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DIGESTÃO DE MICRORGANISMOS EM LARVAS DE *AEDES AEGYPTI*: ASPECTOS FISIOLÓGICOS E MOLECULARES

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Digestão de microrganismos em larvas de *Aedes aegypti*: Aspectos fisiológicos e moleculares

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ORIENTADOR: Prof. Dr. Fernando Ariel Genta

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Rio de Janeiro, 09 de outubro de 2018

Aos meus pais: Antônio e Déa, dedico este trabalho como fruto do amor
e da dedicação de ambos ao longo de 30 anos.
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"*O sucesso nasce do querer, da determinação e persistência em se chegar a um objetivo. Mesmo não atingindo o alvo, quem busca e vence obstáculos, no mínimo fará coisas admiráveis".*

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"Que darei eu ao Senhor, por todos os benefícios que me tem feito? "

(Salmos 116:12)

"Não a nós, Senhor, não a nós, mas ao vosso nome dai glória, por amor de vossa misericórdia e fidelidade".

(Salmos 115:1)

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DIGESTÃO DE MICRORGANISMOS EM LARVAS DE AEDES AEGYPTI: ASPECTOS FISIOLÓGICOS E MOLECULARES

RESUMO

Aedes aegypti é o vetor dos vírus dengue, zica, chikungunya e febre amarela urbana. Atualmente estima-se que 3,6 bilhões de pessoas vivam em áreas com risco de transmissão do vírus dengue. Estudos sobre a biologia das larvas de mosquito ainda são escassos, por essa razão larvas de *Ae.aegypti* foram utilizadas como modelo experimental nesse trabalho. O genoma de *Ae.aegypti* possui seis genes codificantes para GHF16, esses genes parecem estar envolvidos na digestão e na imunidade das larvas. Larvas de *Ae.aegypti* são capazes de ingerir e se desenvolver em uma dieta contendo apenas células de leveduras, essas células são lisadas por beta-1,3-glucanases. Para entender melhor os hábitos nutricionais das larvas, que são consideradas detritívoras e pouco seletivas em sua dieta, alimentamos larvas de *Ae.aegypti* com diversas classes de microrganismos. Leveduras (*Saccharomyces cerevisiae* e *Pseudozyma* sp.), bactérias (*Serratia marcescens*, *Escherichia coli*, *Bacillus* sp., *Asaia* sp., *Ochrobactrum* sp. e *Staphylococcus aureus*) e microalgas (*Chlorella* sp. e *Arthrospira platensis*) foram testadas como fonte alimentar e parâmetros fisiológicos como tempo de desenvolvimento das larvas, taxas de pupação, metamorfose, emergência e mortalidade dos adultos foram observados. Larvas alimentadas com leveduras parecem se desenvolver melhor e apresentam curvas de sobrevivência próximas ao da dieta padrão Tetramin®. Apesar das dietas contendo bactérias e microalgas apresentarem um desenvolvimento tardio, larvas de *Ae.aegypti* conseguem se estabelecer até a fase adulta. A análise morfométrica alar dos adultos, indicou que larvas alimentadas com diferentes microrganismos produzem mosquitos menores e com variações na forma das asas quando comparados ao controle. As quantificações dos valores nutricionais apresentaram um perfil variado e indicaram que dietas contendo microalgas e leveduras possuem uma quantidade maior de proteínas e açúcares totais comparada à dieta com bactérias, o que pode interferir no tempo de desenvolvimento dos insetos. Insetos alimentados com leveduras também possuem reserva energética (proteínas, carboidratos, glicogênio e lipídios) maior que os insetos alimentados com bactérias e microalgas. Além de aspectos bioquímicos e fisiológicos, uma análise global dos diferentes compartimentos intestinais das larvas de *Ae.aegypti* foi realizada através de pirosequenciamento. O intestino de larvas L4 foi fragmentado em intestino médio anterior, médio posterior, posterior, intestino completo e intestino sem os cecos gástricos. A análise dos transcritos mais abundantes varia em cada compartimento intestinal. Foi possível confirmar e descrever a compartimentalização do processo digestivo em larvas de *Ae.aegypti* com uma técnica alternativa. Pesquisas sobre nutrição larval propiciam a aquisição de valiosas informações sobre criação laboratorial especializada, impulsionam investigações de potenciais larvicidas e fornecem conhecimento sobre a quantidade e a qualidade do alimento ingerido pelas larvas e como isso afeta seu desenvolvimento geral.

PALAVRAS-CHAVE: *Aedes aegypti*, dieta, nutrição, sequenciamento, compartimentos intestinais

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DIGESTION OF MICROORGANISMS IN LARVAS DE AEDES AEGYPTI: PHYSIOLOGICAL AND MOLECULAR ASPECTS

ABSTRACT

Aedes aegypti is the vector of dengue viruses, zica, chikungunya and urban yellow fever. It is currently estimated that 3.6 billion people live in areas at risk of dengue virus transmission. Studies on the biology of mosquito larvae are still scarce, therefore larvae of *Ae.aegypti* were used a experimental model in this work. The *Ae.aegypti* genome has six genes encoding GHF16, these genes seem to be involved in the digestion and immunity of the larvae. *Ae.aegypti* larvae are able to ingest and develop on a diet containing only yeast cells, these cells are lysed by beta-1,3-glucanases. To better understand the nutritional habits of the larvae, which are considered detritus and not selective in their diet, we fed larvae of *Ae.aegypti* with several classes of microorganisms. Yeasts (*Saccharomyces cerevisiae* and *Pseudozyma* sp), bacteria (*Serratia marcescens*, *Escherichia coli*, *Bacillus* sp., *Asaia* sp., *Ochrobactrum* sp. and *Staphylococcus aureus*) and microalgae (*Chlorella* sp and *Arthrospira platensis*) were tested as food source and physiological parameters as development time larvae, pupation rates, metamorphosis, emergence and adult mortality were observed. Yeast-fed larvae appear to develop better and have survival curves close to the standard Tetramin® diet. Although the diets containing bacteria and microalgae present a late development, *Ae.aegypti* larvae can establish themselves until adulthood. The adult morphometric analysis indicated that larvae fed different microorganisms produced smaller mosquitoes and with variations in wing shape when compared to control. The quantification of the nutritional values presented a varied profile and indicated that diets containing microalgae and yeasts have a higher amount of proteins and total sugars compared to the diet with bacteria, which can interfere in the development time of the insects. Yeast-fed insects also have higher energy reserves (proteins, carbohydrates, glycogen and lipids) than insects fed with bacteria and microalgae. In addition to biochemical and physiological aspects, a global analysis of the different intestinal compartments of *Ae.aegypti* were performed by pyosequencing. The intestine of L4 larvae was fragmented in the mid-anterior, mid-posterior, posterior, complete intestine and intestine without gastric cecum. The analysis of the most abundant transcripts varies in each intestinal compartment. It was possible to confirm and describe the compartmentalization of the digestive process in *Ae.aegypti* larvae with an alternative technique. Research on larval nutrition promotes the acquisition of valuable information on specialized laboratory creation, promotes investigations of potential larvicides, and provides insight into the quantity and quality of the food ingested by the larvae and how this affects their overall development.

