permissive to DENV through a mechanism that involves the down-regulation of gut trypsin transcripts and inhibition of enzymatic activity in the midgut epithelium. Trypsins have an antagonistic role in DENV infection. Decrease of trypsins abundance results in increased susceptible to DENV infection. Additional files.

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agrees with our studies showing that silencing of a closely related trypsin, the AAEL013714, also results in a greater susceptibility to DENV infection. QTL mapping of loci that determine permissiveness to DENV also indicated the likely involvement of trypsin genes (***Bosio et al., 2000***).

We have recently demonstrated that heat-stable factors secreted by a mosquito-associated *P. chrysogenum* fungus can render *Anopheles gambiae* mosquitoes more susceptible to the malaria parasite *Plasmodium* when present in the midgut tissue (**Angleró-Rodríguez et al., 2016**). We showed that this phenomenon was attributed to the up-regulation of an ornithine decarboxylase gene which in turn results in a suppression of nitric oxide –dependent parasite killing. While ingestion of *Tsp_PR* also resulted in a strong up-regulation of the *Ae. aegypti* ornithine decarboxylase gene, heat inactivation of its secreted factors abolished its ability to modulate DENV infection. Exposure of *An. gambiae* to *Tsp_PR* also rendered it more permissive to *Plasmodium* infection, but exposure of *Ae aegypti* to *P. chrysogenum* did not affect its permissiveness to DENV. Finally, exposure of *An. gambiae* to *P. chrysogenum* did not result in a down-regulation of blood digestive enzymes (**Angleró-Rodríguez et al., 2016**). These results indicate that that the two fungi influence infection with the virus and the parasite through different factors and mechanisms in their respective mosquito vectors. While both *Tsp_PR* and *P. chrysogenum* appears to secrete a factor that influences *An. gambiae* susceptibility to the malaria parasite, only *Tsp_PR* produces the factor that render *Ae. aegypti* more susceptible to DENV.

Our findings may have significant implications for the epidemiology and transmission of DENV by *Aedes* mosquitoes in the field. Fungi are abundantly associated with field mosquitoes (**da Costa and de Oliveira, 1998**; **da S Pereira et al., 2009**; **Marti et al., 2007**); our studies show that mosquitoes can acquire *Talaromyces* sp. fungus through sugar feeding and that it will successfully colonize the mosquito midgut for at least 25 days. Whether the exposure of mosquitoes to *Tsp_PR* in the field results in a greater permissiveness to DENV is unknown and difficult to assess, but greater permissiveness is most likely dependent on the intensity of the fungal exposure and the success of the fungus in persisting in the gut of various mosquito populations. A greater susceptibility of mosquitoes to DENV as a result of *Tsp_PR* exposure could translate into an enhanced transmission. However, this possibility remains to be addressed experimentally, perhaps by correlating the presence of *Tsp_PR* and DENV in field-caught mosquitoes in dengue-endemic areas. The observed impact of *Tsp_PR* on mosquito egg development in the ovary and the delayed degradation of the blood meal may suggest that exposure of mosquitoes to the amounts of the *Tsp_PR* secretome used in this study would impose a fitness cost.

In summary, we show how the mosquito mycobiota can influence *Ae. aegypti* vector competence for DENV by physiologically modulating midgut enzymes, one of the first barriers the pathogen encounters during infection (**Figure 10**). Our study represents a significant step toward the understanding of fungi-mosquito interactions and their possible implications for the transmission of DENV and perhaps other arboviruses.

Materials and methods

Key resources table

Reagent type (species) or resource	Designation	Source or reference	Additional information
strain, strain background (*Aedes aegypti* Rockefeller strain)	Rock	other	From Johns Hopkins University
strain, strain background (*Aedes aegypti* Orlando strain)	Orl	other	From Johns Hopkins University
cell line (*Aedes albopictus* C6/36)	C6/36	ATCC CRL-1660	
cell line (Baby hamster kidney cells (BHK-21))	BHK-21	ATCC CCL-10	
biological sample (*Talaromyces sp.*)	*Tsp_PR*	this paper	Collected from a wild-caught mosquito from Naguabo, Puerto Rico
biological sample (*Penicillium chrysogenum*)	*P. chrysogenum*	PMID 27678168	
biological sample (Dengue virus 2 strain New Guinea C (NGC)	DENV	PMID 18604274	

Continued on next page

Continued

Reagent type (species) or resource	Designation	Source or reference	Additional information
biological sample (*Plasmodium falciparum*)	*P. falciparum*	PMID 27678168	From Johns Hopkins Malaria Research Institute
Low Input Quick Amp Labeling kit		Agilent Technologies	
RNeasy Mini Kit		QIAGEN	
MMLV Reverse Transcriptase kit		Promega	

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the Animal Care and Use Committee of the Johns Hopkins University (Permit Number: M006H300). Mice were only used for mosquito rearing as a blood source, according to approved protocol. Commercial anonymous human blood was used for DENV infection assays in mosquitoes, and informed consent was therefore not applicable. The Johns Hopkins School of Public Health Ethics Committee approved this protocol.

Cell culture and mosquito rearing

The *Ae. albopictus* cell line C6/36 (ATCC CRL-1660) was maintained in MEM (Gibco) supplemented with 10% FBS, 1% L-glutamine, 1% non-essential amino acids, and 1% penicillin/streptomycin. Baby hamster kidney cells (BHK-21, ATCC CCL-10) were maintained on DMEM (Gibco) supplemented with 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin, and 5 µg/mL plasmocin (Invivogen, San Diego, CA). C6/36 cells and BHK-21 cells were incubated in 5% CO_2 at 32˚C and 37˚C, respectively. *Ae. aegypti* mosquitoes were maintained on a 10% sucrose solution at 27˚C and 80% relative humidity with a 14:10 hr light:dark cycle.

Fungus treatments

Tsp_PR was grown on Sabouraud glucose agar (SGA) and identified as described in (***Angleró-Rodríguez et al., 2016***); spores were collected in PBS, counted, and resuspended in a 10% sucrose solution containing 1×10^9 spores. For fungal filtrate, spores were collected in a 10% sucrose solution and kept in a rocker machine overnight at 4˚C. The next day, the solution was centrifuged at 470 rcf to collect the supernatant, which was passed through a 0.2-micron filter to remove fungus mycelia and spores. The heat-inactivated filtrate was treated in the same way, but the supernatant was incubated for 2 hr at 95˚C. Adult female *Ae. aegypti* (3–4 days old) were starved for 6 hr and fed with the appropriate fungal treatment for 48 hr.

DENV infection

DENV2 strain New Guinea C (NGC) was propagated in C6/36 cells, and titers were determined on BHK-21 cells by plaque assay. Mosquito infections with DENV were carried out as previously described (***Das et al., 2007***). In brief, DENV2-NGC was propagated in C6/36 cells for 6 days. Virus suspension was mixed 1:1 with commercial human blood and supplemented with 10% human serum and 100 µM ATP. Mosquitoes were infected via an artificial membrane feeder at 37˚C for 30 min. Midguts were dissected and individually collected at 7 days post-infection.

Plasmodium infection and oocyst enumeration

Plasmodium falciparum infections were performed following a standard protocol (***Dong et al., 2006***). At 48 hr post-feeding on fungi or filtrate, mosquitoes were fed on an NF54W strain gametocyte culture mixed with human blood, through a membrane feeder at 37˚C. Engorged mosquitoes were maintained at 27˚C for up to 8 days. *P. falciparum*-infected mosquito midguts were dissected and stained with 0.1% mercurochrome, and oocyst numbers were determined using a light microscope.

Plaque assay

BHK-21 cells were seeded in 24-well plates the day before the assay. The next day, individual midguts were homogenized in DMEM with 0.5 mm glass beads using a Bullet Blender (NextAdvance). Homogenates were centrifuged at 18,400 rcf and the virus-containing suspensions were 10-fold serially diluted, and 100 uL of each dilution were inoculated onto 80% confluent BHK-21 plates. Plates were rocked for 15 min at room temperature and then incubated for 45 min at 37°C and 5% CO_2. After the incubation, 1 mL of DMEM containing 2% FBS and 0.8% methylcellulose was added to each well, and plates were incubated for 5 days. Plates were fixed and stained for 30 min with a 1:1 methanol/acetone and 1% crystal violet mixture. Then, plates were washed with water and the plaque-forming units counted.

Microbial enumeration

Mosquitoes were surfaced-sterilized for 1 min in 70% ethanol and rinsed twice with 1X PBS. Mosquitoes were dissected, and five midguts were pooled in a microcentrifuge tube containing 150 µl of sterile PBS. Midguts were homogenized with a pestle and plated on LB agar for bacterial enumeration or Sabouraud agar with an antibiotic cocktail of penicillin/streptomycin and gentamicin for fungal enumeration (whole mosquitoes or midguts were collected for this procedure). Plates were incubated at room temperature for 48 hr for bacteria and 4 days for fungus, and then plates were counted as colony-forming units (CFU). *Bacterial growth inhibition assay*. Was performed using a disc diffusion test, in which a sterile filter paper disk soaked in a fungus secretome solution or an antibiotic cocktail as a control, was placed over a bacterial culture on LB agar medium. Plates were incubated for 24 hr at 32°C and the inhibition zone were evaluated.

Aseptic mosquitoes

Adult female mosquitoes were maintained on 10% sucrose solution containing 75 µg/mL gentamicin sulfate and 100 units (µg)/mL of penicillin-streptomycin. Treatment was carried out for 4 days. To validate the efficiency of antibiotic treatment, midguts from control untreated and antibiotic treated mosquitoes were subjected to CFU assays. Mosquitoes were treated with the bacteria-free fungus secretome for 2 days, and then maintained on antibiotic-treated sucrose after the DENV infection.

Genome-wide microarray-based transcriptome profiling

Transcriptome assays were conducted and analyzed as reported previously with a custom-designed full genome *Ae. aegypti* Agilent-based microarray platform (***Sim et al., 2013***; ***Xi et al., 2008***). In brief, 200 ng of total RNA per replicate was used to synthesize Cy3 or Cy5-labeled cRNA probes using a Low Input Quick Amp Labeling kit (Agilent Technologies). Probes from midguts of *Tsp_PR*-treated mosquitoes were individually hybridized against probes from untreated mosquitoes as a control. The arrays were scanned with an Agilent Scanner. Transcription data were processed by beginning with background subtraction of the median fluorescent values, normalized with the LOWESS normalization method. Cy5/Cy3 ratios from replicate assays were subjected to *t*-tests at a significance level of $p<0.05$ using TIGR MIDAS and MeV software. Transcript abundance data from all replicate assays were averaged with the GEPAS microarray preprocessing software and transformed to a logarithm (base 2). Self-self hybridizations have previously been used to determine a cutoff value for the significance of gene regulation on these microarrays of 0.75 in $\log_2$ scale (***Yang et al., 2002***). Three independent experiments were performed. Numeric microarray gene expression data are presented in ***Table 1***, ***Supplementary file 1***.

Oogenesis assays

Mosquitoes we exposed or not exposed to the *Tsp_PR* secretome for 48 hr, after they had received a blood meal. Fully engorged females from both groups were collected and maintained for 6 days; their ovaries were then dissected in PBS, and oogenesis was microscopically evaluated. Through microscopic evaluation of the follicle development in the ovary, we assigned an arbitrary score of 0 for small round follicles, 1 for intermediate size follicles, and 2 for fully developed follicles, with the elongated shape of normal mature eggs.

Body weight measurements

Mosquitoes were fed with non-infectious blood for 20 min, then fully-engorged mosquitoes were individually collected and placed in individual round-bottom conical tubes and incubated without sugar or water. The conical tube weight was measured prior to collecting the mosquito. The weight of mosquitoes at 1 hr post-infection was calculated by subtracting the empty tube weight from the total weight. At 48 hr mosquitoes were cold anesthetized and weighted directly to the analytical balance without the tube to avoid accumulated excreta.

Trypsin activity assay

Mosquito endogenous trypsin activity assay

Mosquitoes were exposed, or not exposed, to the *Tsp_PR* secretome for 48 hr, after they were fed an artificial blood meal (40% PBS, 50% FBS, 1 mM ATP, and 2 mg of phenol red) to avoid interference with the assay (***Brackney et al., 2008***). At 24 hr post-blood meal, 10 midguts per group were dissected, collected in 50 µL of buffer solution (50 mM Tris-HCl, pH 8.0, with 10 mM $CaCl_2$), and homogenized on ice with a pestle. Supernatants were collected after a high-speed centrifugation (18,400 rcf) at 4˚C. Trypsin activity assays were performed using the synthetic colorimetric substrate N_α-benzoyl-D,L-arginine-p-nitroanilide hydrochloride (BApNA) (Sigma). The reaction mixtures, each containing 5 µl of midgut extract and 1 mM BApNA, were then incubated at 37˚C for 5 min. Absorbance values were measured in a plate reader at A405 nm.

In vitro trypsin inhibition assay

A reaction mixture was made using equal volumes of *Tsp_PR* filtrate and commercial trypsin (105 µM) (Gibco). The mixture was incubated at 27˚C for 3 hr. The reaction solution was prepared by adding increasing volumes of the 1:1 mixture to the buffer solution and 1 mM BApNA, to a final volume of 200 uL. Absorbance was measured as described above.

dsRNA-mediated gene silencing

The *trypsin* genes were depleted from adult female mosquitoes using established RNA interference (RNAi) methodology (***Sim et al., 2013***). Mosquitoes injected with *GFP* dsRNA were used as a control and RNAi assays were repeated three times. Gene silencing was verified by qRT-PCR at 3 days post-injection using RNA extracted from five whole mosquitoes per independent experiment. The primers to produce PCR Amplicons for dsRNA synthesis and qRT-PCR are presented in ***Supplementary file 2***.

Quantitative RT-PCR

Mosquito samples were collected in RLT buffer (QIAGEN), and then stored at −80˚C until extraction. Total RNA was extracted using the RNeasy Mini Kit (QIAGEN); samples were treated with Turbo DNase (Ambion) before reverse transcription with a MMLV Reverse Transcriptase kit (Promega) according to the manufacturer's instructions. The cDNA was then used to determine gene expression by quantitative PCR using SYBR Green PCR Master Mix (Applied Biosystem). The transcript abundance of trypsin was compared to the expression of the ribosomal protein gene S7 as a normalization control. qPCR primers were designed to amplify the mRNA transcript outside the dsRNA region. However, groups where trypsins were silenced simultaneously the qPCR primers for a given trypsin crossed detected the injected dsRNA of other highly similar trypsins making unable the evaluation of silencing efficiency.

Statistical analysis

To compare DENV titers between groups, *P*-values were calculated using Generalized Linear Regression (GLM) with experiment-clustered robust variance estimates to account for potential within-experiment correlation of outcomes (***Rogers, 1993***). The models included different link functions for various outcomes: identity link was used to compare DENV PFU and bacteria CFU, log-link with Poisson distribution was used for modeling infections and score 0 to 2 for oogenesis assays and log-link with Negative Binomial distribution was used to model number of *Plasmodium falciparum* oocysts. The models included an indicator variable for treatment with only two levels or multiple indicator variables for multiple treatment groups. Wald test *P*-values are reported in the results. Survival was

analyzed using the Log-rank (Mantel-Cox) test in Graphpad Prism. See source data file for the summary of the statistics.

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Author contributions

Yesseinia I Angleró-Rodríguez, Conceptualization, Data curation, Formal analysis, Validation, Investigation, Visualization, Methodology, Writing—original draft, Writing—review and editing; Octavio AC Talyuli, Benjamin J Blumberg, Seokyoung Kang, Celia Demby, Alicia Shields, Jenny Carlson, Natapong Jupatanakul, Investigation, Methodology; George Dimopoulos, Conceptualization, Resources, Supervision, Funding acquisition, Project administration, Writing—review and editing

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Ethics

Animal experimentation: This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the Animal Care and Use Committee of the Johns Hopkins University (Permit Number: M006H300). Mice were only used for mosquito rearing as a blood source, according to approved protocol.

Decision letter and Author response

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Author response https://doi.org/10.7554/eLife.28844.023

Additional files

Supplementary files

- Supplementary file 1. Table shows all the genes that had differential mRNA abundances (Tsp_PR exposed/non-exposed) over or under the significance cutoff value of ±0.75 Log_2.

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- Supplementary file 2. Primer sequences used for dsRNA synthesis and qPCR. Sequences underlined corresponds to T7 promoter

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- Transparent reporting form

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PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Regulation of midgut cell proliferation impacts *Aedes aegypti* susceptibility to dengue virus

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Abstract

Aedes aegypti is the vector of some of the most important vector-borne diseases like dengue, chikungunya, zika and yellow fever, affecting millions of people worldwide. The cellular processes that follow a blood meal in the mosquito midgut are directly associated with pathogen transmission. We studied the homeostatic response of the midgut against oxidative stress, as well as bacterial and dengue virus (DENV) infections, focusing on the proliferative ability of the intestinal stem cells (ISC). Inhibition of the peritrophic matrix (PM) formation led to an increase in reactive oxygen species (ROS) production by the epithelial cells in response to contact with the resident microbiota, suggesting that maintenance of low levels of ROS in the intestinal lumen is key to keep ISCs division in balance. We show that dengue virus infection induces midgut cell division in both DENV susceptible (Rockefeller) and refractory (Orlando) mosquito strains. However, the susceptible strain delays the activation of the regeneration process compared with the refractory strain. Impairment of the Delta/Notch signaling, by silencing the Notch ligand Delta using RNAi, significantly increased the susceptibility of the refractory strains to DENV infection of the midgut. We propose that this cell replenishment is essential to control viral infection in the mosquito. Our study demonstrates that the intestinal epithelium of the blood fed mosquito is able to respond and defend against different challenges, including virus infection. In addition, we provide unprecedented evidence that the activation of a cellular regenerative program in the midgut is important for the determination of the mosquito vectorial competence.

Author summary

Aedes mosquitoes are important vectors of arboviruses, representing a major threat to public health. While feeding on blood, mosquitoes address the challenges of digestion and

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preservation of midgut homeostasis. Damaged or senescent cells must be constantly replaced by new cells to maintain midgut epithelial integrity. In this study, we show that the intestinal stem cells (ISCs) of blood-fed mosquitoes are able to respond to abiotic and biotic challenges. Exposing midgut cells to different types of stress, such as the inhibition of the peritrophic matrix formation, changes in the midgut redox state, or infection with entomopathogenic bacteria or viruses, resulted in an increased number of mitotic cells in blood-fed mosquitoes. Mosquito strains with different susceptibilities to DENV infection presented different time course of cell regeneration in response to viral infection. Knock-down of the Notch pathway in a refractory mosquito strain limited cell division after infection with DENV and resulted in increased mosquito susceptibility to the virus. Conversely, inducing midgut cell proliferation made a susceptible strain more resistant to viral infection. Therefore, the effectiveness of midgut cellular renewal during viral infection proved to be an important factor in vector competence. These findings can contribute to the understanding of virus-host interactions and help to develop more successful strategies of vector control.

Introduction

The mosquito *Aedes aegypti* is a vector of several human pathogens, such as flaviviruses, including yellow fever (YFV), dengue (DENV) and zika (ZIKV), and thus this mosquito exerts an enormous public health burden worldwide [1,2]. During the transmission cycle, these insects feed on volumes of blood that are 2–3 times their weight, and the digestion of this large meal results in several potentially damaging conditions [3]. The digestion of blood meal requires intense proteolytic activity in the midgut and results in the formation of potentially toxic concentrations of heme, iron, amino acids and ammonia [4]. The midgut is also the first site of interaction with potential pathogens, including viruses, and supports a dramatic increase in intestinal microbiota after blood feeding [5,6]. To overcome these challenges, the ingestion of a blood meal is followed by several physiological processes, such as formation of a peritrophic matrix (PM) [7,8] and down-regulation of reactive oxygen species (ROS) production. In addition, the midgut epithelium is the first barrier that viruses must cross in the mosquito to achieve a successful viral cycle (reviewed in [9]). Thus, in order to ensure epithelial integrity and the maintenance of midgut homeostasis, the midgut epithelium must fine tune key cellular mechanisms, including cell proliferation and differentiation.

In both vertebrate and invertebrate animals, the gut epithelia have a similar basic cellular composition: absorptive enterocytes (ECs) that represent the majority of the differentiated cells and are interspersed with hormone-producing enteroendocrine cells (ee). The intestinal stem cells (ISCs) and enteroblasts (EB) account for the progenitor cells, responsible for replenishing the differentiated cells that are lost due to damage or aging [10–14]. In *A. aegypti*, description of the different cellular types and functions started with identification and basic characterization of absorptive (ECs) and non-absorptive cells (ISC, EB, and enteroendocrine cells) [15]. To date, the study of division properties of the ISCs in this vector species remains limited to the description of the division process during metamorphosis [16].

Several conserved signaling pathways are known to be involved in midgut tissue renewal and differentiation. Comparative genomic analysis of some of these pathways has been done between *Drosophila melanogaster* and vector mosquitoes [17,18], but functional studies in *Aedes*, under the context of tissue regeneration, are still necessary. Notably, the Notch signaling pathway regulates cell differentiation in the midgut of both mammals and *D. melanogaster*.

In this fruit fly, loss of function of Notch is attributed to the increase of intestinal cell proliferation and tumor formation [19]. However, it has already been shown that depletion of Notch in *D. melanogaster* ISCs also leads to stem cell loss and premature ee cell formation [20]. Accordingly, disruption of Notch signaling in mice has resulted in decreased cell proliferation coupled with secretory cell hyperplasia, whereas hyperactivation of Notch signaling results in expanded proliferation with increased numbers of absorptive enterocytes [21], as also observed in *D. melanogaster* [20].

In the fruit fly, the ingestion of cytotoxic agents, such as dextran sodium sulfate (DSS), bleomycin or paraquat, or infection by pathogenic bacteria can stimulates cell turnover, increasing the midgut ISC mitotic index [18,22]. Similar to that, it has been recently shown that cell damage produced by ingestion of several stressors also induced intestinal cell proliferation in sugar-fed *Aedes albopictus* [23]. Likewise, viral infections can trigger cellular responses, such as apoptosis or autophagy, in different infection models [24–27]. However, the interplay between intestinal cell proliferation and pathogen transmission has been a neglected subject in the literature.

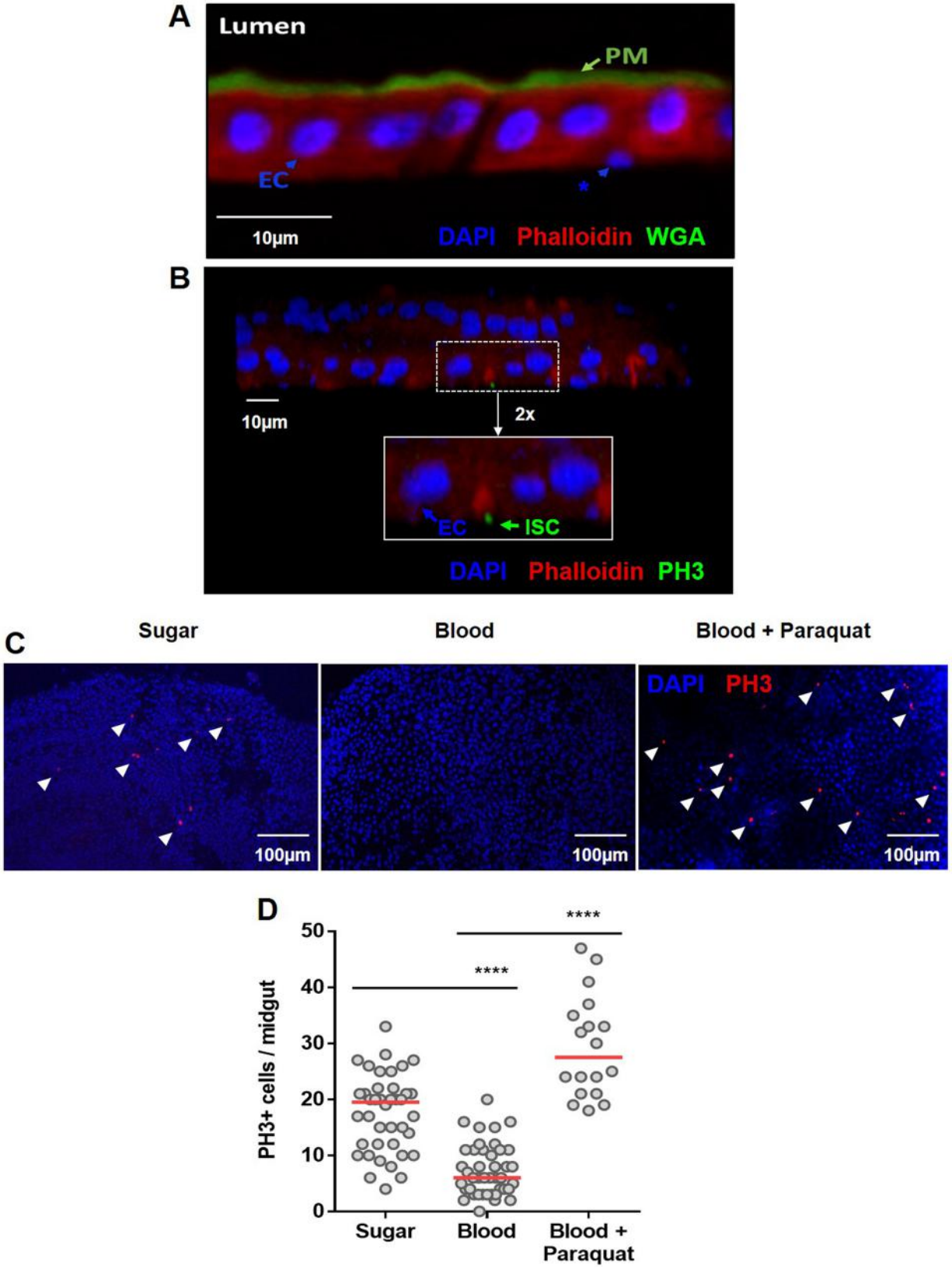
In this study, we have characterized the dynamics of *A. aegypti* intestinal epithelium proliferation during blood meal digestion in response to oxidative stress, bacterial infections, and viral infections. We have also shown that two mosquito strains with different DENV susceptibilities [28] presented differences in cell mitotic rates after viral infection. Finally, our results indicate for the first time that the ability to replenish midgut cells by modulation of cell renewal involves the Delta-Notch signaling and is a key factor that influences *A. aegypti* competence to transmit DENV. We show that the cell proliferation rates influences mosquito infection and vector competence for DENV.

Results

Aedes aegypti adult females acquire DENV and other arboviruses during the blood feedings that are needed to complete the reproductive cycle of the mosquito. To characterize the epithelial adaptation to this event, we first evaluated the cellular response to the blood meal itself. Upon ingestion, the blood induces dramatic changes in the Red strain mosquito midgut at a chemical, microbiological and physiological level. We attempted to dissect each of these challenges, to understand the delicate balance of the factors that play a role in the intestinal microenvironment in which the arbovirus has to thrive in order to pass to the salivary gland and be transmitted.

Characterization of the adult intestinal cells and their regenerative capacity in *A. aegypti* adult midgut epithelium

The tissue homeostasis of the midgut depends on the ability to replenish the damaged cells, and this depends on the presence of ISCs. Due to the lack of specific markers for progenitor cells for *A. aegypti*, we used morphological and physiological parameters to define the presence of ISCs in the adult females. Progenitor cells are well characterized for their basal positioning and being diploid, different to the apical localization of differentiated cells and the polyploidy of enterocytes. Both cell types were clearly distinctive, as well as the peritrophic matrix, in the midgut epithelium of blood-fed adult females (**Fig 1A**). The further characterization of ISC's was performed with phospho-histone 3 antibodies, to specifically mark cells undergoing mitosis. In **Fig 1B**, it can be observed the two monolayers of the *A. aegypti* midgut, where ECs are clearly distinguishable and the PH3+ cell is found, with nuclei corresponding to the diploid size, located basally. Clearly, not every ISC present in the tissue is going to be found undergoing mitosis, but the presence of PH3+ cells, undoubtedly characterizes such cells as ISCs.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Sugar
Blood
Blood + Paraquat

Fig 1. General structure of the midgut epithelium of *Aedes aegypti* and modulation of cell proliferation upon blood meal. The midgut epithelia from a blood-fed *A. aegypti* females were fixed in PFA and in **(A)** sections of 0.14 μm were stained with WGA-FITC (green), red phalloidin (red) and DAPI (blue). The peritrophic matrix (PM), intestinal lumen (Lumen), polyploid enterocytes (EC) and basally localized–putative proliferative cells (*)–are visible. In **(B)**, confocal image (z-stack of 0.7 μm slides (20X)) of the two monolayers of the midgut of a blood-fed female, 5 days post feeding, stained with PH3 mouse antibody (green), DAPI (blue), and phalloidin (red)–Inset (2x): polyploid enterocytes (EC) are PH3-positive ISC (ISC) are visible. **(C)** Mosquitoes were fed on a sugar solution (10% sucrose), blood or blood supplemented with 100μM of the pro-oxidant paraquat. The insect midguts were dissected 24 hours after feeding and immunostained for PH3. Representative images of mitotic (PH3-labeled) cells (red) in the epithelial midgut of animals fed on sugar, blood or blood supplemented with paraquat are shown. The nuclei are stained with DAPI (blue). The arrowheads indicate PH3+ cells. **(D)** Quantification of PH3-positive cells per midgut of sugar, blood or blood plus paraquat-fed mosquitoes for sugar and blood and 18 for blood-paraquat fed midguts. The experiments were performed on Red Eye mosquito strain. The medians of at least three independent experiments are shown (n = 40 for sugar and blood and n = 18 for paraquat supplemented blood). The asterisks indicate significantly different values, **** $P<0.0001$ (Student's t-test).

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To evaluate the homeostatic cell proliferation of the *Aedes aegypti* midgut, we observed the number of cells undergoing mitosis in adult females. After a blood meal, the midgut epithelium showed a lower number of cells undergoing mitosis (phospho-histone 3 positive; PH3+) compared with that of sugar-fed insects **(Fig 1C and 1D)**. To test if this decrease in mitotic cells was due to progenitor cell impairment, we fed insects with blood supplemented with the pro-oxidant compound paraquat. The midgut epithelium responded to an oxidative challenge by increasing mitosis **(Fig 1C and 1D)**, indicating that the intestinal stem cells maintained the ability to divide and replenish damage cells after an insult at blood-fed conditions.

Peritrophic matrix reduces cell proliferation induced by microbial infection

A hallmark of blood digestion is the formation of the peritrophic matrix (PM), a chitin and protein-rich non-cellular layer secreted by the midgut epithelium [7,8]. The mosquito type-I PM surrounds the blood bolus, limiting a direct contact between the epithelium, the blood meal and the indigenous microbiota, thereby playing a similar function as the vertebrate digestive mucous layer. Ingestion of blood contaminated with bacteria allows close contact of these microorganisms to the midgut epithelium before PM formation, which is completed formed only a few hours (14 to 24 hours) after a blood meal [7]. In fact, oral infection with sub-lethal concentrations of the non-pathogenic *Serratia marcescens* or the entomopathogenic *Pseudomonas entomophila* bacteria resulted in a significant increase in mitosis of the epithelium cells **(Fig 2A and 2B).** The increased cell turnover was also observed when heat-killed *P. entomophila* was provided through the blood, indicating that molecules derived from these entomopathogenic bacteria are sufficient to trigger the cell proliferation program, not necessarily requiring tissue infection **(Fig 2B)**. In this case, tissue damage may at least partially be attributed to the lack of cell membrane integrity promoted by Monalysin, a pore-forming protein produced by *P. entomophila* [29].

Supplementation of blood with diflubenzuron (DFB), a chitin synthesis inhibitor [30], leads to the inhibition of PM production, exposing the gut epithelium directly to the luminal content **(S1 Fig)**. Consequently, DFB administration resulted in elevated numbers of mitotic cells **(Fig 2C)**. The co-ingestion of antibiotics completely abolished this effect of DFB on cell proliferation (Fig 2C), demonstrating that in the absence of the microbiota, the lack of the peritrophic matrix did not result in elevated mitosis. These results indicate that not only oral infection with pathogenic bacteria, but also the proliferation of the resident microbiota (by inhibition of PM in this case), in contact with the epithelium, can trigger the midgut proliferative program.

Exposure of *D. melanogaster* enterocytes to bacteria results in ROS production as a microbiota control mechanism. However, the oxidative species produced as a result of bacterial presence can also cause damage to the midgut cells [31–34]. When mosquitoes were fed with blood

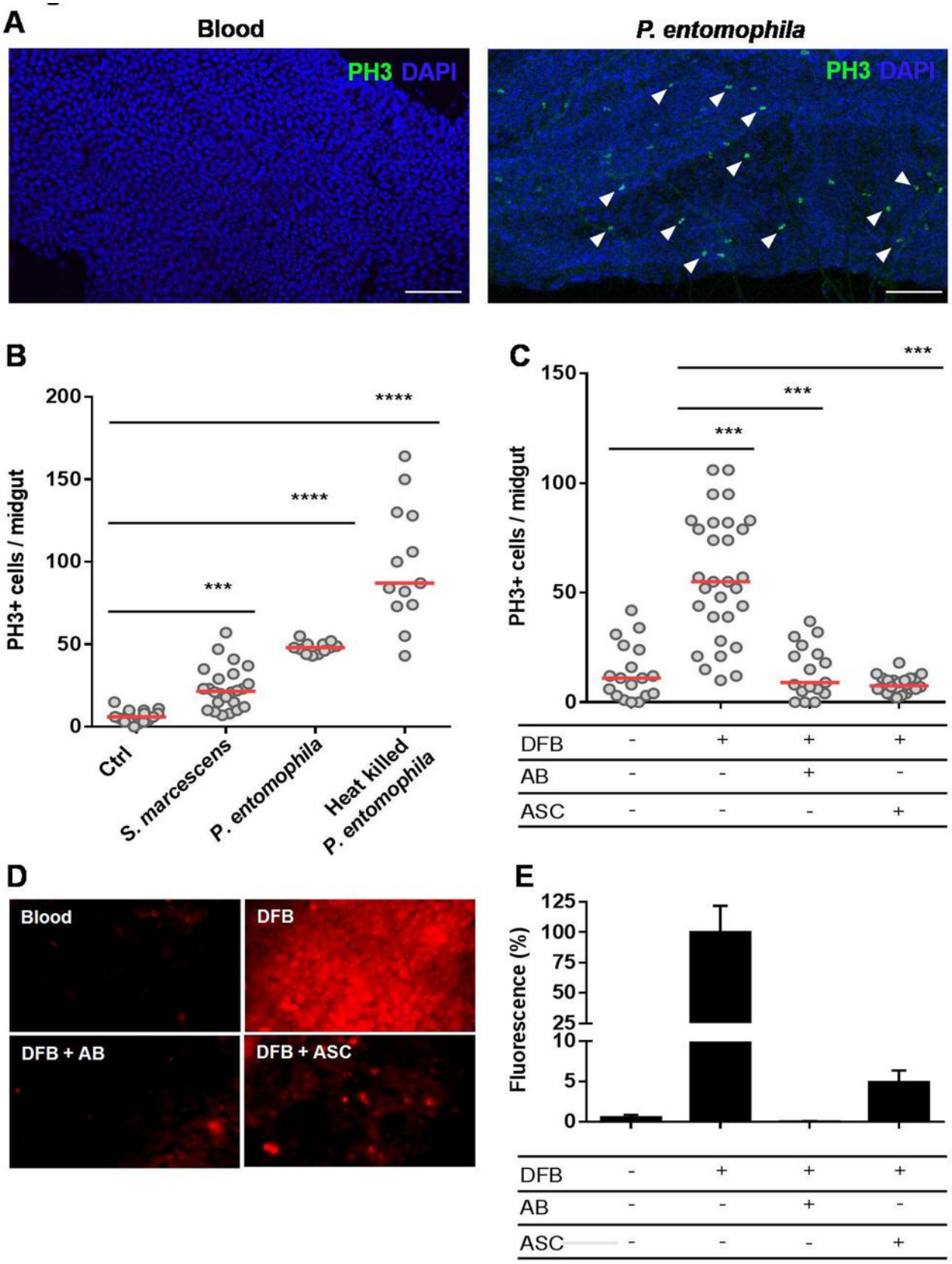
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Fig 2. The peritrophic matrix shapes intestinal homeostasis by limiting contact of the gut epithelium with the microbiota and preventing ROS production. Red strain mosquitoes were fed on normal blood or blood infected with non-pathogenic *S. marcescens* or entomopathogenic *P. entomophila* bacteria. Another group of mosquitoes was fed blood supplemented with heat-killed *P. entomophila*. The midguts were dissected 24 hours after feeding and immunostained for PH3. **(A)** Representative images of PH3-labeled mitotic cells (green) of the midgut epithelium 24 h after a naïve blood meal or blood infected with *P. entomophila*. The nuclei are stained with DAPI (blue). The arrowheads indicate PH3+ cells. Scale bar = 100 μm **(B)** Total PH3-positive cells were quantified from the midguts of mosquitoes fed on naïve and bacteria-infected blood (n = 25) or heat-inactivated *P. entomophila*. (n = 12). The medians of three independent experiments are shown. The asterisks indicate significantly different values *** $P<0.001$ and **** $P<0.0001$ (Student's t-test). **(C)** Inhibition of PM formation results in a significant increase of progenitors cells under mitosis. The mosquitoes were fed blood or blood supplemented with diflubenzuron (DFB), DFB plus an antibiotic cocktail (AB) or DFB plus 50 mM ascorbate (ASC). The midguts were dissected 24 hours after feeding, and the mitotic indices were quantified by counting PH3+ cells. The medians of at least three independent experiments are shown (n = 30). The asterisks indicate significantly different values *** $P<0.001$ and **** $P<0.0001$ (Student's t-test). **(D)** Assessments of reactive oxygen species in the midguts were conducted by incubating midguts of insects fed as in **(C)** with a 50 μM concentration of the oxidant-sensitive fluorophore DHE. **(E)** Quantitative analysis of the fluorescence images shown in (D) were performed using ImageJ 1.45s software (n = 7–9 insects).

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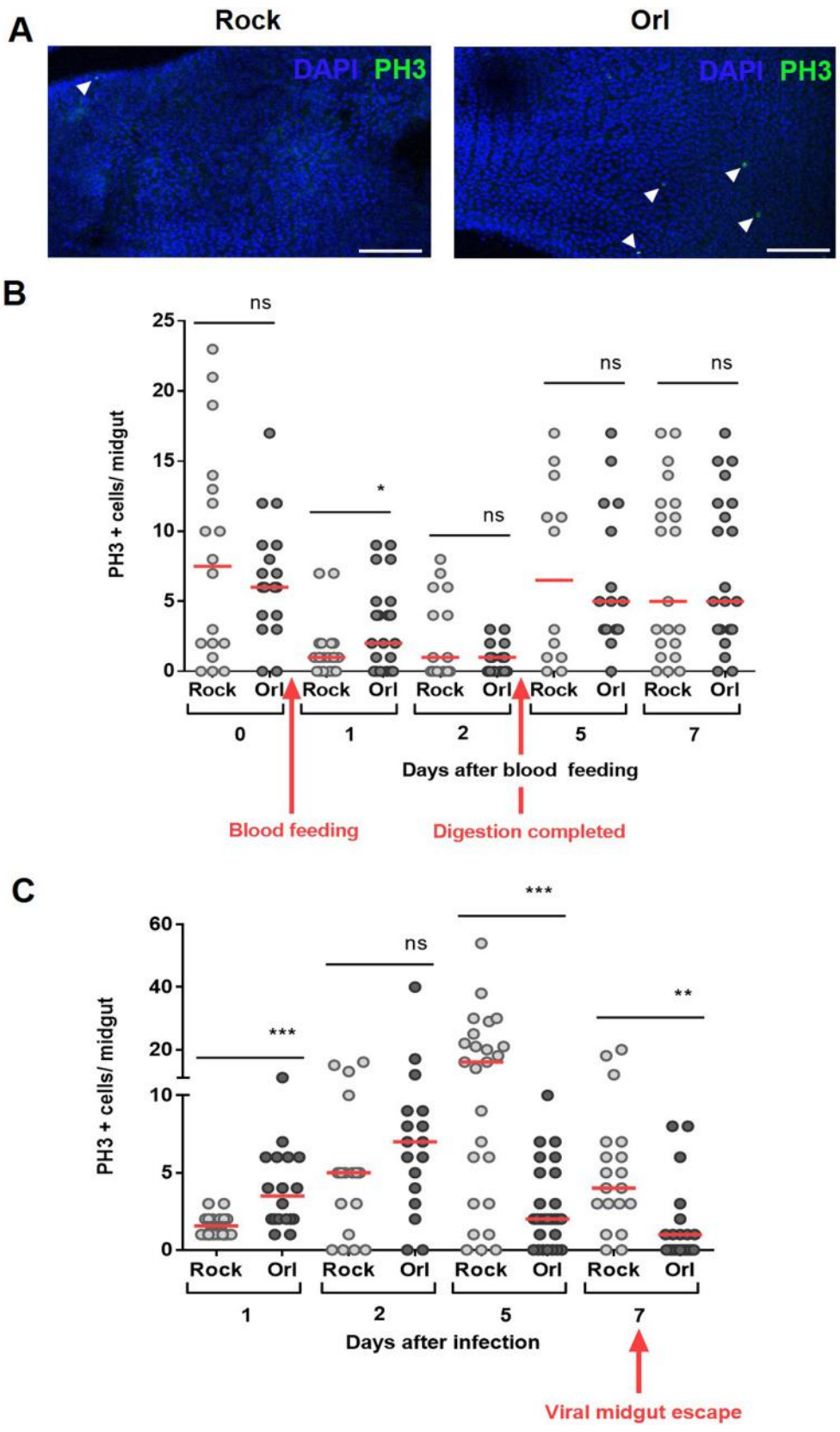
supplemented with DFB together with the antioxidant ascorbate (ASC), the mitosis levels dropped significantly (**Fig 2C**). The ROS production by the midgut epithelium was assessed by fluorescence microscopy using the fluorescent oxidant-sensing probe dihydroethidium (DHE). As shown in **Fig 2D and 2E**, the midguts of DFB-fed mosquitoes exhibited a high fluorescence signal, indicating an intense production of ROS. The intensity of the fluorescence signal of the DFB-treated midguts was significantly reduced upon ascorbate supplementation of the blood meal. Similarly, the suppression of microbiota with antibiotics dramatically reduced ROS levels. These results suggest a mechanism linking PM impairment to ISC proliferation, indicating that the direct exposure of the midgut epithelium to microbiota activates the production of ROS as part of an immune response.

Infection with dengue virus affects midgut epithelia regeneration

The role of epithelial tissue regeneration of the midgut upon viral infection has not been investigated in mosquitoes. Thus, we decided to evaluate the gut regeneration pattern of two mosquito strains that are known to exhibit different susceptibilities to DENV infection [28]. In basal conditions, i.e. sugar fed, all the strains used in this study presented no difference in the number of cells under mitosis (**S2 Fig**)**.** However, after 24 hours of taking a non-infected blood meal (day 1), the DENV refractory Orlando (Orl) strain presented a higher number of mitotic cells compared with the susceptible Rockefeller (Rock) strain (**Fig 3A and 3B**)**,** indicating that the refractory strain is naturally more proliferative than the susceptible one under these conditions. In the following days, both strains showed similar time course profiles of mitotic activity. Upon ingestion of DENV-infected blood, the refractory Orlando strain showed an increase of mitotic cells, peaking at the second day post blood meal (**Fig 3C**). Subsequently, these midguts showed low numbers of cells in mitosis throughout the remaining course of infection, reaching a similar number as non-infected midguts. In contrast, the susceptible Rockefeller strain showed a delayed regenerative response, only reaching the maximum rate at five days after infection (Fig 3C). These results suggest that the midgut cells of refractory mosquitoes are able to respond more promptly to the early events of infection.

To test whether the differences in gut homeostatic responses between the two strains could be a determinant of refractoriness/susceptibility, we disturbed the homeostatic condition of ISCs by silencing *delta* expression. The Notch ligand Delta (Dl) is an upstream component of the Notch pathway that is involved in cell division and differentiation. The *delta* gene is expressed in adult ISC cells. Thus, accumulation of Delta is used as a marker of ISCs in *D. melanogaster* [19]. Furthermore, Delta expression is induced by infection in the *D. melanogaster* midgut [35]. The efficiency and duration of Delta silencing by RNAi are shown in Fig 4A and S3 Fig, respectively. Silencing *delta* led to a significant reduction in mitosis in both mosquito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Days after blood feeding
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Days after infection
Viral midgut escape

Fig 3. Dengue virus infection impacts midgut homeostasis in a strain specific manner. **(A)** Blood feeding induces different levels of PH3 positive cells in the midgut of the susceptible (Rock) and refractory (Orl) strains 24 hours after the meal. Representative images of PH3 labeling in both strains 24 hours after the blood meal. The nuclei are stained with DAPI. The arrowheads indicate PH3+ cells. Scale bar = 100 μm. **(B)** Mosquitoes from the two strains were blood fed and at day zero (non blood-fed) or at different days after feeding, the midguts were dissected and immunostained for PH3. The red arrows indicate the time of blood feeding and the time in which the digestion is completed (after blood bolus excretion). In **(C)** the mosquitoes were fed on DENV2-infected blood and mitotic-cell counting was performed at different days after infection. The red arrow indicates the time of DENV escape from the midgut to hemocoel. The medians of at least three independent experiments are shown (n = 30). The asterisks indicate significantly different values * $P<0.05$ ** $P<0.01$ and *** $P<0.001$ (Student's t-test).

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strains (**Fig 4B and 4C**). Interestingly, silencing of *delta* did not have an effect on infection susceptibility in the Rockefeller strain (**Fig 4D**). In contrast, it significantly increased susceptibility of the Orlando strain to DENV infection, as observed by the increased viral titers in the *delta*–silenced refractory strain compared with the dsGFP-injected group (**Fig 4D**). Conversely, when the susceptible strain was pre-treated with DSS, a known inducer of midgut cell damage, and thereby ISC proliferation [18] and **S4 Fig**, a significant reduction was seen in both DENV infection intensity (**Fig 4E**) and prevalence (**Fig 4F**) in the midgut, compared with non-treated mosquitoes. Similar results were observed when DSS-treated Rock mosquitoes were infected with DENV4 isolates (**S5 Fig**). These data clearly indicate that the ability of midguts to respond at the cellular level, via regeneration of epithelial cells, modulates the success of viral infection of *A*. *aegypti*. Furthermore, these results show for the first time that the mosquito processes required to replenish damaged cells and control tissue homeostasis are determinants of vector competence.

Discussion

Cell renewal is known to be the basis of midgut epithelial integrity in model animals such as fly and mice [12]. Given the importance of the midgut epithelium in mosquitoes, where this tissue is effectively the first barrier that arboviruses affront to complete the transmission cycle [9], we decided to address the question of how this epithelium replenish its cells during the different challenges of blood feeding and infection. Previous descriptive reports of epithelial cell structure, function and midgut remodeling during metamorphosis [15,16,36] have shed some light on this process in mosquitoes, suggesting that the cell types described in other organisms, such as *D*. *melanogaster*, are also found in *A*. *aegypti*. Amongst the fully differentiated cells, the enterocytes were clearly distinguishable by their large nuclei size, abundance and localization [10,11]. However, due to the current lack of mosquito specific markers for other differentiated and progenitor cells, like ee's and EB's, only recently these cells were identified in mosquitoes larvae [37]. Nonetheless, ISC hallmark capacity is to undergo mitosis, which can be marked using antibodies for phosphorylated histone 3. This allowed us to successfully identify the presence of ISC in the epithelium, and to quantify the number or cells dividing in the different conditions evaluated (**Fig 1A and 1B**).

In the life history of mosquitoes, blood feeding represents a dramatic change from a sugar diet to ingestion of a large protein-rich meal. This transition imposes challenges to midgut homeostasis that are not faced by non-hematophagous insects. Knowledge about the mechanisms involved in the maintenance of midgut cellular integrity and homeostasis upon blood feeding or stress conditions is limited not only for *A*. *aegypti*, but also for other important vectors. In this study, we show unique properties of the mosquito midgut, suggesting that the regulation of epithelial cell proliferation is tightly regulated to allow proper handling of both chemical and biological sources of stress, including DENV infection, that occur during and

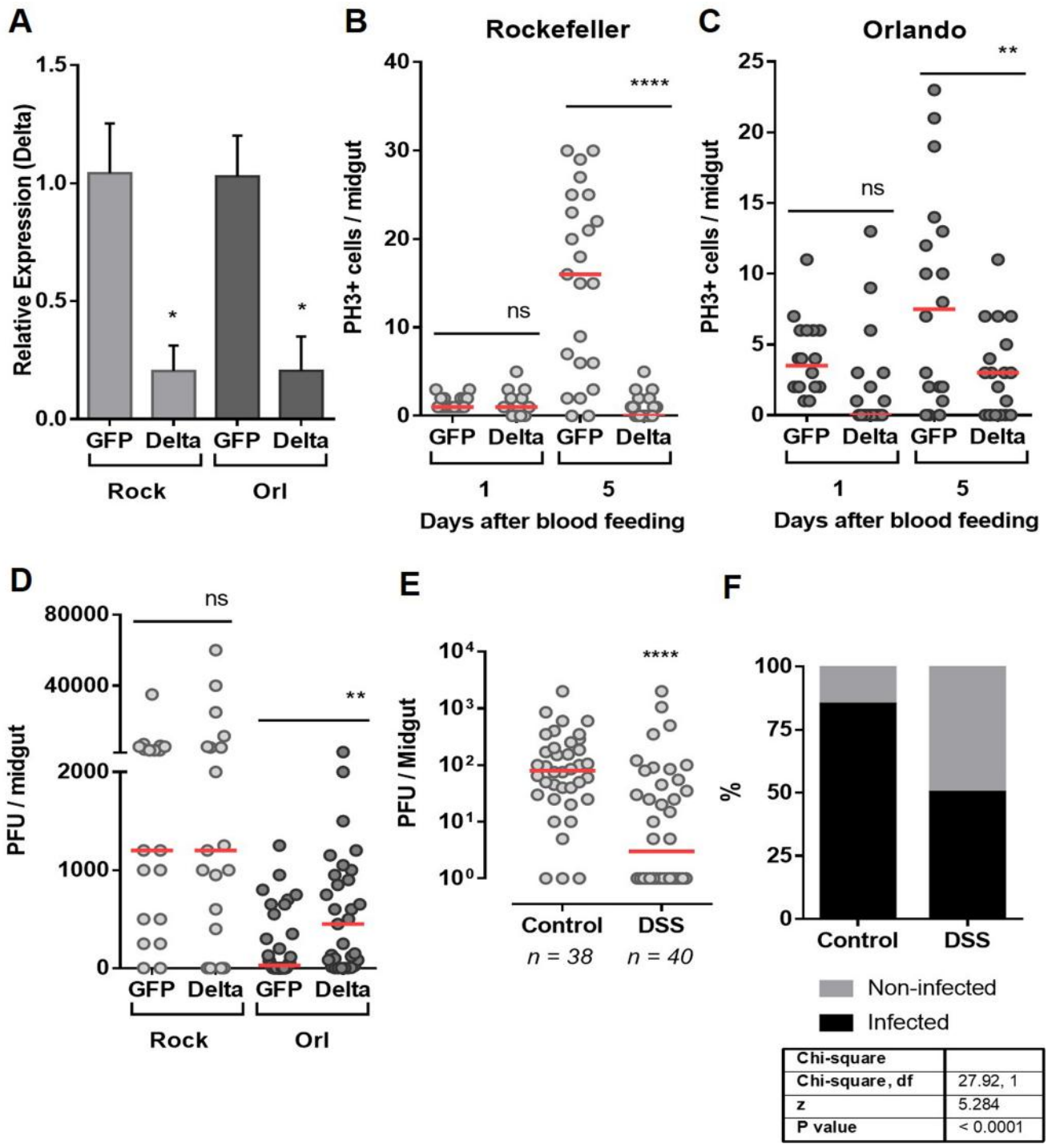

Fig 4. Interference in gut homeostatic response impacts vector competence. (A). The midguts of dsRNA-injected Rockefeller and Orlando mosquitoes were dissected 24 days after a blood meal for silencing quantification of Delta, the ligand of Notch. Total PH3-positive cells were quantified from midguts of silenced Delta or control (GFP) mosquitoes from the Rockefeller **(B)** or Orlando **(C)** strains, both 1 and 5 days after blood meal. **(D)** dsRNA-Injected mosquitoes were fed DENV2-infected blood, and 5 days after the infection, the midguts were dissected for the plaque assay. **(E)** The susceptible (Rockefeller) mosquitoes were pre-treated with the tissue-damaging dextran sulfate sodium (DSS) accordingly to material and methods section. Twelve hours after the end of the DSS treatment, the mosquitoes were fed with DENV-2-infected blood. After 5 days, the midguts were dissected for the plaque assay. **(F)** The percentage of infected midguts (infection prevalence) was scored from the same set of data as in (E). The medians of at least three independent experiments are shown. n = 20–25 in (A), (B) and (C); n = 20–26 in (D) and n = 40 in (E). Statistical analyzes used were: Student's t-test for (A), (B) and (C); Mann-Whitney U-tests were used for infection intensity (D and E); and chi-square tests were performed to determine the significance of infection prevalence analysis (F). $^{*}P<0.05$, $^{**}P<0.01$, $^{****}P<0.0001$.