KEY-WORDS: *Aedes aegypti*, diet, nutrition, sequencing, intestinal compartments

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LISTA DE SIGLAS ABREVIATURAS:

CHKV- Vírus Chikungunya

DAGs- diacilgliceróis

DALY- Disability-Adjusted Life Year (Ano de Vida Ajustado por Incapacidade)

DENV- Vírus Dengue

DUOX- Oxidase Dupla

E.C – Classe enzimática (Enzyme Commission)

EGI-Dengue - Estratégia de Gestão Integrada Para Prevenção e Controle da Dengue

FA- Febre Amarela

FAU- Febre Amarela Urbana

FD- Febre do Dengue

FHD- Febre Hemorrágica da Dengue

FUNASA- Fundação Nacional de Saúde

HClO- Ácido Hipocloroso

IA- Intestino Anterior

IM- Intestino Médio

IMD- Via da Imunodeficiência

IP- Intestino Posterior

JH- Hormônio Juvenil

MP I - MP do Tipo I

MP II - MP do Tipo II

MP- Matriz Peritrófica

NADPH- Dinucleótido de Nicotinamida e Adenina

NCBI- National Center for Biotechnology Information

NGS- Sequenciamento de Nova Geração

OMS- Organização Mundial de saúde

OPAS- Organização Pan-americana de saúde

PAHO- Pan American Health Organization (Organização Pan-americana de saúde)

PAMs- Peptídeos Antimicrobianos

PCR- Reação em Cadeia da Polimerase

PNHs- Primatas Não Humanos

RIDL- Libertação de Insetos carregando um gene Letal Dominante

ROS- Espécies Reativas de Oxigênio

SCD- Síndrome do Choque da Dengue

SIT- Técnica do Inseto Estéril

TAGs- Triacilgliceróis

WHO- World Health Organization (Organização Mundial de Saúde)

YFV- Vírus da Febre Amarela

ZIKV- Zika Vírus

1. Introdução

1.1. Insetos: Biodiversidade e Relevância

Os insetos são uma manifestação exuberante das muitas e variadas formas de vida encontradas na Terra. Esses animais estão inseridos no subfilo Hexapoda e constituem a maioria das espécies do filo Arthropoda (Figura 1.1), representando cerca de 58-67% da biodiversidade global conhecida (Wheeler 1990; Grimald e Engel 2005). Determinar quantas espécies biológicas de insetos e outros artrópodes terrestres existem torna-se minucioso e controverso devido ao elevado nível de sinonímia entre as espécies. Em geral, as proporções derivadas do número de espécies em insetos são realizadas através de estimativas e aproximações em razão do vasto número de espécies e da sua distribuição irregular. Cálculos para estimar a riqueza total de espécies em insetos podem variar de cerca de 2 a 80 milhões de espécies (Stork 1988 e 1990; Hodkinson e Casson 1991; Erwin 2004). A média das estimativas atualizadas sugerem que podem existir aproximadamente 5,5 milhões de espécies de insetos, sendo 1,5 milhão dessas espécies apenas de besouros e 1,3 a 1,5 milhão de outros artrópodes terrestres (Stork 2017 Figura 1.1b). Dos valores médios citados, apenas 1.013,825 milhão de espécies em insetos foram descritas até agora (Zhang 2011), sendo assim, 80-90% das espécies ainda estão por ser descritas.

A maior concentração de espécies em insetos encontra-se em áreas tropicais do globo. Um hectare de floresta amazônica contém mais de 100.000 espécies de artrópodes (Erwin 2004), das quais cerca de 85% são insetos (May 1998). Este valor é mais de 90% do total de espécies descritas em regiões neoárticas. É fascinante observar que animais com uma aparência tão modesta sejam tão bem-sucedidos. Os insetos são capazes de habitar virtualmente todos os ambientes terrestres e de água doce no planeta, apenas não se tornando igualmente predominantes nos mares e oceanos (Rosenberg 1986; Grimald e Engel 2005). Características como a posse de asas, metamorfose completa, tamanho reduzido e plasticidade alimentar têm sido frequentemente citadas como fomentadoras da enorme riqueza de espécies em insetos.

Cada inseto tem traços biológicos individuais, papel ecológico, distribuição e interações dentro da comunidade local e pode diferir em hábito e aparência tanto de seus similares quanto de toda a extensão da espécie para refletir as influências e condições locais. Cada espécie é, portanto, um mosaico de variedade física e constituição genética que pode levar à ambiguidade taxonômica e ecológica em insetos. A capacidade de modificação e adaptação são alternativas para assegurar a integridade física e maneiras pelas quais os insetos possam evoluir e persistir no ambiente (Dajoz 1998; Moczek 2010; Stork 1990).

Em geral, insetos atuam como fonte alimentar para outros táxons, facilitadores para interações interespecíficas, na ciclagem de nutrientes, composição, estrutura e fertilidade do solo por tunelamento e nidificação, transmissão de organismos patogênicos, polinização, dispersão de sementes e na manutenção da comunidade biótica (Carpenter 1928; Richard e Sherman 1977; Majer 1987; Miller 1993). Quase qualquer representação de uma teia alimentar no ecossistema terrestre ou de água doce apresenta os insetos como um componente fundamental para conservação do equilíbrio ecológico e sua perda pode afetar a complexidade e a abundância de outros organismos (Carpenter 1928; Shurin et al 2005). Além de serem utilizados como fonte de alimento por outros animais, os insetos também constituem uma fonte de calorias e proteínas para os seres humanos em muitas regiões do mundo (Bodenheimer 1951; DeFoliart 1989). Cerca de 500 espécies em mais de 260 gêneros e 70 famílias de insetos são conhecidos por serem comestíveis (DeFoliart 1989; Groombridge 1992).

O processo de polinização por insetos é a base para a história evolutiva de plantas com flores, abrangendo pelo menos 135 milhões anos (Crepet 1979, 1983). Aproximadamente 85% das angiospermas são polinizadas por insetos (Grimaldi e Engel 2005). A polinização por insetos, também é de grande valor econômico na indústria de fruticultura e agricultura (Free 1993; Proctor *et al.* 1996; Winfree et al 2009; Cock et al. 2012). O benefício anual das abelhas para os consumidores agrícolas dos Estados Unidos é da ordem de 1,6 a 8,3 bilhões de dólares (Southwick e Southwick 1992). Por outro viés, o impacto de insetos prejudiciais, classificados como pragas ou nocivos, pode ser devastador para os recursos naturais existentes e para saúde humana. Insetos potencialmente destrutivos podem devastar vegetações inteiras o que ocasiona sérios prejuízos para o ambiente e para economia mundial. No Brasil, existem inúmeras espécies que podem afetar de forma negativa nossa agricultura e horticultura, a lagarta do cartucho do milho (*Spodoptera frugiperda),* a broca do feijão (*Zabrotes subfasciatus)* e a broca da cana (*Diatraea saccharalis)* são alguns exemplos das principais pragas encontradas no país. A maioria das espécies nocivas para a agricultura não são compostas por espécies nativas, mas sim, por espécies exóticas que foram introduzidas a um novo ecossistema, geralmente sem seus agentes naturais de controle biológico (Pimentel 2002). A introdução de espécies exóticas pode custar de 5 a 8 bilhões de dólares anualmente (Pimentel 2002).

A transmissão de agentes infecciosos por insetos provavelmente aflige mais os seres humanos que a qualquer outro grupo animal (Leach 1940; Grimaldi e Engel 2005). A Organização Mundial da Saúde acredita que 17% de todas as doenças infecciosas humanas são transmitidas por insetos vetores (WHO 2017). Os mosquitos destacam-se nesse contexto, por serem considerados os animais mais mortais do planeta, responsáveis pela morte de aproximadamente

725.000 a 1.000.000 pessoas por ano (Gates 2014). De todas as doenças infecciosas transmitidas por insetos, malária e dengue apresentam as maiores taxas de ocorrência no mundo (Alvardo e Bruce-Chwatt 1962; Murray 2013). Mais de 400 milhões de pessoas adoecem a cada ano com malária e cerca de 1 a 3 milhões morrem, principalmente crianças menores de 5 anos (Marshall 2000; Miller e Greenwood 2002). Cerca de 390 milhões de pessoas são infectadas por dengue anualmente e a doença encontra-se endêmica em 128 países do mundo (Brady *et al.* 2012; Ferreira *et al*. 2012; WHO 2012; Bhatt *et al.* 2013).

Algumas características de certos insetos os tornam modelos úteis para pesquisas biológicas (Wigglesworth 1976). Estudo com insetos produziram grandes avanços em nossa compreensão da biomecânica, mudança climática, biologia do desenvolvimento, ecologia, evolução, genética, paleolimnologia e fisiologia (Akre *et al*. 1992). Insetos desenvolveram características únicas no reino animal, apresentando mais variedades de formas de desenvolvimento do que qualquer outra classe de organismos (Kause 1960; Boswell e Mahowald 1985; Sander *et al.* 1985). Dado o impacto significativo desses animais para o mundo e, como consequência, sobre nossas vidas, a compreensão das relações estabelecidas pelos insetos torna-se primordial para a conservação da biodiversidade natural, pesquisas biológicas gerais, manutenção da produção frutífera e forrageira e o manejo de espécies nocivas.

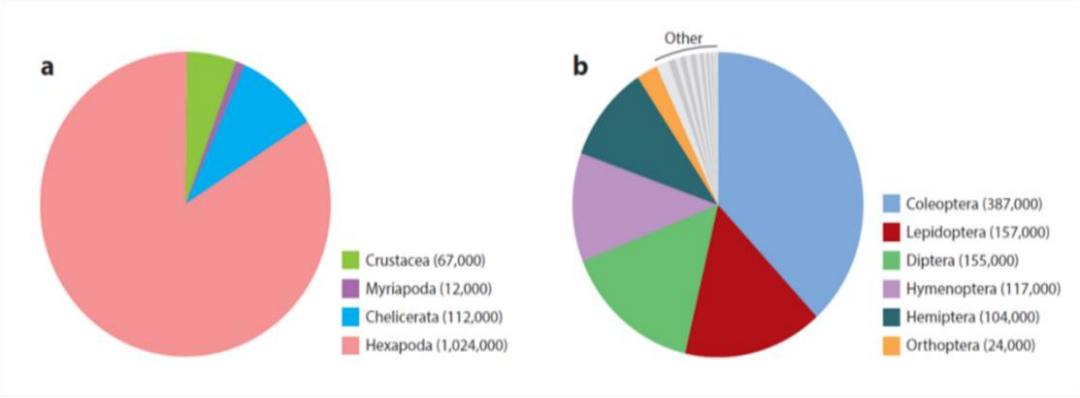

Figura 1.1. Proporções relativas de espécies nomeadas em **(a)** os quatro subfilos que constituem o filo Arthropoda e **(b)** as ordens das espécies descritas nos insetos com os valores entre parênteses (Storck 2017).

1.2. *Aedes aegypti*: Importância Médica, Histórico de Controle e Ciclo Evolutivo

Aedes (*Stegomya*) *aegypti* (Linnaeus, 1762) (Reinert *et al.* 2009) é o principal vetor dos vírus dengue (Rodhain e Rosen 1997; Simmons *et al*. 2012), febre amarela urbana (Jentes et al 2011), chikungunya (Pialoux *et al*. 2007, Leparc-Goffart *et al*. 2014) e Zika (Campos *et al.* 2015, Freitas *et al*. 2016). Os altos índices de mortalidade e a substancial morbidade das arboviroses transmitidas por *Ae.aegypti* determinam sua grande importância médica atual e seu crescente potencial como vetor nos próximos anos (Weaver e Reisen 2010). Os mosquitos do gênero *Aedes* são taxonomicamente classificados na ordem Diptera, subordem Nematocera, família *Culicidae*, tribo *Aedini* e possuem cerca de 931 espécies distribuídas em 78 subgêneros (Reinert *et al*. 2009, Wilkerson *et al*. 2015). No Brasil encontram-se quatro subgêneros: *Ochlerotatus*, *Stegomyia*, *Howardina* e *Protomacleaya*. (Forattini 1996).