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after blood digestion. Based on these findings, we suggest that this regulation of midgut homeostasis is an important determinant of viral infection dynamics in the vector gut.

In *A*. *aegypti*, the maximal digestion rate is attained 24 hours after a blood meal [38]. Despite the dramatic increase of the microbiota, approximately 1000 times the levels before a meal [5], mosquitoes seem to maintain midgut epithelial cell turnover controlled (**Fig 1C and 1D**). One explanation for this is the physical separation between the bolus and the epithelium by the PM. The PM is a thick extracellular layer composed mostly of chitin fibrils and glycoproteins that is gradually formed from 12–24 hours after a blood meal and surrounds the blood bolus, creating a physical separation from the midgut epithelium [7,8]. To preserve homeostasis, the PM establishes a selective barrier, permeable to nutrients and digestive enzymes but acting as a first line of defense against harmful agents. We show here that when the midgut epithelium was exposed to pathogenic bacteria ingested with the blood meal, thus before PM formation, there was a marked increase of mitosis (**Fig 2B**). More importantly, inhibition of the PM formation also resulted in elevated mitotic cell counts (**Fig 2C**). Treating insects with antibiotics abolished the mitosis upregulation promoted by chitin synthesis inhibition, further demonstrating that the contact of the blood bolus itself was not the determining factor to the increase mitotic cell numbers, but instead, the consequent exposure of the gut epithelium to the indigenous bacterial microbiota present in the lumen was the predominant event that elicited this response. In this way, the compartmentalization of the bolus may allow the enterocytes to minimize their exposure to deleterious agents, and it results in reduced need to shed and replenish damaged cells.

ROS production by midgut cells represents a major innate immunity effector mechanism that is involved in the control of the microbiota. However, ROS can also damage host cells, and thus, a proper balance between ROS production and microbial suppression is essential for the health of the host itself [31–34,39]. Here, we show that production of ROS was activated when PM formation was blocked and that this effect can be prevented by antibiotics (**Fig 2D**). Therefore, we propose that the signaling mechanism that leads to increased mitosis after exposure to indigenous bacteria is the production of ROS by the intestinal cells, as a defensive, yet possibly damaging, response (**Fig 2**).

The midgut epithelial cells are the first to support viral replication within the mosquito vector and several studies have addressed the immune response of the mosquito to the virus [40]. Additionally, it is well-established that changes in ROS production in the midgut impact not only innate immunity responses against bacteria, but can also affect the mosquito ability to transmit human pathogens [5,41–44]. Despite this comprehensive knowledge about infection-related processes that occur within midgut cells, little is known about the cell turnover prior to and after infection. It was our intention to evaluate if this natural process of the midgut epithelium was different between mosquito strains with different degrees of susceptibility to DENV. Rockefeller (Rock) and Orlando (Orl) strains are susceptible and refractory strains respectively; however, under normal (sugar fed) conditions, they possess similar levels of mitotic cells (**S2 Fig**). Interestingly, the Orl strain possesses higher levels of mitosis than the Rock strain 24 hours after the blood meal (**Fig 3A and 3B**). This increased number of mitotic cells, is restricted to this specific time window, as 48 hours after the feeding, the numbers are no longer significantly different. This fact becomes relevant when the timeline is superposed to the timeline of the initial viral infection [45]. This becomes more apparent, when the numbers of mitotic cells on the susceptible Rock strain increase after 5 days, in a consistent timeline to the establishment of a successful infection with higher levels of infected cells, which is not observed in Orl strain that constrains the infection. In day 7, when the viruses normally leave the midgut to infect other tissues [45], the mitotic rate is reduced to levels compared of non-infected sugar-fed midguts in both strains (Fig 3C). Transcriptomic analyses of mosquito

strains with different degrees of susceptibility to DENV revealed that some genes associated with cellular proliferation, growth and death are differentially expressed in refractory strains, upon DENV infection [46–49]. However, this has not been directly associated to midgut regeneration in these studies. In addition, the increased expression and activation of a variety of apoptotic cascade components in the midgut after viral infections implicate apoptosis as part of the *A*. *aegypti* defense against arboviruses [24,25,27]. Altogether, these studies pointed to the significant importance of cell replenishing in the midgut epithelium to vector competence. Because of that, we decided to target the Notch pathway through RNAi; to disturb the normal regenerative process of the epithelium. Amongst the proteins involved in this pathway, the ligand Delta was an excellent candidate for RNAi because it is upstream of the Notch signaling pathway and is considered a marker of ISC [19]. Induction of RNAi by injection of dsDelta in adult females led to the silencing of the Notch ligand Delta and resulted on reduced cell division (**Fig 4B and 4C**), as previously reported by Guo and Ohlstein (2015) in *D*. *melanogaster* and by VanDussen et al (2012), in mice. As knockdown of Delta resulted on increased DENV2 viral titers in refractory strain (**Fig 4D**), this suggested that cell regeneration is also a contributing factor to the modulation of viral infection and consequently to refractoriness. In addition to this result, we pre-treated mosquitoes of the susceptible strain (Rockefeller) with DSS, to induce cell division. Likewise, we found that the increase in mitosis was able to expand refractoriness of these mosquitoes. Our data shows for the first time that the ability to replenish the epithelial differentiated cells, by ISC engagement in tissue regeneration, is an important aspect of the mosquito's antiviral response in these strains. Furthermore, these results revealed that the involvement of the Notch signaling pathway in midgut cell proliferation is also conserved in *A*. *aegypti*. Additional work is required to further determine the involvement of the other cell types and to detail the mechanism by which Delta-Notch signaling interferes in midgut cell proliferation in the midgut of *A*. *aegypti*. Very recently, it has been shown that both Delta and Notch transcriptions were induced in midgut of DENV2-primered mosquitoes [50], suggesting that this pathway is important to the vector defense against DENV infection. The role of other pathways previously shown to regulate progenitor cell and differentiation in *D*. *melanogaster* and mammalians, such as the Hippo, JAK-STAT and other pathways, may also reveal key connections between intestinal cell replenishment and vectorial competence. The development of specific markers for each *A*. *aegypti* epithelial cell type would allow the evaluation of the fate of the new cells produced after ISC division, which could also give important insights on the entire process of midgut regeneration.

The first 24–48 h after ingestion of virus infected blood are considered the most critical for determining vector competence of a given mosquito (reviewed in [51]). Accordingly, we propose that the mitotic events in the early stages of infection (e.g., 24 h after viral ingestion) occur when the number of infected cells is still low and the capacity to eliminate damaged cells prevents viral spreading, and therefore must be effective to limit the infection. The number of mitotic cells of the refractory strain midgut at this initial time point is higher than in the susceptible strain, implicating this as a likely determinant for refractoriness (**Fig 4A and 4B**). The differences observed in the total number of mitotic cells and in the pattern of recovery between Rockefeller and Orlando strains may suggest more extensive damage in the midgut of the susceptible mosquitoes caused by virus infection. However, the correlation between viral infection progression, cell damage and regenerative responses in the early infection remains to be investigated. In addition, it is also of great importance to investigate the impact of midgut cell renewal on the cellular mechanisms that have been associated with the overcoming of the midgut escape barrier, leading to the dissemination of arboviruses and impacting the vector competence, such as disassembly of basal lamina [52], apoptosis [53] or midgut conduits [54].

In conclusion, our data suggest that the midgut infection by DENV is favored by delayed midgut renewal in a permissive mosquito strain and that refractoriness would be supported, at least partly, by the capacity to promptly activate the ISC division program. At the present time, dengue, chikungunya and zika viruses are widespread across the globe, and the understanding of the multiple factors affecting virus infection within the mosquito is crucial. The fact that faster cell renewal could be related to refractoriness adds up a new factor to be considered among the many determinants of vector competence and opens up the spectrum of the vector physiological events that are important when studying viral transmission. Future research is required to test if other DENV refractory field strains also possess differential tissue homeostatic properties and if a similar mechanism will occur in other arboviral infections. These findings reveal a new path towards a better understanding of vector competence, and may support the development of alternative strategies of virus transmission control.

Finally, these results highlight that the rate of midgut cell renewal should be taken into account when choosing mosquito strains for vector control strategies that use population replacement, such as SIT or *Wolbachia* based methodologies.

Materials and methods

Ethics statement

All experimental protocols and animal care were carried out in accordance to the institutional care and use committee (Comitê para Experimentação e Uso de Animais da Universidade Federal do Rio de Janeiro/CEUA-UFRJ) and the NIH Guide for the Care and Use of Laboratory Animals (ISBN 0–309-05377-3). The protocols were approved under the registry CEUA-UFRJ #155/13. All animal work at JHU was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH), USA. The protocols and procedures used in this study were approved by the Animal Care and Use Committee of the Johns Hopkins University (Permit Number: M006H300) and the Johns Hopkins School of Public Health Ethics Committee.

Rearing of *Aedes aegypti* mosquitoes

The *Aedes aegypti* (Red Eye strain) were raised at the insectary of UFRJ under a 12-hour light/dark cycle at 28˚C and 70–80% relative humidity. The adults were maintained in a cage and given a solution of 10% sucrose *ad libitum* unless specified otherwise. The *A*. *aegypti* (Rockefeller and Orlando strains) were raised at the insectary of JHU under a 12-hourlight/dark cycle, at 27˚C and 95% humidity. The adults were maintained in a cage and given a solution of 10% sucrose *ad libitum*. The adult females were dissected at different times after blood feeding for the experiments.

Mosquito antibiotic treatment

The mosquitoes were rendered free of cultivable bacteria by maintaining them on a 10% sucrose solution with penicillin (100 u/mL), and, streptomycin (100 μg/mL) from the first day post-eclosion until the time of dissection post blood feeding.

Mosquito meals

The *A*. *aegypti* mosquitoes from the Red Eye strain (four- to seven-days-old) were artificially fed with heparinized rabbit blood. The feeding was performed using water-jacketed artificial feeders maintained at 37˚C and sealed with parafilm membranes. The insects were starved for

4–8 hours prior to the feeding. Unfed mosquitoes were removed from the cages in all the experiments.

The oxidative challenge was provided by addition of 500 μM of paraquat (ChemService, West Chester, PA, USA) to the blood meal. As an antioxidant treatment, 50mM of ascorbic acid (neutralized to pH 7.0 with NaOH) was also added to blood. The mosquitoes were orally infected by *Serratia marcescens* BS 303 strain or *Pseudomonas entomophila* L48 strain at a concentration of 10^5 bacteria/mL of blood. Briefly, overnight cultures were used either live or after heat inactivation. Inactivation of *P. entomophila* was done by heating at 98˚C for 1 hour. Live and heat-killed bacteria were all pelleted after OD600 measurements to achieve final concentration of 10^5 bacteria/mL of blood. The media supernatant was discarded and the cell pellet was resuspended in blood previous to the mosquito feeding. The compound diflubenzuron (DFB) (0.4 g/L), a well-known chitin synthesis inhibitor, was added to the blood meal to prevent the peritrophic matrix establishment [30].

To stimulate ISC proliferation and midgut regeneration [18], the mosquitoes were fed with 1% DSS (dextran sulfate sodium salt 6.5–10 kDa, Sigma, St. Louis, MO, USA) dissolved in 10% sucrose for 2 days before infection. Twelve hours prior to infection, the DSS-sucrose solution was substituted with a 10% sucrose solution to remove residual DSS from the midgut content. The control mosquitoes were fed with 10% sucrose only. The infection with DENV was carried out as described in the following sections.

Proliferation and mitotic cells quantification

The quantification of mitosis in whole midgut tissues was performed by PH3 labeling as described elsewhere [55]. Briefly, female adult mosquitoes were dissected in PBS. Midguts were fixed in PBS with 4% paraformaldehyde for 30 minutes at room temperature. Samples were washed in PBS for 2 times of 10 minutes each. Then the tissues were permeabilized in PBS with 0.1% X-100 (for 15 min at room temperature) and blocked in a blocking solution containing PBS, 0.1% Tween 20, 2.5% BSA and 10% normal goat serum for at least 30 min at room temperature. All samples were incubated with primary antibody mouse anti-PH3 (1:500, Merck Millipore, Darmstadt, Germany). After washing 3 times of 20 minutes each in washing solution (PBS, 0.1% Tween 20, 0.25% BSA), samples were incubated with secondary goat anti-mouse antibody conjugated with Alexa Fluor 488 or 546 (Thermo Fisher Scientific, MA, USA) for at least 1 hour at room temperature at a dilution of 1:2000. DNA was visualized with DAPI (1mg/ml, Sigma), diluted 1:1000. The gut images were acquired in a Zeiss Observer Z1 with a Zeiss Axio Cam MrM Zeiss, and the data were analyzed using the AxioVision version 4.8 software (Carl Zeiss AG, Germany). Representative images were acquired using a Leica SP5 confocal laser-scanning inverted microscope with a 20X objective lens. Images were processes using Las X software.

WGA and phalloidin staining

Midguts from insects that were fed on naive blood or blood with DFB were dissected 24 h after feeding and fixed in 4% paraformaldehyde for 3 h. All of the midguts were kept on PBS-15% of sucrose for 12 h and then in 30% sucrose for 30 h. After a 24-h infiltration in OCT, serial microtome 14-lm-thick transverse sections were obtained and collected on slides that were subsequently labeled with the lectin WGA (Wheat Germ Agglutinin; a lectin that is highly specific for N-acetylglucosamine polymers) coupled to fluorescein isothiocyanate (FITC). The slides were washed 3 times in PBS buffer containing 2 mg/mL BSA (PBSB). The samples were then incubated in 50mM NH4Cl/PBS for 30 min; in 3% BSA, 0.3% Triton X-100 PBS for 1 h; and in PBSB solution with 100 mg/mL WGA-FITC (EY Laboratories) for 40 min. The slides

were then washed three times with PBSB and mounted with Vectrashield with DAPI mounting medium (Vector laboratories). The sections were acquired in an Olympus IX81 microscope and a CellR MT20E Imaging Station equipped with an IX2-UCB controller and an ORCAR2 C10600 CCD camera (Hammamatsu). Image processing was performed with the Xcellence RT version 1.2 Software.

Midguts from insects that were fed on blood alone or blood with DENV-2 were dissected 5 days after feeding and fixed in 4% paraformaldehyde using the same protocol as for mitotic cell quantification. After the secondary antibody incubation washes, 30 min incubation with phalloidin 1:100 (1uL) in 98uL blocking solution, along with the DAPI (1:100) was done at room temperature protected from light. Samples were washed twice, for 5 minutes (stationary, room temperature, protected from light) in 0.5mL washing solution and then onto slides with VectaShield. Images (z-stack of 0.7 μm slides) were taken on a Zeiss LSM700 laser scanning confocal microscope at the Department of Cell Biology at JHU with a 20X objective lens and processed using Zeiss Zen Black Edition software.

ROS detection in the midgut

The mosquito midguts were dissected in PBS 24h after feeding and incubated with 50μM of dihydroethidium (hydroethidine; DHE; Invitrogen) diluted in Leibovitz-15 media supplemented with 5% fetal bovine serum for 20 min at room temperature in the dark. The incubation media was gently removed and replaced with a fresh dye-free media. The midguts were positioned on a glass slide, and the oxidized DHE-fluorescence was observed by a Zeiss Observer Z1 with a Zeiss Axio Cam MrM Zeiss using a Zeiss-15 filter set (excitation BP 546/12; beam splitter FT 580; emission LP 590) (Carl Zeiss AG, Germany) [5,56].

RNA extraction and qPCR analysis

For the qPCR assays, the RNA was extracted from the midgut using TRIzol (Invitrogen, CA, USA) according to the manufacturer's protocol. The complementary DNA was synthesized using the High-Capacity cDNA Reverse transcription kit (Applied Biosystems, CA, USA). The qPCR was performed with the StepOnePlus Real Time PCR System (Applied Biosystems, CA, USA) using the Power SYBR-green PCR master MIX (Applied Biosystems, CA, USA). The Comparative Ct method [57,58] was used to compare the changes in the gene expression levels. The *A*. *aegypti* ribosomal S7 gene was used as an endogenous control [59]. The oligonucleotide sequences used in the qPCR assays were S7 (AAEL009496-RA): S7_F: GGGACAAATC GGCCAGGCTATC and S7_R: TCGTGGACGCTTCTGCTTGTTG; Delta (AAEL011396), Delta_Fwd: AAGGCAACTGTATCGGAGCG and Delta_Rev: TATGACATCGCCAAACG TGC.

Gene silencing

Two- to three-day old mosquito females (Rockefeller and Orlando) were cold anesthetized and 69 nL of 3 μg/μL dsRNA solution was injected into the thorax. Three days after injection, the mosquitoes were infected with DENV. Mosquito midguts were collected after 24h for real time PCR and after 5 days for mitosis assay or DENV infection analysis. The HiScribe T7 *in vitro* transcription kit (New England Biolabs) was used to synthesize the dsRNA. The unrelated dsGFP was used as a control, and the silencing efficiency was confirmed through qPCR. To generate dsDelta, the following oligonucleotides (containing the T7 polymerase-binding site) were used:

dsDelta_Fwd: GTAATACGACTCACTATAGGGAGCAAGCCTAACGAGTGCAT

dsDelta_Rev: GTAATACGACTCACTATAGGGTTCCTTCTCACAGTGCGTCC

Dengue virus propagation and mosquito infections

The DENV-2 (New Guinea C strain) was propagated for 6 days in C6/36 cells maintained in complete MEM media supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% non-essential amino acids and 1% L-glutamine. The virus titer was determined by plaque assay as 10^7 PFU/mL [60]. The females were infected through a blood meal containing: one volume of virus, one volume of human red blood cells (commercial human blood was centrifuged and the plasma removed), 10% human serum and 10% 10 mM ATP. Unfed mosquitoes were removed from the cages. The midguts were dissected at 5 days post-blood meal and stored individually in DMEM at -80˚C until used.

For DENV-4 (Boa Vista 1981 strain) propagation, the virus was cultivated 6 days in C6/36 cells maintained in Leibovitz-15 media supplemented with 5% fetal bovine serum, 1% non-essential amino acids,1% penicillin/streptomycin and triptose (2.9 g/L) [61]. The virus titer was determined by plaque assay as 10^7 PFU/mL. The females that were pre-treated with DSS or regular sucrose (control) were infected using one volume of rabbit red blood cells and one volume of DENV-4. The midguts were dissected at 7 days after infection and stored individually in DMEM at -80˚C until used.

Plaque assay

The plaque assay was performed as previously described [28]. The BHK-21 cells were cultured in complete DMEM media, supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% L-glutamine. One day before the assay, the cells were plated into 24 wells plates at 70–80% confluence. The midguts were homogenized using a homogenizer (Bullet Blender, Next, Advance) with 0.5mm glass beads. Serial dilutions (10 folds) were performed, and each one was inoculated in a single well. The plates were gently rocked for 15 min at RT and then incubated for 45 min at 37˚C and 5% CO_2. Finally, an overlay of DMEM containing 0.8% methylcellulose and 2% FBS, was added in each well, and the plates were incubated for 5 days. To fix and stain the plates, the culture media was discarded and a solution of 1:1 (v:v) methanol and acetone and 1% crystal violet was used. The plaque-forming units (PFU) was counted and corrected by the dilution factor.

Statistical analysis

Unpaired Student's t-tests were applied where comparisons were made between two treatments or two different mosquito strains, as indicated in the figure legends. Mann-Whitney U-tests were used for infection intensity and chi-square tests were performed to determine the significance of infection prevalence analysis. All statistical analyses were performed using GraphPad 5 Prism Software (La Jolla, United States).

Supporting information

S1 Fig. Peritrophic matrix formation after a meal. Midguts of females that were fed on blood alone or blood with DFB. Insects were dissected and fixed, and sections 0.14 μm were stained with WGA-FITC (green) and DAPI (blue). The peritrophic matrix is indicated by a white arrow. (L) indicates the luminal side where the PM forms.
(TIF)

S2 Fig. Comparison of sugar *Aedes aegypti* fed strains. *A. aegypti* Red-eye, Rockefeller and Orlando strains were collected 5 days after emergence. All PH3 positive cells in the anterior midgut were counted. No significant difference was observed between the groups. Klustal-

Wallis test.
(TIF)

S3 Fig. Duration of the silencing effect after Delta dsRNA injection. Midguts of females that were injected with dsRNA for the Delta gene were dissected at 3 and 5 days after blood feeding and the RNA extracted for gene expression analysis. dsLacZ was used as experimental control of non- related dsRNA.
(TIF)

S4 Fig. DSS treatment increases migdut mitosis. Rockefeller mosquitoes were pre-treated with the tissue-damaging dextran sulfate sodium (DSS) for 3 (three) days and blood fed. After 24 hours, total PH3-positive cells were quantified from midguts of Control (sugar fed) or DSS (1% DSS in the sugar solution) mosquitoes.
(TIF)

S5 Fig. DSS treatment decreases both DENV4 infection intensity and prevalence. (A) Rockefeller mosquitoes were pre-treated with the tissue-damaging dextran sulfate sodium (DSS) and infected with DENV-4. After 5 days, the midguts were dissected for the plaque assay. (B) The percentage of infected midguts (infection prevalence) was scored from the same set of data as in A. Statistical analyzes used were Mann-Whitney U-tests for infection intensity (A) and chi-square tests for the infection prevalence analysis (B). ** $P<0.01$.
(TIF)

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The redox-sensing gene *Nrf2* affects intestinal homeostasis, insecticide resistance, and Zika virus susceptibility in the mosquito *Aedes aegypti*

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Production and degradation of reactive oxygen species (ROS) are extensively regulated to ensure proper cellular responses to various environmental stimuli and stresses. Moreover, physiologically generated ROS function as secondary messengers that can influence tissue homeostasis. The cap'n'collar transcription factor known as nuclear factor erythroid-derived factor 2 (Nrf2) coordinates an evolutionarily conserved transcriptional activation pathway that mediates antioxidant and detoxification responses in many animal species, including insects and mammals. Here, we show that Nrf2-mediated signaling affects embryo survival, midgut homeostasis, and redox biology in *Aedes aegypti*, a mosquito species vector of dengue, Zika, and other disease-causing viruses. We observed that AeNrf2 silencing increases ROS levels and stimulates intestinal stem cell proliferation. Because ROS production is a major aspect of innate immunity in mosquito gut, we found that a decrease in Nrf2 signaling results in reduced microbiota growth and Zika virus infection. Moreover, we provide evidence that AeNrf2 signaling also controls transcriptional adaptation of *A. aegypti* to insecticide challenge. Therefore, we conclude that Nrf2-mediated response regulates assorted gene clusters in *A. aegypti* that determine cellular and midgut redox balance, affecting overall xenobiotic resistance and vectorial adaptation of the mosquito.

Aedes aegypti is an invasive mosquito species and represents a significant threat worldwide because of its ability to transmit dengue and, more recently, chikungunya and Zika viruses (1–3). The main strategies to eliminate the mosquito are based on vector control methods, such as removal of potential breeding sites and the use of insecticides, which has been intensified since the 1980s due to continuous dengue outbreaks. However, given the increasing insecticide resistance in vector populations, these control measures are becoming ineffective (4, 5). Significant efforts have been devoted to developing novel strategies for mosquito control, including the use of genetically modified mosquitoes carrying genes that make them refractory to arbovirus infection (6, 7). Because the midgut is the initial site of infection in the mosquito, it also constitutes the ideal site for overexpressing/activating candidate antiviral effectors (8, 9).

Reactive oxygen species (ROS)[2] are implicated in direct killing of pathogens, increased tissue damage, and a variety of cell signaling processes. In mosquitoes, ROS-mediated signaling has been especially studied in association with the control of malaria parasite development in the midgut of *Anopheles* mosquitoes (10, 11) and less intensively investigated regarding viral replication in *A. aegypti* (12, 13). The cap'n'collar transcription factor, also named nuclear factor-erythroid 2 p45-related factor 2 (Nrf2), coordinates the regulation of drug detoxification, GSH homeostasis, and NADPH regeneration with oxidative stress. Nrf2 is structurally and functionally conserved from insects to humans (14), and it heterodimerizes with the small Maf transcription factors to bind a consensus DNA sequence (the antioxidant response element (ARE)) and regulate gene expression (15–17). Nrf2 therefore plays a pivotal role in cellular adaptation to ROS and xenobiotics (14). More recently, functions beyond stress response have been attributed to Nrf2, such as control of energy metabolism and stem cell regulation (18–20).

Here, we show that this ancient redox-sensitive Nrf2 pathway has a pivotal role in stress responses in the adult mosquito and embryonic survival. *Nrf2* depletion alters the redox balance in the midgut, which results in intestinal stem cell proliferation, microbiota growth impairment, and lowered viral infection. AeNrf2 also impacts metabolic adaptation to insecticide resistance. We discuss the importance of this pathway for the overall control of arboviral transmission.

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This article contains Tables S1–S4 and Figs. S1–S4.

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[2] The abbreviations used are: ROS, reactive oxygen species; PH3, phosphohistone 3; ISC, intestinal stem cell; ARE, antioxidant response element; gRNA, guide RNA; DHE, dihydroethidium; qPCR, quantitative PCR; GST, glutathione *S*-transferase; DMEM, Dulbecco's modified Eagle's medium.

Results

The *cap'n'collar* locus in *Drosophila melanogaster* produces multiple alternatively spliced transcripts, giving rise to three main Cnc proteins, CncA, CncB, and CncC, of different sizes and domain compositions. All of these include the COOH-terminally located bZIP DNA-binding and dimerization domain (21). There are two proteins in *A. aegypti*, AAEL015467 and AAEL005077, with sequence similarity to the *D. melanogaster* Cnc. Both are identical at their C-terminal regions (Neh domains 1, 3, and 6), but only AAEL005077 has an N-terminal sequence that contains domains similar to Neh2, -4, and -5. These latter domains are important for transactivation and redox regulation in the *D. melanogaster* (CncC) and mammalian (Nrf2) homologs (Fig. S1*A* and Fig. S2). We therefore designated AAEL005077 as AeNrf2/CncC and AAEL015467 as AeNrf2/CncA because the latter has higher similarity to the shorter Cnc A isoform (Fig. S2). We focused on AeNrf2/CncC (hereafter referred to as AeNrf2) for genetic targeting and characterization of the associated phenotypes because of its greater similarity to CncC, previously described as a redox-regulated transcription factor (22).

Nrf2-knockout mice are viable and fertile, although they exhibit increased sensitivity to environmental stressors (14). We attempted to generate somatic loss-of-function mutants to investigate the role of AeNrf2 in mosquito development by injecting Cas9 protein and *in vitro*-transcribed guide RNAs (gRNAs) targeting AeNrf2 and *KMO* (kynurenine monooxygenase) gene into early-stage embryos (60–75 min after egg deposition). *KMO* was targeted as a positive control for Cas9 activity (23). In contrast to the results in mice, AeNrf2 knockouts in *A. aegypti* result in decreased embryonic survival (Table S1). Embryonic survival rates following multiplex injections of one *KMO* gRNA together with an increasing number (1, 2, and 5) of different *Nrf2*-targeted gRNAs were 19.1, 8.6, and 6.8%, respectively. DNA lesions in the *KMO* gene were confirmed and associated specifically with the *KMO* gRNA target site. However, the majority (75%) of the Nrf2 gRNA target sequences analyzed had a much lower efficiency rate (≤30%) for Cas9-induced mutations, and >50% of the analyzed individuals had no insertions or deletions detected in their pooled DNA sequences (Table S2). Taken together, these results support the conclusion that the disruption of *Nrf2* probably produces an embryonic lethal phenotype, and this results in no mutations being detected in later developmental stages.

Nrf2 connects antioxidant response and redox balance

Vertebrate Nrf2 and *D. melanogaster* CncC regulate the basal and inducible expression of antioxidant and detoxifying genes (22, 24). We conducted a series of experiments to test whether the mosquito ortholog has a similar role. Paraquat-induced expressions of GSH *S*-transferase (*GSTX2*, AAEL010500), cytochrome P450 (*CYP6M11*, AAEL009127), and members of the GSH biosynthesis pathway (namely glutamate cysteine ligase (*GCLC*; AAEL008105) and glutathione synthetase (AAEL009154)) were impaired in the midgut by RNAi-mediated knockdown of *Nrf2* (Fig. 1). Furthermore, Nrf2 homologs coordinate transcriptional activation induced by electrophilic

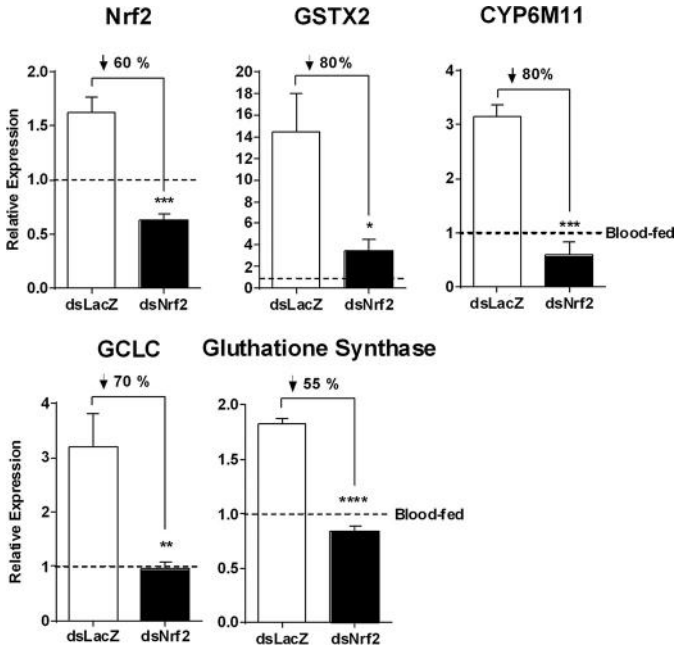


Figure 1. AeNrf2 regulates stress response genes. Mosquitoes were injected with dsRNA for *Nrf2* and *LacZ* (a control dsRNA). 48 h postinjection, mosquitoes were fed with either blood or blood supplemented with 1 mM paraquat. After 24 h, midguts were dissected for RNA extraction and mRNA quantification by qPCR. Shown is knockdown efficiency in the mosquito midgut injected with dsNrf2 (*Nrf2*); *Nrf2*-knockdown effect in GSH *S*-transferase (*GSTX2*), cytochrome P450 (*CYP6M11*), glutamate cysteine ligase (*GCLC*), and GSH synthetase. Gene expressions are relative to tissues from dsLacZ blood-fed injected mosquitoes (evidenced by the *dashed line*). Results are for pools of at least three independent experiments. *Error bars*, S.E. Statistical analyses were made by Student's *t* test. ***, $p < 0.001$.

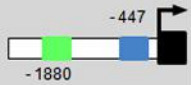
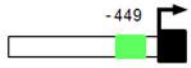
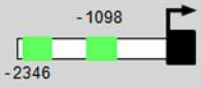
reagents through the ARE (22, 24, 25). A bioinformatics *de novo* motif discovery (26), on *GSTX2* and *CYP6M11* DNA regions, identified two putative ARE motifs that have high sequence similarity to Nrf2/Maf binding sites from the publicly accessible DNA motif JASPAR database (27) (Fig. S1*B*). This consideration was based on the hypothesis that sequence similarity reflects functional analogy. These putative AREs, of 14 and 19 base pairs, were named AeARE1 and AeARE2, respectively, and were found in tandem repeats in the promoter regions of both *GSTX2* and *CYP6M11* (Table 1). Furthermore, a search using previous transcriptomic profiling data from *A. aegypti* cells under heme or paraquat stimulation (28) found that ~70% of the up-regulated transcripts have *cis*-located elements with significant matches to either AeARE1 or AeARE2 in their promoters (Table S3). These include some genes that are not directly involved with detoxification processes, such as energy metabolism genes. This finding indicates that a large fraction of heme and paraquat-induced transcriptional responses are associated with Nrf2 signaling.

We used HPLC quantification to evaluate the redox state of the gut by assaying fluorescent products of a ROS-sensitive probe, dihydroethidium (DHE), following *Nrf2* knockdown. *Nrf2* disruption resulted in significantly increased ROS levels in the midgut even after a regular blood meal (Fig. 2*A*). Taken together, these findings support the conclusion that transcriptional control of key cytoprotective genes through the Nrf2/ARE axis is an ancient mechanism conserved in evolution and

Table 1

Nrf2 target genes analyzed in this study

Shown is a summary of transcription data available for each (with references) and a schematic representation of Nrf2-binding sites (AREs) identified in their promoter regions, ARE1 and ARE2, represented in blue and green, respectively. Numbers indicate the position relative to the transcriptional initiation site.

	Gene	Transcriptomic Upregulation Data	Nrf2 Binding Site
AAEL005424	Actin binding protein KEAP1	▪ Natural pyrethroid-resistant populations (59). ▪ Dengue virus-infected female mosquitoes (86).	
AAEL010500	Glutathione s-transferase GSTX2	▪ Heme- and paraquat-treated *A. aegypti* cells (28). ▪ Natural pyrethroid-resistant populations from the Caribbean (56).	
AAEL009127	Cytochrome P450 CYP6M11	▪ Heme-treated *A. aegypti* cells (28). ▪ Detoxification tissues in response to different xenobiotic exposures (50).	
AAEL014893	Cytochrome P450 CYPBB2	▪ Natural pyrethroid-resistant populations from the Caribbean (56). ▪ Laboratory selected insecticide-resistant strains (57).	
AAEL008105	Glutamate Cysteine Ligase GCLC	▪ Heme-treated *A. aegypti* cells (28). ▪ Natural pyrethroid-resistant populations (59).	
AAEL009154	Glutathione Synthase	▪ Heme-treated *A. aegypti* cells (28). ▪ Xeniobiotic pre-exposed permethrin-resistant strains (87). ▪ DENV2-infected resistant mosquitoes (88).	

important for maintenance of the redox balance in the midgut of the mosquito.

Nrf2 signaling is involved with insecticide resistance in A. aegypti

The Nrf2 pathway is constitutively active in insecticide-resistant strains of *D. melanogaster* (29) and we hypothesized that AeNrf2 signaling could contribute to enhanced insecticide resistance of certain mosquito populations. Two Brazilian field populations with distinctive susceptibility to pyrethroid and organophosphate insecticides, Caseara (Susceptible, S) and Oiapoque (Resistant, R), and a control laboratory strain (Rockefeller) were used to investigate basal expression of selected putative Nrf2 target genes and of *Nrf2* itself. Although *Nrf2* had similar transcript expression levels among the tested populations, Kelch ECH–associating protein 1 (*Keap1*), *GSTX2*, and *CYP6BB2* were expressed at significantly higher levels in the resistant Oiapoque strain, when compared with the susceptible Caseara strain (Fig. 3*A*).

dsRNA silencing of *Nrf2* in 2-day-old Oiapoque (R) females resulted in a concomitant decrease in the accumulation of the transcripts of *Keap1*, *GSTX2*, and *CYP6BB2* (Fig. S3). These mosquitoes were also more sensitive to the insecticide malathion exposition than the ones that had been injected with dsLacZ control (Fig. 3*B*). The mortality for the *Nrf2*-silenced cohorts was 3.7-fold higher compared with controls. These results support the conclusion that activation of the Nrf2 signaling pathway is important to confer tolerance to malathion toxicity.

Nrf2-mediated ROS controls ISC proliferation, microbiota growth, and viral replication in the midgut

Because redox state influences cellular proliferation in high-turnover tissues (30, 31), we investigated the mitosis rate in the intestinal stem cells (ISCs) in the midgut. Phosphohistone H3 (PH3) is detected only in mitotic cells; hence, specific antibodies can be used in assays to determine the fraction of cells undergoing division. Knockdown of *Nrf2* increased the number of PH3-positive ($PH3^+$) cells in midguts either after a regular blood meal or when insects were fed with blood supplemented with paraquat (Figs. 2*B* and 3*C*). This supports the conclusions that increasing intracellular ROS levels promotes ISC proliferation and that AeNrf2 may limit mitotic stimulation by maintaining a reduced intracellular environment and preventing oxidative damage.

A vigorous oxidative burst response is employed as a defense against bacteria in barrier epithelia like the intestinal epithelium (32, 33). We therefore evaluated whether changes in the midgut redox balance imposed by *Nrf2* silencing would prevent the indigenous microbiota proliferation that takes place after a blood meal (34). Determination of bacterial load by qPCR using 16S ribosomal target sequences (35) showed that blood-fed dsNrf2-injected mosquitoes have lower microbiota levels in the midgut, similar to control dsLacZ-injected mosquitoes fed with paraquat (Fig. 4*A*). These results clearly indicate that Nrf2-mediated redox regulation in the midgut exerts profound effects on the growth and development of gut-associated bacteria.

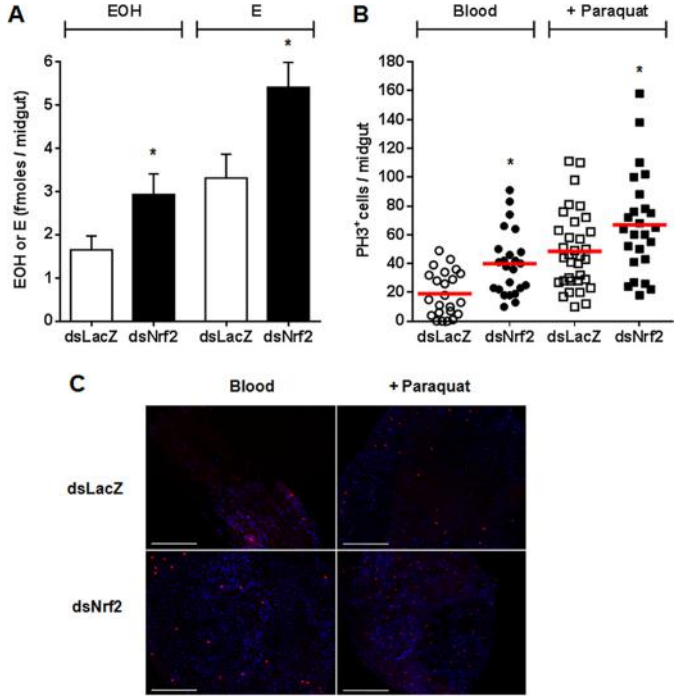


Figure 2. AeNrf2 influences redox and proliferative states of mosquito midgut. *A*, superoxide radical production in the midguts of dsLacZ or dsNrf2-injected mosquitoes 24 h after blood feeding, as measured by HPLC fractionation of DHE oxidation products 2-hydroxyethidium (*EOH*) and ethidium (*E*). Midguts were incubated with DHE (100 μM) in PBS/diethylenetriaminepentaacetic acid for 30 min, extracted with acetonitrile, dried, resuspended in PBS, and analyzed by HPLC. *B*, ISC proliferation rates were assessed as the frequency of phosphorylated histone H3-positive (PH3+) cells. Quantification of mitotic PH3+ cells in individual midguts. Results are for pools of at least three independent experiments. *Error bars*, S.E. Statistical analyses between dsLacZ- and dsNrf2-injected groups were made by Student's *t* test. *C*, representative images of midguts of dsLacZ- or dsNrf2-injected mosquitoes, 24 h after feeding with blood or blood supplemented with 1 mM paraquat, immunostained with anti-PH3 (*red*) and stained with 4′,6-diamidino-2-phenylindole (*blue*). *, $p < 0.05$; ***, $p < 0.001$.

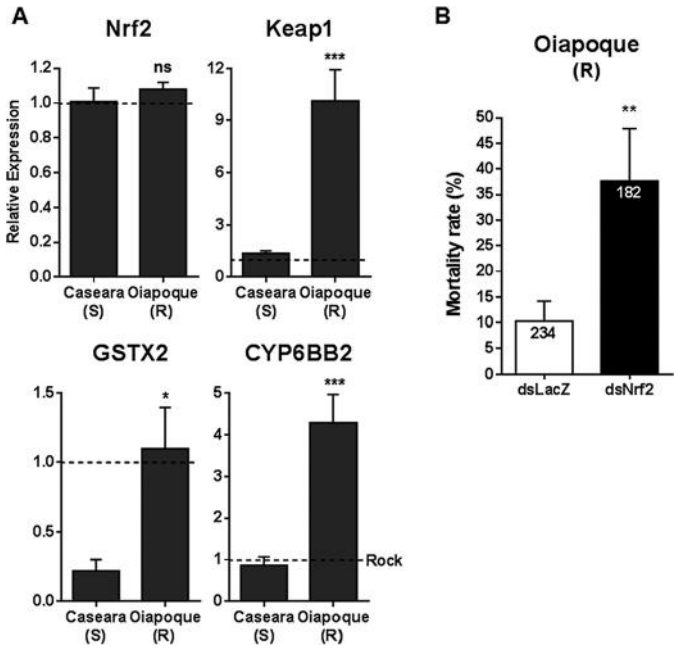


Figure 3. Nrf2 signaling is important for insecticide metabolic resistance. *A*, relative basal expression of *Nrf2* and Nrf2 target genes; comparison between two natural populations: the susceptible (*S*) Caseara and the resistant (*R*) Oiapoque strains against the laboratory strain Rockefeller (*Rock*, *dashed lines*). *B*, effect of silencing *Nrf2* on insecticide-induced mortality. Mosquitoes from the resistant Oiapoque strain were injected with dsRNA for *Nrf2* and *LacZ* (control dsRNA) and 48 h postinjection were exposed to insecticide-impregnated papers (90-min exposure to 2 g/liter malathion). Mortality was recorded 24 h later. Results are presented as percentage mortality for each group. The number of mosquitoes assayed is indicated at the *top* of each *bar*. Results are for pools of at least three independent experiments. *Error bars*, S.E. Statistical analyses were made by Student's *t* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; *ns*, nonsignificant; *ns*, not significant.

To test the hypothesis that changes in the redox balance in the mosquito could influence the midgut infection by a relevant human pathogen, we challenged *Nrf2*-silenced or paraquat-fed *A. aegypti* with Zika virus and measured the number of pfu per midgut (infection intensity) and the number of infected midguts (infection prevalence) 4 days after feeding of virus-contaminated blood. We observed a significant reduction in both infection intensity and prevalence (Fig. 4, *B* and *C*, respectively). The number of noninfected mosquitoes increased ~3-fold in the *Nrf2*-silenced group and 5-fold in the paraquat-fed group. We interpret that this reveals that redox alterations, either by reduction in antioxidant capacity through *Nrf2* knockdown or direct feeding with a pro-oxidant molecule, can reduce arboviral infection of the midgut.

Discussion

Ranges of metazoan species cope with oxidative burden by activating the transcription factor Nrf2, a major regulator of cytoprotective responses to electrophilic stresses. We analyzed a range of phenotypes that result from ablation of this gene in the mosquito vector, *A. aegypti*. A role for this gene and its pathway was discovered for early development, adult midgut biology, insecticide resistance, gut microbiome, and vector competence for an arbovirus. This complexity supports a vital role of this gene in the biology of this insect.

Nrf2 is a key regulatory protein in the redox signaling pathway and, together with small Maf proteins, stimulates transcription through binding to the AREs in the 5′-end regulatory regions of target genes (21). This response is limited by Keap1, a repressor protein that binds to Nrf2, keeping it in the cytosol and promoting its degradation by the ubiquitin proteasome pathway (21). Nrf2 is released upon oxidation of specific Keap1 cysteine thiol residues that are sensitive to the cell redox status, and this leads to increased expression of target genes. The Neh2 domain at the N terminus of the Nrf2 protein contains motifs that bind to the Keap1 Kelch domain, which negatively regulates the transcriptional activity of Nrf2 (21, 36). We found a relatively low level of conservation for the Neh2 domain in AeNrf2, even when compared with the *D. melanogaster* homolog, in contrast with the high identity values found for other domains. Nevertheless, the *A. aegypti* genome contains a *Drosophila* Keap1 homolog (AAEL005424) with a high degree of similarity, including the BTB domain, which is essential for Nrf2-specific negative regulation by Keap1 (37, 38). In contrast, the Neh6 domain, which has high levels of identity to other Nrf2 homologs, has been shown to promote Keap1-independent negative regulation of Nrf2 in vertebrates (39). These data, together with the presence of corresponding ARE motifs in the promoter regions of key detoxification genes and a well con-

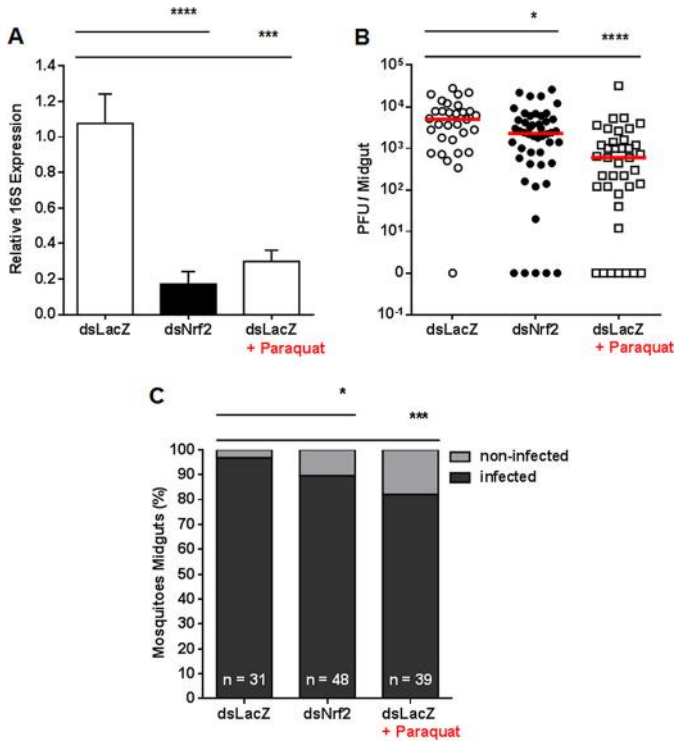


Figure 4. Silencing of AeNrf2 decreases gut bacterial microbiota and Zika virus midgut infection levels. *A*, culture-independent evaluation of midgut natural microbiota in dsLacZ or dsNrf2-injected mosquitoes, 24 h after feeding with blood or blood supplemented with 1 mM paraquat, through qPCR for bacterial ribosomal 16S RNA. Results are for pools of at least three independent experiments. *Error bars*, S.E. Statistical analyses were made by one-way analysis of variance. *B*, dsRNA-injected females were fed on blood contaminated with 10^7 pfu/ml Zika virus, and 4 days after feeding, the number of pfu was determined in the midgut. *C*, the percentage of infected midguts (infection prevalence) was scored from the same set of data as in *B*. Mann–Whitney U tests were used for infection intensity (*B*), and χ^2 tests were performed to determine the significance of infection prevalence analysis (*C*). *, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$.

served, stress-inducible (28) Nrf2 partner Maf protein (15, 40) in the mosquito genome (AAEL011739), show that the AeNrf2/Maf/ARE axis is an active redox-sensitive transcriptional regulatory pathway in *A. aegypti*.

Nrf2 regulates the expression of proteins that collectively promote cell survival following exogenous stress. This protective role of Nrf2 is also demonstrated by inverse genetics. Our attempts to generate gene knockouts in *A. aegypti* through the previously validated CRISPR/Cas9-induced mutagenesis (23, 43) led us to conclude that AeNrf2 encodes a protein vital for embryogenesis in this mosquito. In contrast to our results, *Nrf2*-knockout mice are viable and do not display any signs of an increased basal oxidation state, but have an impaired ability to respond upon challenge (41, 42). In *D. melanogaster*, the longest splice variant, CncC, shows the highest conservation with functionally important domains of Nrf2. Interestingly, it has been suggested that *Drosophila* CncC performs functions that in mammals are attributed to Nrf2 but can also exert others that are typical of Nrf1 (knockout of which yields an embryonic lethal phenotype in mice) (21).

In contrast, we achieved successful *Nrf2* knockdown in adult insects, showing that this transcription factor regulates the basal and stress-inducible expression of a battery of genes encoding key components of the GSH-based antioxidant systems and drug-metabolizing isoenzymes. This signaling pathway is important also for thioredoxin-based antioxidant response, as well as multidrug resistance–associated efflux pumps (44). GSH *S*-transferase (*GST*) is a canonical Nrf2 transcriptional target, having a prototypic ARE in its promoter (16, 22). Similarly, the Nrf2 pathway is associated with cytochrome P450–mediated metabolic resistance to insecticides in *D. melanogaster* (29, 45) and more recently in other insects, such as beetles (46, 47) and aphids (48). It was shown in *A. aegypti* that detoxification enzymes, namely members of the GST and cytochrome P450 families, contribute to increased levels of resistance to insecticides (49–51). Specifically for the *CYP* genes studied here, the CYP6M subfamily has been functionally characterized for their role in insecticide resistance in anophelines; *Anopheles gambiae* CYP6M2, whose best hit in the *A. aegypti* genome is CYP6M11, has been shown to metabolize permethrin and deltamethrin (52, 53). Furthermore, heterologously expressed *A. aegypti* CYP6BB2 exhibited strong metabolic activity for permethrin (51). This same gene was also pointed out as a solid candidate for imidacloprid metabolism based on gene expression data and substrate binding predictions (54). Constitutively activated *Nrf2* controls insecticide resistance in *D. melanogaster* through up-regulation of cytochrome P450 and GSH transferase genes (29, 45).

Regarding insecticide resistance, microarray analyses (55, 56) and RNA-seq studies (57–59) in *A. aegypti*-resistant strains revealed genomic changes (including polymorphism, copy number variation, and gene amplification events) in detoxification enzymes, such as P450s, esterases, and GSTs, that validated the use of these transcripts as genetic markers for resistance. Taken together, these findings suggest that insecticide resistance is not only conferred via multiple resistance genes, but also that regulation of transcription is a key factor in resistance gene amplification (reviewed in Ref. 5). We investigated the transcriptomic database of resistant *A. aegypti* populations present in Faucon *et al.* (59), the most recent and geographic diverse thus far, in a search for ARE-containing genes (Table S4). Surprisingly, we found that ~70% of the transcripts up-regulated in these populations presented AeARE1 and/or AeARE2 in their promoters, implicating this cis-regulatory element as a relevant genomic marker of the resistance phenotype in natural populations. Our results further suggest that a field population naturally resistant to insecticides (Oiapoque) presents an inherently elevated transcriptional regulation of the targets, considering that the large control of the pathway is at the level of the transcription factor protein stability. Hence, one cannot exclude additional components that could allow this constitutive activation in resistant mosquitoes (*e.g.* proteins involved in proteasomal degradation, co-factors, histones, and chromatin remodeling proteins). However, the contribution of Nrf2 itself is demonstrated by the elevated basal (unstressed, sugar-fed) expression of previously identified Nrf2 targets. Additionally, the increased mortality of dsNrf2-injected resistant mosquitoes in response to malathion exposition is probably due to reduced expression of several genes that are downstream to this transcription factor, including the ones herein investigated.