A forma ancestral desse mosquito, designado como *Ae.aegypti formosus*, se desenvolveu em florestas da África sub-saariana, se alimentando do sangue de mamíferos florestais ou primatas não humanos e ovipondo em buracos de árvores e outros ecótopos naturais com água (Weaver e Reisen 2010, Powell e Tabachnick 2013; Tabachnick 2013). Atualmente, essa forma silvestre ainda pode ser encontrada em florestas africanas (Lounibos 1981; Powell e Tabachnick 2013). É provável que a domesticação e a dispersão do mosquito ao redor do mundo, tenha ocorrido em mais de uma ocasião (Brown *et al*. 2011, Moore *et al*. 2013, Powell e Tabachnick 2013). Historicamente, *Ae.aegypti* pode ter sido transportado para as Américas e o Mediterrâneo durante o período colonial em navios que praticavam o tráfico de escravos na costa Africana, causando diversas epidemias, principalmente em zonas portuárias (Christophers 1960;

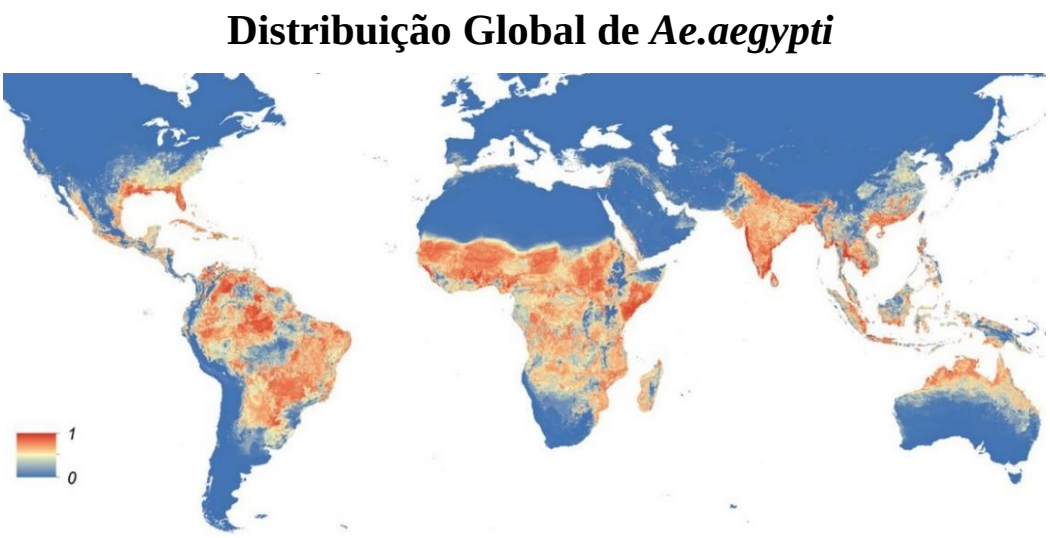

Figura 1.2. Mapa global da distribuição prevista de *Ae.aegypti*. O mapa mostra a probabilidade de ocorrência (de 0 azul para 1 vermelho) em uma resolução espacial de 5 km × 5 km. (Kraemer *et al*. 2015).https://doi.org/10.7554/eLife.08347.004

Tabachnick 1991; Lounibos 2002; Bryan *et al.* 2004; Wilder-Smith e Gubler 2008; Weaver e Reisen 2010; Weaver 2013; Halstead 2015). A movimentação das tropas que lutavam durante a segunda guerra mundial foi um fator agravante para a expansão global do mosquito e o estabelecimento de epidemias (Wilder-Smith e Gubler 2008). *Ae.aegypti* são mosquitos cosmopolitas e estão amplamente dispersos nos trópicos e subtrópicos (Nelson 1986; Braks *et al.* 2003; Scott e Takken 2012; Brown *et al.* 2014; Gloria-Soria *et al*. 2016). O sucesso do avanço geográfico dessa espécie pode ser em grande parte devido a rápida urbanização (IOM 2003a; Gubler 2011; Charrel *et al.* 2014; Kraemer *et al.* 2015; Figura 1.2).

O crescimento demográfico e a globalização proporcionam uma disponibilidade sem precedentes de hospedeiros humanos como fonte nutricional e um ambiente propício para a propagação de *Ae.aegypti*, que apresenta um elevado grau de sinantropia (Brown *et al.* 2011; Moore *et al.* 2013; Powell e Tabachnick 2013). As grandes áreas urbanas estão interligadas por viagens e relações comerciais, o que acaba por promover o transporte da espécie (Gubler 2011). O mosquito pode ser transportado na forma de ovos em pneus e outros recipientes para novas regiões e consequentemente serem expostos à água, ou como adultos em veículos terrestres e aéreos (Lounibus 2002; Whelan *et al*. 2012). As viagens em aeronaves são especialmente eficientes para o transporte de *Ae.aegypti* e suas arboviroses em todo o mundo (Lounibus 2002; Gubler 2011; Whelan *et al*. 2012). Tal potencial e disponibilidade de transportes ameaçam a sustentabilidade do controle de vetores. Desvendar a estrutura de reprodução e a dispersão do *Ae.aegypti* é fundamental para o desenvolvimento de estratégias eficazes de monitoramento e controle do vetor assim como, essencial para compreensão da dinâmica de transmissão das arboviroses.

O combate ao *Ae.aegypti* foi legitimado no Brasil a partir do século XX (Braga e Valle 2007). Entre o final da década de 1940 e a década de 1950 o mosquito foi erradicado em quase todos os países das Américas (Soper 1964). Juntamente com a Organização Pan-americana de saúde (OPAS/PAHO) e a Organização Mundial de saúde (OMS/WHO), o Brasil logrou êxito na campanha de erradicação continental e obteve a eliminação do vetor no ano de 1955 (Franco 1969; Donalísio 1999; Löwy 1999; Funasa 2002). Apesar do sucesso alcançado com a campanha de eliminação, no ano de 1967 confirmou-se a reintrodução do *Ae.aegypti* em alguns estados do país (Nobre *et al.* 1994). No ano de 1973 o mosquito voltou a ser erradicado, mas por pouco tempo (Funasa 2002). Devido ao insucesso da erradicação do mosquito nos países vizinhos, falhas no monitoramento epidemiológico e bruscas alterações sociais e ambientais, no ano de 1976, o mosquito reapareceu nos estados do Rio grande do Norte e Rio de Janeiro (Braga e Valle 2007). Nos anos subsequentes, várias medidas foram adotadas objetivando uma nova erradicação do

vetor, no entanto, o aumento do número de casos de dengue e a expansão da espécie por todo o território nacional tornaram essas medidas inviáveis (FUNASA 2001). Em julho de 2001, os planos de erradicação foram abandonados e os esforços voltaram-se para o controle da proliferação de *Ae.aegypti* (Silva et al 2002; San-Martin e Brathwaite-Dick 2007). Em 2003, alterações no núcleo dos programas nacionais de controle de dengue foram realizadas e introduziu-se a Estratégia de Gestão Integrada para Prevenção e Controle da Dengue (EGI-Dengue). Além do controle do vetor baseado no uso de agentes químicos, a EGI-Dengue propõe a inserção da educação em saúde para a comunidade, atendimento médico básico aos pacientes e gerenciamento de atividades coordenadas nas diferentes esferas. Em todos os estados do Brasil e no Distrito Federal, os serviços de saúde pública foram incentivados a utilizar o EGI-Dengue como ferramenta de prevenção e combate à dengue (OPAS 2001; OPAS 2003; San-Martin e Brathwaite-Dick 2007; Valle D, Pimenta DN; Cunha RV 2015).

Os programas atuais de controle do mosquito geralmente incluem atividades para reduzir o número de larvas e adultos de *Ae.aegypti*. Para o controle dos estádios larvais, a redução dos criadouros com larvicidas químicos ou biológicos são bastante utilizados e buscam manter a população de mosquitos abaixo dos níveis limiares e assim, interromper a transmissão de arboviroses (Gubler 2005; Eisen *et al*. 2009). Essa estratégia, porém, não tem sido totalmente sustentável. Os programas geralmente são mal gerenciados ou não recebem apoio financeiro a longo prazo das agências governamentais. A redução dos criadouros do mosquito torna-se ainda mais dispendiosa devido ao surgimento da "sociedade descartável", onde os locais de reprodução para *Ae.aegypti* se acumulam rapidamente e são quase onipresentes. Os larvicidas de origem química ou biológica possuem uma elaboração custosa, e os locais de reprodução do mosquito podem ser de difícil acesso. Com relação aos adultos, a extrema endofilia da fêmea do mosquito complica os esforços para controle (Edman *et al.* 1992; Harrington *et al*. 2001; Bonds 2012). A pulverização ao ar livre de inseticidas durante surtos de arboviroses possui um alcance limitado devido à penetração deficiente nas construções feitas de concreto e à exposição indiscriminada de mosquitos a doses subletais de inseticidas tem aumentado a evolução da resistência de *Ae.aegypti* a esses compostos (Macoris *et al*. 1999; Braga *et al.* 2004, Lima *et al*. 2003, Cunha *et al*. 2005, Montella *et al.* 2007, Macoris *et al.* 2018). As orientações do PNCD juntamente com o EGI-Dengue, confirmam que o controle mecânico de *Ae.aegypti* (o extermínio manual dos criadouros) é o método mais eficiente atualmente, eliminando tanto indíviduos de populações resistentes como os não resistentes. A eliminação dos criadouros é um método de controle preventivo e deve ser feito de maneira recorrente ao longo do ano. O uso do controle de origem química para extermínio das larvas é recomendado como recurso complementar, para aplicação em criadouros

que não possam ser eliminados mecanicamente. O controle vetorial em adultos não deve ser usado como método de prevenção, podendo ser indicado quando necessário para locais específicos com surtos intensos.

Com a eficácia cada vez mais limitada dos métodos de controle baseados em inseticidas, avanços recentes em técnicas moleculares estão sendo utilizados para o controle de *Ae.aegypti*. Técnicas como produção em massa de Insetos Estéreis (SIT) e o método de controle RIDL (Libertação de Insetos carregando um gene Letal Dominante) são baseados na construção de sistemas genéticos altamente específicos, versáteis e poderosos (Dick *et al*. 2005, Dick e Hendrichs 2005, Alphey *et al.* 2010). Várias espécies-chave de *Aedes* já foram transformadas, seja por métodos de DNA recombinante usando vetores de transposon (Coates *et al.* 1998, Jasinskiene *et al*. 1998, Rodrigues *et al*. 2006, Labbé *et al.* 2010, Fraser 2012) ou por infecção artificial com *Wolbachia*, um grupo diversificado de bactérias intracelulares (Xi *et al.* 2005 e 2006, Chambers *et al.* 2011, O'Neill 2018). O uso dessas tecnologias inovadoras possibilita o desenvolvimento de novas ferramentas baseadas na genética para controlar as principais doenças transmitidas pelo mosquito.

Uma das principais características do *Ae.aegypti* é a sua capacidade de prosperar em áreas densamente povoadas que não contam com abastecimento de água, gestão de resíduos e saneamento adequados (Honorio *et al.* 2009). Com a domesticação e a urbanização, a espécie passou a usar como habitat reservatórios aquáticos internos e externos em habitações humanas, conseguindo explorar uma vasta opção de reservatórios artificiais, como vasos, tanques de água, garrafas e pneus (Kay *et al.* 2000, Cunha *et al.* 2002; Jansen e Beebe 2010). Fontes aquáticas subterrâneas, como esgotos e galerias pluviais também podem ser utilizadas como abrigo pelo vetor (Barrera *et al.* 2008). A criação ao ar livre pode permitir o aumento do número de populações da espécie e proporcionalmente dificultar a implementação de métodos de controle (Jansen e Beebe 2010; Saifur *et al.* 2012). Fatores climáticos podem desempenhar um papel determinante na sobrevivência do vetor, replicação viral e nos ciclos infecciosos (Reiter 2001; Gubler *et al.* 2001; Patz e Reisen 2001; Wilder-Smith e Gubler 2008). O aumento da temperatura global pode acrescer a sobrevida e/ou propiciar a migração do vetor para áreas geográficas anteriormente não endêmicas nos próximos anos (Hales *et al.* 2002). Condições meteorológicas como a taxa de pluviosidade, podem afetar a abundância e a produtividade dos locais de reprodução do vetor, mas a preferência do *Ae.aegypti* por recipientes de água artificial permite que essa espécie não dependa exclusivamente de chuvas para o desenvolvimento larval (Jansen e Beebe 2010). A forte associação com o ambiente doméstico torna o mosquito menos sensível aos fatores climáticos desfavoráveis que poderiam dificultar a sua dispersão. As fêmeas preferem

ovipor em locais próximos a habitações humanas e os ovos apresentam uma alta capacidade de resistência à dessecação em períodos de seca (Consoli e Lourenço-de-Oliveira 1994; Juliano *et al*. 2005; Rezende *et al*. 2008; Medeiros *et al*. 2009; Vargas *et al*. 2014; Farnesi *et al.* 2017).

Similar aos outros culicídeos, *Ae.aegypti* possui desenvolvimento holometábolo e seu ciclo evolutivo compreende as fases de ovo, quatro estádios larvais (L1-L4), pupa e adulto (Figura 1.3). O tempo de desenvolvimento do *Ae.aegypti* pode durar em média cerca de 7 a 14 dias a partir da eclosão dos ovos até a emergência dos primeiros adultos, porém, essa estimativa de tempo pode variar de acordo com as condições ambientais bióticas e abióticas vivenciadas durante o período ovo-larval. Sob baixas temperaturas e escassez de alimento o 4° estádio larval pode perdurar por várias semanas até conseguir alcançar a massa crítica necessária para desencadear o processo de pupação (Consoli e Lourenço-de-Oliveira 1994; Braks *et al.* 2004; Telang *et al*. 2007). Todos os estádios larvais são aquáticos e não competem por nicho, recursos e habitat com as formas adultas. As larvas costumam ingerir detritos, microrganismos e matéria orgânica particulada (Merrit *et al*. 1992; Clements 2000). As pupas permanecem no ecótopo aquático até o surgimento dos adultos e não se alimentam. Os adultos são terrestres, alados e nutrem-se através da fitofagia. A fêmea adulta do mosquito apresenta hábito alimentar hematofágico, utilizando-se das proteínas e outros componentes nutricionais presentes no sangue para dar início ao desenvolvimento dos seus ovaríolos e a maturação dos ovos (Consoli e Lourenço-de-Oliveira 1994; Forattini 1996; Clements 2000).