Recently, it has been shown that in the malaria vector *A. gambiae*, Nrf2 partner Maf protein expression correlates with the expression of a limited transcript set, detoxification-related, such as cytochromes P450 and GSTs, indeed suggesting a regulatory role for this transcription factor in insecticide resistance (60). Here, we propose a more comprehensive role for Nrf2 signaling in mosquito physiology. The use of insecticide by humankind is a very recent event in the evolutionary history of insects, and xenobiotic resistance comes from recruitment of ancestral highly conserved detoxification pathways that evolved as detoxification pathways directed to plant and microbe-borne allelochemicals. In addition, the same mechanisms contribute to protection against redox insult promoted by endogenous sources of free radicals, which generate a wide variety of electrophilic compounds with biological activity. Among the classes of genes induced in a previously described *A. aegypti* cellular transcriptomic response toward heme and paraquat, a large fraction is accounted for by stress response genes (28). However, transcripts from assorted functional groups in this database presented AeAREs in their promoters; these included antioxidant and phase II detoxifying enzymes, which are classically regulated by the Nrf2 pathway and have been associated with protective responses in the midgut of blood-feeding arthropods (61–65) as well as sequences that regulate glucose metabolism and transmembrane transporters, indicating a role for the Nrf2 signaling in the energy metabolic adaptation of *A. aegypti* to environmental stress, as has been suggested for other models (20, 66, 67). Moreover, in the midgut of *A. aegypti*, ROS levels are dramatically reduced after blood ingestion through a heme-driven mechanism (34). Collectively, our results show that Nrf2 signaling regulates batteries of genes involved in various aspects of cytoprotective and metabolic functions through associated AREs, and this is probably an important component of the heme-mediated protective response to avoid oxidative stress.

Modulation of the redox balance in the intestinal epithelium has been shown to induce proliferation of stem cells (68, 69), being a major regulator of tissue homeostasis and an essential feature of midgut physiology, characterized by very high rates of cell turnover. We observed an increased mitosis in response to feeding with the ROS generator paraquat and in *Nrf2*-knockdown mosquitoes, similarly to the pattern for redox-modulated ISC proliferation found in flies, in which loss of CncC results in increased ROS accumulation, accompanied by enhanced proliferation (70), suggesting that the regulation of stem cell function by the intracellular redox milieu is an evolutionarily conserved phenomenon. ROS levels in the gut epithelia are also shown to play an important role in controlling bacterial growth (32, 33, 71). In mosquitoes, changes in ROS production in the midgut not only impact innate immunity responses against bacteria, but can also affect their ability to transmit human pathogens (11, 34, 72, 73). The Nrf2 pathway has been shown to participate as a signaling conduit between the resident microbiota and the eukaryotic host, mediating beneficial effects in the gut (74). Here we present evidence for an Nrf2-mediated redox control on the mosquito's midgut microbiota. Furthermore, Nrf2 signaling depletion, rather than a collateral bacterial alteration, seems to be the primary event causing change in the redox state (Fig. S4).

In mammals, Nrf2-dependent gene expression has been associated with a number of pathologies that are caused or exacerbated by oxidative stress. Specifically, for DENV-mediated response, it has been recently reported that Nrf2 is directly activated by DENV in mononuclear phagocytes, transcriptionally signaling for inflammatory activation (75). Regarding data discussed here, Nrf2 has been implicated in the control of Zika virus infection (76), suggesting an important role for the induction of these intracellular mediators in retarding flavivirus replication. As shown here, *Nrf2* silencing can limit Zika virus infection, an effect that can be emulated by paraquat, suggesting that the permissive role of the Nrf2 pathway could be accounted by its homeostatic function in the redox balance of the midgut. In contrast, in a recent study, Oliveira *et al.* (77) demonstrated that a blood-induced antioxidant response mediated by the enzyme catalase facilitates the establishment of Dengue virus (but not Zika virus) in the midgut of *A. aegypti*. Mosquitoes evolved a redundant antioxidant protection strategy to prevent oxidative stress following blood intake to adapt to the ingestion of large amounts of heme, an unavoidable consequence of blood digestion (34, 63). Here we present evidence that the antioxidant mechanisms under Nrf2 control, while contributing to redox balance and tissue homeostasis of the midgut, also have an infection-permissive effect, affecting the so-called midgut infection barrier for Zika virus. This also indicates that the redox milieu of the mosquito can diversely impact the distinct virus this vector can transmit, and these differences deserve further investigation. Because the *A. aegypti* midgut is an initial site of contact between virions and mosquito cells, genetic alterations capable of improving the midgut infection barrier might be considered as potential tools for preventing the establishment of the infection and can be used as targets for disease transmission control strategies (9, 78).

Overall, our results reveal that AeNrf2-mediated signaling is a major pleiotropic regulator for midgut homeostasis, which affects environmental and stress-related responses in mosquitoes, and can transcriptionally control several genes that directly or indirectly affect their vectorial competence (Fig. 5).

Materials and methods

Ethics statement

All animal care and experimental protocols were conducted in accordance with the guidelines of the Committee for Evaluation of Animal Use for Research (Federal University of Rio de Janeiro, CAUAP-UFRJ) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocols were approved by CAUAP-UFRJ under registration number IBQM155/13. Dedicated technicians in the animal facility at the Instituto de Bioquímica Médica Leopoldo de Meis (UFRJ) carried out all protocols related to rabbit husbandry under strict guidelines to ensure careful and consistent animal handling.

Mosquitoes

A. aegypti (Red Eye strain) were raised in a mosquito rearing facility at the Federal University of Rio de Janeiro, Brazil,

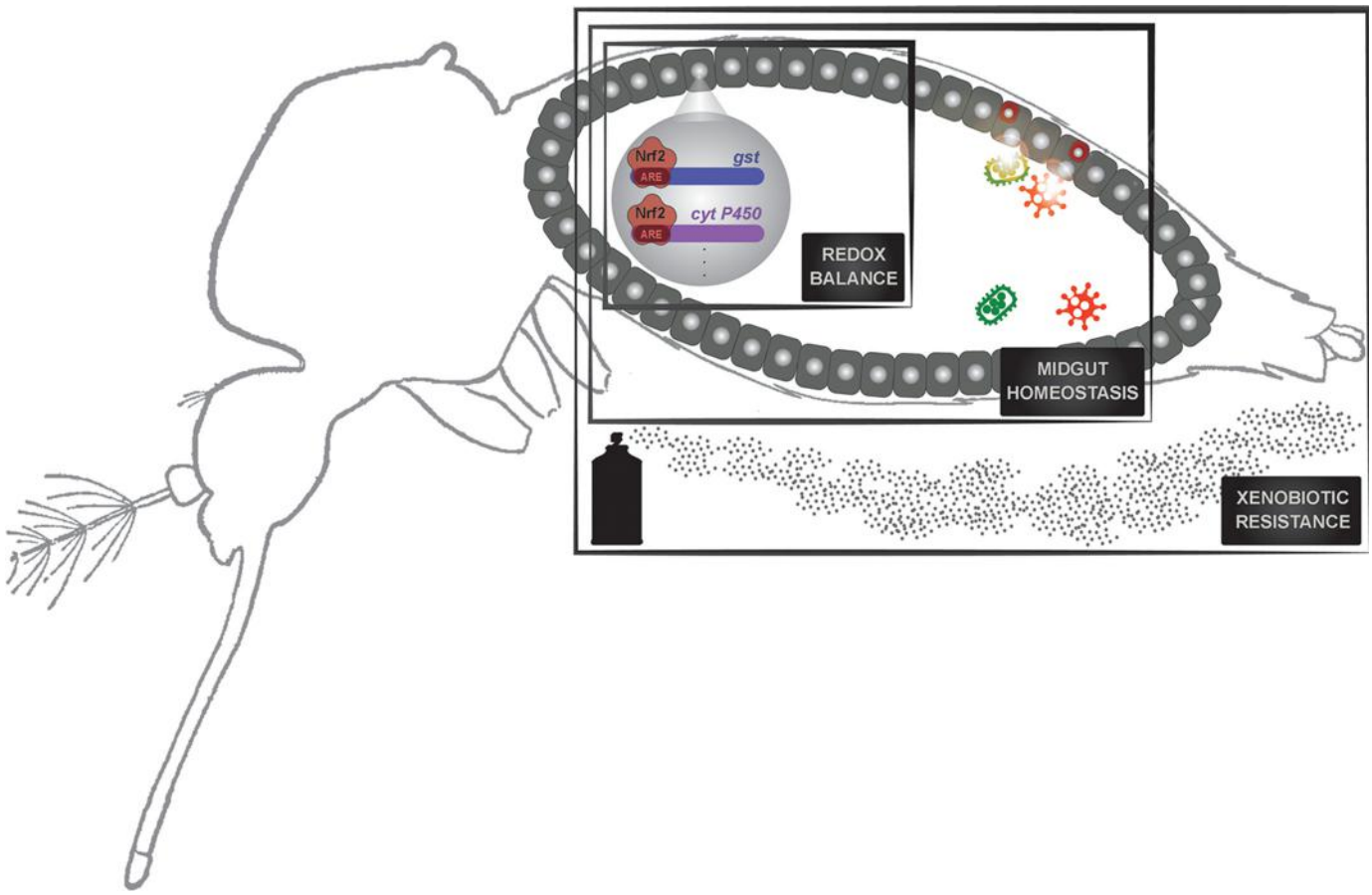


Figure 5. Schematic overview of Nrf2 signaling in mosquito physiology. Through a comprehensive transcriptional enhancement, Nrf2, bound to the ARE sites, regulates cellular redox balance. In a broader tissue context, Nrf2 signaling is able to coordinate midgut homeostasis by balancing signaling/damaging ROS and thus fine-tuning microbiota growth, viral replication, and stem cell proliferation. In consequence, Nrf2 can also systemically influence mosquitoes' xenobiotic tolerance and emerge as a central regulator of various processes important for vectorial adaptation of *A. aegypti*.

under a 12-h light/dark cycle at 28 °C and 70–80% relative humidity. Larvae were fed with dog chow, and adults were maintained in a cage and given a solution of 10% sucrose *ad libitum*. Females 4–7 days posteclosion were used in the experiments.

For insecticide resistance experiments, mosquitoes were maintained at Laboratório de Fisiologia e Controle de Artrópodes Vetores, Instituto Oswaldo Cruz, FIOCRUZ, Rio de Janeiro, Brazil. Laboratory-reared (F6 generation) field populations of *A. aegypti* from Caseara (TO, Brazil) and Oiapoque (AP, Brazil) were used in the insecticide bioassays as models for susceptibility and resistance, respectively, to both pyrethroid and organophosphate insecticides. The Rockefeller reference strain was used as an additional susceptibility control.

When mentioned, mosquitoes were artificially fed with heparinized rabbit blood with or without 1 mM paraquat, a pro-oxidant compound used to induce oxidative stress. Feeding was performed using water-jacketed artificial feeders maintained at 37 °C sealed with parafilm membranes. Female midguts were dissected 24 h after feeding for RNA sample preparation.

Nrf2 gene knockdown by RNAi

dsRNA was synthesized from templates amplified from cDNA of whole mosquitoes using specific primers containing a T7 tail (see supporting material). The *in vitro* dsRNA transcription reaction was adapted from a tRNA transcription protocol (79). Briefly, reactions were performed at 37 °C for 12 h in a buffer containing 40 mM Tris·HCl (pH 8.0), 22 mM $MgCl_2$, 5 mM DTT, 2 mM spermidine, 0.05% BSA, 15 mM guanosine monophosphate, a 7.5 mM concentration of each nucleoside triphosphate, amplified template DNA (0.1 μg/μl), and 5 μM T7 RNA polymerase. The transcribed dsRNA was treated with DNase at 37 °C for 30 min and precipitated using 1:10 (v/v) 3 M sodium acetate, pH 5.2, and 1 M isopropyl alcohol. The pellet was washed twice with 70% ethanol and then eluted in water to reach a final concentration of 3 μg/μl. Mosquitoes were injected intrathoracically with the dsRNA (0.4 μg) with a microinjector (Nanoinjector, Drummond) and were either blood-fed or used in insecticide bioassays 48 h later. The *LacZ* gene was used as a nonrelated dsRNA control and was amplified from a plasmid containing a cloned *LacZ* fragment.

RNA isolation and quantitative real-time PCR analysis

Total RNA was isolated from insects at different developmental stages, whole bodies of adult males and females, and dissected midgut epithelia ovaries, heads, thoraces, and abdomens (carcass) of blood-fed females using TRIzol (Invitrogen). Complementary DNA was synthesized using the High-Capac-

ity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA).

Quantitative gene amplification (qPCR) was performed with the StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA) using the Power SYBR Green PCR Master Mix (Applied Biosystems). The comparative *Ct* method (80) was used to compare RNA abundance. The *A. aegypti* ribosomal protein 49 gene (*Rp49*) was used as an endogenous control (81). The assessment of midgut bacterial growth was performed through qPCR for bacterial ribosomal 16S RNA. All oligonucleotide sequences used in qPCR assays are available in the supporting material.

HPLC analysis of DHE products

To provide specific quantitative assessment of ROS levels, we performed HPLC fractionation of DHE oxidation products (82). Briefly, after incubation with 100 μM DHE, the dissected midguts were opened with forceps and washed in PBS to remove intestinal contents. Pools of 15 gut epithelia each were frozen in liquid N_2, homogenized in 100% acetonitrile (500 μl), sonicated, and centrifuged at 13,000 $\times$ *g* for 10 min. The resulting supernatant was dried under vacuum (SpeedVac SVC100 Savant), and the resulting pellet was stored at −70 °C until use. The dried samples were resuspended in PBS containing 100 μM diethylenetriaminepentaacetic acid (Sigma) and injected into an HPLC LC-10AT device (Shimadzu, Tokyo) equipped with a diode array (SPD-M10A) and fluorescence detectors (RF-20A). Chromatographic separation of DHE oxidation products was performed using a NovaPak C18 column (3.9 $\times$ 150 mm, 5-μm particle size) equilibrated in solution A (10% acetonitrile and 0.1% TFA) at a flow rate of 0.4 ml/min. After sample injection, a 0–40% linear gradient of solution B (100% acetonitrile) was applied for 10 min, followed by 10 min of 40% solution B, 5 min of 100% solution B, and 10 min of 100% solution A. The amount of DHE was measured by light absorption at 245 nm, and the DHE oxidation products, hydroxyethidium and ethidium, were monitored by fluorescence detection.

Immunostaining and microscopy

Mosquito midguts were dissected and fixed with 4% paraformaldehyde. After washing with PBS containing Triton X-100, immunostaining was done with primary antibody mouse anti-PH3 (Merck Millipore, 1:500) and secondary antibody Alexa Fluor 546–conjugated anti-mouse (1:2000). Nuclei were stained with 4′,6-diamidino-2-phenylindole (Sigma) (83).

Quantification of whole-midgut mitosis was performed by counts of individual nuclei marked by the PH3, using a Zeiss Observer Z1 fluorescence microscope equipped with a Zeiss Axio Cam MrM using a Zeiss-15 filter set (excitation BP 546/12; beam splitter FT 580; emission LP 590) under a ×20 objective. Representative images were acquired in an Olympus IX81 microscope and a CellR MT20E imaging station equipped with an IX2-UCB controller and an ORCAR2 C10600 CCD camera (Hamamatsu). Image processing was performed with the Xcellence RT version 1.2 software. Optical slices (0.1 μm) were generated.

Viral infections

A Brazilian strain of Zika virus from Pernambuco was kindly provided by Dr. Laura Helena Vega Gonzales Gil (Centro de Pesquisas Aggeu Magalhães, Fundação Oswaldo Cruz, Brazil) and characterized by Coelho *et al.* (84). Infection procedures were performed as described previously (74). Briefly, viral stocks were propagated in C6/36 cells maintained in Leibovitz-15 medium supplemented with 5% fetal bovine serum, 1% nonessential amino acids, 1% penicillin/streptomycin and tryptose (2.9 g/liter). Culture supernatants containing viral particles were harvested, centrifuged, aliquoted, and stored at −70 °C until use. Viral titers were determined by plaque assay as 2×10^7 pfu/ml. Females were infected in an artificial blood meal containing a 1:1 mix of rabbit red blood cells and L-15 medium containing Zika virus. Midguts were dissected at 4 days post-blood meal and stored individually in DMEM at −70 °C until assayed.

Plaque assays

Zika plaque assays were performed (as described (74)) in Vero cells maintained in DMEM supplemented with sodium bicarbonate, 1% L-glutamine, 10% fetal bovine serum, and 1% penicillin/streptomycin. Midgut tissue was disrupted to release viral particles by vortexing the tubes for 10 min at room temperature. The samples then were centrifuged at 10,000 $\times$ *g* at 4 °C, and 10-fold serial dilutions were performed and each one inoculated in a single well. The plates were gently rocked for 15 min at room temperature and then incubated for 45 min at 37 °C and 5% CO_2. Finally, an overlay of DMEM, containing 0.8% methylcellulose and 2% FBS, was added to each well, and the plates were incubated for 5 days (at 37 °C and 5% CO_2). Culture media were then discarded, and a solution of 1:1 (v/v) methanol and acetone and 1% crystal violet was used to fix and stain the plates. pfu were counted and corrected by the dilution factor.

Bioassays

Previously dsRNA-injected mosquitoes from different strains (Rockefeller, Caseara, or Oiapoque) were submitted to a dose discriminator bioassay (48 h after the injection) to evaluate their profile of susceptibility to the organophosphate, malathion. The test was adapted from the World Health Organization recommended protocol and consisted in confining mosquitoes in acrylic chamber tubes lined internally with Whatman grade No. 1 papers that had been previously impregnated with malathion (840 μl of a 2 g/liter solution, resulting in 0.1 g/m^2 in the paper) (85). Approximately 20 females, ~4 days posteclosion were exposed to the insecticide for 90 min and then transferred to insecticide-free rescue tubes. Mortality was evaluated 24 h later. Two to three replicates were used, and each experiment was repeated three times.

Statistical analysis

All analyses were performed with the GraphPad Prism statistical software package (Prism version 6.0, GraphPad Software, Inc., La Jolla, CA). *Asterisks* indicate significant differences (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; *ns*, not

significant.), and the type of test used in each analysis is described in its respective figure legend.

Author contributions—V. B.-R., O. A. C. T., A. J. M., A. A. J., P. L. O., and G. O. P.-S. conceived and designed the experiments; V. B.-R., O. A. C. T., and L. C. performed the experiments; V. B.-R., O. A. C. T., A. J. M., and G. O. P.-S. analyzed the data; A. J. M., A. A. J., P. L. O., and G. O. P.-S. contributed reagents/materials/analysis tools; and V. B.-R. and G. O. P.-S. wrote the paper.

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Zika infection decreases *Aedes aegypti* locomotor activity but does not influence egg production or viability

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BACKGROUND Zika has emerged as a new public health threat after the explosive epidemic in Brazil in 2015. It is an arbovirus transmitted mainly by *Aedes aegypti* mosquitoes. The knowledge of physiological, behavioural and biological features in virus-infected vectors may help the understanding of arbovirus transmission dynamics and elucidate their influence in vector capacity.

OBJECTIVES We aimed to investigate the effects of Zika virus (ZIKV) infection in the behaviour of *Ae. aegypti* females by analysing the locomotor activity, egg production and viability.

METHODOLOGY *Ae. aegypti* females were orally infected with ZIKV through an artificial feeder to access egg production, egg viability and locomotor activity. For egg production and viability assays, females were kept in cages containing an artificial site for oviposition and eggs were counted. Locomotor activity assays were performed in activity monitors and an average of 5th, 6th and 7th days after infective feeding was calculated.

FINDINGS No significant difference in the number of eggs laid per females neither in their viability were found between ZIKV infected and non-infected females, regardless the tested pair of mosquito population and virus strain and the gonotrophic cycles. Locomotor activity assays were performed regardless of the locomotor activity in ZIKV infected females was observed, in both LD and DD conditions.

MAIN CONCLUSIONS The lower locomotor activity may reduce the mobility of the mosquitoes and may explain case clustering within households reported during Zika outbreaks such as in Rio de Janeiro 2015. Nevertheless, the mosquitoes infected with ZIKV are still able to disseminate and to transmit the disease, especially in places where there are many oviposition sites.

Key words: Zika virus - *Aedes aegypti* - locomotor activity - egg production - egg viability

The Zika virus (ZIKV) is an arbovirus belonging to the *Flaviviridae* family, first isolated in 1947 in Uganda, West Africa from a sentinel rhesus monkey.[1] Since then, sporadic human cases were reported in Asia and Africa. The first large Zika epizooty reported occurred in Micronesia in 2007.[2]

ZIKV was previously believed to cause only a mild and self-limiting illness, but it has emerged as a new public health threat after the explosive epidemic in Brazil in 2015, specially due to the increased severe congenital malformations (microcephaly) and neurological complications reported in the country.[3] This new epidemiological scenario led the World Health Organization to declare the Zika epidemic as a Sanitary Emergency in Public Health in February of 2016.

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ZIKV is transmitted by several species of mosquitoes of the family Culicidae, mainly *Aedes aegypti*.[4] Besides being the single confirmed natural ZIKV vector during the Pan-American epizootic,[5] *Ae. aegypti* is also the only vector for several other arboviruses circulating in the New World, such as the four Dengue virus (DENV) serotypes,[6] Chikungunya virus[7] and Yellow Fever virus[8].

The knowledge of the physiological, behavioural and biological features of the vector, such as preference and frequency of haematophagy in humans, egg laying, gonotrophic discordance and resistance to desiccation[9] may help the understanding of arbovirus transmission dynamics and elucidate the role of those parameters impacting vector capacity.

The haematophagous and anthropophagic behaviours are crucial to females' egg maturation. A single *Ae. aegypti* female can lay from 100 to 200 eggs per batch, and multiple times throughout her lifetime after each blood meal.[10] Eggs are laid usually on container surfaces near water, preferentially in shaded places[9] and their embryonic development and egg viability is directly related to environmental temperature[11].

Concerning activity patterns mosquito species are classified as diurnal, crepuscular and nocturnal.[12] *Ae. aegypti* is considered a diurnal and crepuscular spe-

cies that can modify the activity pattern according to changes in the physiology and viral infection.[12] Accordingly, DENV-2 artificially-infected *Ae. aegypti* females showed increased locomotor activity, potentially effecting the infection kinetics and disease transmission.[13] Moreover, the number of eggs laid by *Ae. aegypti* females orally challenged with DENV-2 may vary during the mosquito lifetime, with a decrease from one gonotrophic cycle to another.[14]

Here, we aimed to investigate the effects of ZIKV infection in the behaviour of *Ae. aegypti* females by analysing the locomotor activity, egg production and egg viability.

MATERIALS AND METHODS

Mosquito populations and rearing - Eggs from *Ae. aegypti* mosquitoes (strain PAEA, Tahiti, French Polynesia) were hatched in plastic trays containing 1.5L of Milli-RO water and approximately 1g of yeast (two tablets of Vitalab®, Brazil). Larvae were fed with the same quantity of yeast, every two days, until pupae development according to Farnesi et al.[11] The pupae were counted and separated in cages (with approximately 400 each) for adult emergence. Males and females mosquitoes were kept together in cages with 10% sucrose solution *ad libitum*. In all experiments, rearing of mosquitoes was carried out in an incubator (Precision Scientific Incubator, USA) at 25ºC, in a photoperiod of 12 hours of light and dark (LD 12:12) and 60-80% relative humidity.

Virus and experimental infection - The ZIKV strain ZIKV/H.sapiens/Brazil/PE243/201 (GenBank accession number KX197192.1) was used for oral experimental infection of *Ae. aegypti* females as previously described.[15] The virus was previously isolated from a febrile patient in the state of Pernambuco and molecularly characterised.[16] Briefly, females were allowed to feed through a membrane attached to an artificial feeder kept at 37ºC for approximately 40 min inside a Biosafety level - 2 (BSL-2) insectary facility. The infectious blood meal consisted of 1:1 mix of rabbit red blood cells and L-15 culture medium containing ZIKV at a final concentration of 10^7 PFU/mL; ATP pH 7.4 at a final concentration of 1 mM was included as a phagostimulant. Control mosquitoes also fed with a similar blood meal, but with a non-infected L15 culture medium.[15] After blood meal, mosquitoes were cold-anesthetised and only the fully engorged females were considered. Viral detection was done by polymerase chain reaction (PCR) (see Supplementary data, Fig. 1, Supporting Information).

Egg production assays in ZIKV infected and uninfected mosquitoes - For gonotrophic cycle assays, approximately 450 females, around two weeks-old, were used per condition (infected or uninfected). We performed three replicates per experiment; each one contained, at least, 50 females that were deprived of sugar prior to one infected or uninfected blood meal (with *Swis*s mice or rabbit blood), for approximately 40 min. In both cases, infected or uninfected blood meal, engorged females mosquitoes were selected (at least, 150 females, in each condition, were used per experiment). After three days, the oviposition was stimulated in cages containing an artificial site for oviposition: plastic container containing 100 mL of filtered water and three strips of rectangular filter paper, 8 cm x 15 cm in size, for two days. In these assays we used the second and third gonotrophic cycles. Three independent experiments were performed.

Eggs viability assays - For eggs viability analysis, the eggs obtained as described above were removed from the oviposition site and placed to dry in a humid chamber. After one week drying, eggs were removed carefully from the filter paper using a brush and counted in the Egg Counter Program (©BioAlg Group, Faro, Portugal). Afterwards, eggs were randomly selected and tested for viability as described below.

Each replica was set up with 50 counted eggs and placed on filter paper to stimulate hatching in Petri dishes containing 50 mL of industrial yeast extract solution 0.15% (weight/volume) for 24 h in a Precision Scientific Incubator (Thermo Fischer) under a constant temperature of 25ºC and 60-80% relative humidity according to Farnesi et al.[11] We analysed eggs viability from the second and third gonotrophic cycles. Each viability experiment analysed contained 600 eggs (300 per gonotrophic cycle, being 150 from uninfected and 150 from ZIKV infected females). This assay was composed by three independent experiments, totalising 1,800 eggs.

Egg production assays in ZIKV infected and uninfected mosquitoes in the other pair vector/virus - In addition, we verified if egg production or viability records would be similar in other pair vector/virus: *Ae. aegypti* (Urca population) and ZIKV (Rio-S1 strain, GenBank accession number KU926310), both originated from Rio de Janeiro and whose vector competence parameters have been previously described to be considerably high.[17] All mosquito treatments, virus titter in the blood meal and other experimental infection procedures were as described above, except that mosquito females from Urca population took a second uninfected blood meal 14 days after being orally challenged by ZIKV. Assessments of egg production or viability was limited to the second gonotrophic cycle.

Locomotor/flight activity assays - *Ae. aegypti* females, around 15 days post emergence, were transferred to four small circular carton cages (60 per cage) lined with micro tulle (8.5 cm of diameter X 9.5 cm of height). Females were deprived of sucrose solution for approximately 10 h prior to a blood meal (ZIKV infected or uninfected blood). Blood feeding followed as described above. ZIKV infected (n = 51) and non-infected *Ae. aegypti* females (n = 54) were individualised in 25 mm glass tubes to analyse locomotion and flight activity. In these tubes, cottons soaked with 10% sucrose solution were placed in one end and the other end was sealed with parafilm. Mosquito tubes were put in locomotor activity monitors (Trikinetics Inc, Waltham, MA, USA) with 32 channels and infrared light beams, where mosquitoes movement could be detected and recorded each time the mosquito passed through the infrared beam.

The experiment lasted, at least, eight days to allow viral dissemination according to Ryckebusch et al.[18] The monitors were kept inside an incubator (Precision Scientific Incubator, USA) at constant temperature of

25°C, in a LD 12:12 regimen (12 h of light followed by 12 h of dark), during seven days and a DD regimen (24 h of constant dark), for one day. The relative humidity was 60-80%. To analyse the locomotor activity, we did an average of 5rd, 6th and 7th days of activity, corresponding to LD condition and 8th DPI, corresponding to a day in DD condition. We performed three independent experiments, with a total of 123 ZIKV infected and 122 control mosquitoes analysed but here we show a representative data with 54 ZIKV infected and 51 control females.

The results were organised and analysed in Excel (Microsoft Office) with parameters previously established[19] and only mosquitoes that were alive at the end of the experiment and positive for ZIKV were considered (See Supplementary data, Fig. 1).

Statistical analysis - The locomotor activity results were analysed, firstly for Shapiro-Wilk normality test. After, we used the parametric *t*-Student test considering the log (N+1) mean of the individual mosquito data every 30 min. Since the mosquito activity data is especially variable, the transformation of the data to logarithm allows their distribution to be more constrained and the average to be less influenced by very low or very high values. In fact, because we have many zeros in the data series, we must use log (N+1) instead of log N. The advantage of using this calculation is that it prevents the masking of data by the effect of very high numbers within a single interval.[20] In the analysis of egg quantification and viability, we first performed the Shapiro-Wilk normality test. When the data showed a normal distribution, we used the parametric *t*-student; when data showed a non-normal distribution, we used non-parametric Mann-Whitney test. Other specific statistical information's are in the figure legend. All analysis were performed using GraphPad Prism 5 (GraphPad Software, San Diego, California, USA) and p value < 0,05 considered statistically significant.

Ethical statement - All the experiments carried out on this study were approved by the institutional Research Ethics Committees IOC/FIOCRUZ #LW34/14 (for feeding on mice) and CEUA-UFRJ 155/13 (for use of rabbit blood).

RESULTS

ZIKV infection has no effect on egg quantity and viability - All ZIKV orally challenged mosquitoes used in this study tested positive (Supplementary data, Fig. 1). To determine whether ZIKV infection affects *Ae. aegypti's* egg production and viability, the second and third gonotrophic cycles were analysed. The overall results showed that, regardless of the gonotrophic cycle (Supplementary data, Fig. 2) and the pair mosquito-virus strain (Supplementary data, Fig. 3), no difference was observed in egg production and viability for ZIKV infected and non-infected *Ae. aegypti* females. Similarly, when we analysed the second and third gonotrophic cycles comparatively, no significant differences were observed either in quantity ($p = 0.4091$ and $p = 0.3496$, respectively) or viability ($p = 0.0773$ and $p = 0.0734$, respectively) (Fig. 1).

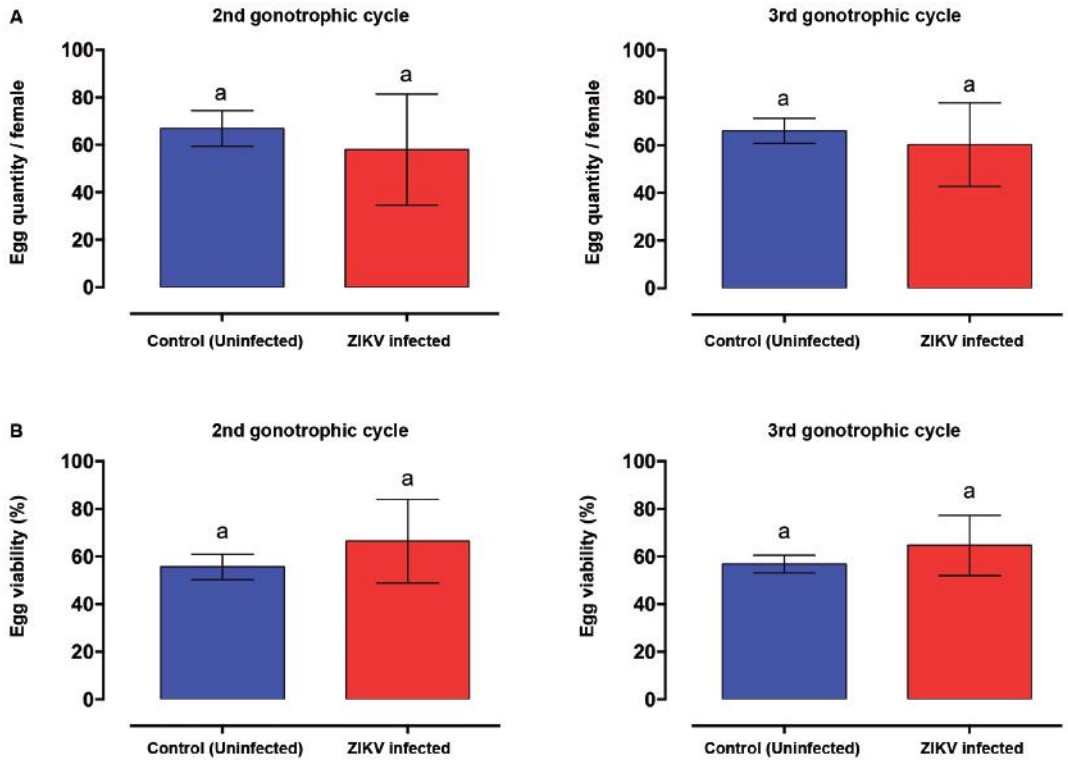

Fig. 1: effect of Zika virus (ZIKV) infection on the eggs quantity (A) and viability (B) of PAEA *Aedes aegypti* females infected with ZIKV PE243, according to the second and third gonotrophic cycles. The lack of significance is represented by p values > 0.05 obtained by using the parametric *t*-Student and non-parametric Mann-Whitney tests, respectively. Error bars represent mean ± s.d of three independent experiments.

ZIKV infection decreases females Ae. aegypti locomotor activity - To evaluate if ZIKV infection could disrupt the diurnal activity pattern of the females, we tested infected and non-infected females for seven days under a LD12:12 cycle. In the first two days of the assay, the activity was very low, probably because of blood digestion. The females became more active from the fourth day on. We focused our analysis on the 5th, 6th and 7th days post infection for a better interpretation. They present a low intensity startle response not controlled by the endogenous clock when lights turn on, which is commonly seen in locomotor activity tests (ZT 0.5, Fig. 2A-B). Comparatively, during the two peaks activity at ZT9 and ZT12, infected *Ae. aegypti* females showed significantly lower locomotor activity than the uninfected ones (a clear difference persisted at ZT5 - ZT13; $p < 0.05$) (Fig. 2B). In both infected and control groups, the major activity peak occurs between ZT 6 to 10 in LD condition. On the 8th day post infection, the mosquitoes went to a constant dark regimen (DD), to verify the influence of the endogenous circadian clock on this altered activity. Contrary to what was observed in LD, in DD conditions the startle response was eliminated, as expected (Fig. 2C). We only observed one activity peak at CT11 (Fig. 2C). The infected females in DD also presented less activity compared to the control ones, although the difference was not statistically significant in this case. After the peak, the activity was drastically reduced in both groups in LD and DD conditions (Fig. 2).

DISCUSSION

Since the Zika outbreak and all the consequences caused by the virus infection in newborns, mainly in Brazil, the major efforts of the scientific community have been focused on the interaction between virus and the vertebrate vector (humans). However, we believe it is also fundamental to investigate in detail the effects of this virus in the insect vector. We aimed to contribute to a major understanding of possible changes caused in the *Ae. aegypti* females behaviour when infected with ZIKV, which will inevitably influence the success of vector control measures.

Recently, *Ae. aegypti* was reported naturally infected by ZIKV.[5] Field and laboratory studies to assess the transmitting success of such an arbovirus to a new host and their offspring are still scarce. Here, we analysed fecundity (i.e., the number of eggs laid by each female) and fertility (the number of viable offspring produced) as well as locomotor activity in ZIKV infected *Ae. aegypti*.

In fecundity analysis, there was no difference between ZIKV infected and uninfected mosquitoes, regardless of the tested pair of mosquito population and virus strain. However, a borderline p value in the statistical analysis ($p = 0.054$) suggests a trend for higher average viability in eggs from infected females (Fig. 1, Supplementary data, Figs 2-3). In fecundity analysis, there were no differences between ZIKV infected and uninfected mosquitoes, even when using another laboratorial approach.[21] In contrast, a previous study showed that DENV infected *Ae. aegypti* females exhibited lower fecundity (egg quantity).[14] Furthermore, lower egg production and hatching were observed when *Ae. aegypti* females were infected by DENV-1 or DENV-2.[14,22] Therefore, our data suggest that the ZIKV infection does not cause damage to the fertility and viability in its main vector *Ae. aegypti*.

The locomotor activity has an important role in arboviruses spread and transmission dynamics. It was previously observed that DENV-2 infection causes an increase in locomotor activity in *Ae. aegypti* during the 24-h period in a LD 12:12 cycle.[13] On the other hand, different alterations in mosquito physiology, like insemination and blood feeding, can also decrease the locomotor activity of *Ae. aegypti* and *Ae. albopictus*.[23] However, different from our previous study with locomotor activity,[13] we performed experimental mosquito infection via oral artificial feeding to mimic as closely as possible the natural kinetics of ZIKV infection and dissemination.

Recently, Ryckebusch et al.[18] showed in the *Ae. aegypti* PAEA strain that the dissemination of the ZIKV occurs between 6th days post infection and 14th days post infection. In our analysis, we consider the interval between 5th and 8th days post infection to test the locomotor activity, to ensure both viral dissemination through the body and mosquito survival.

The very low activity soon after blood feeding was already previously described in mosquitoes and it is independent of the viral infection. It is related to blood metabolism, necessary to perform the oviposition, which normally occurs three days after the blood meal.[12]

The females of both groups started to exhibit a clear diurnal pattern of activity from the fourth day on. In addition to the traditional activity peak at ZT12 in LD, our mosquitoes also showed a peak at ZT9. Interestingly, this seems to be a characteristic of PAEA, since Lima-Camara et al.[13] had already observed an increase in activity near ZT9 in this strain of *Ae. aegypti*. However, ZIKV infection in the *Ae. aegypti* PAEA strain caused a decrease in the activity pattern during the whole light phase in comparison to the control group throughout the analysed days in LD condition (Fig. 2A-B). This is the opposite of what Lima-Camara et al.[13] observed for mosquitoes infected with DENV-2. Thus, it is possible that these viruses influence the behaviour of *Ae. aegypti* by different molecular targets. Furthermore, in DD condition, which means an absence of environmental conditions, both ZIKV infected and uninfected females presented one peak at CT11 (Fig. 2C). Since *Ae. aegypti* has an activity period of approximately 22-23h in DD,[19] it is likely that the peak observed at ZT12 in LD has advanced to CT11 under constant conditions (DD). On the other hand, the observed peak at ZT9 in LD does not present a corresponding peak in DD, an indication that it could be controlled by a masking effect, free from the influence of the clock (for more details regarding the masking mechanisms see Clements[12]).

However, although our locomotor activity data suggest circadian clock involvement, it is possible that Zika infection does not initially need to recruit the central clock in the brain to produce the observed effects. That is because our experiments was continued up until to the ninth activity day (8th day post infection) and according to Ryckebusch et al.,[18] virus dissemination to head

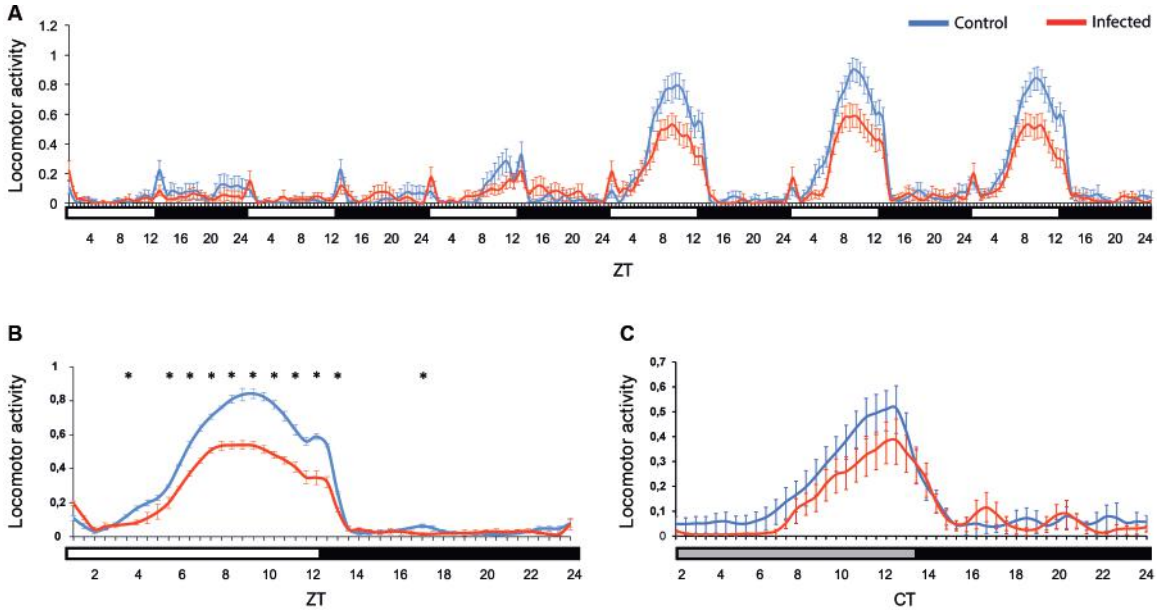

Fig. 2: locomotor activity of *Aedes aegypti* females infected by Zika virus (ZIKV). Locomotor activity of control (blue line, n = 51) and infected *Ae. aegypti* females (red line, n = 54). The mosquitoes were observed from the second to the seventh days post infection (DPI) in LD12:12 (A). The first DPI was not included because we considered them to be still adapting to the system. We also show an average profile in LD (the graph referred only to 5th, 6th and 7th DPI) (B). On the 8th DPI the mosquitoes were kept in constant darkness (DD) (C). Bars below the graphics indicate the light regime: white = lights on in LD cycles, grey = lights off in DD ("subjective day"), black = lights off in LD or DD ("subjective night"). ZT: *Zeitgeber* time within a light/dark cycle experiment; ZT0: time the light turns on; ZT12: time the light turns off; CT: circadian time in DD. Error bars were shown for each 30 min interval. Asterisks represent the significance of the *t*-Student test, where $p < 0.05$.

tissues and salivary glands occurs only from the 14th day post infection. Insects have central and peripheral clocks. The former are responsible for directing the main circadian behaviours and the others are located in several tissues of the body, being fundamental for the modulation of the central clock.[12] Microarray studies estimate that thousands of mosquito genes are controlled in the head and body by circadian clocks.[24,25] Thus, it is quite feasible that Zika infection is able to modulate the behaviour of vectors by the influence of peripheral clocks in the initial days of infection.

Lately, Gaburro and collaborators[21] using microelectrodes to record electrical activity in mosquito primary neurons culture and pools of females to analyse the locomotor activity in Zika infected mosquitoes, showed an increase in spiking activity of the neuronal network and in diurnal locomotion activity compared to uninfected females. Our results are quite different from those found recently by Gaburro et al.[21] It is worthy to mention that we used a validated approach in behaviour studies[13,19,20,23] in which the individual insects are isolated from host odours and inter-specimens communication. These cues may cause a bias and interfere in the overall activity of mosquitoes.

It is noteworthy that the ZIKV has a specific characteristic not described in other viruses of the same family: high tropism by brain tissues of the mosquito.[21] Moreover, when the transcriptome of Zika infected mosquitoes is compared to DENV infected ones, the majority of mosquito genes (61%) that presented a modification in expression (up- or downregulation) are those of Zika infected group. These data suggest that there is a remarkable difference in the mosquito response to these viruses, which could lead to very different physiological and behavioural responses.[26]

The occurrence of Zika epidemics in recent years may be due to several factors, and the non-vector borne forms of transmission cannot be excluded, like in different humans fluids.[27] In Brazil, another determinant may greatly influence the success of ZIKV transmission and maintenance, such as mosquito behaviour, climatic and environmental factors.[28] Moreover, the high mosquito infestation index[29], abundant vector breeding sites and deteriorated infrastructure may also be influencing the transmission of diseases, such as dengue and Zika.[30] Our data show that Zika infection affects neither egg production nor viability and decreases mosquito locomotor activity. These alterations do not seem to negatively influence Zika transmission, once the majority of positive cases tested in the 2015 outbreak in Rio de Janeiro clustered within households.[30] Thus, despite the lower activity, mosquitoes infected with ZIKV are still able to disseminate and transmit the disease, keeping the population, especially because their fertilily and fecundity are not alterated for this infection.

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AUTHORS' CONTRIBUTION

KPP, RVB, LCF and RLO - Conceptualisation; RVB, MHOS, RLO and LCF - methodology; KPP, MEBR, LCF, OATC, RTF and SSC - validation; RVB, KPP, RTF, LCF, MEBR, RLO and SSC - formal analysis; KPP, MEBR and SSC - investigation; RVB, MHFS and RLO - resources; KPP, MEBR and LCF - writing - original draft; RVB, LCF, RLO and MHFS - writing - review, editing and supervision; RVB, MHFS and RLO - project administration and funding acquisition.

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Non-canonical transcriptional regulation of heme oxygenase in *Aedes aegypti*

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Heme oxygenase (HO) is a ubiquitous enzyme responsible for heme breakdown, which yields carbon monoxide (CO), biliverdin (BV) and ferrous ion. Here we show that the *Aedes aegypti* heme oxygenase gene (AeHO – AAEL008136) is expressed in different developmental stages and tissues. AeHO expression increases after a blood meal in the midgut, and its maximal transcription levels overlaps with the maximal rate of the further modified *A. aegypti* biglutaminyl-biliverdin (AeBV) pigment production. HO is a classical component of stress response in eukaryotic cells, being activated under oxidative stress or increased heme levels. Indeed, the final product of HO activity in the mosquito midgut, AeBV, exerts a protective antioxidant activity. AeHO, however, does not seem to be under a classical redox-sensitive transcriptional regulation, being unresponsive to heme itself, and even down regulated when insects face a pro-oxidant insult. In contrast, AeHO gene expression responds to nutrient sensing mechanisms, through the target of rapamycin (TOR) pathway. This unusual transcriptional control of AeHO, together with the antioxidant properties of AeBV, suggests that heme degradation by HO, in addition to its important role in protection of *Aedes aegypti* against heme exposure, also acts as a digestive feature, being an essential adaptation to blood feeding.

Aedes aegypti is a vector of important human viral diseases such as yellow fever and dengue, and more recently has also been associated with the large-scale emergence of viruses such as Chikungunya[1] and Zika[2]. Both adult males and females feed on nectar, but only females require the ingestion of large amounts of vertebrate blood, as a nutritional source for oogenesis. Blood is a protein-rich diet that elicits rapid signaling responses that include nutritional and endocrine regulation[3]. Since hemoglobin is the main blood protein, like other hematophagous animals, mosquitoes face a unique circumstance regarding its digestion, resulting in the formation of large amounts of heme[4]. Besides its relevance in many physiological processes, free heme is able to amplify formation of reactive oxygen species (ROS)[5] and to destabilize the membrane structure leading to cell lysis[6]. Due to the potential toxicity of free heme, the intracellular levels of this molecule are strictly controlled by the balance of the specific biosynthesis and degradation pathways[7].

In most eukaryotic cells, heme oxygenase (HO) catalyzes the degradation of heme, cleaving its alpha-meso carbon bridge to yield equimolar quantities of biliverdin IX alpha, CO and free iron[7]. Free iron is promptly sequestered into ferritin and, in mammals, biliverdin is subsequently converted to bilirubin through the action of biliverdin reductase[8]. Thus, heme oxygenase is a major component of cellular response against stress.

Very little is known about the cellular mechanisms involved in heme degradation in blood-feeding insects. The heme degradation pathway has only been described in the mosquito *A. aegypti*[9] and in the hemipteran *Rhodnius prolixus*[10], vector of Chagas disease. In both cases heme degradation process displays several peculiarities when compared to vertebrates pathway. In general, these distinctive features relate to the regular ingestion of heme associated with reproduction. Further modifications of biliverdin, with the conjugation of hydrophilic molecules, facilitates the completion of heme degradation and the removal of massive amounts of otherwise hydrophobic biliverdin molecules[4].

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In *R. prolixus*, we have previously shown the existence of a unique pathway for oxidative heme degradation, which produces a modified dicysteinyl-biliverdin IX gamma as the main end-product[10], and not the BV IX alpha as in the vertebrates. In the mosquito *A. aegypti*, heme enzymatic degradation has also been shown to occur and, differently from all other previously studied organisms, the produced biliverdin is further modified by conjugation of two glutamine residues, leading to the formation of an excretable biglutaminyl-biliverdin IX alpha (or *Aedes aegypti* biliverdin, AeBV)[9].

Thus far, three isoforms of HO, HO-1, HO-2 and HO-3 have been described. HO-2 isoform is constitutively expressed, HO-1 is induced by multiple stress stimuli whereas HO-3, only described in *Rattus novergicus*, is considered to be catalytically inactive[11]. In insects, HO have only been functionally analyzed in the fruit fly *Drosophila melanogaster*[12] and the hematophagous mosquito *Anopheles gambiae*[13]. However, the physiological relevance of HO and of its product BV IX as antioxidant components and the regulation of HO expression in the context of adaptation to hematophagy have not been investigated so far.

Taking into account that heme degradation is a priority for cells that are exposed to its high levels, the present study sets to fill the gap in the characterization of this relevant pathway pathway in the blood feeding mosquito *A. aegypti*. Here we examined, for the first time in a hematophagous insect, the expression of the Heme oxygenase, the key enzyme responsible to the heme degradation. The *A. aegypti* HO (AAEL008136) gene encodes a predicted protein with high degree of similarity with classical heme oxygenase sequences. The transcriptional profile was evaluated in parallel with the formation of AeBV, its antioxidant product. Against all odds, our results show that the *A. aegypti* HO gene is transcriptionally regulated by a nutrient-sensing pathway and not by stress or heme imbalance, as described for other organisms up to now.

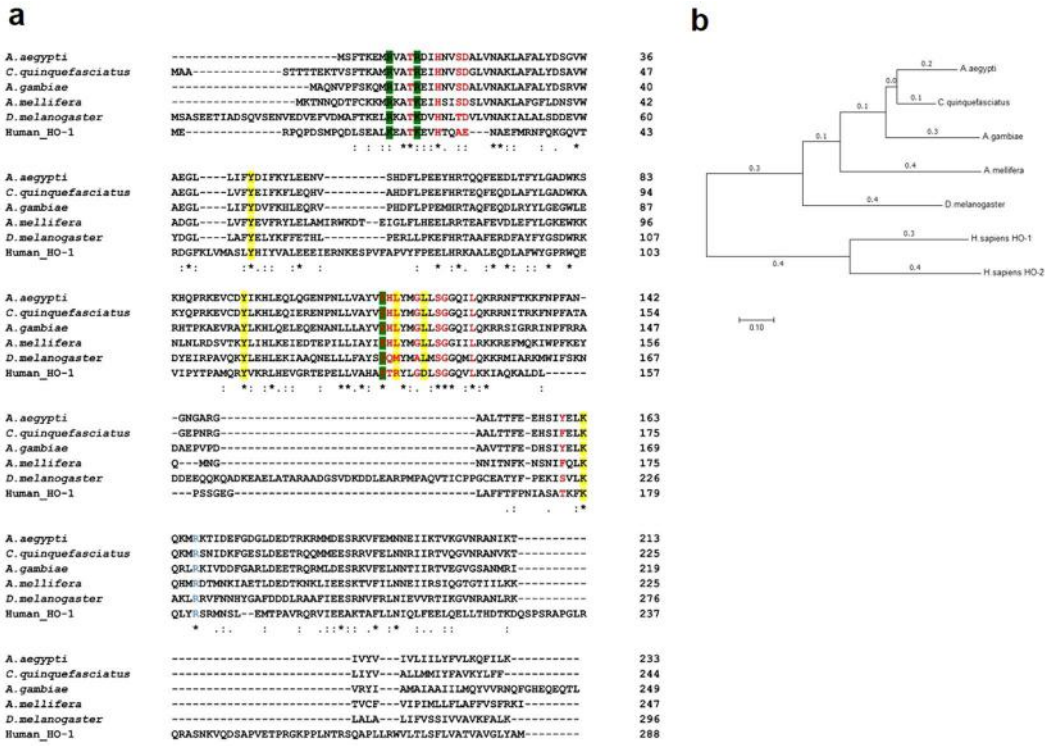
Results

The *Aedes aegypti* heme oxygenase (AeHO). The gene sequence for heme oxygenase from *A. aegypti* (GenBank accession number AAEL008136) has a 988 bp ORF, which encodes a predicted 233 amino acid polypeptide (Fig. 1a). Only one HO-1 predicted ortholog was found in the *A. aegypti* genome (VectorBase Genome Assembly: AaegL5), suggesting that this mosquito expresses only one isoform of HO. No evidences of more than one transcript were found in the microarrays and RNA-Seq experiments results deposited in the VectorBase database (www.vectorbase.org). The deduced amino acid sequence of AeHO showed high similarity to sequences of heme oxygenases from other insects and also to the inducible human isoenzyme HO-1 (Fig. 1b). The percentage of identities/similarities between AeHO and HO amino acid sequences in other insects and humans are shown in Fig. 1c. From the alignment we were able to identify conserved residues relevant for enzymatic activity and for building of the heme pocket of the catalytic site[14], such as the proximal heme ligand His25. Most of the residues involved in the interaction with the heme propionate residues localized in the amino terminal portion are also conserved. In HO-1, a hydroxyl group is the distal heme ligand and establishes hydrogen bonding to Gly139 and Gly143, which are conserved in insect HOs, with the exception of *Drosophila melanogaster* HO. The fly HO lacks a proximal heme ligand resulting in the production of a broad spectrum of BV isomers (α, β, δ)[12].