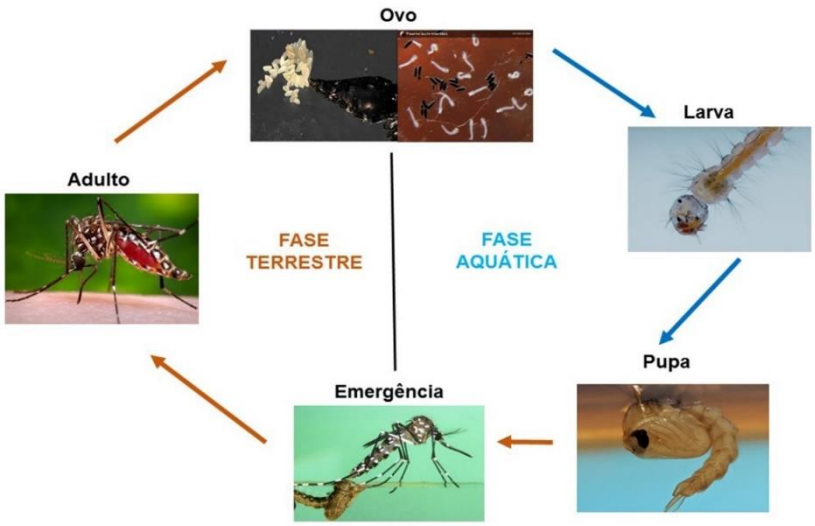

Figura 1.3. Ciclo de vida de *Ae.aegypti* da eclosão do ovo ao mosquito adulto. Fonte: Genilton Vieira, Planeta Invertebrados (Walter Ishikawa), Max Malmann fotos, exame.abril.com.br e James Gathany (CDC).

O *Ae.aegypti* possui um comportamento estritamente sinantrópico e antropofílico, preferindo se alimentar de hospedeiros mamíferos humanos (Turell *et al.* 2005), mesmo na presença de hospedeiros alternativos (Saifur *et al*. 2012; figura 1.4). A domesticação da espécie possibilita a busca de hospedeiros e descanso da espécie dentro das residências (Consoli e Lourenço-de-Oliveira 1994; Weaver e Reisen 2010). *Ae.aegypti* é mais ativo em períodos diurnos e crespusculares vespertinos (Consoli e Lourenço-de-Oliveira 1994). O oportunismo observado na espécie possibilita que os locais de reprodução sejam encontrados a menos de 100 m das habitações humanas ou em regiões mais afastadas (Reiter 2010; Saifur *et al*. 2012). Durante o ciclo gonotrófico, as fêmeas se alimentam várias vezes e a necessidade de mais de um repasto sanguíneo (discordância gonotrófica) para a produção adequada de ovos, resulta em mudanças drásticas na competência vetorial de *Ae.aegypti* (Christophers 1960; Lourenço-de- Oliveira 2005; Weaver e Reisen 2010; Scott e Takken 2012). Sem dúvidas, um dos maiores infortúnios da humanidade foi a domesticação e posterior urbanização do vetor *Ae.aegypti*. O mosquito desenvolveu uma trajetória evolutiva associada ao homem, essa aptidão aumentou o potencial de transmissão de patógenos e impactou toda a saúde pública global. Desse modo, um esforço conjunto deve ser realizado, unindo iniciativas públicas, privadas, governamentais ou não para prevenção das arboviroses e a criação e/ou aperfeiçoamento de métodos de controle e vigilância, capazes de interromper a dispersão e o desenvolvimento do vetor.

Figura 1.4. Fêmea adulta de *Ae.aegypti* alimentando-se de sangue humano. Fonte: James Gathany, Center for Disease Control Public Health, University of Florida.

1.3. Arboviroses transmitidas por *Ae.aegypti*

Arbovírus são capazes de se repilcar em hospedeiros vertebrados e artrópodes hematófagos e se perpetuam na natureza principalmente através da transmissão biológica entre os artrópodes e os hospedeiros vertebrados suscetíveis. A transmissão das partículas infecciosas virais ocorre através da picada desses artrópodes (Clements 2012). Existem aproximadamente 545 espécies de arbovírus classificadas e aproximadamente 150 estão relacionados com doenças transmissíveis ao homem (Gubler 2001; Cleton *et al*. 2012; Lopez *et al*. 2014). Os arbovírus representam 8 famílias e 14 gêneros (Karabatsos 1985). Os gêneros que causam doenças infeciosas em humanos e animais pertencem a cinco famílias virais: *Togaviridae*, *Flaviviridae*, *Bunyaviridae*, *Rhabdoviridae* e *Reoviridae* (WHO 1985; Gubler 1998; Lopes *et al*. 2014). Dentre as doenças infecciosas emergentes e reemergentes conhecidas, as arboviroses transmitidas por culicídeos do gênero *Aedes* tem se destacado no cenário epidemiológico mundial. Arboviroses como: dengue, febre amarela, chikungunya e Zika devem ser monitoradas constantemente devido à morbidade e os altos índices de mortalidade relatados (Gubler 2002). Nesse capítulo, iremos abordar as principais arboviroses transmitidas por *Ae.aegypti*.

1.3.1. Dengue

Dengue ou febre do dengue é considerada a mais importante doença viral transmitida por mosquito do mundo (WHO 2012). Assemelha-se à malária na distribuição geográfica devido a sua crescente incidência nos trópicos e subtrópicos e pode ser mais significativa em termos de morbidade e impacto econômico (Figura 1.5) (Gubler 2002; Guzman *et al*. 2010; Gubler 2011; Brady *et al*. 2012; Bhatt *et al*. 2013). A organização mundial de saúde estima que até 3,97 bilhões de pessoas vivam atualmente em áreas onde o vírus da dengue têm potencial para ser transmitido (WHO 2013; Bhatt *et al*. 2013; Stanaway *et al.* 2016). O principal vetor da dengue é o *Ae aegypti* (Halstead *et al*. 2008; WHO 2013; Guzman e Harris 2015); entretanto, o vírus também pode ser transmitido por outras espécies tais como: *Ae albopictus* e *Ae polynesiensis.* O *Ae. albopictus* é um vetor de importância secundária e encontra-se mais associado a transmissão da doença no meio rural/periurbano (Huber *et al*. 2000; Gratz 2005; Effler *et al*. 2005; WHO 2011; Barcelos 2014). Na Polinésia, a espécie *Ae. polynesiensis* pode ser apontada como vetor da dengue no ciclo urbano/periurbano em caráter local (Rosen *et al*. 1954).