The AeHO expression and heme degradation. In order to address the importance of this gene in the mosquito *A. aegypti*, we initially investigated its broad transcriptional profile, that included the non-hematopagous stages (larvae, pupae and adult males) of mosquito, as HO is expressed in many tissues in vertebrates, including the ones that are not intensively involved in heme detoxification. AeHO is expressed at different developmental stages as well as in many tissues of blood fed females (Fig. 2a,b). As the trigger of oogenesis event is strictly dependent of blood ingestion and digestion in this insect, we compared the expression of AeHO in midguts and ovaries of blood-fed females. The AeHO transcript levels increased in midgut and ovaries after a blood meal (Fig. 2c,d). In the midgut, AeHO transcripts increased between 24 h and 42 h after the blood meal (Fig. 2c), a period of maximum heme concentration in the midgut lumen[15], whereas the maximal expression of AeHO in the ovary occurred between 48 h and 72 h, when the oogenesis is getting into the end in females mosquitoes (Fig. 2d). The amount of AeBV, the end product of the heme degradation pathway, increased in the gut until 24 h after the blood meal, then declined (Fig. 2e) as a result of its appearance in the insect feces (Fig. 2f). Due to the relevance of biliverdin production as a result of HO activity after blood ingestion, we confined the further analyses to the midgut of blood-fed females.

Antioxidant activity of AeBV. It is well known that biliverdin (BV) and bilirubin (BR) are reducing molecules, acting as antioxidants *in vitro* and *in vivo*. Since in mammals BV is rapidly converted to BR by biliverdin reductase, most studies have been performed with BR. However, in *A. aegypti*, BV produced by cleavage of the heme porphyrin ring is further converted to a biglutaminyl-biliverdin IX alpha (AeBV)[9]. Similarly to BV IXα, AeBV showed antioxidant activity, as demonstrated by the delay of fluorescence decay caused by B-PE oxidation induced by the addition of the pro-oxidant ABAP. (Fig. 3a). The antioxidant activity of AeBV was also dose-dependent (Fig. 3b).

Considering that the midgut epithelium is potentially subjected to an oxidative challenge imposed by blood digestion and the release of high amounts of heme in the lumen, the antioxidant capacity of AeBV could be vital to protect midgut proteins from oxidation. In fact, as shown in Fig. 3c, protein oxidation was very extensive in the midgut extracts incubated in the presence of t-BOOH and heme, when compared to non-incubated samples. However, it could be partially reduced by the presence of AeBV at both tested concentrations. Similar reductions were observed with the samples treated in the presence of BV IXα and TROLOX, two well-known antioxidants. These results suggest that AeBV may represent a physiological antioxidant system, protecting midguts against protein oxidation generated by heme.



c

Species	*A. aegypti*	*C. quinquefasciatus*	*A. gambiae*	*A. mellifera*	*D. melanogaster*	Human_HO-1
A. aegypti	100	75.86	62.23	46.09	40.77	25.34
C. quinquefasciatus	75.86	100	63.71	48.73	41.8	29.57
A. gambiae	62.23	63.71	100	42.02	37.66	25.11
A. mellifera	46.09	48.73	42.02	100	36.13	28.09
D. melanogaster	40.77	41.8	37.66	36.13	100	26.18
Human_HO-1	25.34	29.57	25.11	28.09	26.18	100

Figure 1. Heme oxygenase amino acid sequence. (**a**) Alignment of HOs from several insects and human HO-1, comparing the most important residues in the heme interaction. Residues that contact to heme (red); hydrophobic residues wall (blue); residues that exhibit interaction with propionates (green box) and polar residues clusters (yellow box). (**b**) Neighbor-Joining phylogenetic tree for insects and human HOs. The optimal tree with the sum of branch length = 3.03131229 is shown. The tree is drawn to scale, with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. (**c**) Percent identity matrix based on alignment (Clustal Omega).

Non-canonical transcriptional control of AeHO. HO is one of the major components of the response against several types of cellular stress. As a rule, mammalian HO-1 is induced by various stimuli such as oxidative stress and inflammation[16]. Surprisingly, in mosquito midguts, addition of paraquat, a compound that results in increased intracellular formation of superoxide anion[17], leads to a decrease in AeHO gene expression (Fig. 4a). In addition, depletion through RNAi of the Nrf2, a master eukaryotic redox-active transcriptional regulator[18] that has been extensively related to the induction of HO-1 expression[19] (Fig. S1), has no effect on the AeHO mRNA levels (Fig. 4b).

Since AeHO transcript levels increase following a blood meal, we sought to verify if heme itself would account for this upregulation. To test this, we fed the insects with a protein-rich chemically defined artificial diet (Substitute Blood Meal, SBM), supplemented or not with heme[20]. Also unexpectedly, heme was not able to induce AeHO expression at these conditions (Fig. 4c).

In mammalian cells, induction of HO-1 has been recently recognized as a mediator of cellular protection against Zika virus (ZIKV) infection[21]. However, in the midgut of ZIKV-infected mosquitoes, AeHO gene expression remains unaltered (Fig. 4d).

Since AeHO in not canonically regulated, yet it is still induced throughout blood digestion, we investigated whether a nutrient sensor – such as the kinase Target of Rapamycin (TOR) – mechanism would be involved. In fact, the blood-meal increase in AeHO expression is partially blocked when protein digestion is impaired by ingestion of SBTI, a trypsin inhibitor (Fig. 5a). We further tested whether the amino acid-sensing target of rapamycin (TOR) pathway is involved in the control of AeHO transcription. In fact, inhibition of TOR, either by

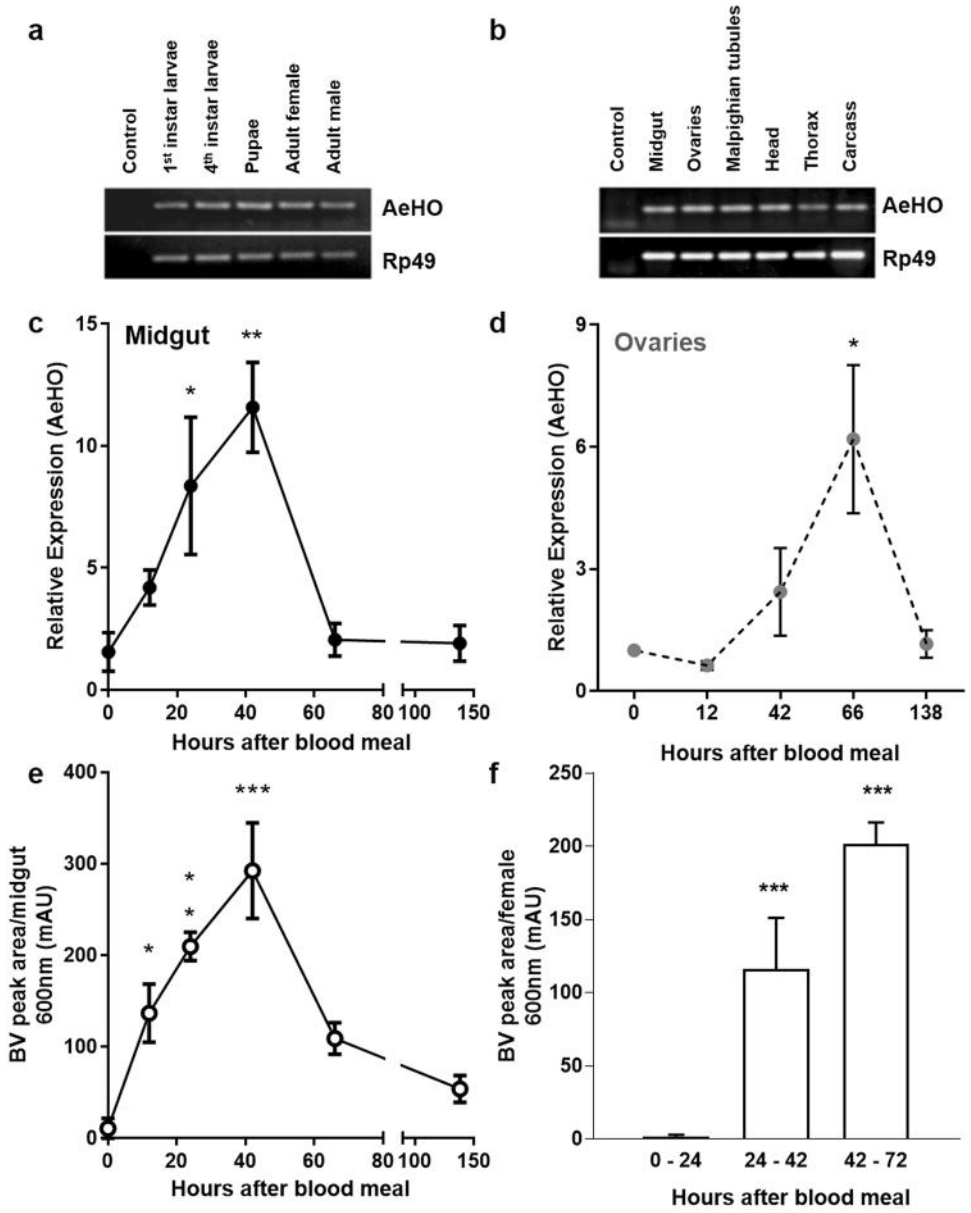


Figure 2. *Aedes aegypti* heme oxygenase - expression profile and production of biliverdin. (**a**) Reverse transcription PCR (RT-PCR) revealing heme oxygenase expression from early developmental stages to adults. (**b**) RT-PCR showing HO expression in female tissues dissected 24 h after the blood meal. Gel images were cropped and processed to improve clarity – original full-length gels are presented in Supplementary Fig. S2. (**c**) Quantitative PCR (Real time qPCR) showing the time course of HO expression in the midgut and (**d**) in the ovary. Rp49 was used as endogenous control (n = 4). (**e**) Time course of AeBV production in the midgut. (**f**) Appearance of AeBV in mosquito feces. Results of (**e**) and (**f**) are pools of at least 3 independent experiments – mAU, 1000x arbitrary units. Error bars indicate the standard error of the mean; one-way ANOVA with Dunnett's post-test with multiple comparisons for (**c**), (**d**), (**e**) and (**f**), where 0 h or 0–24 h after the blood meal samples served as control.

rapamycin (Fig. 5b) or by RNAi specific TOR depletion (Figs 5c and S1) was able to decrease blood-meal induced AeHO expression. These findings indicate that amino acid stimulation of the TOR regulatory cascade increases AeHO levels in the mosquito midgut upon blood digestion.

Discussion

HO belongs to a large family of stress proteins whose transcriptional regulation also responds to varied types of adverse environmental conditions[16]. HO catalyzes the first and rate-limiting step in the oxidative degradation of heme (Fe-protoporphyrin-IX) to produce carbon monoxide, free iron, and biliverdin-IXα[22]. Large amounts of heme are produced in the gut of hematophagous insects, due to proteolysis of dietary hemoglobin. Given that proper heme degradation is essential to avoid pro-oxidizing environments in cells, it is expected that HO may

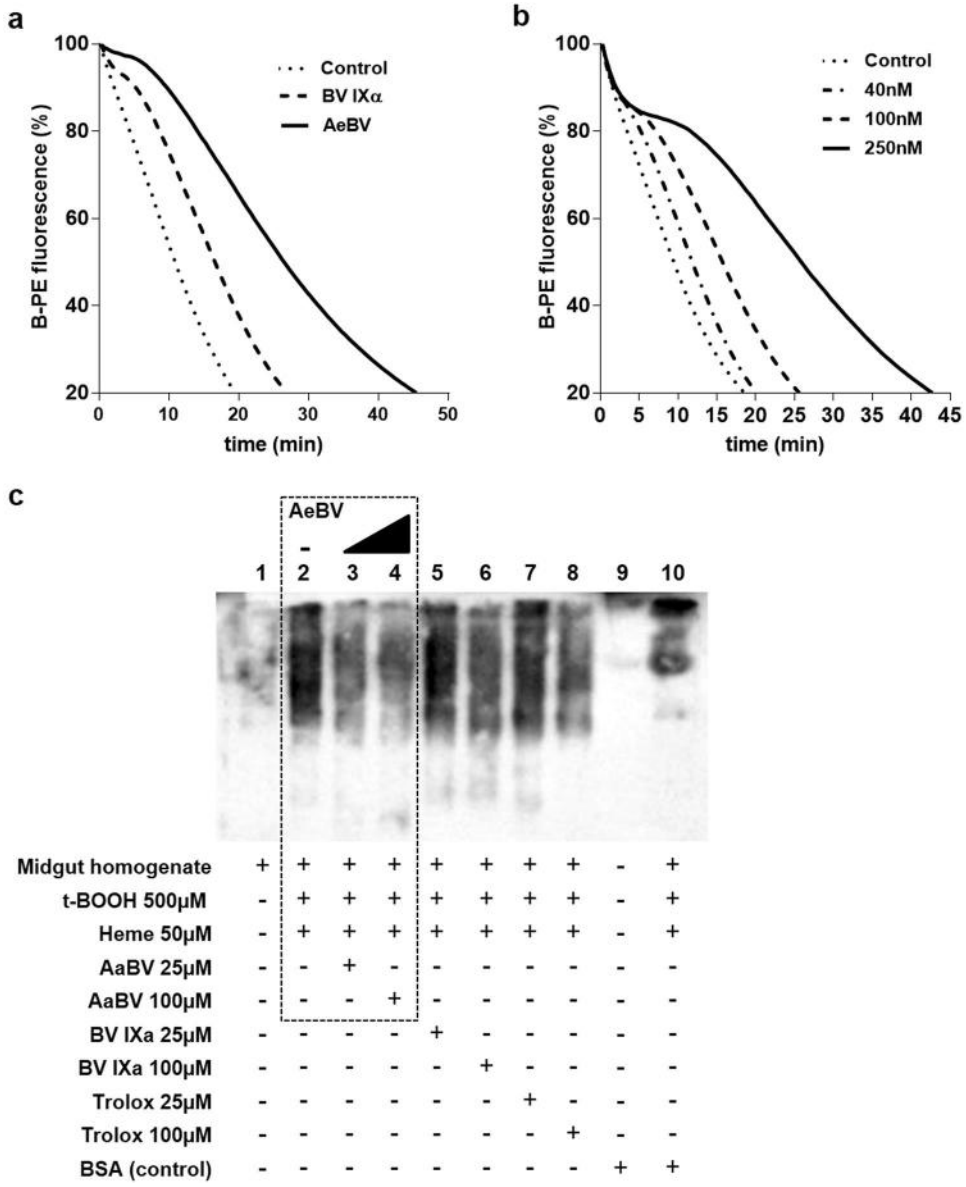


Figure 3. Antioxidant activity of AeBV. *B*-Phycoerythrin (B-PE) oxidation, in a prooxidant system, can be measured by the loss of its natural fluorescence in a fluorimeter. (**a**) Biliverdin IX-α and AeBV inhibit *B*-Phycoerythrin (B-PE) oxidation initiated by 4 mM ABAP, at the same concentration (250 nM). (**b**) Effect of AeBV is dose-dependent. (**C**) AaBV protects the midgut against protein oxidation *in vitro*. Midgut homogenates were incubated with heme and t-butyl hydroperoxide in the presence or absence of AaBV (evidenced in dashed square) – western-blot of *A. aegypti* midguts homogenate after incubations, using specific antibodies against protein carbonyl-DPNH products. Gel image was cropped and processed to improve clarity – original full-length blot/gel is presented in Supplementary Fig. S2.

be relevant in the adaptation of blood-feeding organisms to their heme-rich diet. Upon binding to the HO apoprotein, the heme molecule serves as the substrate and catalytic cofactor in its own degradation, and is generally considered as the prototypical inducer of HO expression[23]. Here we show that the transcriptional profile of AeHO matches the appearance of biliverdin – that has antioxidant properties – in the mosquito midgut. However, AeHO appears to be under an unexpected mode of transcriptional control, being regulated by a nutrient-sensing pathway instead of the canonical stress-response pathway.

While three isoforms of HO have been identified in mammals[8], only a single gene coding for a HO is found in the *Aedes aegypti* genome. The identified gene AAEL008136, here named *Aedes aegypti* heme oxygenase (AeHO), encodes a polypeptide with basic residues that are considered essential to allow enzymatic cleavage of the porphyrin ring (Fig. 1). There is a high degree of evolutionary conservation of the heme-degrading enzymes with HO homologues identified in bacteria, fungi, and plants[24–26]. Generally, the mechanism of HO-catalyzed heme cleavage into an α-isoform of biliverdin, CO and iron is conserved between mammals and these organisms[19]. Among insects, however, there are some notable differences. The *D. melanogaster* HO-1 homolog (dHO), in

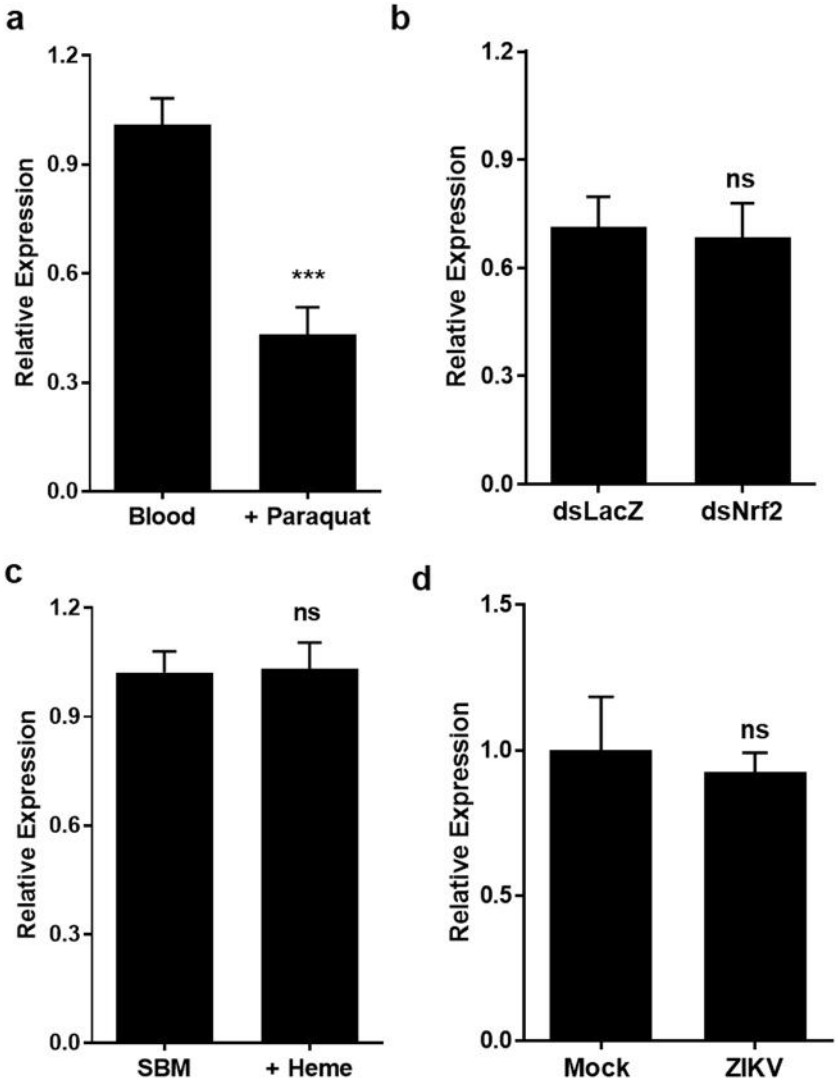


Figure 4. AeHO expression is not increased upon oxidative and viral challenges. Midgut expression of HO by real-time qPCR in (**a**) mosquitoes fed with blood supplemented or not with 500 µM paraquat; (**b**) paraquat-fed (1 mM) dsNrf2-injected mosquitoes; (**c**) mosquitoes fed with substitute blood meal (SBM) with or without 50 µM heme; (**d**) mosquitoes infected with ZIKV (compared to mock control). Results are pools of at least 3 independent experiments. Error bars indicate the standard error of the mean. Statistical analyses were performed by Student's t-test.

contrast to mammalian HOs, is not alpha-specific, producing also the IX beta and IX delta isomers of biliverdin. Furthermore, the reaction rate is slower than that of mammalian HOs[12]. In blood-feeding insects, the tetrapyrole end products of heme degradation have acquired unique features important to facilitate their elimination. After a blood meal, in the gut of *R. prolixus* and *A. aegypti*, biliverdins conjugated to specific amino acids, cysteine and glutamine, respectively, are generated[9,10]. These further modified structures increase the solubility of biliverdin, converting it to a molecule easier to excrete. This suggests that heme degradation in blood-feeding insects evolved to be specialized in the production of massive amounts of biliverdin, generated upon blood digestion. We have demonstrated that heme degradation produces high amount of AeBV molecules that show significant antioxidant activities *in vitro* (Fig. 3). Considering that AeBV is produced and excreted in the midgut lumen during the course of digestion (Fig. 2e), we can suggest that heme degradation by HO represents a primary defense mechanism against heme toxicity: first, by reduction of the prooxidant heme levels and second by the production of a hydrosoluble antioxidant compound that may protect the gut epithelium against the oxidative challenge triggered by blood digestion.

HO is expressed even in stages and tissues that do not face high levels of heme in the diet or environment (Fig. 2a,b), suggesting that it is required to basal heme metabolism of that stage/tissue, probably been involved in the controlled of the intracellular free iron levels or in the production of CO that can act as a second messenger[16]. A similar result was obtained by a previous group, revealing that HO expression is required to the normal development of the non-hematophagous fruit fly *D. melanogaster*[27].

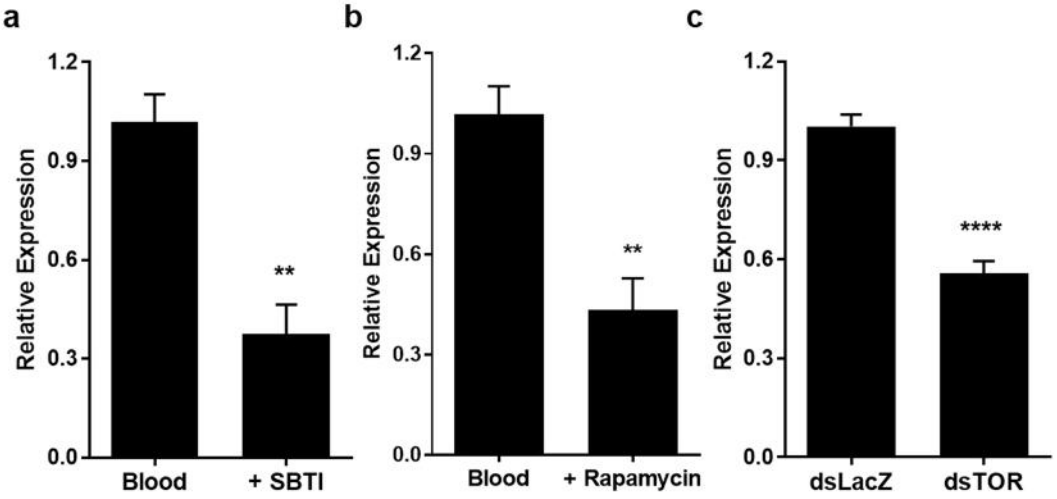


Figure 5. Amino acid-induced TOR regulatory cascade increases AeHO levels upon blood digestion. Midgut expression of HO by real-time qPCR in (**a**) mosquitoes fed with blood supplemented or not with 100 µM SBTI; (**b**) mosquitoes fed with blood supplemented or not with 20 µM rapamycin, and (**c**) blood-fed dsTOR-injected mosquitoes. Results are pools of at least 3 independent experiments. Error bars indicate the standard error of the mean. Statistical analyses were made by Student's t-test.

Maximal expression of AeHO in the midgut occurs from 24 h to 42 h after the blood meal (Fig. 2c), when most of the ingested protein has already been degraded[28]. Monitoring the production of AeBV showed that this end product of the heme degradation pathway increased in the gut almost linearly until 24 h, when it starts to decline, which can be explained by its appearance in the feces (Fig. 2e,f). The total amount of AeBV (in the midgut content and in the feces) remains relatively constant after 24 h post blood meal, suggesting that the period of most intense enzymatic activity of the AeHO is, in fact, occurring simultaneously with the release of heme from dietary hemoglobin. The peritrophic matrix (PM) is a protective barrier with heme-binding activity, secreted by the midgut epithelium, composed by chitin, acidic polysaccharides and proteins, which are synthesized *de novo* in response to blood feeding[29,30]. During the initial hours of blood digestion, it is possible that the growing PM is not able to keep pace with the rate of hemoglobin hydrolysis due to elevated trypsin activity. Therefore, if the PM has not yet fully developed its capacity to isolate heme during the initial steps of digestion, a significant amount of heme would reach the midgut epithelium and AeHO activity would be responsible for counteracting heme toxicity. Unfortunately, all attempts to produce AeHO gene silencing by RNAi were unsuccessful (not shown).

Recent work has confirmed the existence of an enzyme with HO activity in *An. gambiae*, and its functional inhibition in adult females (by means of chemical inhibitors) has led to a dose dependent decrease in oviposition[13]. This correlates well with the data presented here, which show an ovary-specific peak of HO expression at the termination of oogenesis (Fig. 2d). Together with our recent report that silencing of HO shows a deleterious effect on oviposition and egg viability in the hematophagous insect *R. prolixus*[31], we hypothesize that the protective role for HO in blood-feeding insects goes beyond digestion, being crucial for their adaptation to anautogeny as a reproductive strategy.

It is well known that the expression of mammalian HO-1 is induced by its substrate, heme, but its expression can also be strongly up-regulated in response to various stimuli related to cellular stress and pro-oxidant signals (reviewed in[32]). In the mosquito midgut, transcription of the HO gene is regulated both by ingestion of the meal and by formation of digestion products, as trypsin inhibition decreases HO transcript levels (Fig. 5a). However, unexpectedly, the ingested heme is not the trigger in this regulatory cascade (Fig. 4c). While this unresponsiveness of HO to heme has been briefly described in cultured *Drosophila* cells before[27], here we consolidate this feature as part of an intricate physiological regulation of the blood digestion process that couples spatiotemporal hemoglobin catabolism, heme degradation and PM formation in the mosquito midgut.

Another unique feature of AeHO is that, in contrast to mammalian HO-1 which is over expressed upon ROS stimuli, AeHO showed an opposite profile when the blood meal was supplemented with a ROS generator molecule, paraquat (Fig. 4a). Nrf2, a basic leucine zipper transcription factor, is a master regulator of the transcriptional response to oxidative stress that, upon activation, regulates the expression of genes coding for anti-oxidant, anti-inflammatory and detoxifying proteins. For most organisms it is generally accepted that HO is one of the key Nfr2 pathway-regulated genes (reviewed in[18]). In *A. aegypti*, silencing of the Nrf2-homolog did not impair AeHO transcription (Fig. 4b) but significantly altered other stress-related genes[33]. Aside from a possible post-transcriptional regulation of this enzyme, as observed for mammals[34], these results support the hypothesis of multi-transcriptional control of AeHO expression.

The mosquito heme degradation system seems to follow some canonical aspects in its operation, but also involves a unique expression control, that seems related to the pressure imposed by a blood feeding habit. A blood meal provides a surplus of heme and iron (an important micronutrient for mosquitoes), coupled with a wide range of changes in the expression of genes related to iron/heme homeostasis. Ferritin (an iron-storage protein)

is transcriptionally induced after a blood meal, contributes to sequester iron released from heme digestion, and accumulates into the eggs, preventing oxidative damage during embryo development (reviewed in[35]). Therefore, heme degradation is supposed to be protective only when coupled to efficient iron sequestration by ferritin, in order to avoid production of reactive oxygen species by Fenton reaction[5]. In fact, in the mosquito, genes encoding ferritin and catalase, that counteract the production of hydroxyl radicals, respectively by isolating iron and scavenging hydrogen peroxide, are differentially regulated in the midgut up to 24 hours after a blood meal[36] and are proposed to be part of a heme and paraquat-induced response[37]. Accordingly, a negative feedback loop seems to be operating in the mosquito - in the presence of high levels of ROS, the midgut cells can decrease heme degradation, by inhibiting HO expression. This might offer some adaptive value, by avoiding increase in the intracellular levels of free iron formed during HO catalysis, which can take part in deleterious reactions. Otherwise, with an impaired redox balance, cleavage of the heme ring by HO would represent a pro-oxidant event for the insect.

The highly conserved target of rapamycin (TOR) pathway plays a critical role in regulation of translation by conveying extracellular nutritional conditions as an amino acid-sensing system[38,39]. TOR signaling is a key pathway linking blood digestion and egg development in *A. aegypti*[40,41]. This regulation is finely tuned to the nutritional requirements of the mosquito, and occurs at transcriptional and post-translational levels[42,43]. Since blood digestion impairment (Fig. 5a) is able to suppress AeHO gene expression, we tested whether this nutrient-sensing pathway could act upstream in the transcriptional regulation of AeHO. In fact, the use of rapamycin together with the blood meal, and the specific TOR depletion by RNAi (Fig. S1) were both effective in down regulating AeHO after feeding (Fig. 5b,c). This amino acid-dependent nutrient signaling has been associated with regulation of transcripts linked to mosquito vitellogenesis and digestion, in the fat body and the midgut, respectively[40,41]. The TOR-mediated cascade includes a GATA factor, which is the specific transcriptional activator of the vitellogenin gene. GATA binds specifically to GATA-binding sites in the proximal promoter region of the vitellogenin gene to activate its expression[44]. Indeed, we also found putative GATA-binding motifs in the promoter region of the AeHO gene (Fig. S1), which could indicate that this factor is the final downstream step in the TOR-mediated regulation of AeHO. Despite the fact that HO-1 in mammals is induced by rapamycin in injury models[45–47], we consider that in such examples this enzyme mediates the protective effects of rapamycin, exerting its expected cytoprotective role. However, TOR signaling in hematophagous insects has a fundamental role in supporting metabolic adaptations important for blood feeding. For instance, in *R. prolixus*, blood meal amino acids decrease ROS levels in the midgut immediately after feeding, through the TOR pathway[48]. In *A. aegypti*, this ROS minimizing event is mediated by heme itself[49], which strongly indicates that coupling the transcriptional regulation of AeHO with protein digestion can be an additional element in the intricate heme-driven response that occurs in the mosquito midgut upon blood feeding.

In mammals HO-1 expression and activity have been associated with anti-inflammatory, antioxidant, anti-apoptotic, and anti-proliferative effects that underlie tissue-protective responses under pro-inflammatory conditions (reviewed in[32]). Additionally, during viral infections, HO-1 has been reported to display noteworthy antiviral activity against a wide variety of viruses, including Zika and dengue viruses (Huang *et al*., 2017 and reviewed in[50]). Despite the fact that ZIKV infection was not capable of altering AeHO gene expression (Fig. 4d), further genetic knockdown studies are required to directly investigate the role of AeHO in the context of mosquito infection and its influence on the redox homeostasis and vectorial adaptation of *A. aegypti*.

Taken together, our results suggest a fundamental and novel role for HO in the mosquito. It is well established that heme degradation has an antioxidant role, not only by removal of the pro-oxidant free heme but also due to the antioxidant properties of biliverdin. Furthermore, we show that in the *A. aegypti* midgut, the heme degradation after a blood meal is fine-tuned through a nutrient-sensing regulation, being as essential as the blood digestion itself. The enzymatic heme degradation by AeHO seems to play a key role in the adaptation of *A. aegypti* to blood feeding, and its unusual transcriptional regulation may act as an evolutionary response to compensate for the massive ingestion of heme (Fig. 6).

Methods

Ethics statement. All animal care and experimental protocols were conducted in accordance with the guidelines of the Committee for Evaluation of Animal Use for Research (Federal University of Rio de Janeiro, CAUAP-UFRJ) and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (ISBN 0-309-05377-3). Dedicated technicians in the animal facility at the Instituto de Bioquímica Médica Leopoldo de Meis (IBQM) carried out all protocols related to rabbit husbandry under strict guidelines to ensure careful and consistent animal handling. The protocols were approved by CAUAP-UFRJ under registry #IBQM155/13.

HO sequence analyses. Multiple sequence alignment was constructed using the Clustal Omega web tool (http://www.ebi.ac.uk/Tools/msa/clustalo/). The evolutionary history was inferred using the Neighbor-Joining method[51] and the analyses were conducted in MEGA7[52].

Mosquitoes. *Aedes aegypti* (Red Eye strain) were raised in a mosquito rearing facility at the Federal University of Rio de Janeiro, Brazil, under a 12 h light/dark cycle at 28 °C and 70–80% relative humidity. Larvae were fed with dog chow[20], and adults were maintained in a cage and given a solution of 10% sucrose *ad libitum*. Four to seven day-old females were used in the experiments.

Mosquito meals. Female mosquitoes were artificially fed with the following different diets: (1) heparinized-rabbit blood with or without 500 µM paraquat or 20 µM rapamycin; (2) Substitute Blood Meal (SBM)[20] with or without 50 µM heme. Feeding was performed using water-jacketed artificial feeders maintained at 37 °C sealed with parafilm membranes. For RNA sample preparation, midguts (15–20) were dissected 24 h after feeding.

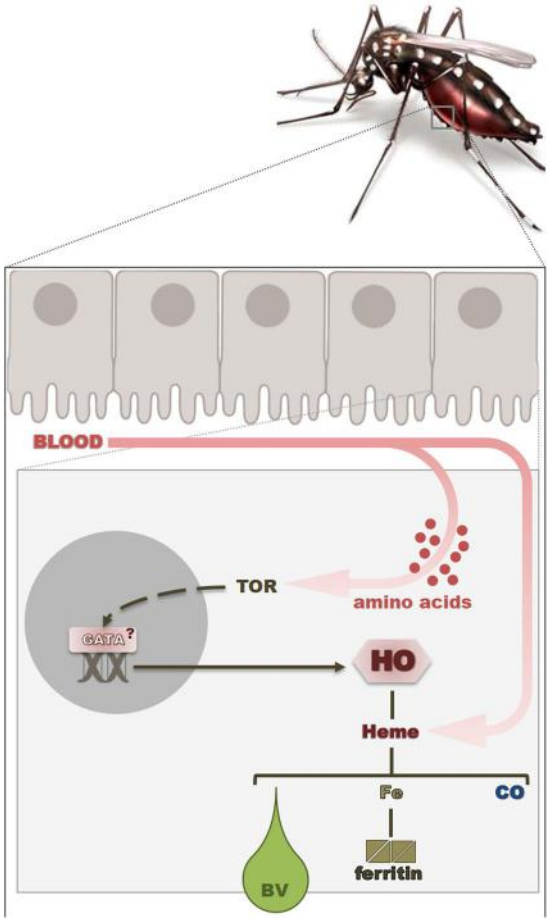


Figure 6. Overview of the non-canonical and coupled with digestion transcriptional regulation of heme oxygenase in *Aedes aegypti* midgut. Schematic representation of a female of *A. aegypti* mosquito after a blood meal and in detail, the inside of a midgut cell – amino acids activate target-of-rapamycin (TOR) pathway, which induces the transcription of AeHO (presumably through the GATA factor) resulting in heme degradation and production of AeBV, thus contributing to gut homeostasis and hematophagic adaptation.

Mosquito gene knock-down by RNAi. Double-stranded RNA (dsRNA) was synthesized from fragments amplified from cDNA of whole mosquitoes using specific primers containing a T7 tail. The *in vitro* dsRNA transcription reaction was adapted from a tRNA transcription protocol[53]. Briefly, it was performed at 37 °C for 12 h in a buffer containing 40 mM Tris•HCl (pH 8.0), 22 mM MgCl2, 5 mM DTT, 2 mM spermidine, 0.05% BSA, 15 mM guanosine monophosphate, 7.5 mM of each nucleoside triphosphate, PCR amplified template DNA (0.1 μg/μL) and 5 μM of a previously purified T7 RNA polymerase. The transcribed dsRNA was treated with DNAse at 37 °C for 30 minutes and precipitated using 1:10 (v/v) 3 M Sodium acetate pH 5.2 and 1 (v/v) of isopropanol. The pellet was washed twice with 70% ethanol and then eluted in water to reach a final concentration of 3 μg/μL. Mosquitoes were injected in the thorax with the double-stranded RNA (0.4 μg) and were blood-fed 48 h post injections. LacZ gene was used as a non-related dsRNA control and was amplified from a plasmid containing a cloned LacZ fragment. The oligonucleotides sequences used in dsRNA synthesis of Nrf2 and TOR can be found elsewhere[33,40].

Viral infections. Infection procedures were performed as described previously (Bottino-Rojas *et al*., 2018). Briefly, Zika viral stocks (ZIKV-BR_{PE}[54]) were propagated in C6/36 cells maintained in Leibovitz-15 medium supplemented with 5% fetal bovine serum, 1% nonessential amino acids, 1% penicillin/streptomycin and tryptose (2.9 g/liter). Females were infected in an artificial blood meal containing a 1:1 mix of rabbit red blood cells and L-15 medium containing Zika virus. Midguts were dissected at 5 days post-blood meal and subjected to RNA isolation.

RNA isolation, conventional and quantitative PCR analysis. Total RNA was isolated from 20–30 insects at different developmental stages (first and third instars larvae, and pupae), whole bodies of adult males and females and from midgut epithelium, ovary, head, Malpighian tubules, thorax and abdomen (carcass) of blood fed females using TRIzol (Thermo Fisher Scientific, Waltham, MA USA) according to the manufacturer's protocol. Complementary DNA was synthesized using the High-Capacity cDNA Reverse transcription kit (Thermo Fisher Scientific, Waltham, MA USA). PCR was performed using Taq DNA polymerase (Thermo Fisher Scientific, Waltham, MA USA) for 35 cycles (30 seconds at 94 °C, 30 seconds at 60 °C and 30 seconds at 72 °C)

in a thermocycler GeneAmp PCR System 2400 (Thermo Fisher Scientific, Waltham, MA USA). Both fragments obtained were about 100 bp in length. PCR products were separated on a 2% agarose ethidium bromide stained gel.

The Real time qPCRs were performed with the StepOnePlus Real Time PCR System (Thermo Fisher Scientific, Waltham, MA USA) using the Power SYBR-green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA USA). The Comparative Ct Method (Livak and Schmittgen, 2001) was used to compare the changes in the gene expression levels. The *A. aegypti* ribosomal protein 49 gene (RP49) was used as reference gene, based on previous data[55]. All oligonucleotides sequences used in qPCR assays are available in the Supplementary Material.

Pigment extraction. For AeBV pigment identification and purification, females were fed with rabbit blood. Fifty midguts were dissected under 50% ethanol, transferred to 10 mM sodium phosphate, 0.15 M NaCl, pH 7.4 (PBS), homogenized and centrifuged for 15 minutes at 12,000 × g. Supernatants were dried under vacuum, kept protected from light, and stored at −20 °C until analysis and purification by HPLC.

HPLC fractionation. HPLC was performed on a Shimadzu CLC-ODS C18 column (15 mm × 22 cm) using a Shimadzu LC-10AT device (Tokyo, Japan), equipped with a diode array detector (SPD-M10A). Chromatography analysis was performed using 5% acetonitrile with 0.05% trifluoroacetic acid (TFA) as solvent, at a flow rate of 0.4 mL/minutes. Before injection, dried samples were diluted in 10% acetonitrile with 0.05% TFA and centrifuged for 15 minutes at 12,000 × g. Ten minutes after sample injection a 40 minutes linear acetonitrile gradient (5–80%) was applied, followed by 20 minutes of 80% acetonitrile. Supernatants were dried under vacuum, and stored at −20 °C protected from light until use as described above. AeBV peak area data were taken for statistical analyses.

B-phycoerythrin oxidation protection assay. Loss of B-phycoerythrin (B-PE) fluorescence as a result of oxidation by peroxyl radicals generated by thermal decomposition of ABAP (2,2′-azobis (2-amidinopropane) dihydrochloride) was performed with a protocol modified from[56]. B-PE (Sigma) stocks were prepared in 0.01 M NH_4HCO_3 pH 5.0 to a final concentration of 2.08×10^{-6} M and stored at 4 °C under light protection. Stock solutions of the pro-oxidant ABAP (40 mM), and of the water soluble antioxidants ascorbic acid, TROLOX (Sigma), and AeBV (1 mM) were freshly prepared in 0.075 M NaH_2PO_4/KH_2PO_4 buffer pH 7.0. BV IXα solution (1 mM) was prepared in DMSO (Sigma). For B-PE oxidation assays with BV IXα, AeBV was also dissolved in DMSO. The B-PE oxidation reaction was started by addition of 10 µL of ice-cold ABAP stock solution to a cuvette containing the reaction mixture (1 mL) composed of B-PE ($1{,}65 \times 10^{-8}$ M) and the antioxidants (250 nM) in 0.075 M NaH_2PO_4/KH_2PO_4 buffer pH 7.0, at 37 °C. Control samples were prepared in the absence of antioxidants. DMSO was added to the controls of BV IXα assays. Fluorescence was monitored in a VARIAN Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, CA, USA) Fluorimeter for 60 minutes, excitation was measured at 540 nm and emission at 565 nm. For data analyses, fluorescence emission values were expressed as percentage of the initial fluorescence of B-PE.

Detection of protein carbonylation by western blot. Heme stock solutions (5 mM) were prepared as following: 3.27 mg of hemin was dissolved in 200 µL of 0.1 N NaOH and vigorously vortexed for 10 minutes. 800 µL of PBS pH7.4 was added followed by vigorous vortexing for 5 minutes. The solutions of 10 mM tert-butylhydroperoxide (t-BOOH) (Sigma) and TROLOX were prepared in Milli-Q water. Biliverdin IX alpha (BV IXα) stocks were prepared in 0.1 M of NaOH. The AeBV stock was prepared as described above. All the solutions were freshly prepared.

A single homogenate (female midguts extract) from 40–50 midguts was prepared as described in the item pigment extraction. We challenged midgut extract samples with a reaction mixture, composed by t-BOOH in the presence of heme, a system that is able to produce alkyl and peroxyl radicals. Carbonyl groups derived from oxidation of midgut proteins were subjected to derivatization with DPNH (2,4-dinitrophenylhydrazine) and detected by western-blot using anti-DPNH antibodies.

Samples of the guts homogenate (30 µg of total protein content) were incubated in PBS, pH 7.4 with 500 µM t-BOOH and 100 µM heme, in the absence or presence of AeBV (25 or 100 µM), for 60 minutes at 37 °C. Samples from the same homogenate were also incubated with 500 µM t-BOOH and 100 µM heme, in the absence or presence of Biliverdin IXa (25 or 100 µM), and the antioxidant TROLOX as controls. The oxidation reactions were stopped by addition of 50 mg/mL of the antioxidant butyl-hydroxytoluene (BHT) and SDS-PAGE loading buffer. BSA incubated with heme and t-BOOH or non-incubated midgut homogenates and BSA samples were used as positive and negative controls, respectively. Samples were subjected to 15% SDS-PAGE and transferred to PVDF membranes. Proteins were derivatized by treatment with 0.1 mg/mL DNPH in 2 N HCL for 30 minutes at RT. After incubation, membranes were washed 3 times with 2 N HCL and 5 times with 100% Methanol. Finally, membranes were rinsed with PBS and incubated with rabbit anti-DPNH antibody (Thermo Fisher Scientific, Waltham, MA USA) diluted 1:5000. Sequentially, anti-rabbit IgG conjugated to horseradish peroxidase and ECL Western blotting detection system was used (Thermo Fisher Scientific, Waltham, MA USA) according to the manufacturer's instructions. This analysis was followed by visualization on X-ray film (Amersham Hyperfilm ECL, Buckinghamshire, United Kingdom). Membranes were kept under light protection during all procedures described above (modified from Wehr and Levine, 2012). BSA (about 2 µg) was used as positive control for the oxidation system. BV IXα and TROLOX (25 or 100 µM) (Sigma) were used as standard antioxidants for this antioxidant capacity assay.

Statistical analysis. All analyses were performed with GraphPad Prism Statistical Software Package (Prism 6.0, GraphPad Software, Inc., San Diego, CA). Asterisks indicate significant differences (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; ns = non-significant) and each used post-test analysis is described in its respective figure legend.

Data Availability

All data generated or analyzed during this study are included in this published article (and its Supplementary Information Files).

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Author Contributions

Conceived and designed the experiments: V.B.R., L.O.R.P., O.A.C.T., G.S., B.C.D., P.L.O., G.O.P.S. Performed the experiments: V.B.R., L.O.R.P., O.A.C.T., G.S. Analyzed the data: V.B.R., L.O.R.P., O.A.C.T., G.S., G.O.P.S. Contributed reagents/materials/analysis tools: B.C.D., P.L.O., G.O.P.S. Wrote the paper: V.B.R., L.O.R.P., G.O.P.S.

Additional Information

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Chikungunya infection modulates the locomotor/flight activity of *Aedes aegypti*

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ABSTRACT

Aedes aegypti mosquito is involved in the transmission of arboviruses such as chikungunya virus (CHIKV). The effects of CHIKV infection on the locomotor/flight behavior of the *Ae. aegypti* mosquito vector was not studied so far, although it represents an essential aspect of virus epidemiology. Here, locomotion/flight activity of infected females were for the first time evaluated by monitors that captured movement by infrared light beams for seven days under light/dark regimen (12 hours of light followed by 12 hours of dark) at 25ºC and 60-80% relative humidity (RH). The results showed that the CHIKV infection caused a significant decrease in the locomotion/flight pattern of *Ae. aegypti* females. It describes an important difference in *Ae. aegypti* behavior and parasite-vector interaction, which may influence CHIKV viral spread and transmission dynamics. Thus, it is of great importance further studies focused on the analysis of other aspects of potential changes on physiology and behavior in infected mosquitoes.

Keywords: *Aedes Aegypti*; Chikungunya Virus; Locomotor Activity; Flight Activity

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INTRODUCTION

Aedes aegypti is a mosquito that belongs to the *Culicidae* family and the *Aedes* genus. This species can be found mainly in tropical and subtropical regions and its life cycle is divided into four stages: egg, four larval instars, pupae and adult. Female mosquitoes are hematophagous insects, feeding preferably on human blood, which is necessary for the maturation of their eggs. *Ae. aegypti* is capable to transmit several pathogens during the blood meal, being the most important vector of arboviruses responsible for serious public health problems, such as dengue (DENV), Zika (ZIKV), yellow fever and chikungunya[1-3].

Chikungunya virus (CHIKV) belongs to *Togaviridae* family and *Alphavirus* genus. This virus was isolated for the first time in 1952 in Tanzania from human serum[4]. Following the occurrence of several isolated outbreaks of this *Alphavirus* around the world, CHIKV reached the Americas in 2013 through the Caribbean and in 2014, the first records of autochthonous transmission occurred in Brazil. Since then, the virus spread throughout causing serious epidemics[5,6].

There are some vaccines that are yet in initial testing phase[7], thus the only way to try to contain CHIKV epidemics is focusing efforts on vector control. For this reason, understanding aspects of the biology and behavior of *Ae. aegypti* is highly encouraged.

In this sense, regarding the behavioral characteristics of this vector and the insects in general, it is known that the circadian clock is involved in the control of behaviors such as locomotion, flight, hematophagy, oviposition activity, among others[8]. More specifically, concerning locomotor activity, it was seen that mosquito species could be classified as having diurnal, twilight and nocturnal habits. The *Ae. aegypti* mosquito has a diurnal and twilight pattern[8,9].

Recently, studies have revealed that infection can modulate the locomotor behavior of *Ae. aegypti*. Infection by DENV2 in *Ae. aegypti* females caused them to increase their locomotor activity during the 24h period under LD 12:12 regimen (LD, which is 12 hours of light followed by 12 hours of dark)[10]. However, ZIKV showed an opposite effect; our group observed that ZIKV infection caused a decrease in locomotor/flight activity of *Ae. aegypti* females in a LD 12:12 and constant dark (DD) regimen[11]. Besides influencing the locomotor behavior, it was observed that arboviruses could modulate other aspects of behavior, such as oviposition[11,12]. Thus, the next step, now, is to focus efforts on studies that describe the behavioral characteristics of this vector when infected with other circulating arboviruses, such as CHIKV.

Here, we aimed to study the effect caused by CHIKV infection on the behavior of *Ae. aegypti* locomotor activity/flight in the LD regimen. This knowledge may serve for better understanding the transmission dynamics of this arbovirus.

METHODS

Mosquito breeding

All assays were performed with *Aedes aegypti* PAEA strain (Tahiti, French Polynesia). Mosquito breeding was realized in a controlled manner in the laboratory during all stages of its life cycle in incubators (Forlab Scientific Incubator, USA) at 25°C (± 1°C) and 60-80% RH, under LD 12:12 regimen, details in[11]. After the emergence of the adults, the females were kept together with the males for about 5 days to ensure insemination.

Blood Feed/Viral Infection

Aedes aegypti females were kept fasted for 6 hours before blood supply (infected with CHIKV or uninfected). Artificial feeding was done for approximately 40 min through a membrane attached to an artificial feeder at 37 °C.

The infectious blood meal consisted of a 1:1 mixture of rabbit red blood cells and L-15 culture medium containing CHIKV (isolate BHI3745/H804709, as described in[5]) with a final concentration of 10^7 PFU/ml; ATP was also included as phage stimulant at pH 7.4 in a final concentration of 1mM. Uninfected control mosquitoes were fed with naïve blood supplemented with L-15 culture media. Uninfected blood meal consisted of the same mixture but deprived of CHIKV.

After feeding, females were cold anesthetized and only the fully engorged were considered for the experiment (details in[11,13]). The entire procedure was performed within a biosafety level 2 insectary facility (BSL-2, Laboratório de Bioquímica de Insetos Hematófagos, Instituto de Bioquímica Médica, UFRJ).

Locomotor/flight activity

After blood feeding, infected and uninfected *Ae. aegypti* females were individually transferred into 25 mm glass tubes. In all tubes we added cotton soaked with 10% sugar solution to ensure mosquitoes feeding during the experiment. The tubes were then positioned within locomotor/flight activity monitors (Trikinetics Inc, Waltham, MA, USA). Each monitor has 32 channels with infrared light beams and the activity is captured every time the mosquito interrupts the beam. In general, two monitors were used for each condition (infected and uninfected) per experiment. We performed three independent experiments totaling 144 CHIKV-infected and 139 uninfected mosquitoes. The monitors were placed inside incubators (ELETROlab Scientific Incubator, Brazil), with the constant temperature (25°C ± 1°C), the humidity ranged from 60 to 80%, in LD12:12.

The movement of mosquitoes was captured every 5 minutes, for seven sequential days. For better representativeness, data were transformed to 30 minutes to the analyzes. Mosquitoes that had no activity during the last 24 hours of the experiment were considered dead and disregarded from analyzes.