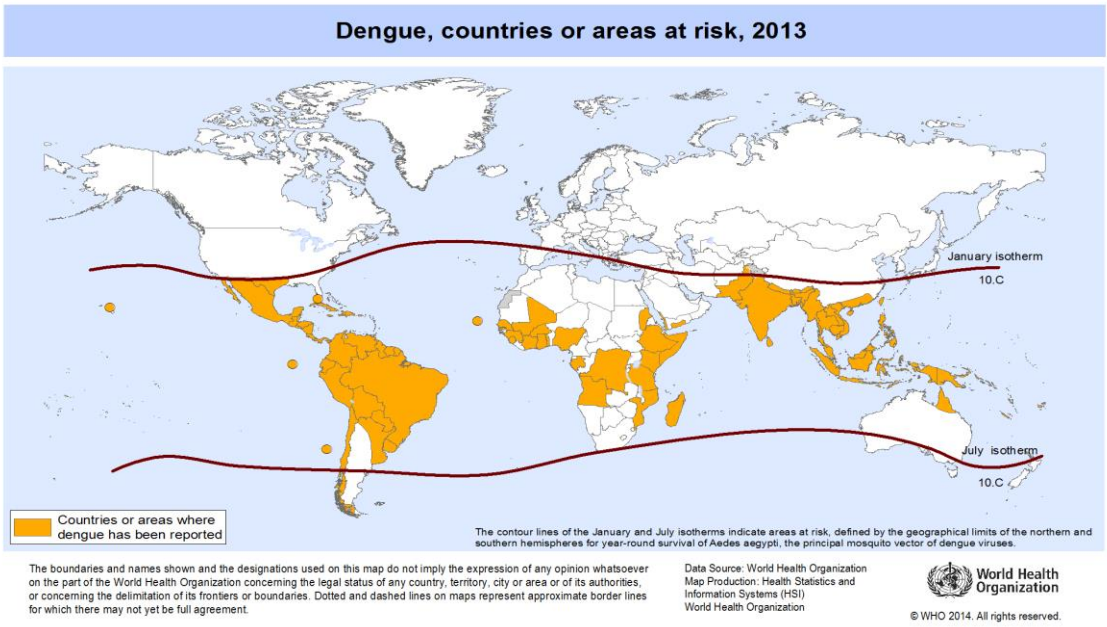

Figura 1.5. Países ou áreas do mundo onde a dengue foi relatada em 2013. Dados coletados pela Organização Mundial da Saúde. Genebra: Organização Mundial da Saúde (WHO). Acessado em 22 de julho de 2018. Copyright © 2012.

Existem cinco sorotipos imunogenicamente distintos do vírus da dengue, todos são originários da família *Flaviviridae* e do gênero *Flavivirus* (Wang *et al*. 2000; Gubler 2002; WHO 2011; Simmons *et al*. 2012; WHO 2013; Normile 2013; Mustafa *et al*. 2015). Os sorotipos são denominados DENV-1, DENV-2, DENV-3, DENV-4 e DENV-5, infecções com qualquer um dos cinco vírus resulta em imunidade vitalícia a esse sorotipo específico (Halstead 1974; Wilder-Smith *et al.* 2010; WHO 2013; Mustafa *et al.* 2015). Os sorotipos DENV-1, DENV-2, DENV-3 e DENV-4 foram individualmente considerados responsáveis por epidemias de dengue urbana e associados a casos de dengue mais graves (WHO 2001; Gibbons e Vaughn 2002). O DENV-5 foi isolado do sangue de um fazendeiro na Malásia em outubro de 2013 e está associado ao ciclo silvestre, diferentemente dos outros quatro sorotipos que participam do ciclo urbano da doença (Normile 2013; Mustafa *et al*. 2015). A causa provável da emergência do novo sorotipo pode dever-se a fatores de recombinação genética, seleção natural ou até mesmo possíveis gargalos genéticos (Mustafa *et al*. 2015).

A dengue é uma doença viral sistêmica aguda que se estabeleceu globalmente em ciclos de transmissão endêmicos e epidêmicos. É considerada uma doença complexa com um amplo espectro de apresentações clínicas, que em muitas ocasiões não são reconhecidas ou são diagnosticadas erroneamente como outras doenças tropicais causadoras de febre hemorrágica (Gubler 1986; Amarasinghe *et al.* 2011). A infecção viral de dengue em humanos é frequentemente assintomática (Simmons *et al*. 2012; Endy*et al.* 2011), mas, dependendo de

fatores como o estado imunológico ou a faixa etária do acometido pode ocasionar uma série de manifestações clínicas, desde febres indiferenciadas menos severas (FD) a doenças potencialmente graves como a febre hemorrágica da dengue (FHD). A FHD pode ser classificada em quatro graus de gravidade, com os graus III e IV sendo definidos como síndrome do choque da dengue (SCD). Essa síndrome é a forma mais severa da doença e pode provocar o óbito do paciente (WHO / TDR 2009). Infecções secundárias de dengue ou cepas virais particularmente virulentas são fatores que podem estar associados à manifestação dos casos mais graves da doença (Rigau-Perez *et al*. 1998; Gubler 2011). Atualmente, não existem terapias antivirais para o tratamento de dengue e a vacina Dengvaxia® licenciada em 2015 pelo Instituto Sanofir, mas não foi completamente eficaz para proteger todas as faixas etárias contra a hospitalização por FD e FHD/SCD (Ferguson *et al*. 2016; Aguiar *et al*. 2016; Sanofi 2017; WHO 2017; Pang *et al*. 2018; The Lancet 2018). A prevenção e o controle mais amplo da dengue dependem atualmente diretamente do controle do vetor (WHO 2009; Maciel-de-Freitas *et al*. 2012).

1.3.2. Febre Amarela Urbana (FAU)

A febre amarela (FA) é uma doença zoonótica com dois principais ciclos de transmissão epidemiológica: silvestre e urbano. O agente etiológico é o vírus da febre amarela (YFV), pertencente ao gênero *Flavivirus* e a família *Flaviviridae*. Tradicionalmente, o YFV é transmitido dentro de um ciclo florestal entre primatas não humanos (PNHs) e mosquitos do gênero *Haemagogus* (vetores primários) e *Sabethes* (vetores secundários). Durante as epizootias de FA, os seres humanos podem ser infectados se residirem perto de florestas ou quando em contato com locais silvestres (Bryan 2004; Gubler 2004; Vasconcelos *et al.* 2004). O ciclo urbano da FA é transmitido aos seres humanos principalmente através da picada do mosquito *Ae.aegypti*, porém a espécie *A. albopictus* também se mostrou suscetível ao YFV (Strode 1951; Couto-Lima *et al.* 2017).