Data analysis

All results were analyzed with Excel software (Microsoft Office). We represented the data compiled from values of Williams' mean[14] every 30-minutes of activity of each day of the experiment. The data were transformed into logarithmic due to a large variation in individual mosquito values, thus avoiding data masking (as explained by[11]).

Statistical analysis was based on the methodology described by[11]. Firstly, were performed the Shapiro-Wilk test to assess whether data is parametric or nonparametric. The data were analyzed for significance through the Mann Whitney statistical test.

Ethical Statement:

All experiments performed in this work were approved by Research Ethics Committees CEUA-UFRJ 149/19 (for rabbit blood use).

RESULTS

CHIKV infection decreases females *Ae. aegypti* locomotor/flight activity

We performed experiments to determine if CHIKV infection influenced *Ae. aegypti* behavior. Figure 1 show the locomotor/flight activity where data were represented by the values of 30-minutes mean activity of each experiment's day. As previously reported[9,10], females of *Ae. aegypti* concentrate most activity during the light phase. A bimodal pattern of activity with a peak during the day (between ZT8 and ZT11) and a peak in the early evening (ZT13.5) were observed in both CHIKV-infected and uninfected mosquitoes. Moreover, as expected, after ZT14, mosquito's activity is drastically reduced, remaining close to zero until late in the evening[9-11].

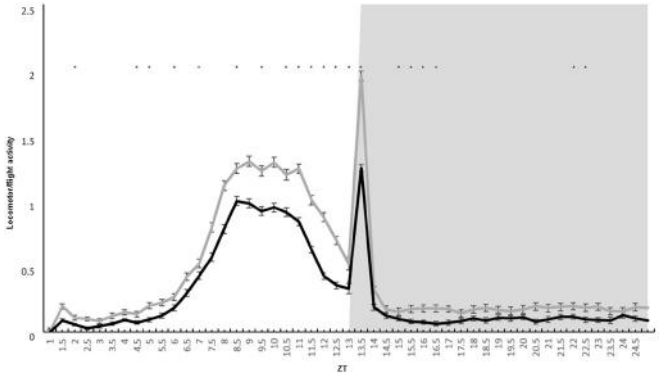

Figure 1. Average locomotor/flight activity of *Aedes aegypti* females infected with CHIKV (n=144, black line) and control females (grey line, n =139) along seven days, under LD12:12. It is possible to observe a significant decrease in the locomotor/flight activity of CHIKV infected females in the last hours of the light phase and in the transition between light and dark. Light area means light phase and grey area means dark phase. Error bars were shown for each 30 min interval. X axis represents the William's mean of locomotor activity and the Y axis represents the *Zeitgeber* Time. Asterisks represent the significance of the Mann-Whitney test, where $p < 0.05$.

Interestingly, we observed that CHIKV infection altered the *Ae. aegypti* locomotor/flight behavior, where which infected females showed a significant decrease in the activity along the light phase, with a more pronounced drop during the highest peaks of activity (between ZT8 and ZT11 and ZT13.5) (Mann-Whitney test, where $p < 0.05$; Figure 1, black line)

DISCUSSION

Knowing that vaccines for CHIKV arbovirus are still under study and as its infection can cause debilitating symptoms, it is necessary to focus on the containment of this virus spread by controlling its *Ae. aegypti* vector[7,15]. For this, more studies should be carried out in order to know more the aspects of the biology and behavior of this species to improve the development of control alternatives.

Regarding the locomotor behavior and physiology of *Ae. aegypti*, recent studies have observed that these aspects could be differently modulated according to the types of arboviruses that infected the mosquitoes. For example, even though they are from the same genus (*Flavivirus*), DENV2 infection increased mosquito's locomotor activity while ZIKV infection caused a decreasing in its locomotor/flight activity[10,11]. Additionally, in relation to other features of vector physiology, it was seen that DENV2 infection decreases the number of eggs laid for females, but ZIKV does not affect this parameter[11,12]. Together, these interesting data show antagonistic behaviors modulated for arboviruses infection of the same genus, which led us to question how it would be the response of the vector against an arbovirus infection of a different family.

Here, we present the first study that focuses on describing the behavior of locomotion/flight of *Ae. aegypti* females when infected with CHIKV arbovirus. We observed a decrease in their locomotor/flight activity, similar to the effect seen when females were infected with ZIKV, despite the viruses belong to different families.

This decrease in activity itself did not negatively influence the spread of the virus in Brazil due to the very large geographical distribution and infestation rates by this mosquito recorded in the country[6,16]. So, although infected females move less than uninfected ones, the huge population of the CHIKV main vector in the country can ensure considerable transmission and spread in the country[16]. In addition to the high population density of *Ae. aegypti* in the Brazilian territory, at least two other factors are likely to help in compensating the lower mobility of infected females in the spread and intensity of transmission: the high vector competence of Brazilian populations to CHIKV and the short extrinsic incubation period of CHIKV (virus can be expectorated by vector within 3 days after a blood meal on a viremic individual[17,18]). As a result, a large number of infected people, reaching 47.830 reported cases in Brazil between 2014 and 2015.

Recently, in 2019, until Entomological Week 34, the Southeast and Northeast regions had the highest values of incidence rate of probable cases, with 94.1 cases/100 thousand inhabitants and 39.3 cases/100 thousand inhabitants, respectively[6,15,19]. However, interestingly, the spread of CHIKV in Brazil was not as fast as expected in a country with a naive population. Despite sharing the same vector (*Ae. aegypti*), the spatial-temporal spread and annual number of cases due to ZIKV and CHIKV, following their invasion in Brazil was quite distinct: while ZIKV infected thousands of people and almost cover the country in one year, CHIKV inexplicably did not do so[11,20]. In 2014, CHIKV was firstly detected in Brazil in two states far apart: one in the north and another in the northeast. Outbreaks or clusters of cases were reported over 2014 in other four states[5,6]. This unexpected epidemiological profile of spread and incidence has not yet been explained. It is possible the alteration of the behavior of the CHIKV infected *Ae. aegypti* reported herein may have that somehow contributed to this phenomenon.

Our findings reinforce the importance of further studies focusing on analysis of the physiology and behavior of *Ae. aegypti* infected with CHIKV, for example, analysis of female fecundity and fertility, daily survival of the vector, aspects of hematophagy, among others. In addition, it is necessary to understand the influence of the circadian clock on this locomotor behavior of CHIKV-infected *Ae. aegypti*, performing experiments in constant dark conditions. Besides, it is important to investigate the effects of infection at the molecular level.

We consider that our data will support the better understanding of how the *Ae. aegypti* behavior is impacted by the viral infection and could highlight some important aspects of host-vector interaction, which in future may serve as a basis for control strategies of this vector mosquito.

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Natural infection by the protozoan *Leptomonas wallacei* impacts the morphology, physiology, reproduction, and lifespan of the insect *Oncopeltus fasciatus*

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Trypanosomatids are protozoan parasites that infect thousands of globally dispersed hosts, potentially affecting their physiology. Several species of trypanosomatids are commonly found in phytophagous insects. *Leptomonas wallacei* is a gut-restricted insect trypanosomatid only retrieved from *Oncopeltus fasciatus*. The insects get infected by coprophagy and transovum transmission of *L. wallacei* cysts. The main goal of the present study was to investigate the effects of a natural infection by *L. wallacei* on the hemipteran insect *O. fasciatus*, by comparing infected and uninfected individuals in a controlled environment. The *L. wallacei*-infected individuals showed reduced lifespan and morphological alterations. Also, we demonstrated a higher infection burden in females than in males. The infection caused by *L. wallacei* reduced host reproductive fitness by negatively impacting egg load, oviposition, and eclosion, and promoting an increase in egg reabsorption. Moreover, we associated the egg reabsorption observed in infected females, with a decrease in the intersex gene expression. Finally, we suggest alterations in population dynamics induced by *L. wallacei* infection using a mathematical model. Collectively, our findings demonstrated that *L. wallacei* infection negatively affected the physiology of *O. fasciatus*, which suggests that *L. wallacei* potentially has a vast ecological impact on host population growth.

Infections induce physiological alterations that can potentially impact host lifespan, development, reproduction, and behavior[1–4]. Combined, these phenotypical alterations may reduce the overall fitness and impair host development[5–7]. Such modifications usually arise in response to microorganism by-products or host adaptation to the infection, or both[8]. Thus, these host physiological alterations may directly or indirectly impact population development and shape their community structure in nature[9,10]. Despite several reports demonstrating disturbances in reproduction and mating behavior related to infection, little is known about the long-term negative impacts of such infections on the population dynamics in the ecosystem[11,12].

Trypanosomatids are protozoans that parasitize all classes of vertebrates, several invertebrates (mostly insects), and plants[13–15]. *Leptomonas* is a genus of the family Typanosomatidae (order Trypanosomatida, class

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Kinetoplastea); this order is solely comprised of flagellated parasites[13,14]. A high percentage of trypanosomatids infect only insects, but the genera *Trypanosoma* and *Leishmania* are considered the most important ones because they cause severe illnesses in humans and often lead to the death of infected patients[15]. *Leptomonas wallacei* is a gut-restricted insect parasite that naturally infects the seed-eater *Oncopeltus fasciatus* (Hemiptera: Lygaeidae)[16–18].

The hemipteran *O. fasciatus* has been used in seminal studies on embryology[19,20], cytogenetics[21,22], biochemistry[23,24], nucleic acids[25] and interaction with its natural or experimental trypanosomatid parasites[26–28]. Some of these studies date back to 1926[26,29] when a natural infection of *O. fasciatus* by *Leptomonas* spp. was first described[26]. Later on, *O. fasciatus* was widely employed in molecular studies that intended to shed light on embryogenic and physiological aspects of this insect[30,31], which has since been considered a pivotal model for studying evolutionary developmental biology[30]. RNA interference (RNAi) approaches have been successfully used to hamper expression and determine the influence of specific genes on sexual and morphological development on *O. fasciatus* species[32,33]. In addition, other studies have been conducted to better understand the relationship between *O. fasciatus* and trypanosomatids[16–18,34–38].

Host responses to infections are often studied in artificial models rather than in natural conditions. Nevertheless, investigations of natural infections are preferable to elucidate their actual impact on host populations[39,40]. In general, infections caused by trypanosomatids affect insect physiology by reducing their reproductive capacity, impairing host development and locomotion, by modifying hosts behavior, and by increasing host mortality[41,42]. Since its first description, *L. wallacei* has not been considered pathogenic towards *O. fasciatus* in short-term infections. Conversely, no data is available for long-term infections[16]. The transmission of *L. wallacei* between insects occurs via the ingestion of cystic forms through coprophagy, as well as by transovum transference of cysts that adhere to the eggs during oviposition; i.e., the newly born nymphs are infected by probing the fecal droplets on eggshells[36]. It was common for the *O. fasciatus* individuals that we used in our laboratory to be naturally infected by *L. wallacei*[16]. Therefore, we generated another colony of *O. fasciatus*, which we raised from disinfected eggs. The novelty of the present study was to compare the *L. wallacei*-free and the naturally infected insects in a controlled environmental laboratory model to determine the long-term effects of natural infection. Hence, we demonstrated that a natural protozoan infection can significantly affect the insect host development and population growth, which could greatly impact the population dynamics of the host species.

Results

***Leptomonas wallacei* infection reduces *Oncopeltus fasciatus* lifespan.** The study of natural infections may help to understand the burden of parasitic diseases on host fitness and population dynamics. Previously, we have demonstrated that *L. wallacei* is vertically transmitted and reaches 100% infection in *O. fasciatus* adults[38]. Here, we used that experimental model to evaluate the impact of *L. wallacei* natural infection on *O. fasciatus*, as compared to non-infected insects. First, we observed the effect of *L. wallacei* on *O. fasciatus* life expectancy. Notably, infected females were more affected by infection than males, as 50% of infected females showed a greatly reduced lifespan, i.e., they died twice as fast as the uninfected ones (Fig. 1A). Moreover, under stress conditions caused by food and water deprivation, infected insects died earlier in life than uninfected insects, although no difference in stress susceptibility between sexes was noted (Fig. 1B,C). These experiments demonstrated that infection impacts host survival in either normal situations or under stress conditions, with higher severity in females, but only in normal conditions. To evaluate the importance of infection status in insect development and mortality, we observed the insects from the time they hatched from eggs into first instar nymphs until they reached the adult stage. We observed a slight delay in time from fifth instar nymph to adult in infected insects, although there was no difference in life expectancy between infected and uninfected nymphs (Fig. S1). Therefore, *L. wallacei* infection also induces a delay in the development of *O. fasciatus*.

***Leptomonas wallacei* induces morphometric alterations in *Oncopeltus fasciatus*.** From the analysis of insect development, we observed severe morphologic alterations in infected insects that were rarely observed in uninfected insects (Fig. 2). To demonstrate these differences graphically, we devised a measurement of overall morphology to compare infected and uninfected insects (see Methods for details, Figs. S2 and S4, Table 1). We observed a significant reduction in the overall size of infected insects (Fig. 2A, see below). Despite the morphological alterations, no differences in weight were observed between infected and uninfected virgin adult insects, which demonstrated that the alterations in overall morphology did not result in weight loss (Fig. S2A). Since *L. wallacei* infection induces severe morphological alterations on *O. fasciatus*, we tried to distinguish the insects by their infection status by applying a principal component analysis (PCA) to morphological features. Scatter plots of the first two principal components (PCs), individually colored by infection status, are shown in Fig. 2B (females) and 2 C (males). For both groups (males and females), PCA resulted in the first PC that explained ~50% of the total variance and was positively correlated with all the original variables (more details in the supplementary text). These analyses showed that there is a clear separation between infected and uninfected females (Fig. 2D, Figs. S4A,B) in the morphometric (feature) space and that this separation is more evident, and thus able to distinguish females by their infection status (Fig. 2B). To clearly demonstrate the association between morphology and infection, we took the first PC as a combined measure to describe insect size and used this variable as a predictor of infection status in a binary generalized linear model (GLM). As shown in Fig. 2D, it was possible to detect a significant association between insect size and infection (see Fig. S4C for the same analysis in the nymph dataset). Moreover, it was possible to observe the association between insect size and infection status and more visibly demonstrate higher severity of alterations in infected females than in males, in the GLM analysis.

Female insects are more susceptible to *Leptomonas wallacei* infection than males. To investigate sex differences in lifespan and the impact of infection on morphological features, we evaluated the presence of parasites in the intestine of males and females. Through the analysis of the relative expression of a *L.*

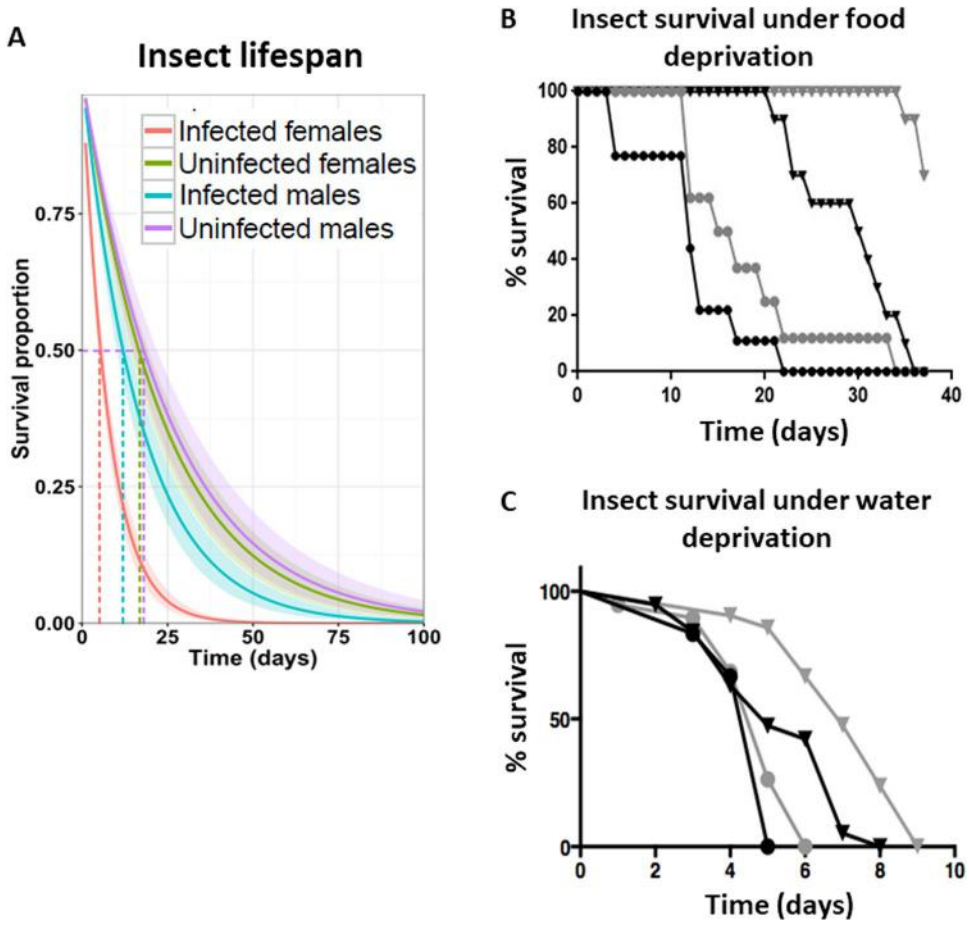

Figure 1. *Leptomonas wallacei* infection reduces *Oncopeltus fasciatus* survival. (**A**) Lifespan of adult insects, fed *ad libitum*, from infected and uninfected colony. Infected males, blue line (n = 12), uninfected males, purple line (n = 18), infected females, red line (n = 27) and uninfected females, green line (n = 18). Lifespan of adult insects maintained submitted to food (**B**) or water deprivation (C). Infected males, black circles (n = 10), uninfected males, black triangles (n = 10), infected females, gray circles (n = 10) and uninfected females, gray triangles (n = 10).

wallacei-specific sequence (16 S rRNA gene), no difference was observed between males and females (Fig. 3D). Nevertheless, promastigote counts were higher in females than in males and the difference observed was noticeably located in the midgut, i.e., the main site of *L. wallacei* infection, and no difference in the hindgut number of parasites was observed (Figs. 3A–C and S5). To visualize the infection micro-environment, we prepared scanning electron micrographs of insect midguts and observed massive amounts of parasites attached to the whole gut wall in infected insects, whereas no parasites and intact intestinal structures were observed in uninfected ones (Fig. 3E,F). Therefore, our images clearly demonstrated that *L. wallacei* induces gut micro-environment alteration when established in the infection site. Moreover, there was no difference in *L. wallacei* relative gene expression between males and females. Despite that, females present more *L. wallacei* promastigotes in the intestine reflected by a robust difference in promastigote forms in the midgut (Fig. 3C).

***Leptomonas wallacei* infection reduces *Oncopeltus fasciatus* reproductive fitness, downregulating *intersex* gene expression.** In the present study, we observed that females were more susceptible than males in terms of changes to their morphology and lifespan. This led us to wonder if this higher susceptibility was related to the cost of carrying eggs during reproduction. We thus tested if *L. wallacei* infection also impacted *O. fasciatus* reproduction. Reproductively active infected females were lighter than the uninfected ones, whereas no difference in weight was observed between males (Fig. 4A). Because females need to carry eggs, we investigated if the observed weight loss was associated with a decrease in reproductive output (Figs. 4B,C, S6A,B). Uninfected females laid more eggs than infected ones and the decrease in the number of eggs laid overtime was slower in uninfected than in infected females (Figs. 4B and S6A for absolute values). We observed a ~27% reduction in laid eggs from infected insects in comparison to uninfected ones. Furthermore, the eclosion rate of eggs laid from uninfected insects was higher than from infected insects (Fig. 4C and Table S1 in data S1, for overall values).

To investigate the reason for the reduction in the number of eggs laid and the eclosion of eggs in infected insects, we evaluated the eggs in the abdomen of females from both colonies. The average egg load of uninfected

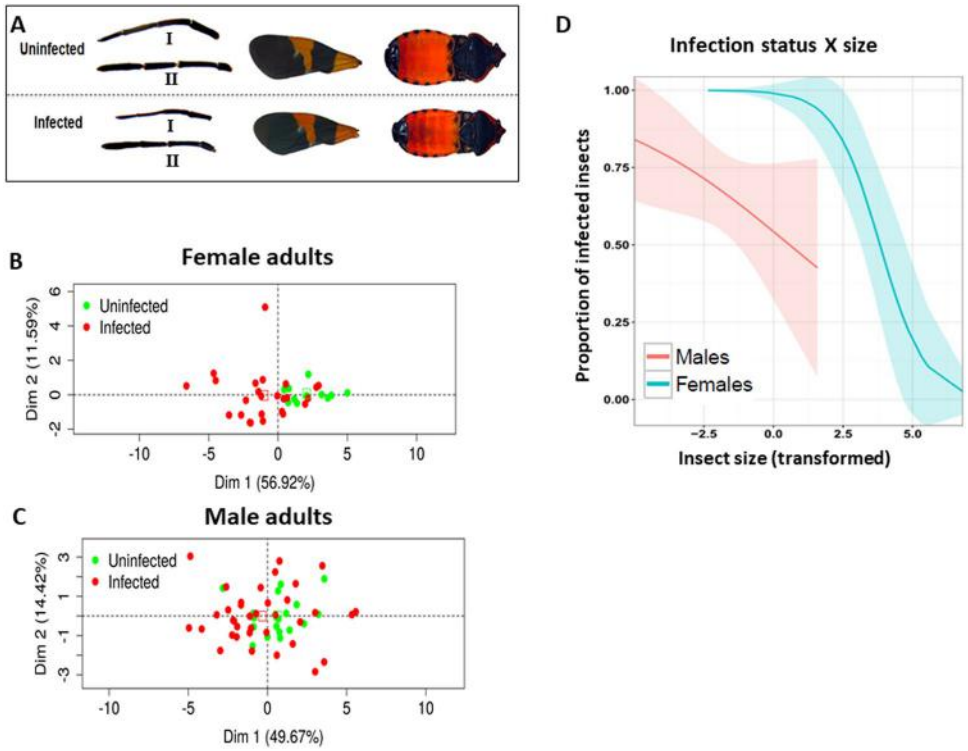

Figure 2. (**A**) *Leptomonas wallacei* infection induces host morphology alterations. Different body parts of uninfected (above) and infected (below) *Oncopeltus fasciatus* side by side for comparison (1- rostrum, 2- antenna, 3- forewing, and 4- body length). Principal components analysis (PCA) plots for the morphometric data in females (**B**) and males (**C**). All morphometric variables were transformed using the PCA, the first and second principal components were plotted, and each point graphically represents a sample (infected-red dots and uninfected-green dots). (**D**) Generalized linear model (GLM) prediction of the probability of being infected. Results of GLM prediction of the probability of being infected in response to the size principal component derived using the PCA for males and females in the adult dataset.

females is 30 eggs; infected females had an average of 23 eggs. Infection by *L. wallacei* reduced the egg load of *O. fasciatus* by almost 25% (S2 Table in S1 data). To verify if there were differences between these loaded eggs, we also evaluated the morphology of eggs from uninfected and infected insects (Figs. 4D and S6B). We observed that ~2.5% of the eggs from infected females showed characteristics of reabsorption, whereas less than 0.5% of the eggs from uninfected females showed these characteristics (Fig. 4E).

After demonstrating the reproductive impact of *L. wallacei* infection on *O. fasciatus* development and reproduction fitness, we investigated the influence of the parasite and infection on the expression of a gene related to all these parameters, the intersex gene (*ix*). When *Ix* expression was evaluated between both colonies, uninfected individuals expressed higher levels of *Ix* than the infected ones (Fig. 5A). In addition, we applied RNAi for the *Ix* gene and the females subjected to *Ix* RNAi protocol showed the same egg reabsorption rates as observed in infected females, albeit the control RNAi had no effect on egg formation (Fig. 5B–D). After demonstrating that the *Ix* gene was essential for ovarian maturation, insects that were subjected to ds*ix* also showed ovarian atrophy (Fig. 5E).

***Leptomonas wallacei* infection impacts *Oncopeltus fasciatus* population dynamics.** To assess the population-level impact of natural infection by *L. wallacei* on its host, we proposed a simple ordinary differential equation-based mathematical model to obtain short-term population projections for infected and uninfected colonies. We used the data and estimates observed in this study to create two separate sets of parameters, one for each scenario (infected and uninfected) and using an initial population size $N(0) = 400$. First, we obtained the ratio of each stage trajectory in the infected scenario relative to its counterpart in the reference scenario. This offered unit-free trajectories, which we can then be studied to determine the impact of infection on the growth of each life stage. Figure 6A shows the results of this analysis and it is notable that the ratios tend to reach zero as time progresses. Interestingly, there was also a temporary increase in the number of females in the infected population, likely driven by the transient rise in nymphs of the fourth instar. This result is most likely a consequence of sampling error in the measurements of development rates (which indicated higher rates of transitions from the third instar to the fourth instar in the infected group), although the possibility that the molting rates may be higher in infected insects still requires investigation. The long-term behavior of the model, however, indicated a clear reduction in population size in the infected scenario, which was confirmed by the overall population projections in Fig. 6B. This analysis showed exponential growth as expected since, apart from infection, all conditions

	Males			Females		
status	Infected	Uninfected	*p*	Infected	Uninfected	*p*
weight (g)	0,04 (0,01)	0,04 (0,00)	0,0178	0,06 (0,01)	0,06 (0,01)	0,0993
lenght (mm)	10,60 (0,51)	10,77 (0,43)	0,165	11,66 (0,56)	12,20 (0,31)	$<0,001$
hindwing lenght (mm)	7,85 (0,43)	8,01 (0,39)	0,1303	8,54 (0,43)	9,00 (0,28	$<0,001$
forewing lenght (mm)	9,69 (0,53)	9,85 (0,48)	0,2243	10,70 (0,57)	11,21 (0,40)	0,002
hindwing area (mm^2)	17,37 (2,04)	18,71 (1,54)	0,0051	19,98 (2,37)	23,58 (1,56)	$<0,001$
forewing area (mm^2)	19,86 (2,06)	20,82 (1,72)	0,0533	23,96 (2,43)	27,09 (2,07)	$<0,001$
rostrum lenght (mm)	5,26 (0,43)	5,33 (0,39)	0,5374	6,03 (0,56)	6,10 (0,31)	0,5964
antenna lenght (mm)	6,29 (0,30)	6,53 (0,33)	0,0051	6,55 (0,34)	7,24 (0,28)	$<0,001$
abdomen width (mm)	3,69 (0,23)	3,78 (0,22)	0,1221	4,13 (0,25)	4,33 (0,17)	0,0043
leg lenght (mm)	7,11 (0,83)	7,65 (0,70)	0,0079	7,50 (0,96)	7,84 (0,62)	0,181
insect area (mm^2)	30,35 (2,96)	31,92 (2,43)	0,026	38,15 (3,51)	41,23 (2,20)	0,0013

Table 1. Morphometric analysis of the insects. Virgin insects (one week after becoming adults) were collected from both colonies, separated by sex and measured. The expressed values are the average values obtained for each parameter. In parenthesis is the standard deviation value for each data group. The weight, body size, total area of the body, membranous wing and hemi-elytra size, membranous wing and hemi-elytra area, rostrum and median leg size, abdomen width and antenna size of the insects were measured. The weight of the insects was obtained using a precision scale and the measurements were obtained using the software Analyzing Digital Images (Museum of Science, Boston). The morphometric variables between the groups were performed using unpaired *t* test.

for insect development and reproduction were optimal. We draw the reader's attention, to the striking difference in population projections between infected and uninfected groups, a gap that increases as time progresses. The results shown in Fig. 6A,B account for the differential mortality between males and females, as this is an important finding of the present study. Similar projections using a combined mortality rate for adults, i.e., a model without sexual differentiation, are presented in Fig. S7 and the gap between the infected and uninfected population albeit present, seemed to be smaller than the one from the model that accounts for differential mortality.

Discussion

The current study presents the broad range of long-term effects of a natural *L. wallacei* infection on *O. fasciatus* fitness, including alterations in life expectancy and gene expression that culminate in reproductive deficits. Here, we propose an association between the morphology and infection status on the effects of this natural on host trypanosomatid infection physiology, as demonstrated by several parameters. In addition, we demonstrated an infection sexual bias, being females more susceptible than males. On average, infected females survived for half the period of time than uninfected females. Several reports have demonstrated that infections caused by insect pathogens impact the insect's lifespan, although sex-biases were not described[45–48].

Infected adults survived for a shorter period of time than the uninfected ones, even though the same was not observed in the early stage nymphs. This might be explained by the increased rate of *L. wallacei* infection over time, as previously demonstrated by our group. Here, we also observed the reduced lifespan of insects subjected to stress conditions and the higher susceptibility of infected insects in terms of a lower life expectancy than uninfected, as has also been demonstrated in several trypanosomatid-host interaction models that have considered sub-pathogens[49]. In nature, insects sometimes live under stress conditions and our results suggest that infected insects, males and females with no sex-bias, were more susceptible to water and food deprivation than uninfected individuals, which might impair the ability of infected hosts to persist in the environment.

In the present study, a massive amount of *L. wallacei* was attached to the gut wall of the host, which might cause disturbances of the digestive physiology and interfere with the normal functioning of this organ and with nourishment. This may explain why infected insects had shorter life expectancies in our model. Similar scenario has been reported for vector interactions with other trypanosomatids, such as *Blastocrithidia triatomae*, *Trypanosoma congolense*, and *Letomonas pyrrhocoris*[50]. The presence of parasites covering the intestinal wall of the host lowers its nutrient uptake through competition, i.e., by producing a mechanical barrier, reducing the contact surface of the microvilli, and disturbing the excretion of the host[50]. The higher number of promastigotes that effectively attach to the insect intestine may explain why females have a shorter life expectancy than males. On the other hand, the qPCR results demonstrated that there was no difference in *L. wallacei* quantification, comparing males with females. However, in qPCR we analyze both promastigotes and cysts of *L. wallacei*, whereas by microscopy, without staining the parasites, we can only see the promastigotes. Also, the classical technique for counting, using a Neubauer hemocytometer, has been employed to quantify *L. wallacei* in whole homogenates or gut contents, but the quantification of parasites in gut samples by microscopic counting is frequently hampered by the presence of a high concentration of debris in the gut, and urate crystals in the hindgut. Few reports in the literature demonstrate infection sex-bias, with notable exceptions being the fungal infections of *Metarhizium matsumae* and *M. anisopliae* in tsetse flies[5,51].

Here, we also demonstrated an evident infection effect on insect morphology as an important aspect of *O. fasciatus* infection. Similar results have been demonstrated in the literature, and further investigation is required to investigate if morphological alterations impacts on *O. fasciatus* behavior[52,53]. In an innovative way,

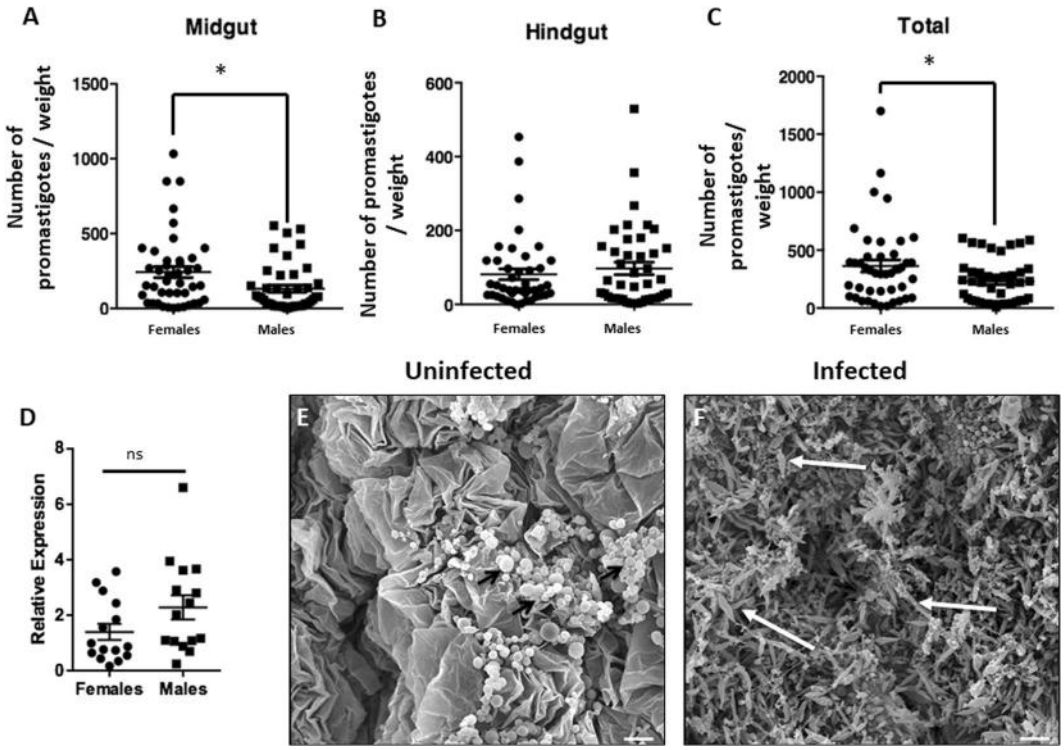

Figure 3. Parasite number in the gut of infected *Oncopeltus fasciatus*. Insect intestine was separated into the midgut and hindgut and promastigote forms counted in a Neubauer chamber (**A,B**). The overall number of promastigotes was obtained using the sum of the number of parasites in the midgut and hindgut (**C**). Insects were collected from the infected colony and their alimentary tracts were dissected for qPCR analysis for comparative *L. wallacei* gene expression (**D**). The hindgut was dissected from an uninfected or infected insect and observed by scanning electron microscopy (**E,F**). No flagellates were observed in the gut of an uninfected insect (**E**), whereas the gut of an infected insect shows a massive presence of flagellates (**F**). Black short arrows show uric acid spherules (**E**)[66] and white long arrows highlight *L. wallacei* promastigotes (**E**). Scale bars = 10 µm.

we delineated a straight parallel between morphology and infection of *O. fasciatus*, which may also be applied to other microorganism-host interaction studies to evaluate morphologic disturbances and determine infection statuses by morphometric parameters. The PCA and GLM data analyses applied here may reveal an opportunity for analysis of the long-term effects of infection on host morphology. Thus, these results provide evidence that *L. wallacei* severely impacts the overall morphology of its host and provides evidence that infected and uninfected insects may be distinguishable by morphometric analysis.

In parasitic infections, energy allocation may be at the center of the alterations because the host requires energy to compete with the microorganisms for nutrients and to increase resource allocation to combat the infection impacting host reproduction (Figs. S8 and S9)[43,44,54]. In the present study, we also demonstrated that *L. wallacei* imposed negative effects on the reproductive fitness of *O. fasciatus*. Compared to uninfected females, a decrease in mass was observed in infected females during reproduction, although no differences in weight were observed between virgin infected and uninfected insects. Similar negative effects have been observed in response to viral infection in flies, associated with reduced rates of digestion and excretion in response to damage caused by infection[55]. *Microsporidium* infection in gypsy moth larvae results in mass loss, which is attributed to a decreased nutrient absorption[56]. Here, weight reduction in infected females could be explained by a combination of both; high number of promastigote forms in the intestine that might disturb the nutrient absorption, and the direct damage caused by infection. We also found that infected *O. fasciatus* presented decreased oviposition, egg loads, and egg eclosion. Oviposition reduction in infected insects has been reported and is associated with *Plasmodium*-mosquito interactions and trypanosomes interaction with *R. prolixus* and tsetse[57,58]. Similar observations in eclosion reduction were related to the depletion of resources for investment in egg load, which would have resulted in nutritional depletion and thus would have affected the composition of the embryo[59,60]. Similarly, we observed an increase in egg reabsorption in infected females, which suggests one reason for the observed reduction in egg load, oviposition, and viability in the infected colony, i.e., due to the reabsorption of eggs, which is a well-reported tradeoff strategy for the maintenance of somatic activities as an adaptation to infections[61–64]. Moreover, we demonstrated that infection manipulated the host *Ix* gene expression, a gene that is crucial for sexual maturation in flies[65]. In addition, when we used the RNAi protocol for the *Ix* gene, the insects developed spontaneous egg reabsorption with the same characteristics of infected insects. Thus, our data suggest that the decrease in reproductive fitness seen in infected insects is a result of egg reabsorption, which in turn is triggered by lower *Ix* gene expression. Thus, the present study argues that reproductively active females have increased infection susceptibility. This may be because females need to deal with the high energetic costs associated with

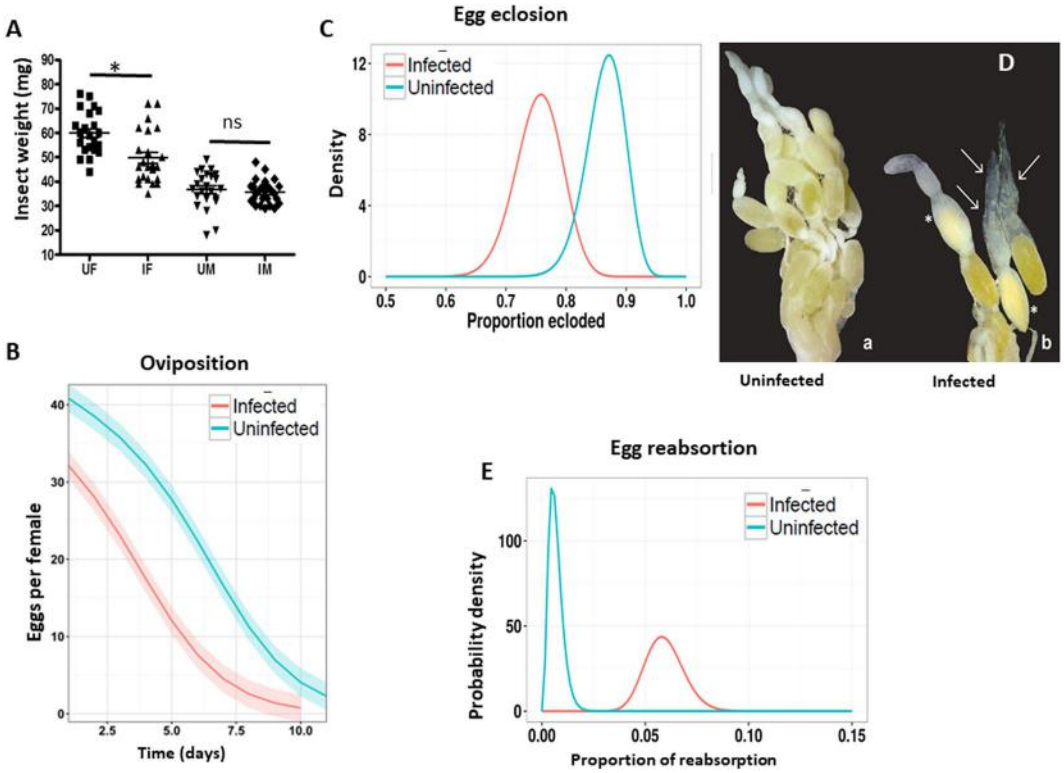

Figure 4. *Leptomonas wallacei* infection induces reproductive fitness reduction in *Oncopeltus fasciatus*. (**A**) Reproductively active adult females and males were collected and weighed using a precision balance (UF-uninfected female, IF-infected female, UM-uninfected male, IM- infected male) * represent significant differences in the one-way ANOVA test. (**B**) Oviposition from both colonies was monitored daily for two weeks and the average daily ovipostion was predicted using the zero-inflated Poisson model. Generalized linear model formulation was used with the R statistical pscl package to compute environmental variables. (Uninfected n = 17 and infected n = 20). (**C**) Egg eclosion was determined by counting the number of ecloded eggs laid from both colonies. The x-axis shows the estimated proportion of eclosion and the y-axis shows the probable density (uninfected n = 90 and infected n = 120). (**D**) Representative images of the morphology of female ovaries from an uninfected or infected female. (**E**) Egg reabsorption was observed by classifying and counting the number of eggs in dissected females. The x-axis shows the estimated proportion of reabsorption and the y-axis shows the probability density (uninfected n = 30 and infected n = 30).

the infection, which decrease the energetic stock that may be allocated for use in reproduction. Although we have evidenced innate immune activation induced by *L. wallacei*, further studies are needed to evaluate the impact of this infection on *O. fasciatus* immunity.

Regarding all the parameters that were evaluated (lifespan, reproduction, and morphology), infected insects showed decreased fitness, which posed an obvious question on whether all the modifications impact the insect population, as observed in other host-parasite models. Hence, we proposed a mathematical model to obtain short-term population projections for both colonies for comparison. The long-term behavior of the model, however, points to a well-defined reduction in population size in the infected scenario, a finding confirmed by the overall population projections. Our analysis showed exponential growth, because apart from infection, all conditions for insect development and reproduction were optimal. Readers should focus on the striking difference in population projections between infected and uninfected groups, a gap that gets bigger as time progresses. This result showed differential mortality between males and females, and was an important finding of the present study. Similar projections using a combined mortality rate for adults, i.e., models without sexual differentiation were also tested and the gap between the infected and uninfected population was albeit present. In our opinion, this constitutes an important finding, as the females bear the eggs, and it might have a differential impact on population dynamics in *O. fasciatus*.

In all scenarios tested in our mathematical models, *L. wallacei* infection can impact the population dynamics of *O. fasciatus*. Nonetheless, it is important to emphasize that our modeling approach presents two important limitations. First, it does not account for infection directly, thus it assumes that populations are either 100% infected or completely parasite-free. Although this constitutes an unrealistic assumption, as about 30% of *O.fasciatus* is infected with monoxenic trypanosomatids in nature, infection rates for this system are very difficult to measure in practice[16]. Since complete infection in a colony seems to occur within a week, our modeling results (which can span over 8 weeks) remain valid. Second, we can only obtain short-term population projections (within ~16 weeks). It would be interesting to investigate the impact of *L. wallacei* infection on the evolutionary dynamics of

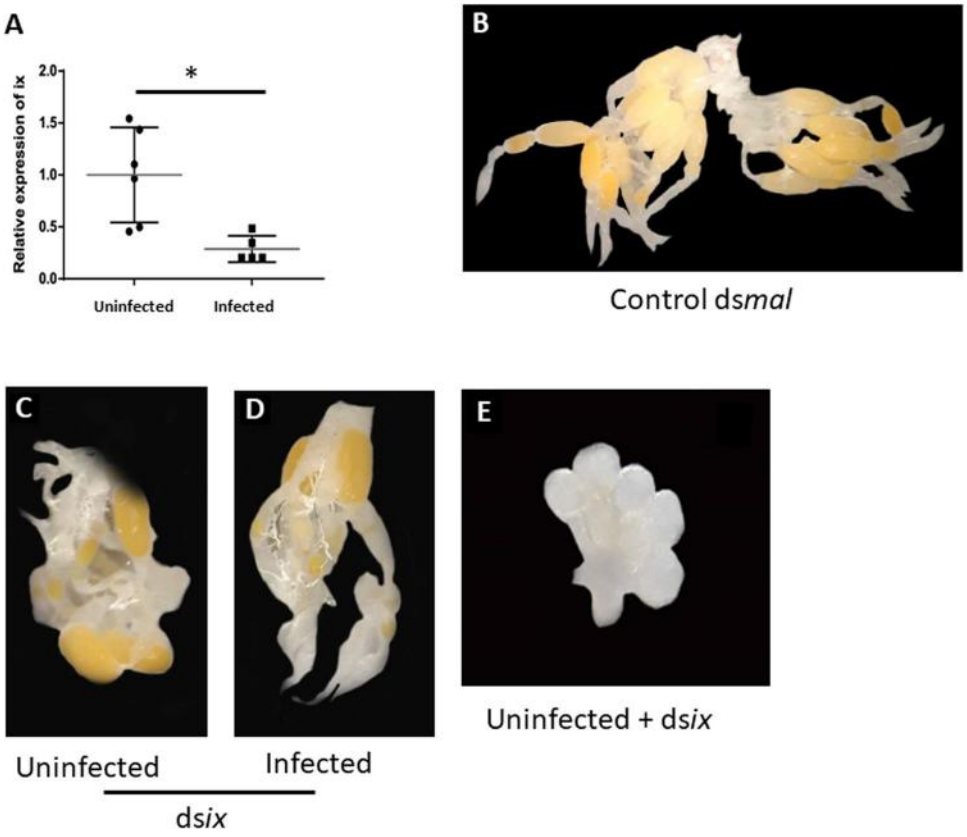

Figure 5. *L. wallacei* infection impacts on *O. fasciatus* intersex (ix) gene expression reproductive organs resulting in reproductive fitness loss. (**A**) Ovarian *ix* gene expression was assessed by qPCR in uninfected and infected females. (**B**) Representative image of ovary of an uninfected insect subjected to control ds*mal* protocol; showing normal ovary morphology. Reproductively active uninfected (**C**) and infected (**D**) insects silenced with ds*ix*. (**E**) Representative image of an ovary from an uninfected female subjected to RNAi with ds*ix*; showing an atrophied ovary morphology. Representative of two independent experiments.

Oncopeltus populations. We hope that further studies specifically targeted at this question will be able to provide the necessary data, which (with slight model modifications), could be used to gain insights into this host-parasite interaction over more extensive temporal scales.

In conclusion, our results demonstrated the importance of a natural infection in a host's life and suggest a new way to evaluate how infection can impact the features and fitness of the host. Also, our model could help further understanding of related insects. Finally, our study concluded that *L. wallacei* had a negative impact on its host's physiology and that such impacts could ultimately affect *O. fasciatus* population regulation.

Methods

Maintenance of *O. fasciatus*. In the present study, we used two colonies of *O. fasciatus*: one was constituted of insects naturally infected with *L. wallacei* and the other was constituted of uninfected insects, which served as the control[38]. To obtain the uninfected colony, eggs collected from the infected colony were decontaminated by 2% sodium hypochlorite treatment for 5 min. After decontamination, the eggs were kept in sterile plastic containers and the newly hatched insects maintained in the same conditions described for the parental colony. In order to avoid recontamination of uninfected colony with *L. wallacei*, these insects have been kept in a different, isolated, room from the parental colony. In order to validate the absence of *L. wallacei* in the uninfected colony all the insects used in the experiments have been checked for the presence of *L. wallacei* in their guts by optical microscopy or PCR. The colonies were maintained under a 12 h light/dark cycle, at 28 °C, and at 65–75% relative humidity, as previously described[16-18,38]. All insects were supplied with sterilized peeled sunflower seeds and fresh mineral water *ad libitum*.

Insect sorting. To obtain virgin insects in the same period of development, fifth instar nymphs were collected from parental colonies, separated into small plastic vessels. These insects were maintained in the same conditions as the parental colony. The insects were observed for the transition to the adult stage. After five days in the adult stage, the insects were used for the experiments.

Development analysis. Insect development in both colonies was observed comparatively. Ten breeding pairs from each colony were separated and allowed to copulate for 1 week. After oviposition, 30 eggs at the same

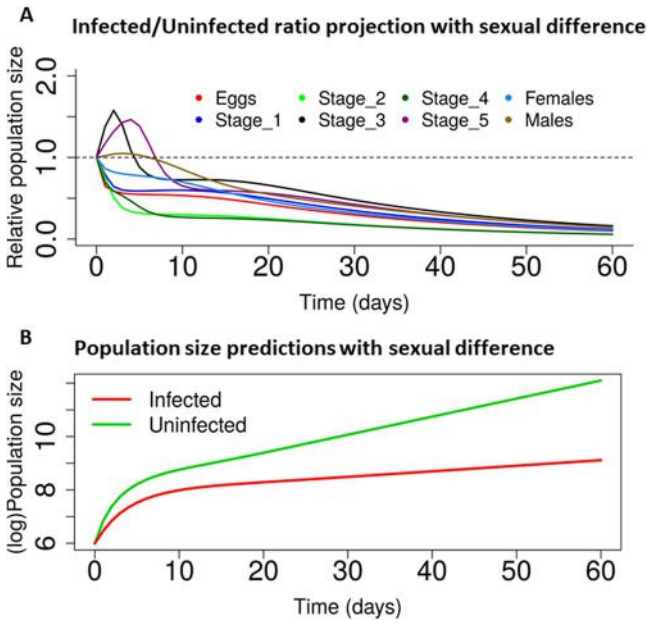

Figure 6. *L. wallacei* affects *Oncopeltus fasciatus* population dynamics with sex-bias. (**A**) Development, lifespan, and reproductive data from infected and uninfected colonies of *O. fasciatus* were combined to model the relative population size of each life stage in the population dynamics considering the sexual differences. (**B**) Population projections for uninfected or infected colonies. Even if infection impacts the population, it is still shown to grow exponentially.

period of maturation, laid by females from both colonies, were separated into small plastic vessels. After eclosion, the insects were maintained under the same conditions as previously described. The insects were observed daily and in each developmental stage, the first and the last insect to molt were registered. With the aim of assessing if the infection influenced the mortality rate of the insects, mortalities were recorded throughout the developmental period[48].

Longevity of the insects. Males and females in adult stages from both colonies were maintained under the same conditions as previously described. The numbers of live and dead insects were recorded daily. The insects used in this experiment were sorted as explained above.

Morphometric analyses. To evaluate the influence of *L. wallacei* infection to insect morphometric parameters, virgin adults and fifth instar nymphs were measured for weight, body length, total area of the body, membranous wing and hemielytra length, membranous wing and hemielytra area, rostrum and median leg length, abdomen width and antenna length. The insects were photographed on graph paper and measured using the software Analyzing Digital Images (program provided by Museum of Science, Boston, MA, USA).

Reproductive analysis. Oviposition and egg load were observed in individually separated copulated, infected and non-infected females. The oviposition was observed for two weeks. Oviposited eggs from both colonies were separated in glass vials and counted daily for eclosion. To observe egg reabsorption and egg load, females were collected after the copulation period and their ovaries were dissected in order to perform morphologic evaluations of the ovarian follicles and for egg counting. Eggs were considered to be in the reabsorption process when possessing an opaque ooplasm and a loss of shape of ellipsoid prolate, which is otherwise a characteristic of normal eggs[61]. All insects were weighed using a precision scale.

***Intersex* gene expression.** Ovaries of insects from both colonies (infected and uninfected n = 24) were dissected in PBS, pH 7.4. RNA was extracted with Trizol (Invitrogen), following the manufacturer's protocol. RNA subsamples (1 μg) were treated with 1 U DNase (Fermentas) in DNA buffer in a total volume of 10 μl and incubated at 37 °C for 30 min. The samples were then incubated at 65 °C for 10 min with 1 μl EDTA 4.5 mM for DNase inactivation. Then, a high-capacity cDNA reverse transcription kit (Applied Biosystems) was used for cDNA synthesis, following the manufacturer's protocol. The qPCR for intersex expression was performed for cDNA qualitative analysis in a final volume 15 μl of 7,5 μl power SYBR green PCR Master Mix (Applied Biosystems), 5 μl cDNA (1:10 diluted), and 350 nM primers: forward: 5′-GAGTAGCCCCGACGAGAAGT-3′ and reverse 5′-ATGCCATGCATTTCCTTAGC-3′. The qPCR reactions were performed using a StepOnePlus Real-Time PCR System (Applied Biosystems), with default settings: 10 min at 95 °C, 40 cycles of 15 s at 95 °C, and 1 min at 60 °C, and finally by a denaturation curve. The ΔΔCt method was comparatively used to analyze the alterations in gene expression. Specific primers for eIF3: forward 5′-CTTCAGCTTCTTTGGGTTGG-3′ and reverse 5′-GAAATGTGGGAAGACCGAGA-3′ were used to normalize the expression levels of the *intersex* gene.

RNAi protocol. *Intersex* gene silencing protocol was performed by RNAi silencing by dsRNA administration by feeding. For specific intersex dsRNA (dsix) synthesis, gene segments were generated by PCR using primers: forward: 5′-TAATACGACTCACTATAGGGCCACCTCAGGAGAAACTGGA-3′ and reverse: 5′-TAATACGACTCACTATAGGGATGCCATGCATTTCCTTAGC-3′. Those templates were used for dsRNA and in the Kit T7 MEGAscript (Thermo Fischer), following the manufacturer's protocol. The dsRNA was then quantified by Nanodrop 1000 v.3.7 (Thermo Fisher Scientific) and dsRNA dissolved in sterile water for insect feeding. For the control group, dsmal was offered to the insects via the water. The dsmal was synthesized as reported for dsix using a template for plasmid Litmus 28i-mal and primers: forward and reverse: 5′-TAATACGACTC ACTATAGGG-3′. Third stadium nymphs (n = 30) were separated and monitored until they reached the forth stadium. The insects were then fasted for two days, after which they were offered the dsix and dsmal in the water for two more days. After reaching the adult stage, the inhibition of the intersex gene expression was evaluated by qPCR as described in the previous section.

Conventional PCR and qPCR for parasite detection. Primers specific for *L. wallacei* and *O. fasciatus* 16 S rRNA gene were designed as previously described[38]. The sequences of the primers designed are as follows: F-Lw 5′-CTTTTGGTCGGTGGAGTGAT-3′ and R-Lw 5′-GGACG-TAATCGGCACAGTTT-3′; F-Of 5′-CAAAATTTGGTTGGGGTGAC-3′ and R-Of 5′-ATC-GAGGGTCGCAAACTCTT-3′. Total RNA was extracted using Trizol (Invitrogen) following the manufacturer's protocol. We have treated the RNA with RNase-free DNase I (Fermentas International Inc., Burlington, Canada), and cDNA was synthesized using the High Capacity cDNA reverse transcription kit following manufacturer's protocol (Applied Biosystems, Foster City, CA). cDNA from whole intestine was PCR-amplified using the PCR master mix (Fermentas International Inc.). The amplification reactions were performed as previously reported by our group[38] in a final volume of 10 µl. Each reaction was performed with 50 ng of DNA sample, 5 µl of PCR Master Mix (Fermentas International Inc., Burlington, Canada) and 350 mM of primers specific for *L. wallacei* or *O. fasciatus*. The PCR was performed as follows: initial denaturation of DNA for 5 min at 94 °C; 40 amplification cycles each consisting of 30 sec at 94 °C, 45 sec at 53 °C for both parasite and insect DNA amplification and 30 sec at 72 °C; and a final step of 5 min at 72 °C for extension of incomplete products. Following PCR, the amplification products were analyzed by electrophoresis in 2% (wt/v) agarose gels that were submitted to ethidium bromide staining and analyzed under ultraviolet light excitation in comparison to GeneRuler TM 100 bp Plus DNA ladder fragments (Fermentas International Inc.). qPCR was performed on a StepOnePlus real-time PCR system (Applied Biosystems) using the Power SYBR Green PCR master mix (Applied Biosystems). We used comparative Ct method to compare gene expression levels and the *O. fasciatus* 16 S rRNA gene was used as an endogenous control.

Number of promastigotes in the intestine. Adult insects from the infected colony were collected and dissected in 200 µl phosphate-buffered saline (PBS, pH 7.4) and the promastigote forms counted in a Neubauer chamber under a Zeiss Axioplan 2 light microscope (Oberkochen, Germany). Each intestinal compartment was macerated in PBS and the number of live promastigotes counted for each insect separately, according to the gut region.

Scanning electron microscopy. Insect guts were dissected in PBS at 4 °C before fixation. Fixation involved immersion in a solution that contained 2.5% glutaraldehyde, 4.0% formaldehyde, 3.7% of sucrose, and 5 mM $CaCl_2$ in 0.1 M cacodylate buffer (pH 7.2) for 2 h at 26 °C. After three washes in 0.1 M cacodylate buffer (pH 7.2), samples were dehydrated using an ethanol series (50, 70, 90, and 100%) and dried using the critic point method in a Balzers CDP-20 apparatus (Balzers Union, Fürstentun Liechstenstein). The micrographs were made using a scanning electron microscope (Jeol JSM-5310).

Statistical analysis and mathematical modeling. We employed a Weibull regression model to estimate survival curves with confidence bands for the four experimental groups (infected males, uninfected males, infected females, and uninfected females). Morphometric data were first analyzed using a principal component analysis (PCA) and then the first principal component (PC) (which retained most of sampling variance) was used as the overall size indicator. This size variable and the insect sex were used as predictors of infection in a binary generalized linear model (GLM) with a logit link function. As many of the daily laid egg counts were zero, a zero-inflated Poisson GLM was employed to model oviposition through time for infected and uninfected females. Posterior distributions for the proportions of egg eclosion and re-absorption were obtained using a binomial likelihood with a conjugate Beta (1, 1) prior.

To integrate all the data collected in this study in a coherent manner, we used data on reproductive fitness and development to parameterize a system of ordinary differential equations that model *Oncopeltus* population growth. Our models were age-structured and we explored models with and without sexual differentiation of adults. Models with sexual differentiation were employed to capture differences in mortality between males and females. The governing equations for the model with sexual differentiation were:

$$E' = oF - eE$$

$$N_1^{'} = eE - d_{1,2}N_1 - m_1N_1$$

$$N_j^{'} = d_{j-1,i}N_{j-1} - d_{j,j+1}N_j - m_jN_j, j = 2, \ldots, 5$$

$$M' = (1 - p_F)d_{5,A}N_5 - m_MM$$

$$F' = p_F d_{5,A} N_5 - m_F F$$

A description of the parameters can be found in Data S1 and Table S3. We used the data collected in this study to construct two sets of parameters, one for an infected and another for an uninfected population scenario. Using this model and a different set of parameters for each scenario we obtained short-term population projections under both scenarios and then compared the resulting trajectories. Further theoretical background on the statistical and mathematical analyses is provided in S1 Data. The R code to perform all the described statistical and mathematical analyses is publicly available at https://github.com/maxbiostat/CODE/tree/master/OncoLeptoModeling.

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Author contributions

Conceived and designed the experiments: A.H.L., F.G.L., L.R.C.V., L.M.F.C., M.H.F.S., F.A.D. Performed the experiments: L.R.C.V., I.C.G., F.S.C., O.A.C.T., F.A.M.S., T.L.A.S., L.A.R., F.A.D., F.G.L. Statistical analysis and mathematical modeling: L.M.F.C., L.B.S., C.J.S. Analyzed the data: A.H.L., L.R.C.V., L.M.F.C., F.G.L., L.S.B., O.A.C.T., M.H.F.S., C.J.S. Wrote the paper: L.R.C.V., A.H.L., L.M.F.C., F.G.L.

Competing interests

The authors declare no competing interests.

Additional information

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Unlike Zika, Chikungunya virus interferes in the viability of *Aedes aegypti* eggs, regardless of females' age

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Chikungunya and Zika are arboviruses transmitted by the mosquito *Aedes aegypti*. Mosquito fecundity and egg viability are important parameters of vectorial capacity. Here we aim to understand, comparatively, the effects of Chikungunya virus (CHIKV) and Zika virus (ZIKV) infections on the fecundity and fertility of young and old *Aedes aegypti* females. Using artificial infection blood feeding experiments we observed that both CHIKV and ZIKV do not alter the number of eggs laid when compared to uninfected females, although the egg fertility significantly decreases in both young and old CHIKV-infected females. There is an upward trend of null females (infertile females) from 2.1% in young to 6.8% in old ZIKV-infected females. Together, our data revealed that CHIKV and ZIKV affects differently *Ae. aegypti* physiology, that may be related to different viral spread in nature.

Chikungunya virus (CHIKV), a member of the *Togaviridae* family, *Alphavirus* genus, was first isolated in 1952 in Tanzania[1]. Emerging and reemerging outbreaks have occurred since its discovery in several regions in Africa, Asia, Indian Ocean islands and Mediterranean areas in Europe[2,3]. In Brazil, the first Chikungunya autochthonous cases occurred in 2014[4]. The virus spread throughout the country[5], being in co-circulation with dengue (DENV) and Zika (ZIKV) viruses during this period[6]. Zika, a virus belongs to *Flaviviridae* family, *Flavivirus* genus, was first isolated in 1947[7]. For 60 years, only sporadic Zika cases were reported in humans, however in 2007 an outbreak occurred in Yap Island, Micronesia[8]. Subsequently, ZIKV expanded throughout the Pacific islands and reached the Americas in 2015, turning into an epidemic in Brazil. It is currently considered a new public health threat[9–11].

Both arboviruses (Zika and Chikungunya) are transmitted by the bite of mosquitoes of genus *Aedes*[7,12,13]. Among them, the *Aedes aegypti* is the main vector in the urban transmission cycle[14–16]. This domestic and anthropophilic species is anautogenous (the females need blood supply for eggs maturation)[17]. After one or more blood feedings, egg maturation occurs in about 3 or 4 days. Each period between blood feeding and egg laying is called gonotrophic cycle (GC)[17–19]. An *Ae. aegypti* female is able to lay approximately 100 eggs per GC[20]. The ingestion of more than one blood meal by mosquito females within a single gonotrophic cycle is called gonotrophic discordance and can occur in *Aedes aegypti* mosquitoes. This feature is involved in vectorial capacity and is very important for the transmission of viruses[21–23].

Comparatively, the biology of the egg phase is less explored than other in the mosquito life cycle. The *Ae. aegypti* eggs can survive for long periods in dry conditions at the end of their embryonic development[17,19,24–26]. This important feature is related to ecological issues such as dormancy, that enables the embryo to survive

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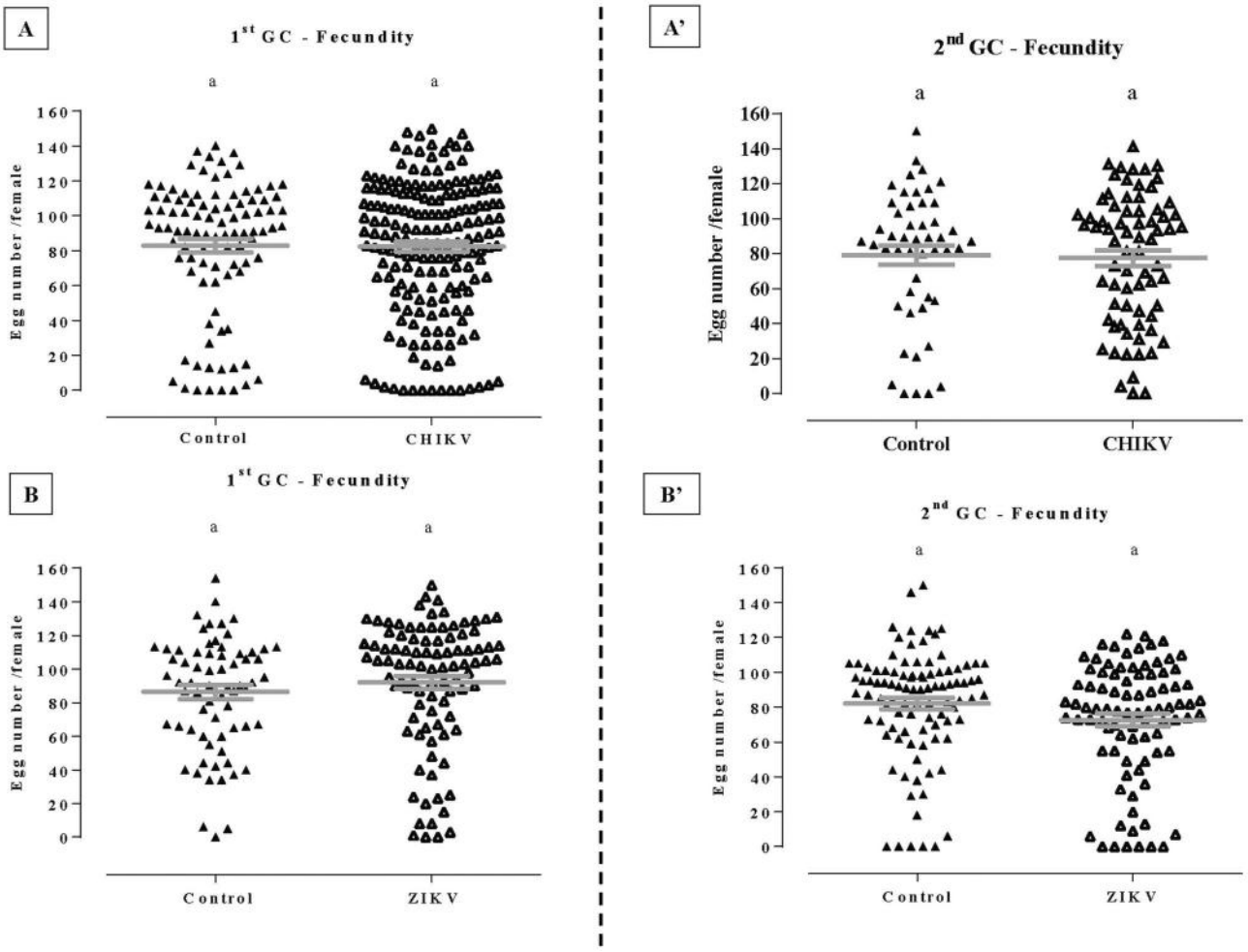

Figure 1. Effect of Chikungunya (CHIKV) and Zika (ZIKV) viruses infections on first gonotrophic cycle (GC) (**A**,**B**) and second GC (**A′**,**B′**) fecundity of *Aedes aegypti* females. The significance is represented by $p < 0.05$ obtained by using the non-parametric Mann–Whitney test. Bars represent mean and ± se of independent experiments.

drought periods[19]. Furthermore, egg resistance to desiccation (ERD) facilitates passive dispersal to new locations being important to epidemiologic features of arboviruses that has *Ae. aegypti* as the main vector[27].

Distinct arbovirus may replicate in different velocities and consequently the time between taking a viremic blood meal and shedding virus into saliva (extrinsic incubation period, EIP) vary. CHIKV reaches the *Aedes* salivary glands faster than DENV2, which directly affects the potential of viral spread[28]. During the EIP, virus replicate in the midgut and in several secondary mosquito tissues following disseminate in the hemocoel prior to be shed into saliva. Thus, arbovirus can interfere in female biology and consequently in their physiology such as fecundity and fertility[29–31]. Hence, the analysis of number of eggs laid by an infected mosquito (fecundity) and their viability (fertility) are also key parameters to comprehend the vectorial capacity.

Our previous studies have shown that ZIKV infection can modulate *Ae. aegypti* females' locomotor activity but did not change significantly the fecundity and fertility neither in the second or third GCs when the female egg laying is clustered[30]. Here we refined our methodology focusing on understand, comparatively, the effect of infection by CHIKV and ZIKV in fecundity and fertility of *Ae. aegypti* females from different ages, analyzed at distinct GC.

Results

CHIKV and ZIKV infection do not change the fecundity. We analyzed whether CHIKV and ZIKV infection influenced in the fecundity in females of different ages following the first or second GCs. Infection rate of randomly chosen samples of mosquitoes orally challenged with viruses was 80% for CHIKV infection and 89.5% for ZIKV infection. . We observed that the number of eggs did not change in any of the tested settings for CHIKV infected (7-days-old or 14-days-old females; $p = 0.88$ and $p = 0.96$, respectively) and non-infected mosquitoes (Fig. 1A,A′). The same was observed when females were infected with ZIKV, where no significant differences were observed in fecundity, independent of the females' age ($p = 0.09$ and $p = 0.0542$, respectively) (Fig. 1B,B′).

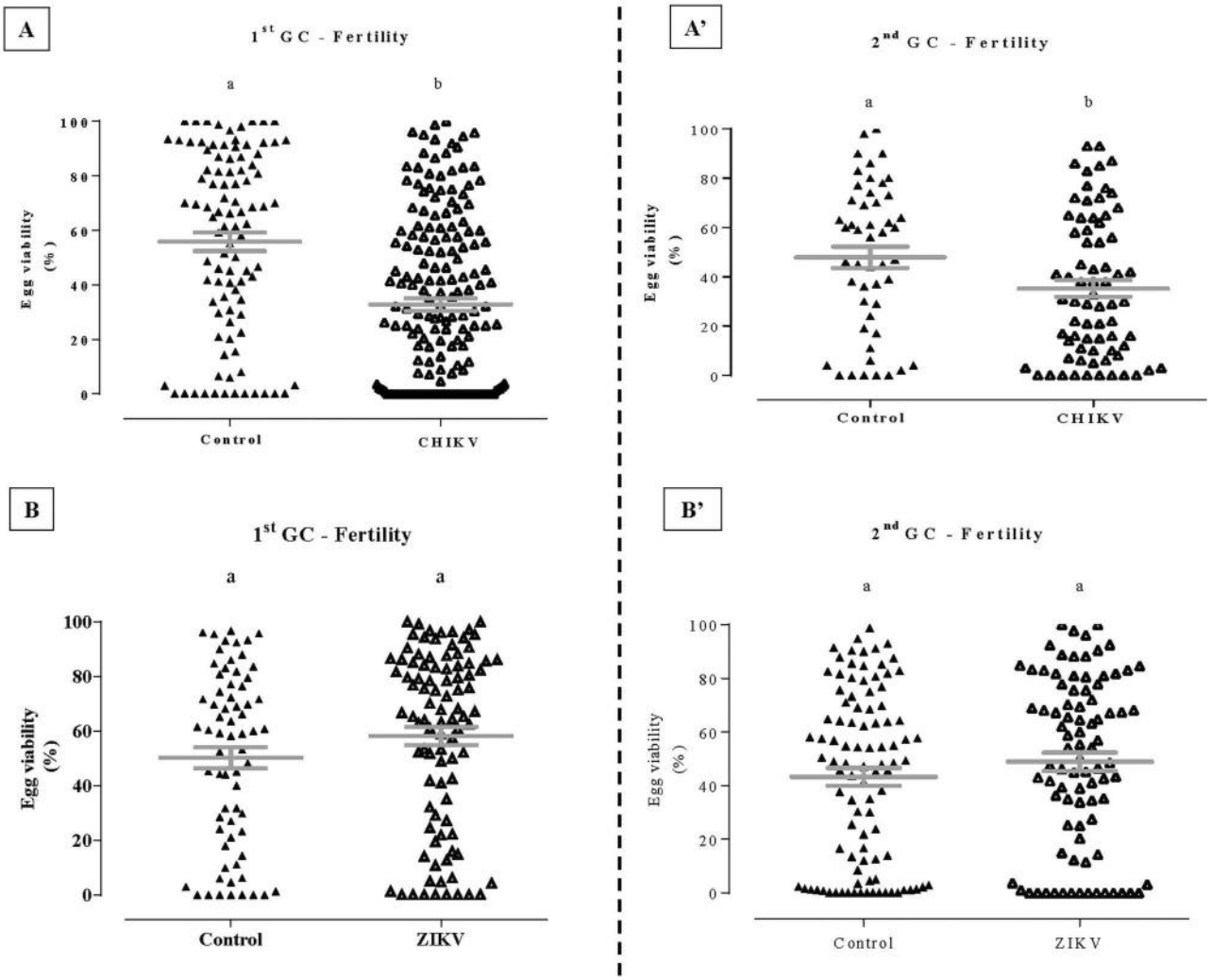

Figure 2. Effect of Chikungunya (CHIKV) and Zika (ZIKV) viruses infections on first GC (**A**,**B**) and second GC (**A′**,**B′**) fertility of *Aedes aegypti* females. The significance is represented by $p < 0.05$ obtained by using the non-parametric Mann–Whitney test. Bars represent mean and ± se of independent experiments.

CHIKV infection affects females' fertility, dissimilar from ZIKV infection. As opposed to the fecundity, the fertility (the quantity of egg hatching) was affected by CHIKV infection. When females were younger (infectious blood meal taken in the 1st GC), the average of fertility in non-infected l was 55.77 (± 3.43) compared to 32.73 (± 2.27) in CHIKV infected females. In addition, when females were older (infectious feeding was performed in the 2nd GC), the average of the control group was 47.88 (± 4.31) and decreased to 35.22 (± 3.37) in the treated group (Fig. 2A,A′) ($p < 0.05$ in both cases).

We evaluated the influence of ZIKV infection in *Ae. aegypti*'s fertility, in the same conditions of CHIKV (females were blood fed in different ages/GCs). We did not observe any significant difference for ZIKV infected and non-infected *Ae. aegypti* females' fertility (Fig. 2B,B′; $p = 0.09$ and $p = 0.35$, respectively).

Fecundity and fertility lose correlation when females are infected with CHIKV. We analyzed the correlation between fecundity and fertility in the first and second GCs of females infected with CHIKV or ZIKV. In the former, we can observe that the 7-days-old and 14-days-old females from the control group showed a positive correlation (Fig. 3A,A′ boxes; r = 0.45 and r = 0.33, respectively; $p < 0.0001$, $p = 0.02$, respectively). On the other hand, correlation data from females infected with CHIKV are different when feeding occurred in the first (young females) or second GC (old females). In the first cycle, the positive correlation observed in the control is maintained (r = 0.30; $p < 0.0001$), however, in the second GC this correlation is lost (r = 0.11, $p = 0.35$) (Fig. 3A,A′).

Analyzing the correlation between fecundity and fertility in ZIKV young and old females, we observed a positive linear relationship in the control group (Fig. 3B,B′ boxes; r = 0.42; r = 0.27, respectively and $p = 0.004$, $p = 0.0065$, respectively). When analyzing infected *Ae. aegypti* females' eggs throughout the ages, this positive correlation is still maintained (Fig. 3: B,B′; r = 0.38; r = 0.40, respectively and $p = 0.0002$, $p = 0.0002$, respectively).

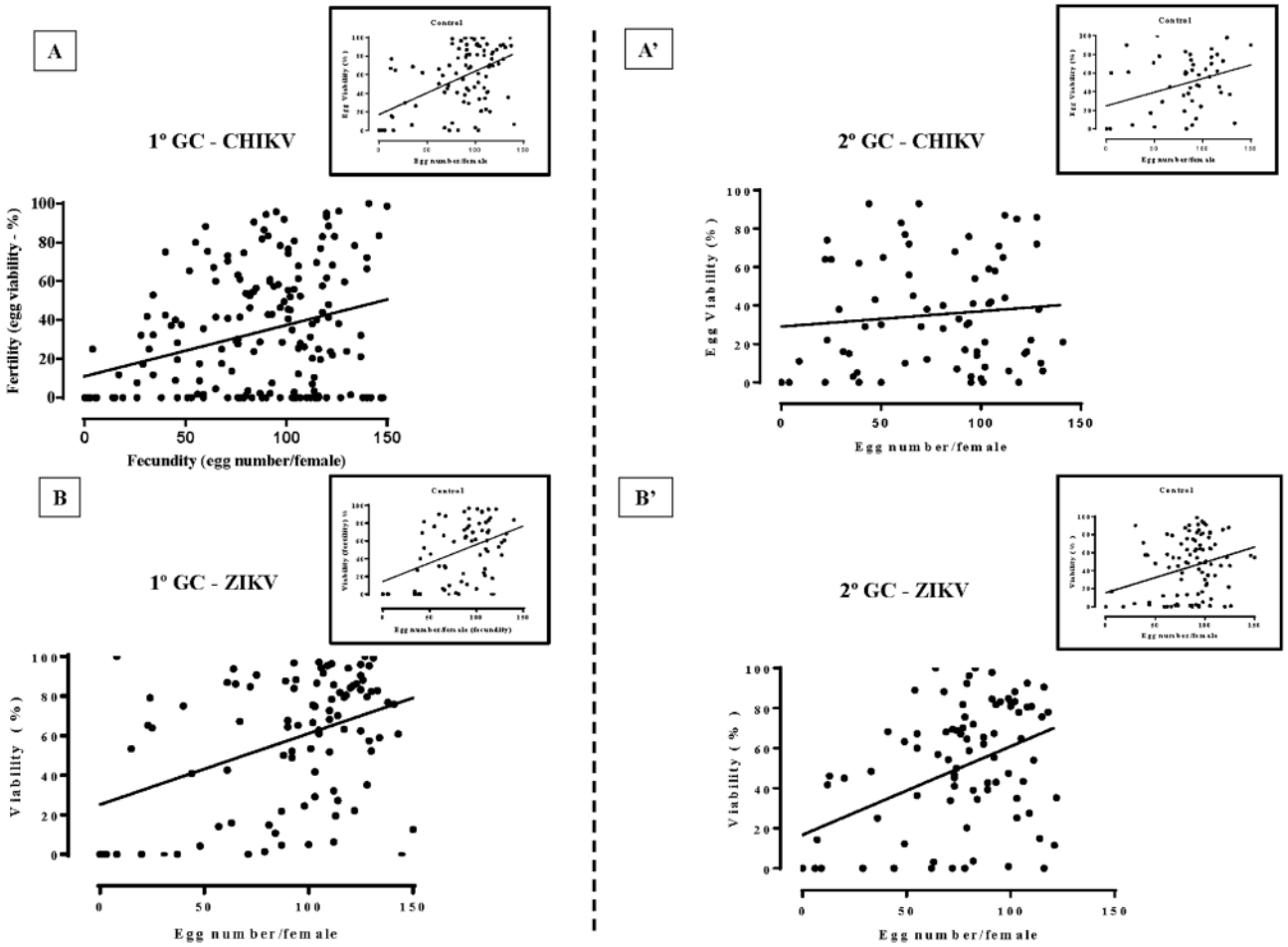

Figure 3. Correlation between fecundity and fertility of *Aedes aegypti* females uninfected and infected with Chikungunya or Zika virus. (**A**): CHIKV—1º gonotrophic cycle (GC); (**A′**): CHIKV—2º gonotrophic cycle (GC); (**B**): ZIKV—1º gonotrophic cycle (GC); (**B′**): ZIKV—2º gonotrophic cycle (GC). All boxes shows controls of each conditions. Analysis was made for Correlation of Spearman, the R number of each correlation is described in the text.

	N	Median	% of efficient females[a]	% of null females[b]
1st gonotrophic cycle				
CHIKV	186	91	49.5	3.8
ZIKV	97	92	64.9	2.1
2nd gonotrophic cycle				
CHIKV	72	87	52.8	2.7
ZIKV	88	91	35.2	6.8

Table 1. Oviposition efficiency of *Ae. aegypti* females infected with CHIKV or ZIKV. *N* number of females. [a]Efficiency was measured according to the control median. [b]Females that did not lay eggs were considered null.

Oviposition efficiency for CHIKV and ZIKV. We considered as oviposition efficiency the percentage of infected females (with CHIKV or ZIKV) that presented an oviposition value equal to or greater than the median value of control females. In the CHIKV infection, we obtained 49.5% of efficient females when feeding was performed in the first GC and 52.8% when it occurred in the second one. For ZIKV infection, oviposition efficiency was of 64.9% and 35.2% for first and second GCs, respectively. Regarding the number of nulls, that is, females that did not lay eggs, we obtained for CHIKV infection 3.8% and 2.7% when virus challenge was performed in the first or second GC, respectively. When analyzing the number of null females for ZIKV infection, the percentage increased from 2.1% in the first to 6.8% in the second GC (Table 1). In the case of ZIKV null females, we observed an increase of infertility in ZIKV infected females in second GC.

Discussion

Vectorial capacity (VC), critical for arboviruses transmission, is the predictable value through a mathematical formula that considers beyond environmental conditions, intraspecific physiological and behavioral parameters, those that will be important to arboviruses transmission in nature[32,33]. The VC contemplates mainly biting behavior (frequency of host contact for blood feeding), mosquito-vector survivorship and population density[32–36]. Population density is very dependent on mosquito fecundity and fertility. *Aedes aegypti* behavior and physiology as well as arbovirus-mosquito interactions are thoroughly studied topics, but additional studies are needed in order to endorse the development of new tools to mosquito control actions.

There are different methods described in the literature to study the fertility of *Ae. aegypti* females[24,37,38]. Here, we used the methodology called synchronized posture that is successful in forcing females *Ae. aegypti* to lay their eggs in short time intervals[24,39]. The calculation of the oviposition time of 90 min was based on a comparison of the average number of eggs per female from previous studies in our group with longer time intervals[37]. In that paper, we showed a female laid an average number of 82 eggs in synchronized postures that lasted 6 h, while we present in this study an average number of 86 eggs per female in postures lasting 90 min. Therefore, we optimized the bioassay and achieved success in a shorter time.

In 2018, Padilha and collaborators[30] tested the effects of ZIKV in fecundity and fertility in clusters of infected and non-infected *Ae. aegypti*. Here we aimed for a more accurate evaluation, where eggs laid by individual females were analyzed for additional parameters such as oviposition efficiency and number of null females, and tested correlation between fecundity and fertility.

We examined whether CHIKV or ZIKV infection could affect the number of eggs laid per females and hatching. We noted that, regardless of female age, CHIKV infection does not alter fecundity, but had significant influence on fertility. It is important to report that our results are different from those observed by Sirisena et al.[31], when using a different methodology from ours. They performed their experiments with different mosquito and virus strains. The oviposition was carried out with females in group and during three GCs, where infective blood feeding occurred only in the first GC. Sirisena et al.[31] found that CHIKV infection caused the group of females to lay fewer eggs than the control in the first and third GCs but not in the second one; the authors did not test for fertility of individual females.

In relation to ZIKV, despite using different methodologies, our data corroborate those of Padilha et al.[30], where ZIKV infection does not cause damage to the overall fertility and viability of *Ae. aegypti*, but with a decreasing tendency ($p = 0.0542$) of egg numbers laid by older females. On the other hand, Petersen et al.[38] considered the first three clutches of eggs individually laid by young (7 days), mature (14 days) and old (21 days) *Ae. aegypti* females, analyzing two aspects of fecundity: the oviposition success and clutch size. These authors observed that ZIKV infected mosquitoes laid fewer eggs than the uninfected ones and the egg production was affected by the age of feeding, once younger females laid more eggs than the older ones; egg viability was not tested. When we analyzed the oviposition success, we considered the percentage of females that presented an oviposition value equal to or greater than the control median, while Petersen et al.[38] considered individual females that laid at least one egg, in a qualitative analysis.

Our results regarding the success of oviposition indicated that CHIKV-infected females showed a small increase of about three percent in the oviposition efficiency when feeding was performed in the second GC. ZIKV-infected females, on the other hand, showed to be less efficient in this GC, showing a greater decrease in almost half of their efficiency percentage. Moreover, in relation to the percentage of null females, those infected with CHIKV presented infertility of 3.8% and 2.7%, when feeding was performed in the first or second gonotrophic cycles, respectively. However, interestingly, the females infected with ZIKV presented a percentage of 2.1% and 6.8%, respectively, indicated that infertility increases when they get older as expected.

Unlike what was reported by previous studies[30,31], in which they used in the second GC the same females that had already had the first oviposition, we aimed to isolate the age factor from the possible drop in viral load over time[31,40]. That is why we performed infective blood feeding only in the first or in the second GC.

The temporal tropism of the virus was taken into consideration when we decided to perform the infective blood feeding, since the females lay eggs about 4 days after feeding. According to Sirisena et al.[31] and Ryckebusch et al.[40], after 4 days of infection, CHIKV and ZIKV have already disseminated over the mosquito's body, although CHIKV does it more quickly. Vega-Rúa et al.[16] showed that CHIKV of distinct genotypes disseminated in 80–100% of *Ae. albopictus* in only 3 days after oral challenge. Le Coupanec et al.[28] described the quick distribution of CHIKV particles in *Ae. aegypti* body, more specifically in the midgut (MG) and salivary glands (SG). The virus was detected in the SG at 4-day post viral exposure, peaking by day 8. The replication kinetics of CHIKV is different from other viruses, such as ZIKV, which seems to disseminate to secondary tissues of the mosquito body in a slower manner, with the peaking at 10–14 days after oral challenge[41,42].

Finally, we observed a positive correlation between the number of laid eggs and the tendency of hatching viable larvae in younger females. Interestingly, CHIKV and ZIKV infection does not change this pattern in *Ae. aegypti*. On the other hand, when females are older, this correlation is lost in CHIKV infected females.

Our data revealed that ZIKV infection may increase infertility as the females get old but did not affect the viability of the eggs, while CHIKV infection affects viability and shows a loss of correlation of viability versus number of eggs. It indicates that a high oviposition efficiency (high number of eggs) does not correspond to a high number of viable larvae. CHIKV and ZIKV affects differently *Ae. aegypti* physiology, which can have relation with the different viral spread in nature. Understanding these parameters of vectorial capacity is crucial to elucidate the arboviruses transmission as well as the infected *Ae. aegypti* biology.

Methods

Mosquito rearing. *Aedes aegypti* mosquito eggs (PAEA strain from Tahiti, French Polynesia[43], maintained in laboratory since 2003) were hatched in plastic trays containing 1.5 L of Milli-RO water and approximately 1 g of yeast (Vitalab, Brazil). First instar larvae were counted and redistributed to new plastic trays (300 larvae per tray) and fed with the same quantity of yeast, every 2 days, until pupae development (according to Farnesi et al.[39]. Pupae were counted and separated in cages (with approximately 400 each) for adult emergence; males and female mosquitoes were kept together (with 10% sucrose solution ad libitum) to allow copulation. For all experiments, mosquitoes were maintained in an incubator (Forlab Scientific Incubator, USA) at 25 ± 1 °C, with a photoperiod of 12 h of light and dark (LD 12:12) and 60–80% relative humidity (RH).

Virus and mosquito oral infection. The ZIKV strain ZIKV/H.sapiens/Brazil/PE243/201 (GenBank accession number KX197192.1) and CHIKV strain BHI3745/H804709[4] were used for oral experimental infection of *Ae. aegypti* females.

These strains were harvested in C6/36 monolayer cells flask for 7 days, in Leibovitz-15 media supplemented with 5% fetal bovine serum, triptose 2.9 g/L, 0.075% sodium bicarbonate, 0.02% L-glutamine, 1% of non-essential amino acids and 1% penicillin/streptomycin at 28 °C according to Oliveira et al.[44]. Cell culture supernatant was collected and centrifuged at 1500*g* for 5 min. The aliquots were kept frozen − 70°C until use.

Both viral titles were determined by plaque assay in Vero cells following 10 × serial viral stock dilution and covered by a layer of DMEM media supplemented with 2% fetal bovine serum, 1% penicillin/streptomycin and 0.8% methylcellulose, incubated for 3 days at 37 °C and 5% CO_2.

The mosquitoes were orally infected with seven ('young females') or fourteen ('old females') days old by blood meal containing 10^7 PFU/ml of CHIKV or ZIKV. The infectious blood meal was prepared by mixing 1:1 of rabbit red blood cells and virus stock and 10% of heat—inactivated rabbit plasma. The mosquitoes were artificially fed using glass artificial feeders, sealed with Parafilm-M membrane stretched, connected to a bath at 37 °C for approximately 40 min, inside a Biosafety level—2 (BSL-2) insectary. Control mosquitoes fed on a similar blood meal, but with a non-infected L15 culture medium[44]. Prior to blood meals, female mosquitoes were deprived of sucrose for approximately 6 h. After blood meal, mosquitoes were cold anesthetized and only the fully engorged females were used.

Viral confirmation. Total RNA from the whole mosquitoes was extracted individually, 4 days after virus infection, using TRIzol (Life Technologies) according to manufacturer's protocol. Viral RNA detection and quantification of Zika and Chikungunya were carried out through RT-qPCR with TaqMan Fast Virus 1-Step Master Mix Kit (Invitrogen, Carlsbad, CA, USA) in QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). For ZIKV detection, reaction was conducted using 0.6 µM forward primer—5′-CTTGGAGTGCTTGTGATT-3′, genome position 3451–3468; 0.6 µM reverse primer—5′-CTCCTCCAGTGT TCATTT-3′, genome position 3637–3620 and 0.8 µM probe—5′FAM-AGAAGAGAATGACCACAAAGATCA -3′TAMRA, genome position 3494–3517. For CHIKV detection, reaction was conducted using 1.1 µM forward primer 5′-TCACTCCCTGTTGGACTTGATAGA-3, genome position 6856–6879; 1.1 µM reverse primer—5′-TTGACGAACAGAGTTAGGAACATACC-3, genome position 6981–6956), and 0.2 µM probe 5´FAM-AGG TACGCGCTTCAAGTTCGGCG-3´, genome position 919–6941[45,46]. Cycling conditions for reactions were the same, as follows: 50 °C for 5 min, 95 °C for 20 s, followed by 40 amplification cycles of 95 °C for 15 s and 60 °C for 60 s.

Gonotrophic cycle assays for fecundity and fertility. For young females assays we used eggs of the first GC. Seven days-old *Ae. aegypti* females were provided with infected or uninfected blood meal as previously described. Oviposition was stimulated 4 days after blood meal as described in Farnesi et al.[39]. About 30 females per condition, in each experiment, were individually isolated in an inverted plastic Petri dish (90 mm in diameter) with wet filter paper (Whatman No. 1) lining the lid. All females were allowed to oviposit for 90 min inside incubators (25 °C), in dark and 60–80% relative humidity conditions. After oviposition, females were released in cages, and randomly separated for posterior viral detection (at least five females per condition). Eggs were stored inside the incubators in a regimen of 12 h of light followed by 12 h of dark (L/D) until the end of embryogenesis.

For old females' assays, the eggs of the first GC of 7-days-old females were discarded as described by Padilha et al.[30]. After that, females returned to the cages provided with sucrose 10% ad libitum until they were 14 days-old when they were fed with infected or uninfected blood meal. Then, they were individualized and stimulated to lay eggs of the second GC as described above. Eggs from second GC were used to the analyses. The eggs dried for 7 days inside incubators (LD12:12; 25 °C and 60–80% RH) and then were counted and tested for viability.

Fertility assays. All the eggs obtained from individual females were tested for viability. Briefly, to stimulate hatching 50 mL of industrial yeast extract solution 0.15% (weight/volume) were added in each Petri dish placed in incubators for 24 h (25 °C, 60–80% RH and photoperiod LD 12:12), according to Farnesi et al.[39]. In general, the assays were made three times independently.

Oviposition efficiency. To evaluate if infection with CHIKV or ZIKV interferes in the efficiency of females to lay eggs, we assumed they were efficient when they could lay a number of eggs equal or higher than the control median, considering the control group of each age and each infection condition. The median of all controls is near to the average of egg laid described to *Ae. aegypti* species[19,20].

Statistical analysis. At first, all physiological data were tested for normality by Shapiro–Wilk test. We applied Mann–Whitney test for fecundity and fertility, as described in each graphic legends. For correlation analysis between fecundity and fertility, was used the Spearman coefficient. All statistical assays were executed using GraphPad Prism 5 (GraphPad Software, San Diego, California, EUA) and p value < 0.05 was considered for significant differences.

Ethical statement. All experiments carried out on this study were approved by the institutional Research Ethics Committees IOC/FIOCRUZ #LW34/14 and CEUA-UFRJ 149/19 (for use of rabbit blood). All experiments were performed in accordance with relevant guidelines and regulations.

Data availability

The data generated and analyzed during the current study are available upon reasonable request to the corresponding author.

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Author contributions

L.C.F. and R.V.B. designed experiments and conceived the project. L.C.F., R.V.B., M.E.B.R. and K.P.P. discussed the data and wrote the original draft. L.C.F., R.V.B. and R.L.O. reviewed the manuscript. M.E.B.R., K.P.P. and A.P.C. carried out infected and uninfected mosquito experiments. O.A.C.T. and A.F.B. performed molecular experiments and confirmed the infections collection. R.V.B. supervised the project. All authors read and approved of the final manuscript.

Competing interests

The authors declare no competing interests.

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PLOS NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Prostaglandins regulate humoral immune responses in *Aedes aegypti*

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Abstract

Prostaglandins (PGs) are immuno-active lipids that mediate the immune response in invertebrates and vertebrates. In insects, PGs play a role on different physiological processes such as reproduction, ion transport and regulation of cellular immunity. However, it is unclear whether PGs play a role in invertebrate's humoral immunity, and, if so, which immune signaling pathways would be modulated by PGs. Here, we show that *Aedes aegypti* gut microbiota and Gram-negative bacteria challenge induces prostaglandin production sensitive to an irreversible inhibitor of the vertebrate cyclooxygenase, acetylsalicylic acid (ASA). ASA treatment reduced PG synthesis and is associated with decreased expression of components of the Toll and IMD immune pathways, thereby rendering mosquitoes more susceptible to both bacterial and viral infections. We also shown that a cytosolic phospholipase (PLAc), one of the upstream regulators of PG synthesis, is induced by the microbiota in the midgut after blood feeding. The knockdown of the PLAc decreased prostaglandin production and enhanced the replication of Dengue in the midgut. We conclude that in *Ae. aegypti*, PGs control the amplitude of the immune response to guarantee an efficient pathogen clearance.

Author summary

Mosquito immune responses work on an on/off switch model, where the recognition of microorganisms turns the switch on, and its clearance turns it off. Besides pathogen derived molecules, no other compounds are known to directly regulate the activation and

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the amplitude of immune responses. Here we described that prostaglandins, lipid mediators of the immunity in vertebrates, also modulate the amplitude of immune responses in mosquitoes as well. Prostaglandins regulate the production of antimicrobial peptides and other effector molecules and directly impact the susceptibility of mosquitoes to bacterial and viral infections. When prostaglandin production is impaired, immune activation is inefficient and renders the mosquito more susceptible to bacterial and Dengue infections.

Introduction

Insects fight infections using humoral and cellular responses. Humoral immune responses comprise the secretion of compounds into the hemocoel. Among those hemolymph-soluble components there are antimicrobial peptides (AMPs), proteolytic enzymes that mediate melanization and coagulation, and control signal transduction, like the activation of the Toll pathway [1]. In cellular immune responses, hemocytes are the main players and mediate phagocytosis, nodulation and encapsulation of microorganisms [2]. Humoral and cellular immune responses are interconnected, since hemocytes produce and secrete humoral components, such as AMPs, CLIP-domain serine proteases and pro-phenoloxydases. Invertebrate immune responses work on an on/off switch model, where the recognition of pathogen-associated molecular patterns (PAMPs) turns the switch on, and its clearance turns it off. In mosquitoes, besides PAMPs, no other molecules are known to directly regulate the activation and the amplitude of immune responses. Toll, the broadly conserved NF-κB pathway, *Immune Deficient* (IMD) and the *Janus Kinase/Signal Transducer and Activator of Transcription* (Jak-STAT) are the main immune pathways activated in the mosquito in response to bacteria, fungal and viral infections [3]. Activation of Toll and IMD leads to the production of AMPs and other effector molecules that participate in the pathogen killing [2]. In mosquitoes, the activation of Jak-STAT increases nitric oxide synthase (NOS) expression and culminates with *Plasmodium* killing [4].

Prostaglandins (PGs) are bioactive lipids derived from arachidonic acid and in insects were originally implicated in egg-laying and reproduction [5, 6]. Later, PGs were recognized as mediators of immune responses in the tobacco hornworm *Manduca sexta* [7, 8], the kissing bug *Rhodnius prolixus* [9], the beet armyworm *Spodoptera exigua* [10] and the mosquito *Anopheles gambiae* [11]. PGs trigger hemocyte spreading behavior, nodule formation, AMP expression and melanization cascade, participating in cellular and humoral responses. Besides reproductive organs, Malpighian tubules and salivary glands [5, 12, 13], prostaglandins can be produced by the midgut tissue in response to the microbiota [11, 14]. Midgut prostaglandins attract hemocytes and are the first signal necessary to establish immune memory in *An. gambiae* [11]. More recently, the first insect PGE2 receptor (MansePGE2R) was characterized in hemocytes of *M. sexta*, opening a new venue for eicosanoid signaling pathway studies in insects [15].

Enzymes from the phospholipase A2 family mediate the first and limiting step for the biosynthesis of PGs [16]. This enzyme cleaves phospholipids containing C20 polyunsaturated fatty acids (PUFAs) releasing arachidonic acid, the main precursor of several bioactive lipids, including PGs. The activity of these enzymes is sensitive to immunomodulators like PAMPs and cytokines [17]. Puzzlingly, cyclooxygenases, the enzymes that use arachidonic acid to generate PGs, are absent in the genome of insects [18]. In *An. gambiae* and the fruit fly *Drosophila melanogaster*, heme peroxidases functionally replace the cyclooxygenases activity necessary for PG synthesis [11, 19]. Classical inhibitors of PG synthesis in mammals, such as acetylsalicylic

acid (ASA) and ibuprofen, can impair the production of PG in insects, being able to inhibit heme peroxidases as well [19].

Although PGs have previously been implicated in the regulation of insect immune responses, their global effect on gene expression of immune components and its implication in viral susceptibility have not been addressed. Here, we show for the first time that, blocking of PG production compromises humoral immune responses in the mosquito *Ae*. *aegypti*, down-regulating Toll and IMD pathways. As a result, mosquitoes become more susceptible to bacteria and Dengue virus infections. We also characterized one more component of PG synthesis in mosquitoes, describing that a cytosolic phospholipase A2 (PLA2c) induced by the microbiota is involved in PG synthesis in the midgut. Knockdown of this PLA2c increases mosquito susceptibility to bacteria and Dengue infections.

Methods

Mosquitoes and cell culture

Aedes aegypti (Red Eye strain) were raised in an insectary at the Federal University of Rio de Janeiro, Brazil, under a 12 h light/dark cycle at 28˚C and 70–80% relative humidity. Larvae were fed with dog chow. Adults were maintained in cages and fed a solution of 10% sucrose *ad libitum*. Four- to seven-day-old females were used in the experiments. *Aedes aegypti* Aag-2 cells were maintained at 28˚C in Schneider´s *Drosophila* medium with L-glutamine (Life Tecnologies, Grand Island, NY) supplemented with 10% Fetal Bovine Serum (FBS) (Cultilab, Campinas, SP) and penicillin (100 u/mL) and streptomycin (100 μg/mL) (LGC biotecnologia, Cotia, SP).

In vitro bacterial and viral infection

For bacterial challenge, cells were incubated with two different heat-killed bacteria as previously described [20]: *Micrococcus luteus*, a Gram-positive bacteria, and *Enterobacter cloacae*, a Gram negative bacteria. Aag-2 cells were incubated with 100 bacteria per cell (10^7 bacteria/well), either heat killed or live, as described for each experiment. Cells were also incubated with 0.5 mg/mL Zymosan A (Sigma-Aldrich, St. Louis, MO), as previously described [21]. For viral infection, cells were infected with Sindbis virus or Dengue virus (DENV) using a MOI (Multiplicity of Infection) of 1, as previously described [22].

Mosquito meals

Mosquitoes were artificially fed with different diets: (1) 10% sucrose (*ad libitum*), (2) rabbit blood, (3) bicarbonate-buffered saline-agarose (BBSA) supplemented with *Serratia marcescens* (Sm). The BBSA solution was composed of glucose (10 mg), 500 mM freshly made bicarbonate buffer pH 7.4 (10 μL), 0.5 mg low melting-point agarose and 100 mM ATP, pH 7.4 (5 μL). The final volume was set to 500 μL with 150 mM NaCl. Feeding was performed using water-jacketed artificial feeders maintained at 37˚C and sealed with Parafilm "M" (Sigma-Aldrich, St. Louis, MO) membrane. For depletion of mosquito's microflora, females were fed with sucrose 10% supplemented with antibiotics, penicillin (100 u/mL) and streptomycin (100 μg/mL) (LGC biotecnologia, Cotia, SP) for 4 days, as previously described [23].

Mosquito Dengue infections and titration by plaque assay

The New Guinea C (NGC) DENV-2 strain was propagated in *Aedes albopictus* C6/36 cells: cells seeded to 80% confluency in 75 cm^2 flasks were infected with virus stock at a multiplicity of infection (MOI) of 3.5, and incubated for 6 days at 32˚C and 5% CO_2. Infected cells were

scraped into solution and lysed to release virus particles by repeated freezing and thawing in dry ice and a 37˚C water bath and centrifuged at 12.000g for removal of cell debris. Virus suspension was mixed 1:1 with commercial human blood and supplemented with 10% human serum. The bloodmeal was maintained at 37˚C for 30 min and then offered to mosquitoes via an artificial membrane feeding system, as described above. After seven days post infection, individual midguts were homogenized in DMEM with a Bullet Blender (NextAdvance), serially diluted, and then inoculated onto BHK cells seeded to 80% confluency in 24-well plates (100 ul per well) for viral titration. Plates were rocked for 15 min at room temperature, and then incubated for 45 min at 37˚C and 5% CO_2. Subsequently, 1 mL of DMEM containing 2% FBS and 0.8% methylcellulose was added to each well, and plates were incubated for 5 days at 37˚C and 5% CO_2. Plates were fixed with a methanol/acetone mixture (1:1 volume) for >1 h at 4˚C, and plaque-forming units were visualized by staining with 1% crystal violet solution for 10 min at room temperature.

Microarray gene expression analysis

Aag-2 cells were seeded to a confluence of 80% in 12-well plates and treated in quadruplicate with the following: (a) Heat-killed Gram negative bacteria *Enterobacter cloacae*; (b) Heat-killed Gram negative bacteria *Enterobacter cloacae* in the presence of 1.5 mM of Acetylsalicylic acid (ASA); or (c) Schneider´s *Drosophila* medium.

After incubation at 28˚C, for 6 hours of conditions (a) and (b), infected and control cells were lysed by the addition of 600 μL of Buffer RLT (Qiagen) and homogenized for 30 s with a rotor-stator homogenizer. RNA was then extracted with the Qiagen RNeasy Mini Kit. Two micrograms of total RNA were used for synthesis of Cy3- and Cy5-labeled cRNA probes and hybridizations were carried out on an Agilent-based microarray platform. Hybridization intensities were determined with an Axon GenePix 4200AL scanner, and images were analyzed with Gene Pix software. Expression data were processed and analyzed as previously described [24, 25]. Self–self-hybridization has been used to determine the cutoff value for the significance of gene regulation on these types of microarrays to 0.78 in log2 scale, which corresponds to 1.71-fold regulation [26]. Numeric microarray gene expression data are presented in S1 Dataset.

RNA Extraction, cDNA synthesis and Quantitative PCR

Total RNA from cells and mosquitoes in all conditions was extracted using the TRIZOL reagent (Invitrogen, St. Louis, MO) following the manufacturer's instructions. RNA was treated with DNAse I (Fermentas, Waltham, MA) and first-strand cDNA synthesis was carried out using High-Capacity cDNA Reverse transcription kit (Applied Biosystems, St. Louis, MO). The amplification efficiency of the experimental set for each gene was tested with serial dilutions of cDNA and was only used if the resultant efficiency was ≥ 90%. Each PCR reaction (15 μL volume) contained diluted cDNA, Power SYBR Green PCR Master Mix (Applied Biosystems, St. Louis, MO) and 300 nM of forward and reverse primers. Quantitative PCR was performed in a StepOnePlus Real Time PCR System (Applied Biosystems, St. Louis, MO) using Applied Biosystems recommended qPCR conditions (20 seconds at 95˚C followed by 40 cycles of 95˚C for 1 second and 20 seconds at 60˚C followed by a melting curve to assure a single product was amplified). The comparative ΔΔCt method was used to evaluate changes in gene expression levels and all standard errors were calculated based on ΔCt as described in Applied Biosystems User Bulletin #2 (http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_040980.pdf). The *A*. *aegypti* ribosomal protein 49 gene, RP-49, was used as endogenous control (accession number AAT45939), based on previous data [27]. Each figure represents at least five biological replicates with three technical

replicates for each sample. Primers used in this manuscript for gene knockdown and expression were listed in S1 Table. For 16S expression in mosquito midguts the following primers were used: 16S forward: TCCTACGGGAGGCAGCAGT and 16S reverse: GGACTACCAGG GTATCTAATCCTGTT.

Quantification of prostaglandins in tissues and cell supernatant

For quantification of prostaglandins, Aag2 cells were incubated in Hanks Balanced Salt Solution with calcium and magnesium (HBSS ++). Cells were also incubated with 1.5 mM ASA in HBSS++ for 1 hour and then the heat-killed bacteria *Enterobacter cloacae* was added to the media. After one or 24 hours, the supernatant was collected and centrifuged at 12.000 rpm to remove cells in the supernatant before the measurement. For tissue quantification of prostaglandins, 10 midguts were dissected and placed in HBSS++ for 1 hour at 28˚C. After this, tissues and the medium were collected, homogenized with a pestle and the homogenate was centrifuged at 12.000 rpm. The supernatant was used for measurement. Prostaglandins were quantitated by a commercial EIA kit (Prostaglandin Screening kit, Cayman Chemical Co., Ann Arbor, MI) according to the manufacturer´s instructions. The prostaglandin screening ELISA kit has a range of detection of 15.6–2000 pg/ml. All the measurements conducted were done with a standard curve to allow the calculations of prostaglandin levels by interpolating the values within the curve. So, the levels of prostaglandins detected are inside the range of detection which relates to the sensitivity of the assay. Alternatively, PG synthesis within Aag2 cells was immunolocalized by EicosaCell assay [28]. Briefly, cells were mixed with water-soluble 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDAC; 0.2% in HBSS containing 1% BSA for 10 min) (Sigma), used to cross-link eicosanoid carboxyl groups to amines in adjacent proteins, and then washed, cytospun onto glass slides and subjected to a blocking step (1%BSA for 30 min), were incubated with rabbit anti-PGs Abs (Cayman Chemicals) overnight and secondary DyLight546 red fluorochrome anti-rabbit IgG (Jackson ImmunoResearch Laboratories) for 1 h. Mounting medium containing DAPI was applied to each slide before coverslip attachment to allow visualization of blue-stained eosinophil nuclei. Images were obtained using an Olympus BX51 fluorescence microscope at 100x magnification and photographs were taken with the Olympus 72 digital camera (Olympus Optical Co., Tokyo, Japan) in conjunction with CellF Imaging Software for Life Science Microscopy (Olympus Life Science Europa GMBH, Hamburg, Germany).

Bacteria proliferation assay and quantitation of Dengue and Sindbis virus

Aag2 cells were placed in a plate and preincubated with ASA 1.5 mM for 2 hours. After this, live *Enterobacter cloacae* were added to the media and after 4 hours of co-incubation, an aliquot of the supernatant was plated in LB agar medium. Plates were placed in an incubator overnight at 37˚C. The number of colonies was counted to evaluate the growth of the bacteria in the supernatant of treated and non-treated cells. Alternatively, Aag2 cells were infected with Dengue or Sindbis virus (Halsted strain or New Guinea C strain) and, after 4 days of infection, an aliquot of the supernatant was collected and RNA was extracted from particles present in the supernatant. Viral RNA was used to synthesize cDNA and the amount of viral RNA was measured by quantitative PCR, using SYBR Green (Applied Biosystems, St.Louis, MO). Viral RNA amount was normalized to the number of cells in each well, counted using Trypan Blue.

Survival curves

Females were previously fed for three days with ASA 5 mM in a sugar solution, *ad libitum*. After that, females were artificially fed with a BBSA solution supplemented with *Serratia*

marcescens (Sm). After growing overnight in liquid LB medium, the bacteria was pelleted, washed, re-suspended in PBS and mixed with BBSA components to a final volume of 1 mL. Fully engorged mosquitoes, taken immediately after feeding, with or without bacteria, were transferred to new cages and scored for survival at different time points.

dsRNA synthesis and gene knockdown

Three to four day old female *Ae*. *aegypti* were cold anesthetized and injected with 69nl of a 3μg/μl dsRNA solution for each phospholipase (PLAc and PLAs). DsRNA was generated from *Ae*. *aegypti* whole body cDNA template using the MEGAscript RNAi kit (ThermoFisher Scientific, Waltham, MA, USA). Specific primers containing a T7 tail were designed for each phospholipase, PLAc (AAEL001523) and PLAs (AAEL009876), and are listed in S1 Table. A 611-bp fragment was amplified for PLAc and a 648-bp fragment was amplified for PLAs. A 218-bp fragment was amplified from LacZ gene cloned into pCRII-TOPO vector using M13 primers to generate a dsRNA control.

Statistical analysis

All analyses were performed with GraphPad Prism statistical software package (Prism 5.01, GraphPad Software, Inc., San Diego, CA). Asterisks indicate significant differences ($^{*}p< 0.05$; $^{**}p<0.01$; $^{***}p<0.001$) and the type of test used in each analysis is described in its respective figure legend.

Results

Gram-negative bacteria and the microbiota modulate PG production in Aag2 cell line and *Aedes aegypti* midgut, respectively

We investigated whether exposing the mosquito *Ae*. *aegypti* to a bacterial challenge would increase the production and secretion of prostaglandins. To test this hypothesis, we stimulated the immune responsive *Ae*. *aegypti* cell line, Aag2, with heat-inactivated Gram-negative bacteria *E*. *cloacae* (Ec) during one or 24 h, and then measured the prostaglandin content in the culture supernatant. Prostaglandin levels were undetectable in the supernatant of non-stimulated Aag2 cells, but upon stimulation, the levels of prostaglandin increased up to 120 pg/mL after 1 h and remained elevated after 24 h (Fig 1A and 1B). In *Drosophila*, an acetylsalicylic acid (ASA)-sensitive COX-like peroxidase mediates prostaglandin synthesis [19]. We observed that Ec-induced prostaglandin secretion was sensitive to ASA in Aag2 cell line as well (Fig 1A and 1B).

To identify the subcellular sites involved with prostaglandin production, we used a previously established technique called EicosaCell [28], which consists of immunostaining of newly synthetized eicosanoid lipid mediators within cells. Aag2 cells were stimulated with heat-killed *E*. *cloacae* and, after 24 h, were stained for prostaglandin detection. In non-stimulated cells, prostaglandins were undetectable, however upon bacterial challenge, we detected prostaglandin in nuclear and perinuclear sites (Fig 1C).

Since Aag2 cells produced PGs in response to bacterial challenge, we investigated whether the *Ae*. *aegypti* midgut could produce PGs. The mosquito midgut is constantly exposed to the natural microbiota, which is composed mainly by Gram-negative bacteria [29–31], that increases up to three orders of magnitude upon blood feeding [23]. Prostaglandin levels were compared between female midguts treated with antibiotics in a regular sugar diet and untreated females. The reduction of microbiota levels strongly suppressed prostaglandin production by the midguts (Fig 1D and S1 Fig). These results suggest that Gram-negative bacteria might be important modulators of prostaglandin production in *Ae*. *aegypti*.

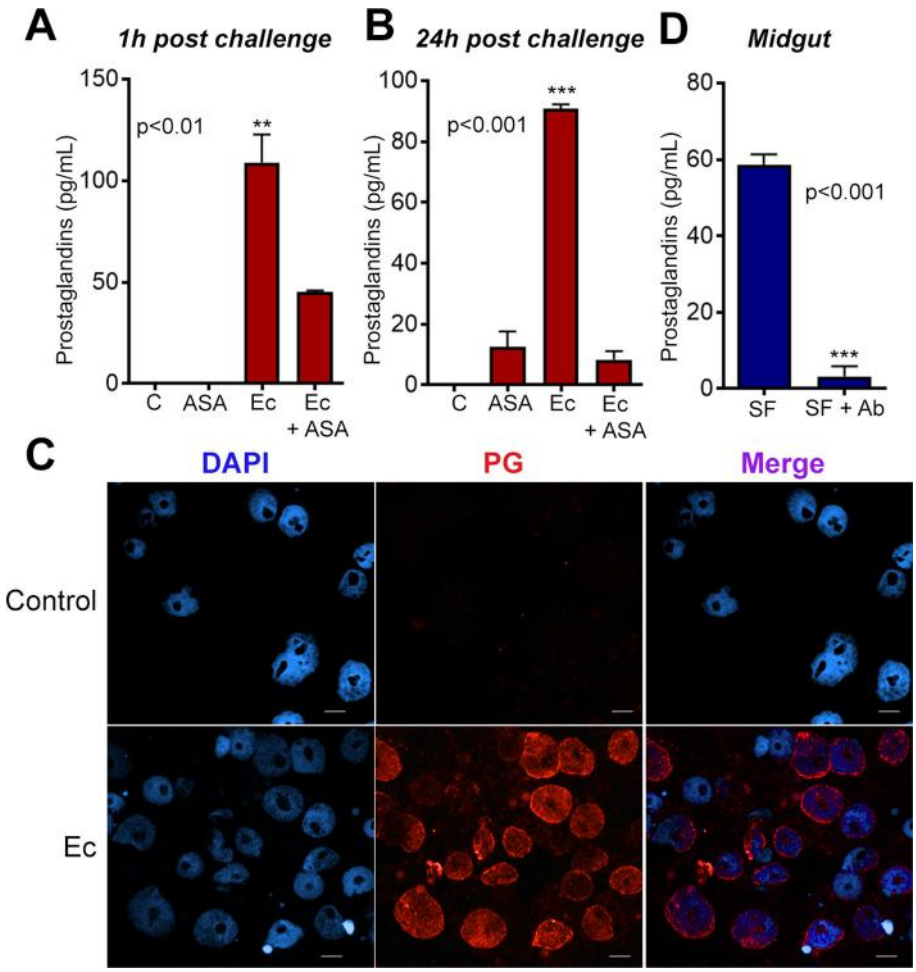

Fig 1. *E.cloacae* (Ec) stimulates PG production in Aag2 cells and *Aedes* midgut cells. (A-B) Aag2 cells were incubated with heat-killed bacteria *E. cloacae* (Gram negative, Ec) in the presence or absence of ASA for 1 hour (A) or 24 hours (B). PG levels in the supernatant of Aag2 cells incubated with heat killed Ec and ASA. (C) PG detection *in situ* (by EicosaCell assay) in Aag2 cells challenged with heat-killed bacteria. (D) PG levels in midguts of *Ae.aegypti* females sugar fed (SF) and antibiotic treated (SF + Ab). Statistical analyses were performed using ANOVA followed by Dunnett's multiple comparison test for figure A and B. In C, statistical analyses were conducted as an unpaired t-test. Error bars represent mean ± SEM. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. Scale Bars = 7μm.

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Inhibition of prostaglandin production suppresses the *Ae. aegypti* immune response against Gram-negative bacteria and virus infections

We evaluated the impact of PG synthesis inhibition on the global gene expression in the cell line Aag2. Aag2 cells were pre-treated with ASA or vehicle for one hour, and then challenged with the heat-killed Gram-negative bacteria, *E. cloacae* (Ec). In vehicle-pretreated Aag2 cells, Ec-challenge induced the upregulation of 361 genes, whereas 822 were down-regulated (Fig 2A, S2 Fig and S1 Dataset). In contrast, in ASA-pretreated, Ec-challenged Aag2, 1008 genes were up-regulated and 1267 genes were down-regulated. 114 genes were commonly up-regulated and 399 down-regulated in both treatments (S2 Fig and S1 Dataset).

Next, we investigated how these treatments affected immune-related genes. In ASA-pretreated Aag2 cells, 99 immune-related genes were suppressed (S2 Dataset). Transcripts for clip-domain serine proteases were strongly down-regulated, followed by transcripts related to the toll pathway, and transcripts for protease inhibitors serpins. Some transcripts of the IMD

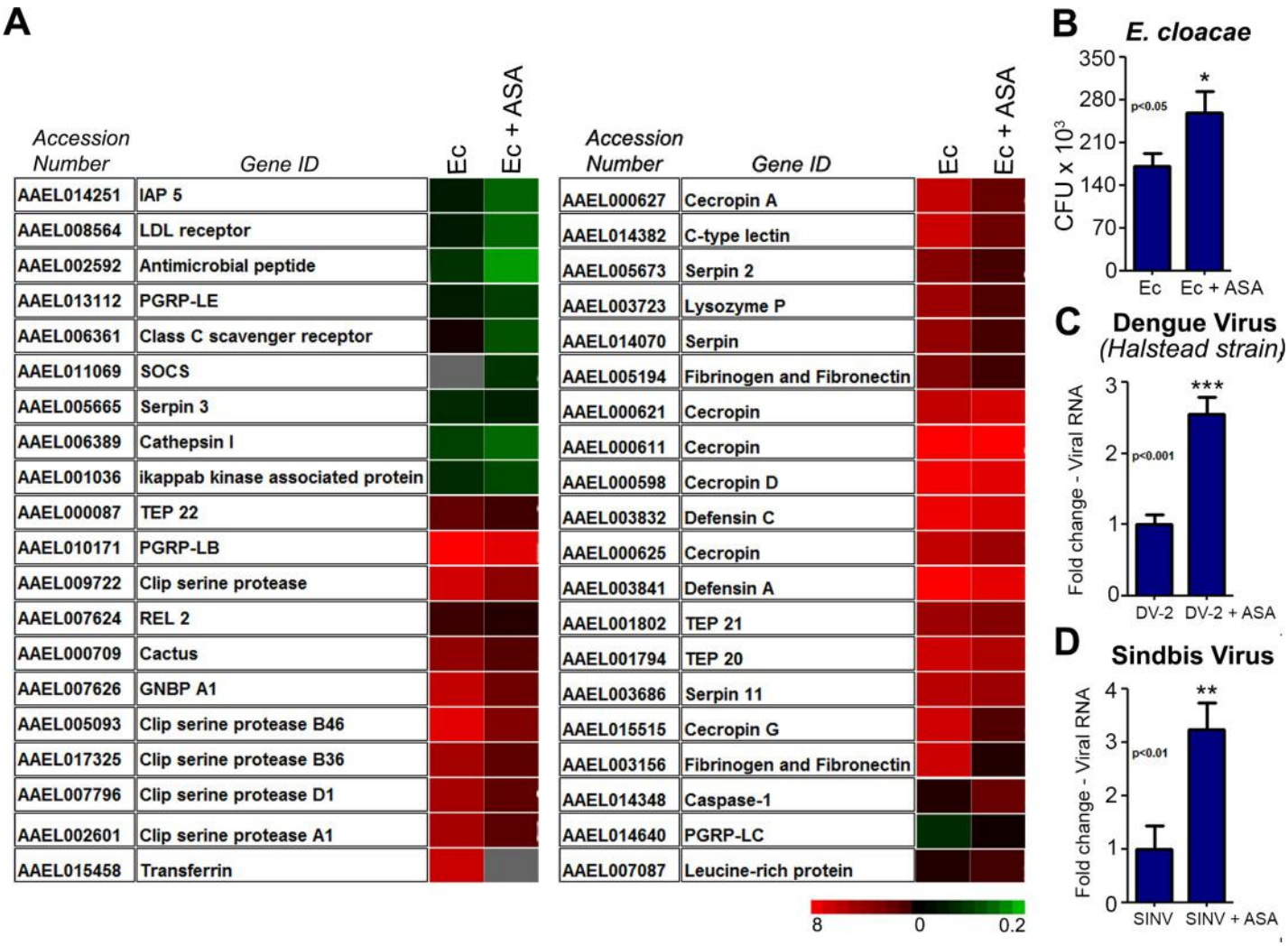

Fig 2. ASA treatment compromises the proper expression of immune genes and the ability to control bacterial and viral infections. (A) Immune related genes modulated by ASA treatment in the microarray analysis. Green color indicates down-regulated genes and red color is referred to up-regulated ones. Complete detailed expression data can be seen in S1 Dataset. A complete list of the immune genes regulated by ASA treatment can be seen in S2 Dataset. (B) Number of CFU recovered from the supernatant of Aag2 cells that were pre-treated or not with ASA before Ec exposure. (C-D) Viral RNA recovered from the supernatant of Aag2 cells infected with Dengue and Sindbis virus after pretreatment with Aag2 cells (C) Dengue virus RNA recovered after 4 days post infection (D) Sindbis virus RNA recovered 2 days post infection. Error bars represent mean ± SEM. Unpaired t-test, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. Each biological replicate corresponds to a well on a plate and at least three independent experiments were performed per assay. Viral RNA amounts were normalized by the number of cells present in the well, which were determined using trypan blue stain.

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pathway were strongly down-regulated, such as the transcriptional factor REL 2, the receptors PGRP-LE and PGRP-LB (*Peptidoglycan Recognition Protein*), and some AMPs, e.g. defensins and cecropins. The negative regulator of the Jak/STAT pathway, SOCS (*Suppressor Of Cytokine Signaling*), was up-regulated by ASA-treatment suggesting a repression of the Jak/STAT pathway. The thioester–containing proteins TEP 20, 22 and 19, putatively involved in pathogen opsonization, were also down-regulated by ASA treatment (Fig 2A). We validated the microarray results by qPCR by measuring the transcript levels of the AMPs defensin A, attacin, cecropin G and D and gambicin (S3 Fig). Following Ec challenge, the expression of all AMPs was significantly increased compared to unchallenged Aag2 cells (S3 Fig). Accordingly, to the microarray and qPCR validation, ASA pretreatment hampered the expression of defensin A,

attacin and cecropin G, while the transcript levels of gambicin were unaltered (Fig 2A, S3 Fig and S1 Dataset). We also observed a reduction on the transcript abundance of defensin A and gambicin when Aag2 cells were pre-treated with ibuprofen–a competitive inhibitor of cyclooxygenase, that has a different mechanism of action from ASA (S4 Fig).

We evaluated whether ASA-treated Aag2 cells became immune deficient and, thereby, more susceptible to infections. To test this hypothesis Aag2 cells were pre-treated with either the inhibitors (ASA or ibuprofen) or vehicle (control), followed by incubation with live Ec. After 4 hours of incubation, supernatants of Aag2 cells previously treated with inhibitors presented significantly higher levels of *E. cloacae* in comparison to supernatant of control cells (Fig 2B and S5A Fig), suggesting a compromised ability of drug-treated cells in fighting infections. To test whether the prostaglandin inhibition-related immune suppression could also affect anti-viral defense, Aag2 cells were treated with ASA or ibuprofen prior to infection with Dengue or Sindbis viruses. In both cases, treatment with ASA increased the amount of viral RNA in the supernatant. Viral RNA from Dengue virus increased 2.5-fold while Sindbis RNA increased about 3-fold (Fig 2C and 2D) four days post infection, showing that prostaglandin production is necessary for the generation of both an antiviral and antibacterial immune response. Also, treatment with ibuprofen and infection with another strain of Dengue virus (New Guinea C) showed the same pattern, culminating with an increase of 3–4 fold in viral RNA (S5B and S5C Fig). To confirm that ASA treatment impairs PG production during a viral infection, we measured PG levels in the supernatant of Aag2 cells infected with Dengue. The levels of PG went from 17 pg/mL in the supernatant of infected cells to non-detectable levels after ASA treatment.

Inhibition of prostaglandin production impairs antimicrobial gene expression in *Ae.aegypti* midgut and increases Dengue viral loads

To confirm these observations in adult mosquitoes, we pretreated female mosquitoes with 5 mM ASA in a sucrose solution for 3 days and fed or not with blood in order to allow growth of the indigenous microbiota. The same pattern observed for cells was observed in mosquitoes. Defensin A, cecropin G and cecropin D, which were down-regulated upon ASA treatment in the microarray analysis, had their expression increased in response to the microbiota growth, but treatment of the females with ASA prevented the up-regulation of this AMPs expression (Fig 3A–3C). Expression of serpin 27A and gambicin were independent of prostaglandin production, both in cells and mosquitoes, and treatment with ASA did not alter their expression in comparison to blood-fed non-ASA treated females as observed in the microarray (Fig 3D and 3E).

To evaluate whether impairment of prostaglandin production would also compromise the efficiency of the immune response in mosquitoes, *Aedes aegypti* females were allowed to feed on a sugar solution containing 5 mM ASA for 3 days prior to feeding on a saline solution (BBSA) containing the entomopathogenic bacteria *Serratia marcescens* ($5x10^8$ bacteria/mL) [32]. The control cohort was not treated with ASA. Mosquito survival was then monitored daily for five days as an indicator of the capacity to cope with bacterial infection and hence immunocompetence (Fig 3F). Treatment of mosquitoes with ASA compromised anti-bacterial defense by increasing the mortality rate from 28% to 49%, in the case of *Serratia marcescens* (Fig 3F). Hence, here we show that prostaglandin production is also required for the generation of a fully functional immune response in mosquitoes.

To evaluate if inhibition of PG synthesis with ASA would increase viral loads also in mosquitoes, *Aedes aegypti* female mosquitoes were pretreated with 5mM of ASA and then, infected with Dengue virus (New Guinea C strain). Seven days post infection, the cohort that received

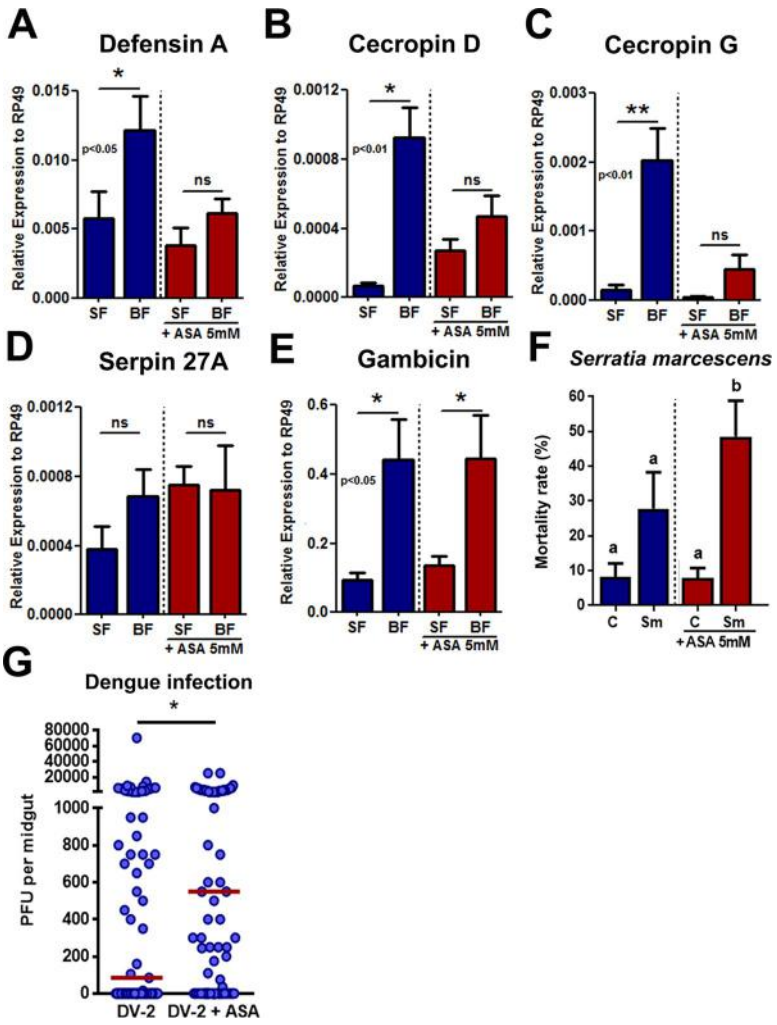

Fig 3. In the midgut, PG is required for proper induction of immune genes after blood feeding, and its inhibition compromises survival after bacterial infection. (A-E) *Aedes aegypti* female mosquitoes were pretreated with a sugar solution supplemented with ASA for three days, before blood feeding. Twenty -four hours after feeding midgut expression of AMPs (A) defensin A, (B) cecropin D, (C) cecropin G, (D) serpin 27A and (E) gambicin was analyzed by qPCR. Statistical analyses were performed using unpaired t-test comparing sucrose and blood groups in each condition. (F) Mortality rate of mosquitoes after feeding with the bacteria *Serratia marcescens* ($5x10^8$ bacteria/mL) with and without ASA sugar pretreatment. (G) Number of infective Dengue units per midgut (PFU) in mosquitoes pre-treated with ASA for 2 days (Dengue New Guinea C strain). (A) to (F) Error bars represent mean ± SEM. (G) Red bars represent the median of each group. (A) to (F) Unpaired t-test, NS ($P>0.05$), $^{*}P\leq0.05$, $^{**}P\leq0.01$; Conditions were compared with its correspondent SF control. (A) to (E) Pools of 10 midguts were used for each biological replicate, at least 3 biological replicates were used per condition. (F) Mortality rate across 6 independent survival curves. (G) Number of PFU per mosquito midgut, each dot represents one individual mosquito (control = 69 and ASA = 74).

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ASA treatment prior to infection presented a 6.5 fold increase in viral particles in comparison to ASA non treated group (Fig 3G).

The expression of the cytosolic Phospholipase A2 (PLA2c) is sensitive to different immune challenges

To further investigate the role of prostaglandins in mosquito immune responses, we targeted components of prostaglandins synthesis pathway and identified in the microarray a cytosolic phospholipase A2 (PLA2c; AAEL001523) that was downregulated by ASA treatment in the

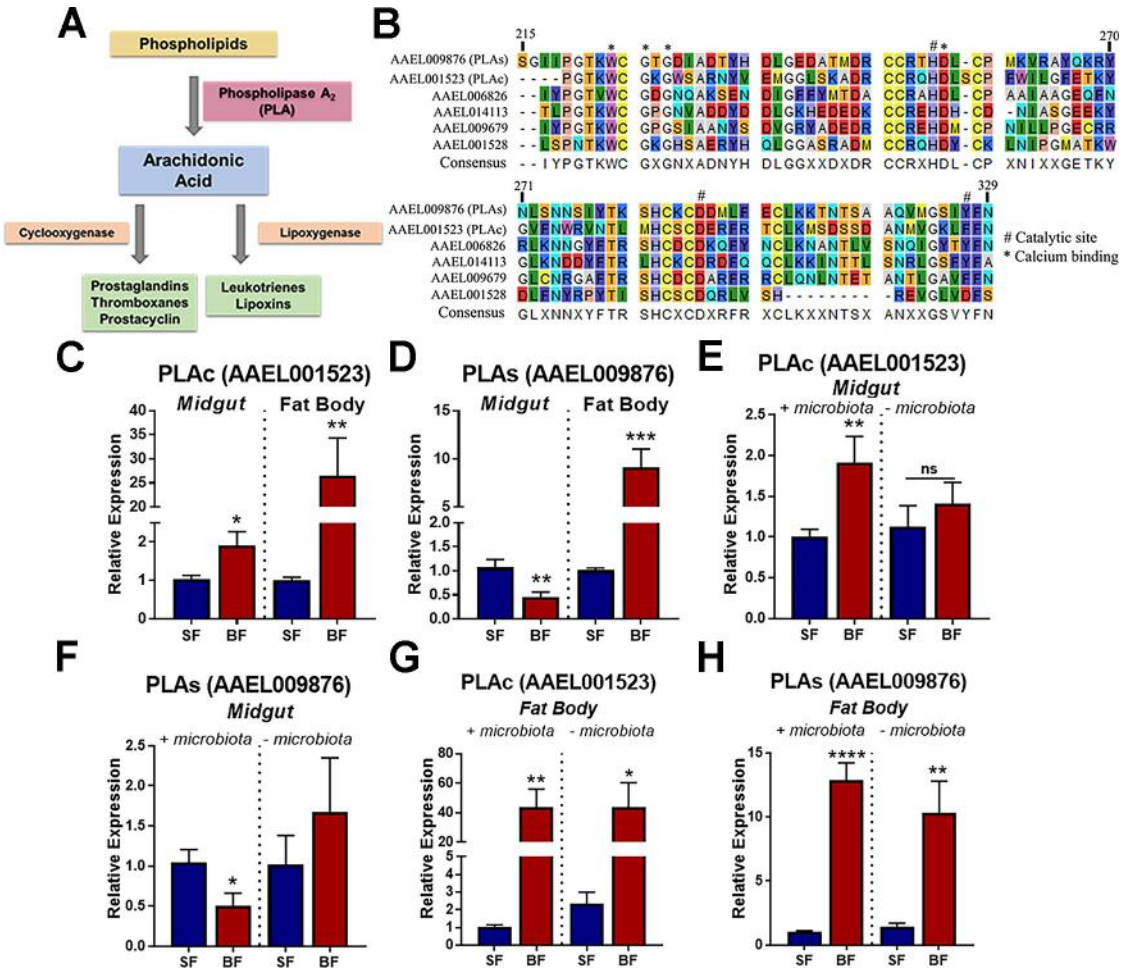

Fig 4. Cytosolic phospholipase A2 (PLA2c) is induced by the microbiota in the midgut after blood feeding. (A) Schematic representation of canonical PG production pathway. (B) Alignment of the amino acid sequences of six phospholipases of *Aedes aegypti*. * represents calcium binding domain; # represents catalytic domain. Gene expression of (C) PLA2c and (D) PLA2s in the midgut and fat body in sugar fed (SF) and blood fed mosquitoes (BF), 24 h post feeding. Gene expression of (E) PLA2c and (F) PLA2s in the midgut with or without the presence of the microbiota. Gene expression of (G) PLA2c and (H) PLA2s in the fat body with or without the microbiota presence. Error bars represent mean ± SEM. Unpaired t-test, NS ($P>0.05$), $^{*}P\leq0.05$, $^{**}P\leq0.01$, $^{***}P\leq0.001$, $^{****}P\leq0.0001$; Conditions were compared with its correspondent SF control. Each biological replicate was a pool of 10 midguts, and each experimental group had at least 3 biological replicates.

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presence of a bacterial challenge (S1 Dataset). Phospholipase A2 (PLA2) mediates the first step in eicosanoid synthesis by converting membrane phospholipids into arachidonic acid that will be converted into prostaglandins, leukotrienes, lipoxins and prostacyclins (Fig 4A). We analyzed the amino acid sequence of six annotated PLA2, including the candidate one, selected from the microarray. All the sequences had conserved residues in the catalytic domain and calcium binding sites (Fig 4B). We selected the PLA2c (AAEL001523) and a secretory PLA2 (PLA2s) (AAEL009876) for further biological characterization. Those candidates were classified as cytosolic and secreted based on the presence of a signal peptide, using signal analysis. Twenty-four hours post feeding, PLA2c was upregulated following a blood meal in the midgut and in the fat body (Fig 4C), while PLA2s was downregulated in the midgut, but upregulated in the fat body (Fig 4D). The upregulation of PLA2s in the midgut following a blood meal was dependent on the presence of the microbiota, since antibiotics treatment abolished its induction (Fig 4E). The microbiota reduction abolished the downregulation of PLA2s on the midgut

(Fig 4F). The expression of both PLA2c and PLA2s in the fat body was not affected by the status of the microbiota (Fig 4G and 4H). We also measured PLA2s and PLA2c expression in Aag-2 cell line challenged with different stimulus: heat killed *Enterobacter cloacae* (gram-negative); *Micrococcus luteus* (gram-positive) and zymosan. PLA2c and PLA2s were induced upon bacterial challenge after 6 h of incubation but not with zymosan (S6A Fig). At 24 h, no PLA2c regulation was observed upon any of the challenges, while PLA2s was induced by heat killed gram-negative bacteria and zymosan (S6B Fig).

The cytosolic Phospholipase A2 modulates the expression of prostaglandin and protects against Dengue infection

Next, to evaluate the functional role of phospholipases in midgut prostaglandin production, we knocked down PLA2c and PLA2s expression using RNAi. Three days after dsRNA injection, PLA2c and PLA2s expression had a 50% reduction in expression, both in sugar and blood fed conditions (Fig 5A and 5B). The silencing of PLA2s significantly decreased in 70%

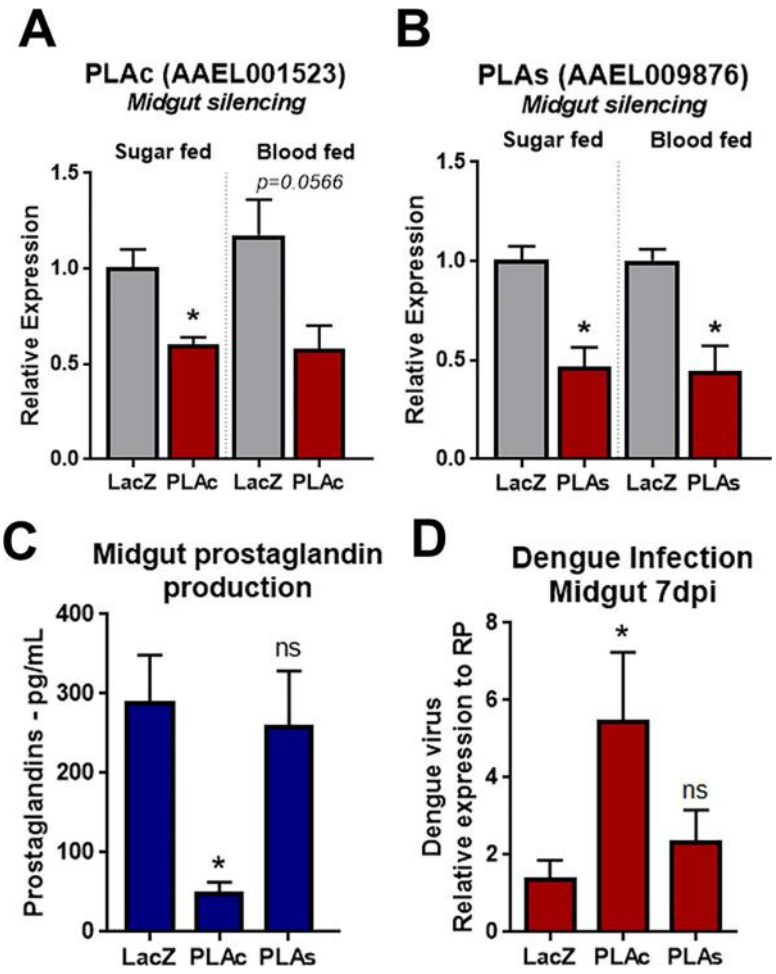

Fig 5. PG production in the midgut is dependent on PLA2c expression and its impairment increases Dengue viral loads. Knock-down efficiency in the mosquito midgut injected with (A) dsPLAc dsRNA and (B) dsPLAs dsRNA, in sugar fed (SF) and blood fed (BF) females. (C) PG levels in the midgut after RNAi silencing of PLAc and PLAs. Mosquitoes injected with dsRNA for LacZ were used as control. (D) Dengue virus RNA levels in the midgut relative to mosquito RP-49 expression. Error bars represent mean ± SEM. Unpaired t-test, NS ($P>0.05$) and $^{*}P\leq0.05$. Each biological replicate was a pool of 10 midguts, and each experimental group had at least 3 biological replicates.

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the production of PG in the midgut, whereas the silencing of PLA2c did not affect PGs production (Fig 5C). We evaluated whether phospholipases and PG production played a role on Dengue infection in the midgut. The knockdown of PLA2c increased the Dengue viral loads (DV2) in the midgut over 5-fold compared to LacZ, whereas PLA2s silencing had no effect on viral loads (Fig 5D).

Discussion

We show that, upon incubation with gram negative bacteria *E. cloacae*, Aag2 cells increase PG production in at least 10-fold, being this PG increase prevented by pre-incubation of the cells with ASA, a cyclooxygenase inhibitor (Fig 1A and 1B). Similarly, when mosquitoes are fed on a sugar solution containing antibiotics, PG levels were also reduced in more than 60-fold (Fig 1C), supporting the hypothesis that in mosquitoes, the microbiota induces the production of PG by the midgut. Previously, PGs were described as key molecules released by the midgut of *Anopheles gambiae* in response to the direct contact of the microbiota with the midgut epithelia. Not only PGs have a chemotactic effect on hemocytes but are also essential for immune memory establishment after *Plasmodium berghei* infection [11].

In vertebrates, the main cellular sites of prostaglandin production are the perinuclear membrane, lipid bodies, phagosomes and endoplasmic reticulum [33]. We demonstrated that, in Aag-2 cells, prostaglandins are mainly synthetized in the nuclear and perinuclear region upon bacterial stimuli (Fig 1D).

The microarray analysis revealed that the upregulation of immune genes induced by Ec challenge is no longer observed if the cells were pretreated with ASA. The expression of effector molecules such as cecropins, defensin, serpins; transcription factors, like REL 2; and regulators of the main immune pathways, such as SOCs and Cactus (IκB homolog), is reduced when prostaglandin synthesis is blocked (Fig 2A). This suggests a global immunosuppression of gene expression of several key immune genes upon ASA treatment even in the presence of bacterial challenge. In the mosquito *Anopheles albimanus*, antimicrobial peptides expression in the midgut is reduced upon eicosanoid inhibition and reverted when PGE_2 is added to the media [14]. Although affecting a wide range of classes of immune genes, from PAMP receptors and intracellular signaling to effector genes, the effect of ASA treatment seems specific, since several immune genes, such as gambicin and serpin27A are not modulated when prostaglandin synthesis is inhibited (Figs 3A–3E and S3). ASA treatment does not prevent the induction of immune genes expression. Instead, the lack of PGs just reduces the amplitude of the response. In vertebrates, PGE_2 has different effects depending on the cell type, it supports acute local inflammation and phagocyte attraction, but also has a suppressive role to control type-1 immune responses and host self-preservation [34]. In the insect *Spodoptera exigua* (beet armyworm), PGE_2 synthesis inhibition suppresses phenoloxidase activity and reduces AMPs expression levels [10]. We confirmed that the inhibition of PG synthesis suppresses several immune genes, including AMPs, leading to a more permissive environment for bacterial and viral replication in mosquitoes. Thus, it seems that in insects, depletion of PGs promotes immune suppression, reducing the effectiveness of the immune response against bacteria and virus.

The microarray findings using Aag2 cells were confirmed by feeding female mosquitoes with ASA in the sugar followed by a blood meal. The lack of prostaglandins prevents the upregulation of defensin, cecropins D and G, while Serpin 27A and gambicin's expression were not sensitive to ASA treatment. Treating Aag2 cells with another inhibitor of PG synthesis, Ibuprofen, a compound that inhibits cyclooxygenase through a different mechanism from ASA, also resulted in antimicrobial peptides expression down-regulation and increase in pathogen

replication, for both Ec and Dengue. Thus, it is unlikely that effects observed with ASA treatment constitute off-target effects of this compound (S4 Fig).

Global immune down-regulation due to PG synthesis inhibition creates a permissive environment for pathogen replication and spread, since those pathways have previously been implicated in the clearance of *Plasmodium sp* and Dengue virus [24, 25, 35, 36]. Inhibition of PG synthesis turns Aag2 cells more permissive to a flavivirus, Dengue-2, to an alphavirus, Sindbis, and to the bacteria Enterobacter cloacae (Fig 2B–2D and S5 Fig). The cells became unable to control infections with multiple pathogens, indicating that PGs affect the proper activation of a general mechanism of fighting infections. Mosquitoes pretreated with ASA in the sugar were also more permissive to Dengue infection in the midgut reproducing the findings obtained with Aag2 cells (Figs 2C and 3G, S5B and S5C Fig). Overall, impairment of PG synthesis leads to a decreased effectiveness in viral replication control, caused by the low expression of immune related genes.

The loss of the ability to control pathogen replication can lead to a decrease in host survival, especially upon bacterial infections where its overgrowth compromises the life of the insect. According to this, mosquitoes pretreated with ASA were more sensitive to Serratia infection and had a mortality rate higher than mosquitoes not treated and infected with the bacteria (Fig 3F). This suggests that the immunosuppression caused by the lack PG production leads to pathogen susceptibility and results in survival reduction.

Although PGs can be detected in mosquitoes [37], insects lack a canonical pathway of PG synthesis, lacking a direct ortholog of cyclooxygenase. A recent study described that heme peroxidases, HPX7 and HPX8, are involved and necessary for PG synthesis in the mosquito *Anopheles gambiae* [11]. Microbiota proliferation induces the expression of HPX7 and HPX8 that results in PG production in the midgut. Here we decided to investigate the upstream components of eicosanoid production, by characterizing the phospholipase A2 that converts phospholipids into arachidonic acid, the first reaction of the pathway. Our results describe a phospholipase directly related to PG synthesis, unraveling one more step of this pathway in mosquitoes. Although the two PLA2 tested have a similar amino acid sequence, especially in catalytic and Ca+2 binding sites, they appear to have distinct biological roles in mosquito physiology. The two phospholipases, PLA2c and PLA2s, were upregulated in the fat body in response to blood feeding (Fig 4C, 4D, 4G and 4H). That could indicate lipid mobilization and secretion in the hemolymph for egg development after the nutritional input of the blood ingestion [38]. Only PLA2c was upregulated in the midgut in response to the blood meal and this increase was microbiota dependent, indicating that bacterial elicitors might be triggering the expression in the midgut (Fig 4C–4F). This could argue that this PLA2c is playing an immune role in the midgut, while the other PLA2s seems to have a stronger regulation in the fat body independently of the microbiota presence. Using gene knockdown through RNAi both PLA2c and PLA2s were successfully silenced in the midgut in both sugar and blood fed mosquitoes (Fig 5A and 5B). Knockdown of PLA2c resulted in the decrease of PG production in the midgut and higher amounts of Dengue RNA (Fig 5C and 5D). Previously, it has been shown that PG levels increase during Dengue infection in the midgut of *Ae*. *aegypti* mosquitoes [37]. PG increase during Dengue infection could indicate an attempt of the mosquito immune response to control viral replication. When PG synthesis is blocked, by either ASA or Ibuprofen treatments or PLA2c knockdown, the mosquito becomes more susceptible to viral replication.

Altogether, our results indicate that prostaglandins are an important component of the immune response in mosquitoes, not being responsible for the activation of the immune response but playing a role in the amplitude of this response. We shed some light on how PGs are responsible for this "fine tuning" of the immune response by providing a genome wide analysis of the effects of PG on *Aedes* gene expression, revealing that PGs may modulate the

expression of several genes from the main mosquito immune pathways, Toll, IMD and Jak/STAT, and probably, as a consequence of this modulation, alter the expression of effector genes. We described a PLA2c that is involved in PG synthesis in the midgut and showed that, when it is silenced, Dengue replication increases. Here we showed using pharmacological and genetic approaches the role of PGs in immune modulation and viral susceptibility in the mosquito *Ae*. *aegypti*.

Supporting information

S1 Fig. Bacterial 16S expression in the midgut of Ae. aegypti upon antibiotic treatment. Quantitative PCR of bacterial 16S mRNA in midguts from sugar (SF) and blood fed (BF) mosquitoes kept on regular sugar or treated for four days with antibiotics (+Ab). Expression was evaluated 24 hours post blood feeding. 16S expression was calculated relative to the mosquito ribosomal protein 49 (RP49) used as an endogenous control.
(TIF)

S2 Fig. Venn diagram of the microarray analysis of Aag2 cells stimulated with *E. cloacae* (Gram Negative, Ec) in the presence of ASA. 361 genes were up-regulated in response to Ec incubation, while 822 were down-regulated. In the presence of Ec and ASA 1008 genes were up-regulated and 1267 were down-regulated. 114 genes were up-regulated in both conditions while 399 were down-regulated. For detailed information on the genes see S1 Dataset.
(TIF)

S3 Fig. ASA treatment impairs AMPs expression in response to bacterial challenge. Gene expression of AMPs identified in the microarray analysis in Aag2 cells challenged with heat-killed Ec in the presence of ASA. (A) Defensin A, (B) Cecropin G, (C) Cecropin D, (D) Attacin and (E) Gambicin. Error bars represent mean ± SEM. ANOVA Dunn's multiple comparison test, NS ($P>0.05$), $^{*}P\leq0.05$, $^{**}P\leq0.01$, $^{****}P\leq0.0001$. Each biological replicate was an individual well from a culture plate, and each experimental group had at least 3 biological replicates. AMPs expression was calculated relative to the expression of the RP-49 gene.
(TIF)

S4 Fig. Inhibition of PG production with ibuprofen leads to a decrease in several immune-related genes after bacterial infection in Aag2 cells. Gene expression of AMPs in response to heat-killed Ec challenge in the presence of ibuprofen. (A) Defensin A and (B) Gambicin. Error bars represent mean ± SEM. ANOVA Dunn's multiple comparison test, NS ($P>0.05$), $^{***}P\leq0.001$. Each biological replicate was an individual well from a culture plate, and each experimental group had at least 3 biological replicates. AMPs expression was calculated relative to the expression of the RP-49 gene.
(TIF)

S5 Fig. In the absence of PG, Aag2 cells capacity of controlling E. cloacae or Dengue virus proliferation is compromised. Aag2 cells were challenged with live Ec and Dengue virus in the presence of prostaglandin synthesis inhibitors, ibuprofen and ASA. (A) Number of CFU in the supernatant of challenged cells incubated with ibuprofen. (B) Viral RNA present in the supernatant of cells infected with *New Guinea C* Dengue 2 strain in the presence of ASA. (C) Viral RNA present in the supernatant of cells infected with *Halstead* Dengue 2 strain in the presence of ibuprofen. Viral RNA amounts in present in the supernatant of the cell culture were normalized by the number of cells present in the well, which were determined using trypan blue stain. Error bars represent mean ± SEM. Unpaired t-test, $^{*}P\leq0.05$, $^{**}P\leq0.01$. Each biological replicate was an individual well from a culture plate, and each experimental group

had at least 3 biological replicates.
(TIF)

S6 Fig. Both cytosolic and secretory PLA are transcriptionally regulated by bacterial and fungal products. Aag2 cells challenged with heat-killed Gram positive (Ml) and negative (Ec) bacteria and zymosan (Zy) a glucan present in fungus surface. Cells were challenged for 6 and 24 hours. (A) Gene expression of PLAc 6 and 24 hours post stimulus. (B) Gene expression of PLAs 6 and 24 hours post stimulus. Error bars represent mean ± SEM. Dunn's multiple comparison test, NS ($P>0.05$), $^{*}P\leq0.05$, $^{**}P\leq0.01$, $^{***}P\leq0.001$, $^{****}P\leq0.0001$. Each biological replicate was an individual well from a culture plate, and each experimental group had at least 3 biological replicates. PLAc and PLAs expression were normalized using the expression of endogenous RP-49 gene.
(TIF)

S1 Table. List of primers used in this study.
(TIF)

S1 Dataset. Gene expression changes of Aag2 cells modulated by bacterial infection in the presence or absence of ASA.
(XLSX)

S2 Dataset. Immune genes modulated by ASA treatment.
(XLSX)

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Zika virus infection drives epigenetic modulation of immunity by the histone acetyltransferase CBP of *Aedes aegypti*

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Abstract

Epigenetic mechanisms are responsible for a wide range of biological phenomena in insects, controlling embryonic development, growth, aging and nutrition. Despite this, the role of epigenetics in shaping insect-pathogen interactions has received little attention. Gene expression in eukaryotes is regulated by histone acetylation/deacetylation, an epigenetic process mediated by histone acetyltransferases (HATs) and histone deacetylases (HDACs). In this study, we explored the role of the *Aedes aegypti* histone acetyltransferase CBP (AaCBP) after infection with Zika virus (ZIKV), focusing on the two main immune tissues, the midgut and fat body. We showed that the expression and activity of AaCBP could be positively modulated by blood meal and ZIKV infection. Nevertheless, Zika-infected mosquitoes that were silenced for AaCBP revealed a significant reduction in the acetylation of H3K27 (CBP target marker), followed by downmodulation of the expression of immune genes, higher titers of ZIKV and lower survival rates. Importantly, in Zika-infected mosquitoes that were treated with sodium butyrate, a histone deacetylase inhibitor, their capacity to fight virus infection tends to be rescued. Our data point to a direct correlation among histone hyperacetylation by AaCBP, upregulation of antimicrobial peptide genes and increased survival of Zika-infected-*A. aegypti*.

Author summary

Pathogens have coevolved with mosquitoes to optimize transmission to hosts. As natural vectors, mosquitoes are permissive to and allow systemic and persistent arbovirus infection, which intriguingly does not result in dramatic pathological sequelae that affect their lifespan. In this regard, mosquitoes have evolved mechanisms to tolerate persistent infection and develop efficient antiviral strategies to restrict viral replication to nonpathogenic levels. There is a great deal of evidence supporting the implication of epigenetics in the modulation of the biological interaction between hosts and pathogens. This study reveals that Zika virus infection positively modulates the expression and activity of *A. aegypti* histone acetyltransferase CBP (AaCBP). This study shows that AaCBP plays a role in the activation of immune-responsive genes to limit Zika virus replication. This first description that Zika virus infection has epigenomic consequences in the regulation of *A. aegypti* immunity opens a new avenue for research on mosquito factors that can drive vector competence.

Introduction

Mosquitoes are primary vectors of a variety of human pathogens throughout the world. *Aedes aegypti* mosquitoes can develop long-lasting viral infections and carry high viral loads, which make them efficient vectors for the transmission of arboviruses such as Zika virus (ZIKV) (Weaver et al., 2016).

Host-pathogen interactions are among the most plastic and dynamic systems in nature. In this regard, epigenetic modifications can offer an accessory source of fast-acting, reversible and readily available phenotypic variation that can be directly

shaped by both host and pathogen selection pressures(Gómez-Díaz et al., 2012; Ruiz et al., 2019). One of the hallmarks in the study of host gene regulation is to elucidate how specific sets of genes are selected for expression in response to pathogen infection.

Understanding the interactions between the mosquito immune system and viruses is critical for the development of effective control strategies against these diseases. Mosquitoes have conserved immune pathways that limit infections by viral pathogens (Angleró-Rodríguez et al., 2017; Tikhe & Dimopoulos, 2021). Mosquito antiviral defense is regulated by RNA interference (RNAi), Janus kinase/signal transducer (JAK-STAT), Toll, the immune deficiency (IMD) and MAPK immune pathways (Angleró-Rodríguez et al., 2017; Asad et al., 2018; Tikhe & Dimopoulos, 2021).

The Toll pathway has been shown to play the most important role in controlling ZIKV infections (Angleró-Rodríguez et al., 2017). In this context, gene expression analysis of ZIKV-infected mosquitoes has indicated that Toll pathway-related genes are highly upregulated in Zika infection when compared to other immune pathways (Angleró-Rodríguez et al., 2017; Tikhe & Dimopoulos, 2021).

Eukaryotic gene expression is controlled by the functions of *cis*-DNA elements, enhancers and promoters, which are bound by transcription factors, in combination with the organization of the chromatin (Barrera & Ren, 2006). Although it is still poorly understood how chromatin-associated processes participate in the regulation of gene transcription in the context of pathogen-vector interactions, it has been previously shown that *Plasmodium falciparum* infection induces significant chromatin changes in the *Anopheles gambiae* mosquitoes (Ruiz et al., 2019). This

study identified infection-responsive genes showing differential enrichment in various histone modifications at the promoter sites (Ruiz et al., 2019).

The transcriptional coactivator CREB-binding protein (CBP), and its paralog p300, play a central role in coordinating and integrating multiple signal-dependent events with the transcription apparatus, allowing the appropriate level of gene activity to occur in response to diverse stimuli (Chan & Thangue, 2001). CBP proteins do not specifically interact with the promoter elements of target genes, but they are recruited to promoters by interaction with DNA-bound transcription factors, which directly interact with the RNA polymerase II complex (Chan & Thangue, 2001; Revilla & Granja, 2009). A key property of CBP is the presence of histone acetyltransferase (HAT) activity, which endows the enzyme with the capacity to influence chromatin activity by the acetylation of histones (Chan & Thangue, 2001).

Consistent with its function as a transcriptional coactivator, CBP plays crucial roles in embryogenesis (Fang et al., 2014), development (Bantignies et al., 2002), differentiation (Bantignies et al., 2002; Fauquier et al., 2018), oncogenesis (Bantignies et al., 2002; Iyer et al., 2004) and immunity (Revilla & Granja, 2009). Although most of these studies have been conducted in mammalian systems, nonvector insect models have also made important contributions to the biological functions of CBP (Kirfel et al., 2020; Li et al., 2018; Sedkov et al., 2003; Tie et al., 2009).

The present work describes the functional characterization of a histone acetyltransferase in an insect vector and provides indications that Zika virus infection exerts epigenomic consequences in regulating *A. aegypti* immunity.

Materials and Methods

Ethics statement

All animal care and experimental protocols were conducted in accordance with the guidelines of the Committee for Evaluation of Animal Use for Research – CEUA of the Federal University of Rio de Janeiro (UFRJ). The protocols were approved by CEUA-UFRJ under the registration number IBQM149/19. Technicians in the animal facility at the Instituto de Bioquímica Médica Leopoldo de Meis (UFRJ) carried out all protocols related to rabbit husbandry under strict guidelines to ensure careful and consistent animal handling.

Mosquito rearing and cell culture

Aedes aegypti (*Liverpool black-eyed* strain) were raised in a mosquito rearing facility at the Federal University of Rio de Janeiro, Brazil, under a 12-h light/dark cycle at 28 ^{o}C and 70-80% relative humidity. Larvae were fed dog chow, and adults were maintained in a cage and given a solution of 10% sucrose *ad libitum*. Females 7-10 days posteclosion were used in the experiments. When mentioned, mosquitoes were artificially fed heparinized rabbit blood. For virus infection, mosquitoes were fed using water-jacketed artificial feeders maintained at 37 ^{o}C sealed with parafilm membranes. Alternatively, mosquitoes were infected by intrathoracic injections of 69 nL containing 60 Plaque Forming Units (PFUs) of ZIKV.

Female midguts or fat bodies were dissected 24, 48, 72 or 96 h after feeding for RNA sample preparation.

The *A. aegypti* embryonic cell line Aag2 was maintained in Schneider medium (Merk) supplemented with 5% FBS (LGC, Brazil) and 1% penicillin/streptomycin/amphotericin B. Aag2 cells were incubated at 28 ^{o}C.

ZIKV infection and virus titration

ZIKV strain Pernambuco (ZIKV strain ZIKV/H.sapiens/Brazil/PE243/201) (Donald et al., 2016) was propagated in the *A. albobictus* C6/36 cell line, and titers were determined by plaque assay on Vero cells. ZIKV was propagated in C6/36 cells for 6 days; virus was then harvested and mixed with a sterile 1% solution at pH 7.1 (2.18 M sucrose, 38 mM KH_2PO4, 72 mM K_2HPO4, 60 mM $_L$-glutamic acid), and stored at -80 ^{o}C. Mosquitoes were intrathoracically infected by microinjections (Göertz et al., 2019) of 69 nL of virus, containing 60 PFUs.

Midguts and fat bodies were dissected, individually collected, and stored at –80 ^{o}C until use for plaque assays. Virus titration was performed as described previously (Sim et al., 2013). Plates were incubated for 4-5 days, fixed and stained with a methanol/acetone and 1% crystal violet mixture, and washed, after which the plaque forming units (PFUs) were counted.

AaCBP gene knockdown by RNAi

Double-stranded RNA (dsRNA) was synthesized from templates amplified from cDNA of adult female mosquitoes using specific primers containing a T7 tail (Supplementary Table 1). The *in vitro* dsRNA transcription reaction was performed following the manufacturer's instructions (Ambion MEGAscript RNAi). Two

different dsRNA products (dsAaCBP1 and dsAaCBP2) were PCR amplified based on the coding sequence of *A. aegypti* CBP (GenBank accession number XP_011493407.2), using the oligonucleotides listed in Supplementary Table 1. The irrelevant control gene luciferase (dsLuc) was amplified from the luciferase T7 control plasmid (Promega). Female mosquitoes were injected intrathoracically (Ramirez et al., 2012) with dsRNA (0.4 μg) with a microinjector (NanoJect II Autonanoliter injector, Drummond Scientific, USA). Injected mosquitoes were maintained at 28 ^{o}C, and 70-80% humidity, with 10% sucrose provided *ad libitum*.

RNA isolation and quantitative real-time PCR

Total RNA was isolated from whole bodies, midguts or fat bodies of adult females, using the RiboPure kit (Ambion) followed by DNase treatment (Ambion) and cDNA synthesis (Superscript III, Invitrogen), following the manufacturer's instructions. Quantitative gene amplifications (qPCR) was performed with StepOnePlus Real-Time PCR System (Applied Biosystems) using the Power SYBR Green PCR Master Mix (Applied Biosystems). The comparative *Ct* method (Livak & Schmittgen, 2001) was used to compare mRNA abundance. In all qPCR analyses, the *A. aegypti* ribosomal protein 49 gene (*Rp*49) was used as an endogenous control (Gentile et al., 2005). All oligonucleotide sequences used in qPCR assays are listed in the Supplementary Table 1.

Western blotting

Protein extracts were prepared as previously described (Coutinho Carneiro et al., 2020; Ribeiro et al., 2012). Briefly, *A. aegypti* total protein extracts were carried out by homogenizing adult female mosquitoes or Aag2 cells in TBS containing a protease inhibitor cocktail (Sigma). Proteins were recovered from the supernatant by centrifugation at 14.000xg, for 15 min. at 4 ^{o}C. Protein concentration was determined by the Bradford Protein Assay (Bio-Rad). Western blots were carried out using secondary antibody (Immunopure goat anti-mouse, #31430). The primary monoclonal antibodies (ChIP grade) used were anti-H3 pan acetylated (Sigma-Aldrich #06-599), anti-H3K9ac (Cell Signaling Technology #9649) and Anti-H3K27ac (Cell Signaling Technology, #8173), according to the manufacture's instructions. For all antibodies, a 1:1000 dilution was used. For normalization of the signals across the samples, an anti-histone H3 antibody (Cell Signaling Technology, #14269) was used.

Statistical analysis

All analyses were performed with the GraphPad Prism statistical software package (Prism version 6.0, GraphPad Software, Inc., La Jolla, CA). *Asterisks* indicate significant differences (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant).

Genome-wide identification of lysine acetyltransferases in *A. aegypti*

We obtained the latest *A. aegypti* proteome and functional annotations (AaegL5.0) from NCBI RefSeq

(https://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/002/204/515/GCF_002204515.2_AaegL5.0). The amino acid sequences of the 23 *D. melanogaster* lysine acetyltransferases (DmKATs) reported by Feller *et al* 2015 were obtained from FlyBase. The KAT orthologs in *A. aegypti* were identified using BlastP (e-value ≤1e-10, identity ≥25% and query coverage ≥50%) (Altschul et al., 1997). We further validated the presence of various acetyltransferase domains in these KATs. Conserved domains were predicted in putative KATs using hmmer (e-value ≤ 0.01; http://hmmer.org/) and the PFAM-A database. Following the nomenclature of DmKATs and the conserved domain architectures, AeKATs were classified into 5 major sub-families: HAT1, Tip60, MOF, HBO1, GCN5 and CBP (Feller et al., 2015). Conserved domain architectures were rendered with DOG (Domain Graph, version 1.0) (Ren et al., 2009). Multiple sequence alignment of AeKATs was performed using using Clustalw (Thompson et al., 1994).

Results

Aedes aegypti has an ortholog of the CREB-binding protein (CBP) and five additional putative histone acetyltransferases

The *A. aegypti* transcriptional coactivator CBP (AaCBP) contains all five canonical structural and functional domains (Fig 1) of the CBP family (Liu et al., 2008). The TAZ, KIX and CREB are the protein interaction domains that mediate interactions with transcription factors. The histone acetyltransferase (HAT) catalytic domain of the CBP enzymes shows high level of conservation among different species (Fig 1), as well as among other HATs from *A. aegypti* (Supplementary Fig 1A

and B). AaCBP also contains a bromo domain, which binds acetylated lysines. The conserved domains are connected by long stretches of unstructured linkers.

Because transcriptional coactivator complexes are intricate structures composed of multiple subunits, and because cooperative assembly of histone acetyltransferases is a rate-limiting step in transcription activation, we aimed to identify other HATs in *A. aegypti*. By searching the latest *A. aegypti* proteome and functional annotations (see Methods), we identified six other putative HATs, all containing the conserved HAT functional domain (Supplementary Fig 1A and B).

ZIKV infection modulates the expression and activity of AaCBP

Mosquitoes naturally acquire viral infections when they feed on blood. In this context, it is well established that in addition to extensive genome-wide transcriptional modulation in mosquitoes after blood meals (Bonizzoni et al., 2011), viral infections can also modulate host cell gene expression and influence cellular function. Thus, we first evaluated whether a blood meal was able to modulate the expression of AaCBP in the midgut or fat body (Supplementary Fig 2). We showed that the expression of AaCBP in both tissues was significantly upregulated by blood meal, reaching its peak of transcription at 24 and 48 h after feeding (Supplementary Fig 2A and B). The stimulated transcription of AaCBP was especially dramatic in the fat body 48 h after a blood meal (Supplementary Fig 2B). Because we were particularly interested in evaluating the functional role of AaCBP in ZIKV infection, we were forced to change our virus infection approach. As an alternative to feeding infected blood, mosquitoes were infected with ZIKV by intrathoracic injections. Importantly, we showed that ZIKV infection was also able to upregulate the

expression of AaCBP in the midgut or fat body, reaching its peak transcription at four days after infection (Fig 2A and B). However, we did not see upregulation when we assayed head and thorax (Fig 2C). A similar phenomenon was observed when we used Aag2 cells, where the expression of AaCBP reached their peaks at 6 and 15 h, post infection, respectively (Fig 2 D). Importantly, we confirmed that the increase in mRNA expression correlated with the increase in AaCBP acetylation activity (Fig 2 E and F). Of note, ZIKV infection enhanced the acetylation activity of AaCBP toward lysine 27 of histone H3 (H3K27ac), but not toward lysine 9 of histone H3 (H3K9ac), (Fig 2F). Considering that H3K27 is the main target substrate for CBP enzymes (Raisner et al., 2018), and that H3K9 is the main substrate of Gcn5 HAT (Karmodiya et al., 2012) (also present in *A. aegypti*; see Supplementary Fig 1A and B), these results point to a specific enhancement of AaCBP by ZIKV infection.

AaCBP plays a role in the defense and survival of ZIKV-infected mosquitoes

To investigate the role of AaCBP in the lifespan of mosquitoes infected with ZIKV, we knocked down the *AaCBP* gene two days before infection and followed their survival rates for 20 days, on a daily basis (Fig 3A). It is important to emphasize that it was mandatory to use intrathoracic injections for ZIKV infection in these experiments. This was due to the fact that *AaCBP* gene silencing was not successfully achieved when a blood meal was utilized (Supplementary Fig 3; of note, we showed that the expression of AaCBP is upregulated by blood meal, and thus, this likely counteracted the effects of the silencing). The levels of silencing of AaCBP at Day 2 post dsRNA injections were 50% in the midgut and 80% in the fat boby, as judged by its mRNA expression and activity (Supplementary Fig 4A and B). Mock-infected

mosquitoes that received dsLuc injections, showed a high rate of survival until Day 15, when survival started to decline, most likely due to normal aging (Fig 3B, black lines. Mock-infected mosquitoes that were silenced for AaCBP revealed a mortality rate of 60% at Day 20 (Fig 3B, blue line), a similar pattern observed for ZIKV-infected mosquitoes that received injections with the dsLuc control (Fig 3B, green line). Importantly, mosquitoes that were silenced for AaCBP followed by ZIKV infections showed a much higher mortality rate than all other groups (Fig 3B, orange line). These results clearly show that even a partial deletion of AaCBP is enough to disrupt the homeostasis of the mosquito (Fig 3B blue line), and when virus infection occurs, the lack of AaCBP becomes enormously detrimental.

To correlate the lack of AaCBP and mortality, with an increase in viral loads, we determined viral titers over time in the midguts and fat bodies (Fig 3C and D, respectively). We clearly saw that AaCBP-silenced mosquitoes did not efficiently fight virus infections in these tissues that are expected to mount strong immune responses against viruses (Angleró-Rodríguez et al., 2017; Cheng et al., 2016; Tikhe & Dimopoulos, 2021). In this respect, our data reconfirmed that antimicrobial peptides (AMPs) are highly upregulated by ZIKV infections in the midgut (Supplementary Fig 5).

AaCBP regulates the expression of antiviral immune-response genes

Because AaCBP-silenced mosquitoes revealed higher viral loads than control-silenced mosquitoes (Fig 3C and D), and knowing the transcriptional coactivator role of CBP enzymes, we questioned whether AaCBP knockdown could have affected the transcription of antiviral immune-response genes. Indeed, qPCR analysis showed that

the lack of AaCBP led to downregulation of the immune-response genes *cecropin D, cecropin G, and defensin C,* in the midgut or fat body (Fig 4A and B, respectively). In addition, the *vago 2* gene was also downregulated in the fat body (Fig 4B). Interestingly, *vago 2* has been shown to be upregulated in *A. aegypti* larvae exposed to dengue virus (DENV) (Vargas et al., 2020), as well as in *A. albopictus* cells infected with DENV (Paradkar et al., 2014). Of note, two members of the RNAi (*si*RNA) pathway, dicer 2 and Ago 2 were not modulated in either tissue of AaCBP-silenced mosquitoes (Fig 4A and B). Importantly, downregulation of antiviral immune-response genes persisted until at least day five-post infection, which matched the kinetics of AaCBP-mediated gene silencing (Supplementary Fig 6A-D).

Histone hyperacetylation induces mosquito immune responses and suppression of ZIKV infection

Histone deacetylation by histone deacetylases (HDACs) is involved in chromatin compaction and gene repression (Shahbazian & Grunstein, 2007). Inhibition of HDACs by sodium butyrate (NaB) leads to histone hyperacetylation and potent gene activation (Kurdistani & Grunstein, 2003). We treated mosquitoes with NaB and showed a significant increase in H3K27 and H3K9 acetylation (Supplementary Fig 7A and B). An increase in total histone H3 acetylation could not be observed (Supplementary FigA and B), which is somehow expected if one considers that a combination of acetylated and nonacetylated H3 might coexist in a specific cell type and/or during a specific period. We next investigated the effect of NaB in ZIKV-infected mosquitoes (Fig 5) and showed that the expression levels of defensin A, defensin C and cecropin D were significantly increased (Fig 5A-C).

Importantly, the effect of NaB treatment consisten tly showed a tendency for a reduction in viral loads (Fig 5D and E)**.** It is important to emphasize that NaB treatment leads to hyperacetylation of all histones. In this respect, we observed hyperacetylation of H3K27, (a CBP target) and H3K9 (a Gcn5 target). However, the acetylation levels of H3K27 were higher than those of H3K9 (Supplementary Fig 7A and B), likely due to the specific enhancement of AaCBP gene expression and activity by ZIKV infection (Fig 2), which was not observed for AaGcn5 activity (Fig 2F, H3K9ac panel). All together, these data suggest a direct correlation between high chromatin decompaction, AMPs overexpression, and strong immunity.

Discussion

Upon pathogen detection, the innate immune system must be able to mount a robust and quick response, but equally important is the need to rein in the cytotoxic effects of such a response. Immune-response genes are maintained in a silent, yet poised, state that can be readily induced in response to a particular pathogen, and this characteristic pattern is achieved through the action of two elements: the activation of transcription factors and the modulation of the chromatin environment at gene promoters. Although the activation route of the immune pathways against viruses is relatively well known in *A. aegypti*, our work is the first to attempt to explore the role of chromatin structure in this process.

The regulation of cellular functions by gene activation is accomplished partially by acetylation of histone proteins to open the chromatin conformation, and strikingly, CBP histone acetyltransferase activity always plays a role in this process

(Dancy & Cole, 2015). One example of signals that ultimately use CBP enzymes as transcriptional coregulators includes the NF-*k*B signaling (Mukherjee et al., 2013).

CBP genes are conserved in a variety of multicellular organisms, from worms to humans and play a central role in coordinating and integrating multiple cell signal-dependent events. In this regard, the *A. aegypti* CBP shows a high degree of homology, notably within the functional domains, with well-characterized human and fly enzymes (Fig 1). Therefore, one might anticipate that AaCBP functions as a transcriptional coactivator in a variety of physiological processes of the mosquito, including innate immunity.

The Toll pathway is an NF-*k*B pathway that plays an important role in immunity in mosquitoes (Angleró-Rodríguez et al., 2017; Cheng et al., 2016; Tikhe & Dimopoulos, 2021). Gene expression analysis of ZIKV-infected mosquitoes has shown that Toll pathway-related genes are highly upregulated in ZIKV infection when compared to other immune pathways (Angleró-Rodríguez et al., 2017). Here, we showed that the expression of AaCBP is also upregulated upon ZIKV infection and that CBP-dependent histone acetylation enables the mosquito to fight viral infections. Although it is not yet clear how AaCBP could limit virus replication, one could envision a molecular role where a particular transcription factor (for example, Rel1) would recruit AaCBP to immune-related gene (for example, AMP genes) promoters and/or enhancers and acetylate histones (for example, H3K27), culminating with chromatin decompaction and gene activation (depicted in our hypothetical model in Fig 6). Indeed, we experimentally demonstrated in part that our model might be correct: 1. We showed that ZIKV-infection potentiates AaCBP-mediated H3K27 acetylation (a mark of gene activation); 2. The lack of AaCBP leads to downregulation of immune-related genes, higher loads of ZIKV virus, and lower rates

of mosquito survival; 3. An inverse phenotype was obtained under H3K27 hyperacetylation. Nevertheless, the AaCBP-bound transcription factor in this signaling pathway has yet to be determined.

Host-pathogen interactions provide a highly plastic and dynamic biological system. To cope with the selective constraints imposed by their hosts, many pathogens have evolved unparalleled levels of phenotypic plasticity in their life history traits (Reece et al., 2009). Likewise, the host phenotype is drastically and rapidly altered by the presence of a pathogen (Edelaar et al., 2021; Rando & Verstrepen, 2007). One important example of these alterations is the manipulative strategy of the pathogen aimed at maximizing its survival and transmission, and one obvious target is the host's immune system. In recent years, the epigenetic modulation of the host's transcriptional program linked to host defense has emerged as a relatively common occurrence of pathogenic viral infections (Paschos & Allday, 2010). Interestingly and importantly, our data reveal that chromatin remodeling by histone acetylation contributes to establishing a resistance in ZIKV-infected *A. aegypti*. In this respect, we showed that ZIKV-infected wild type *A. aegypti* resisted to infections better than ZIKV-infected-AaCBP-silenced mosquitoes, whose survival was drastically compromised (Fig 3B). Thus, our data point to an important role of AaCBP in maintaining *A. aegypti* homeostasis through fine-tuning the transcriptional control of immune genes.

Our work has attempted to explore the epigenetic nature of virus-vector interactions. We have focused on epigenetic events that initiate changes in the vector nucleus, likely involving the cooperation between transcription factors and chromatin modifiers to integrate and initiate genomic events, which culminate with the limitation of Zika virus replication.

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Author contributions

A.M.A, I.C.A.S., P.L.O., T. M. V. AND M.R.F. conceived and designed the experiments; A.M.A., I.C.A.S., A.R.R.V., M.A.P., and K.C.M. performed the experiments; L.M.H. prepare and titrate Zika virus stocks; A.M.A, I.C.A.S., V.C.C., O.A.C.T., T. M. V., P.L.O., and M.R.F analyzed the data. A.M.A and M.R.F wrote the paper.

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Figure legends

Figure 1. Overview of AaCBP protein domain conservation. Schematic representation of the full-length AaCBP protein (XP_011493407.2), depicting the conserved functional domains: the TAZ domain (orange), KIX domain (pink), bromo domain (blue), HAT domain (purple) and CREB domain (green). The full-length CBP from *Homo sapiens* (NP_001420.2), *Apis melifera* (XP_026294861.1) and *Drosophila melanogaster* (AAB53050.1) are also shown for comparison. The percentages of similarity of the CBP-HAT domains are shown within the purple boxes.

Figure 2. ZIKV infection modulates the expression and activity of AaCBP. Adult female mosquitoes as well as Aag2 cells were infected with ZIKV at a MOI of 2.0, and 60 PFUs, respectively. Mosquito infections were performed by intrathoracic injections. The expression of AaCBP in mosquitoes (A-C) or Aag2 cells (D) was measured by qPCR on the indicated tissues and days post infection. E and F. Western blot of total protein extract from Aag2 cells infected with ZIKV virus, or mock-infected at different time points. Monoclonal antibodies against H3K9ac, H3K27ac, H3 panacetylated, or H3 (as a loading control) were used. The intensity of the bands was quantified by densitometry analysis plotted as a graph using ImageJ (NIH Software). Western blotting was performed on 3 independent biological replicates and

one representative is shown here. Error bars indicate the standard error of the mean; statistical analyses were performed by Student's *t* test. *, $p < 0.05$; **, $p < 0.01$.

Figure 3. *AaCBP* gene knockdown compromises the lifespan of ZIKV-infected mosquitoes. A. One hundred adult female mosquitoes were injected intrathoracically with double-stranded RNAs for the AaCBP or Luc control gene, two days before Zika infections (by intrathoracic injections). Survival of the mosquitoes was monitored on a daily basis, until Day 20. B. Survival curve of mosquitoes that were not injected, mock-infected mosquitoes that were silenced for dsLuc or dsAaCBP, and mosquitoes that were silenced for dsLuc or dsAaCBP and infected with ZIKV. C and D. Midguts or fat bodies from silenced mosquitoes were assessed for virus infection intensity at different time points. Each dot or square represents the mean plaque-forming units (PFUs) per two midguts or fat bodies (totaling 20 midguts or fat bodies examined) from five independent experiments. Bars indicate the standard error of the mean; tatistical analyses were performed by Student's *t* test. *, $p < 0.05$; **, $p < 0.01$.

Figure 4. The expression of antiviral immune-response genes is reduced in ZIKV-infected mosquitoes silenced for AaCBP. A and B. Fifty adult female mosquitoes were silenced for AaCBP, and after two days, mosquitoes were infected with ZIKV. The expression of cecropin D, cecropin G, attacin, serpin, defensing C, Dicer 2, Argonaut 2 and vago 2 in the midgut (A) or fat body (B) was measured by qPCR four days post infection. Silencing levels of AaCBP in both tissues are shown (first bars in Panels A and B). qPCR was performed from 3 independent biological replicates. Bars indicate the standard error of the mean; statistical analyses were performed by Student's *t* test. *, $p < 0.05$.

Figure 5. Histone hyperacetylation in ZIKV-infected mosquitoes induces immune responses and suppresses virus infection. A-E. Fifty adult female mosquitoes were infected with ZIKV and treated with 0.5 M NaB for three days. A-C. The expression of defensin A, defensing C, and cecropin D, in whole mosquito tissues was measured by qPCR. qPCR was performed on 3 independent biological replicates. D and E. Midguts or fat bodies from ZIKV-infected mosquitoes treated with NaB were assessed for infection intensity at 3 or 7 days post infection. Bars indicate the standard error of the mean; statistical analyses were performed by Student's *t* test. *, p <0.05; **, $p < 0.01$.

Figure 6. Proposed model for the role of AaCBP-mediated histone acetylation in suppressing ZIKV infection in *A. aegypti*. Zika infection signals the functional activation of transcription factors (TF) that translocate to the nucleus where they activate the expression of immune-related genes to fight the virus infection. Chromatin decondensation is mandatory for gene activation. Therefore, AaCBP is likely recruited by TFs to target promoters, where it exerts its histone acetyltransferase activity, leading to an open state of the chromatin at these promoter sites.

Supplementary Table 1. List of primers used in this study.

Supplementary Figure 1. Protein domain and alignment of putative histone acetyltransferases from *A. aegypti*. A. Genome identification of putative lysine acetyltransferase (KAT) homologs from *A. aegypti*, AaHAT1 (XP_001651817.1), AaTip60 (XP_021706851.1), AaMOF1 (XP_001658578.2), AaMOF2 (XP_021711683.1), HBO1 (XP_021701314.1), GCN5 (XP_0016566424.1) and AaCBP itself. Functional domains are indicated above each box. B. Protein sequence alignment of the HAT domains from the putative *A. aegypti* KATs. Amino acids in red show identity or conservation among all 7 HAT domains.

Supplementary Figure 2. Blood meal upregulates the expression of AaCBP. Mosquitoes were fed with blood over different time courses and mRNA quantification by qPCR was performed in the midgut or fat body. The results in A and B are pools of at least 3 independent experiments, plotted using samples from sugar-fed mosquitoes as reference. Error bars indicate the standard error of the mean; statistical analyses were performed by Student's *t* test. *, p <0.05; **, p < 0.01; ***, p <0.001.

Supplementary Figure 3. Silencing efficiency of AaCBP after a sugar or blood meal. Two days after feeding on sugar or blood, the *AaCBP* gene was knocked down and its expression was measured two days after silencing. qPCR was performed from 3 independent biological replicates. Bars indicate the standard error of the mean; statistical analyses were performed by Student's *t* test. **, p < 0.01.

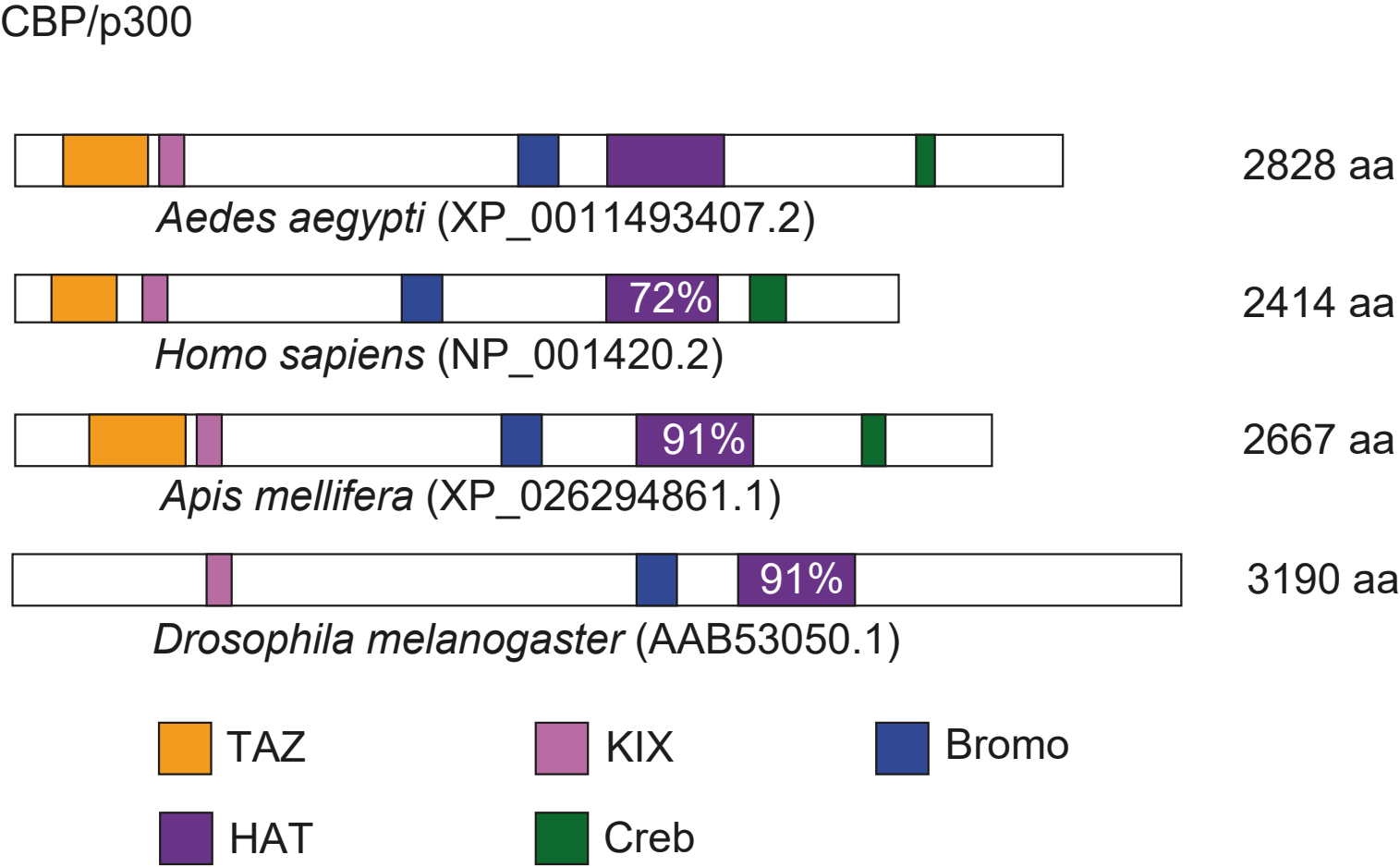
Supplementary Figure 4. Silencing of AaCBP in adult female mosquitoes by intrathoracic injections of dsRNAs. The expression (A) and activity (B) of AaCBP were used to evaluate the efficiency of gene knockdown. A. The mRNA levels of

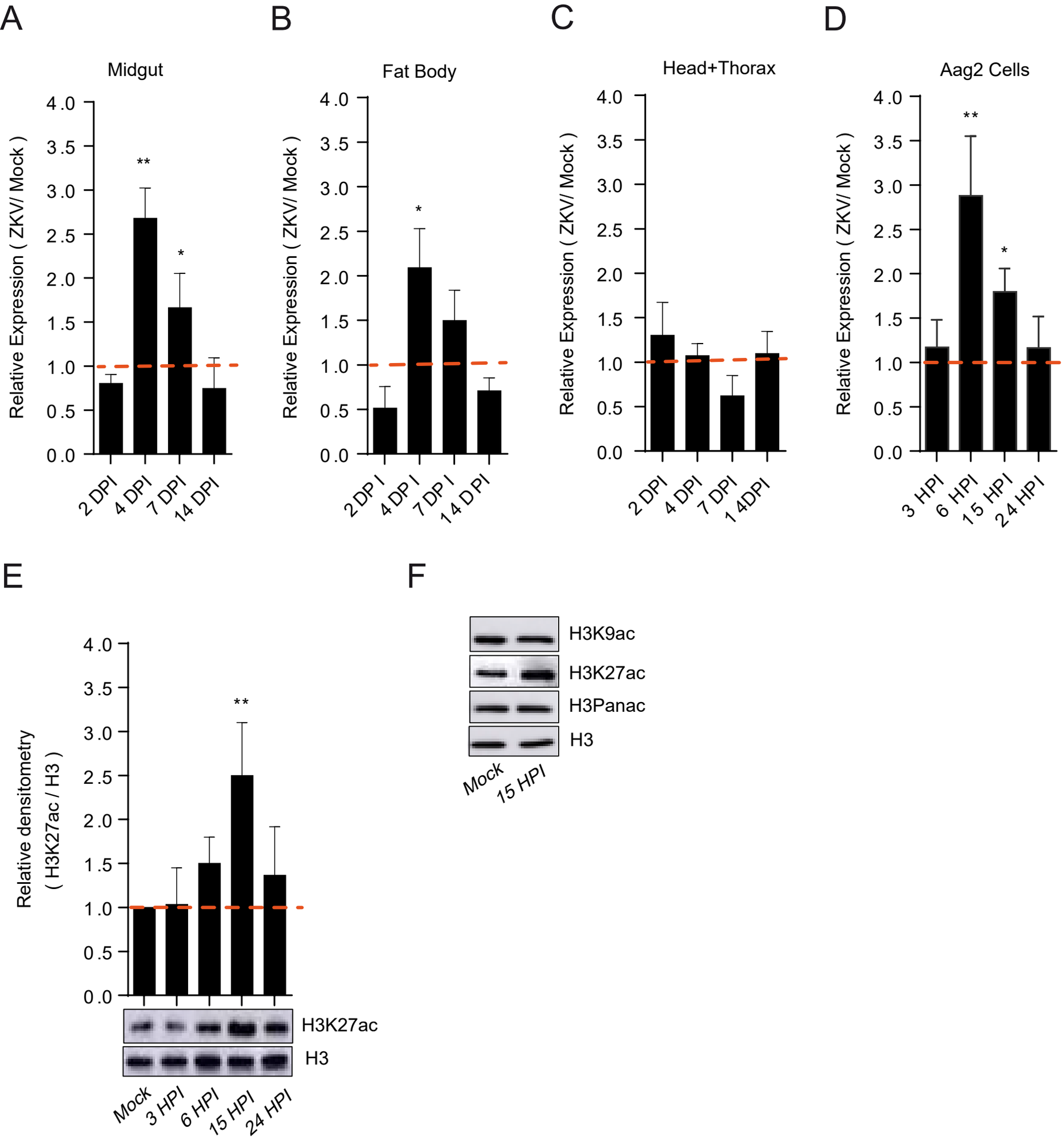
AaCBP in the midgut, ovary, fat body or whole mosquito were quantified by qPCR at 48 h postinjection with dsCBP or dsLuc. Silencing efficiency was determined by the ratio between mRNA levels of AaCBP-silenced versus dsLuc-injected mosquitoes. B. Western blot of 10 µg of total protein extract of dsCBP- or dsLuc-injected-mosquitoes. Monoclonal antibodies against acetylated- or nonacetylated histone H3 (loading control) are indicated. The intensity of the bands was quantified by densitometry analysis plotted as a graph using ImageJ (NIH Software). Western blotting was performed on 3 independent biological replicates and one representative is shown here. Bars indicate the standard error of the mean; statistical analyses were performed by Student's *t* test. *, $p < 0.05$.

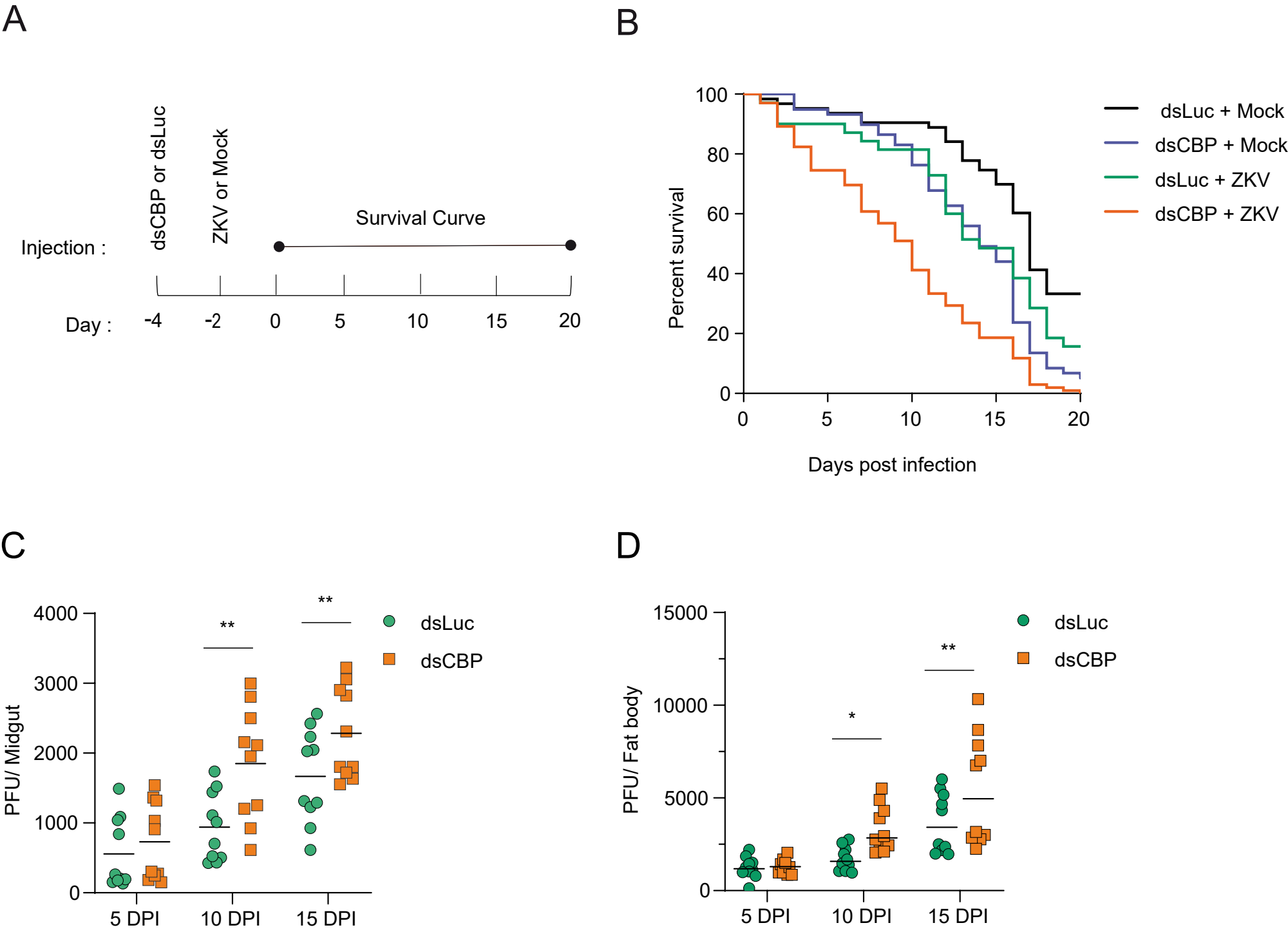
Supplementary Figure 5. ZIKV infection upregulates the expression of antimicrobial peptides in the midgut of *A. aegypti*. Fifty adult female mosquitoes were infected with ZIKV after feeding on infected blood and the expression of cecropin D, cecropin G, attacin, gambicin, serpin and defensin C was measured by qPCR four days post infection. qPCR was performed from 3 independent biological replicates. Bars indicate the standard error of the mean.

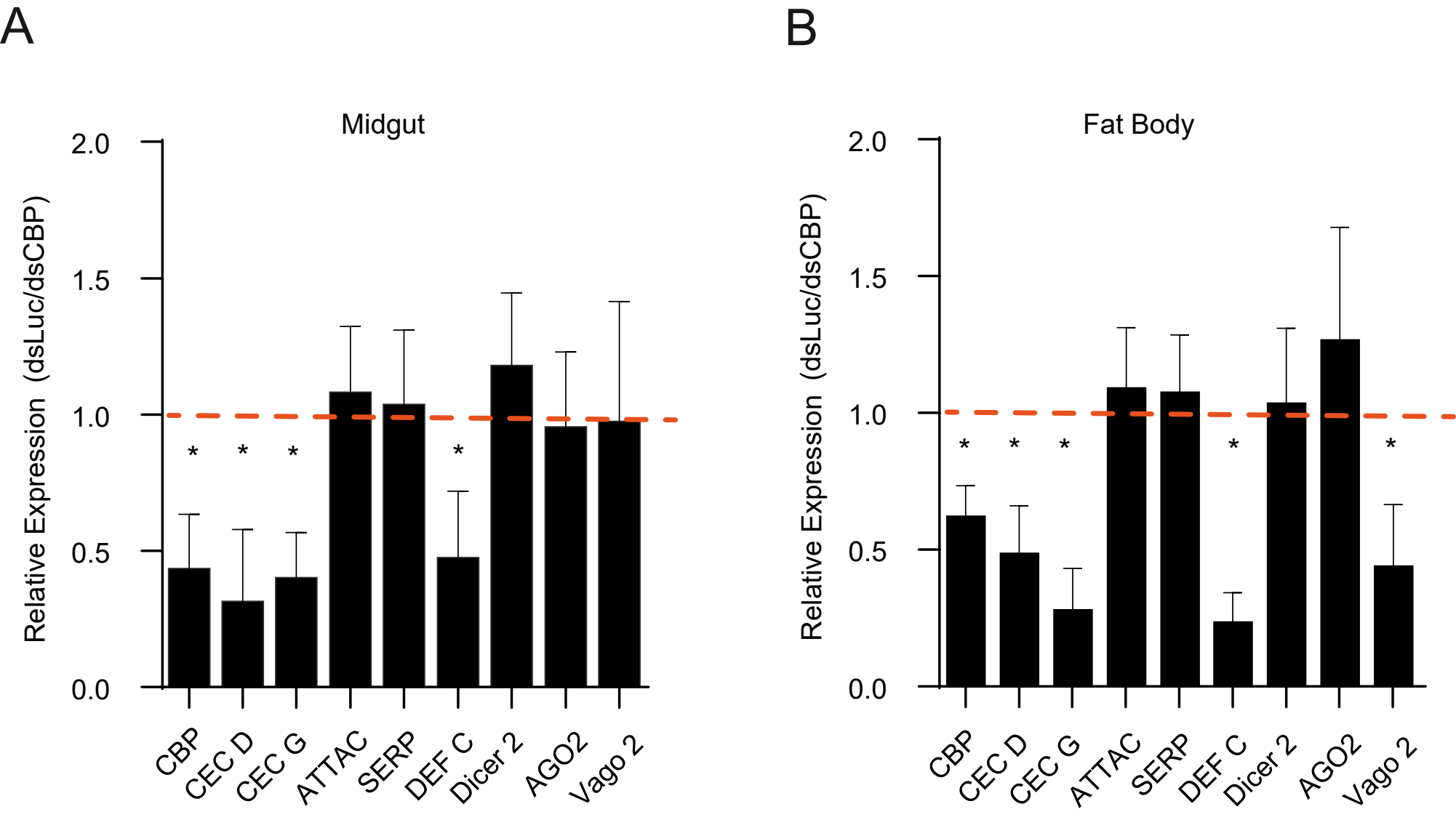
Supplementary Figure 6. Silencing of AaCBP is sustainable and efficient until five days of ZIKV infection. Fifty adult female mosquitoes were infected with ZIKV for 1, 5, 7 or 10 days and the expression levels of AaCBP itself, cecropin G, defensin or vago in the fat body were measured by qPCR on 3 independent biological replicates. Bars indicate the standard error of the mean; statistical analyses were performed by Student's *t* test. *, $p < 0.05$.

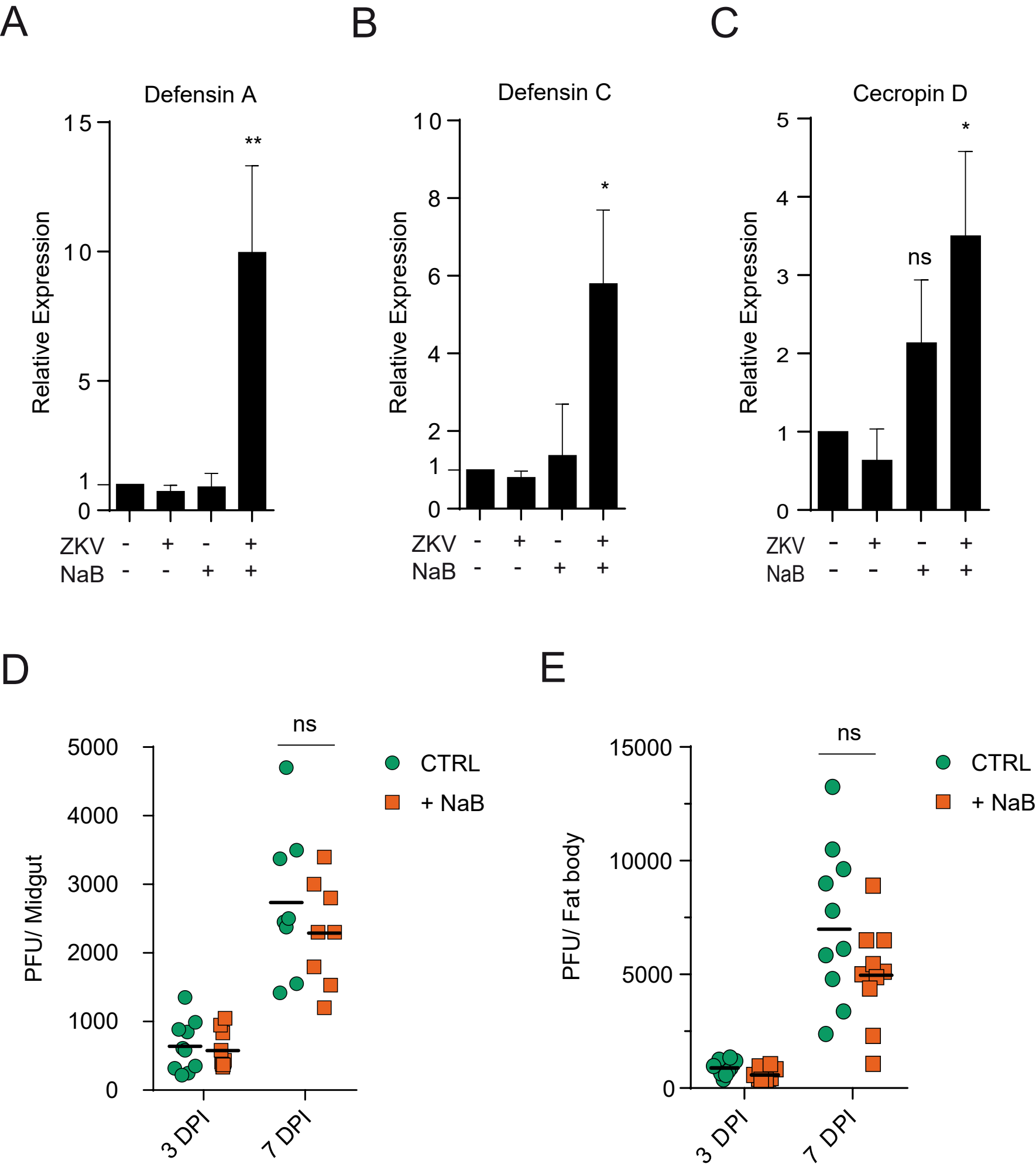
Supplementary Figure 7. Histone deacetylase inhibition leads to histone hyperacetylation in *A. aegypti*. Ten adult female mosquitoes were intrathoracically injected with PBS, 0.25 M (17.5 pmol), or 0.5 M (35 pmol) of sodium butyrate (NaB). Four days after treatment, 10 µg of total protein extract from 10 mosquitoes was used for histone acetylation analysis. Western blotting with monoclonal antibodies against H3K9ac, H3K27ac, H3 panacetylated, or H3 (as loading control) was performed. The intensity of the bands was quantified by densitometry (lower panel) analysis plotted as a graph using ImageJ (NIH Software). Western blotting was performed on 3 independent biological replicates and one representative is shown in panel A. Error bars in Panel B indicate the standard error of the mean; statistical analyses were performed by Student's *t* test. *, $p < 0.05$.

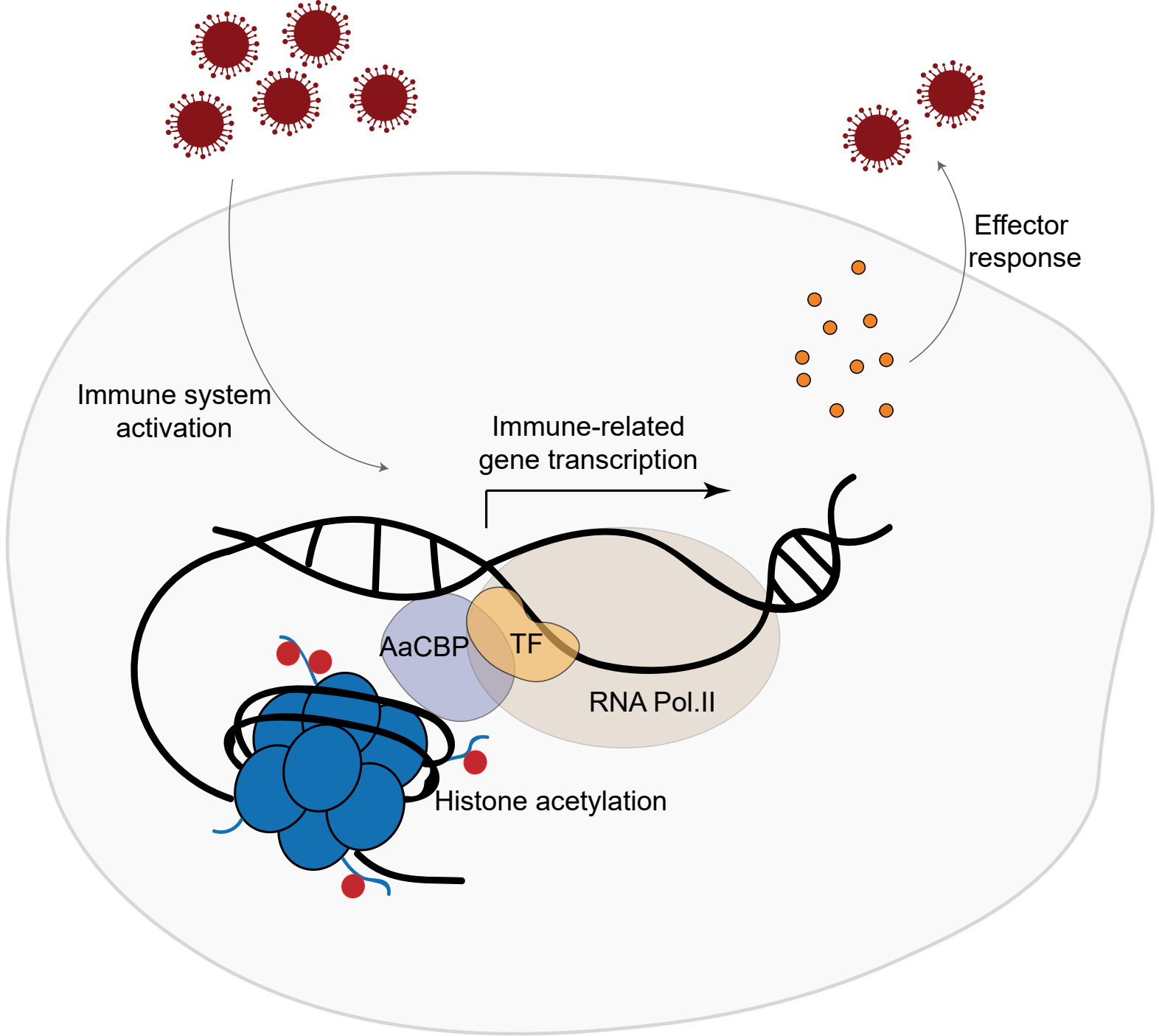

CBP/p300
2828 aa
Aedes aegypti (XP_0011493407.2)
72%
2414 aa
Homo sapiens (NP_001420.2)
91%
2667 aa
Apis mellifera (XP_026294861.1)
91%
3190 aa
Drosophila melanogaster (AAB53050.1)
TAZ
KIX
Bromo
HAT
Creb


A
Midgut
Relative Expression (ZKV/ Mock)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
*
2 DPI
4 DPI
7 DPI
14 DPI
B
Fat Body
Relative Expression (ZKV/ Mock)
*
2 DP I
4 DP I
7 DP I
14 D PI
C
Head+Thorax
Relative Expression (ZKV/ Mock)
2 DPI
4 DPI
7 DPI
1 4DPI
D
Aag2 Cells
Relative Expression (ZKV/ Mock)
**
*
3 HPI
6 HPI
15 HPI
24 HPI
E
Relative densitometry
(H3K27ac / H3)
**
H3K27ac
H3
Mock
3 HPI
6 HPI
15 HPI
24 HPI
F
H3K9ac
H3K27ac
H3Panac
H3
Mock
15 HPI


A
dsCBP or dsLuc
ZKV or Mock
Survival Curve
Injection :
Day :
-4
-2
0
5
10
15
20
B
Percent survival
Days post infection
dsLuc + Mock
dsCBP + Mock
dsLuc + ZKV
dsCBP + ZKV
C
PFU/ Midgut
dsLuc
dsCBP
5 DPI
10 DPI
15 DPI
D
PFU/ Fat body
dsLuc
dsCBP
5 DPI
10 DPI
15 DPI


A
Midgut
Relative Expression (dsLuc/dsCBP)
2.0
1.5
1.0
0.5
0.0
*
*
*
*
CBP
CEC D
CEC G
ATTAC
SERP
DEF C
Dicer 2
AGO2
Vago 2
B
Fat Body
Relative Expression (dsLuc/dsCBP)
2.0
1.5
1.0
0.5
0.0
*
*
*
*
*
CBP
CEC D
CEC G
ATTAC
SERP
DEF C
Dicer 2
AGO2
Vago 2


A
Defensin A
Relative Expression
15
10
5
1
0
**
ZKV - + - +
NaB - - + +
B
Defensin C
Relative Expression
10
8
6
4
2
1
0
*
ZKV - + - +
NaB - - + +
C
Cecropin D
Relative Expression
5
4
3
2
1
0
*
ns
ZKV - + - +
NaB - - + +
D
PFU/ Midgut
5000
4000
3000
2000
1000
0
ns
CTRL
+ NaB
3 DPI
7 DPI
E
PFU/ Fat body
15000
10000
5000
0
ns
CTRL
+ NaB
3 DPI
7 DPI


Effector
response
Immune system
activation
Immune-related
gene transcription
AaCBP
TF
RNA Pol.II
Histone acetylation

Curriculum Vitae

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Formação Acadêmica

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