Nas Américas, a febre amarela urbana (FAU) foi um grande problema de saúde pública do século XVIII ao início do século XX, apresentando diversas epidemias urbanas. No entanto, o desenvolvimento de duas vacinas atenuadas na década de 1930, bem como um programa de controle continental do vetor *Ae.aegypti* na década de 50, levou à erradicação do ciclo urbano da doença (Gubler 2004). A FA é uma febre hemorrágica altamente letal e apesar da disponibilidade de vacinas eficazes, continua sendo um importante problema de saúde pública na África e na América do Sul, com uma incidência anual de cerca de 200.000 casos e 30.000 mortes (Vasconcelos *et al*. 2016).

O elevado número de indivíduos não vacinados que visitam áreas selváticas, onde o ciclo silvestre de FA já existe, e retornam às áreas urbanas infestadas de mosquitos do gênero *Aedes*, representam um perigo real de ressurgimento da FAU. Nos últimos anos, um número crescente de casos de FA tem sido relatados em muitas cidades brasileiras onde a floresta permanece com reservatórios de PNHs, e a presença de mosquitos silvestres próximo a habitações humanas é constante (Massad *et al.* 2001). Em 2016, um surto de FA caracterizado como uma epizootia silvestre, ocorreu em Minas Gerais, Brasil (WHO 2017). No período de julho/2017 a fevereiro/2018, foram confirmados 723 casos de febre amarela no país, com 237 óbitos. Ao todo, foram notificados 2.867 casos suspeitos, sendo que 1.359 foram descartados e 785 permanecem em investigação, com uma taxa de letalidade de 34% (Ministério da Saúde). Em outubro de 2017, a maioria dos grandes parques da cidade de São Paulo estava fechada para visitação devido à morte de macacos *Alouatta guariba* infectados com FA (Estadão 2017). Mutações detectadas na sub-linhagem viral 1E (Genótipo da América do Sul I) do YFV, estão relacionadas às mortes dos PNHs e à ocorrência de FA em humanos (de Souza *et al*. 2010; Nunes *et al*. 2012; Bonaldo *et al*. 2017; Gómez *et al*. 2018).

Até meados de fevereiro de 2018, o Estado de São Paulo registrou 202 casos de FA em humanos com 79 óbitos laboratorialmente confirmados. Nenhum desses casos recentes relatados no Brasil foram atribuídos ao mosquito *Ae.aegypti*, o vetor responsável por todos os surtos urbanos na primeira metade do século passado. Os mosquitos infectados com YFV pertenciam a duas espécies: *Haemagogus leucocelaenus* e *Hg. Janthinomys (*Gómez *et al.* 2018*)*. Após estes casos infecciosos, houve uma redução importante na incidência de notificações em regiões epizoóticas do Brasil (PAHO 2017). A transmissão da FAU, no entanto, ainda ocorre continuamente dentro e fora da África, fato demonstrado pelos surtos em Angola no ano de 2016 (4.000 casos suspeitos e 600 mortes), Costa do Marfim em 2017, República Democrática do Congo em 2015-16 e China em 2016, áreas densamente povoadas e infestadas de mosquitos do gênero *Aedes* (WHO 2014; ProMED 2014; Woodall e Yuill 2016; Ling *et al.* 2016; Kraemer *et al*. 2016).

A FA tem se expandido para as regiões sul e sudeste do Brasil e consequentemente aproximado-se de cidades densamente povoadas e altamente infestadas com *Ae.aegypti* (Carvalho *et al*. 2014; Romano *et al*. 2014; Health *et al*. 2015). Por essa razão, existe um risco baixo, mas não negligenciável de ressurgimento de FAU em áreas endêmicas de dengue (Massad *et al*. 2018). O risco iminente dependerá da probabilidade de que pelo menos um humano infectado apareça em locais onde ocorra proliferação da população de *Ae.aegypti*. Além disso, a competência vetorial dos mosquitos na transmissão de YFV será um fator determinante para a transmissão da

FA e o ressurgimento da FAU (Massad *et a*l. 2018). Alguns estudos reportaram, que a competência vetorial do *Ae.aegypti* para transmissão da FAU é menor do que para transmissão de dengue (Massad *et al*. 2001; Couto-Lima *et al*. 2017). Este fato e a cobertura vacinal relativamente alta de grandes regiões do território brasileiro, pode explicar porque a FAU urbana não reapareceu nessas localidades. No entanto, as áreas costeiras do Brasil, incluindo a região Sudeste atualmente fortemente afetada por epizootias da FA, não estão incluídas nos programas de vacinação de rotina do Ministério da Saúde do Brasil. A pouca cobertura vacinal nessas regiões, associado à alta densidade de mosquitos e um grande número de epizootias que se mantêm recorrentes nestas áreas não vacinadas, representam um sério risco de ressurgimento da FAU nessas regiões (Possas *et al*. 2018). Então, estimar o risco de reaparecimento da FAU é primordial para projetar uma estratégia de vacinação ótima considerando a cobertura atual de vacinação, a sua eficácia e o risco de eventos adversos da atual vacina contra FA (Massad 2005).

1.3.3. Chikungunya

O vírus Chikungunya (CHIKV) é um alphavírus pertencente à família *Togaviridae* transmitido por mosquito que causa uma doença febril aguda caracterizada por artralgia severa (Sudeep e Parashar 2008). Originário da África, o vírus pode circular em complexos ciclos zoóticos entre PNHs e mosquitos do gênero *Aedes* (Kunar et al 2010, Weaver *et al*. 2014). Os principais vetores do CHIKV em áreas urbanas e periurbanas, são os mosquitos *Ae.aegypti* e *Ae. albopictus*, respectivamente (Honorio *et al.* 2009; Nasci *et al*. 2014). As viagens internacionais e a expansão global dos vetores, principalmente do *Ae.aegypti*, aumentaram a capacidade do vírus se espalhar para novas regiões onde as condições ambientais são permissivas à transmissão viral (Vega-Rúa *et al*. 2014). O CHIKV foi descrito pela primeira vez em 1954 na Tanzânia e estima-se que mais de 4 milhões de casos tenham ocorrido em todo o mundo nos últimos 12 anos (Powers 2016). No final de 2013, as primeiras infecções por CHIKV adquiridas localmente nas Américas foram relatadas na ilha de Saint-Martin, no Caribe (Leparc *et al*. 2014; Cassadou *et al.* 2013). Até o momento, apenas mosquitos *Ae.aegypti* foram implicados na transmissão de chikungunya nas Américas e o vírus se espalhou sucessivamente para outras ilhas infestadas pelo vetor (Morrion *et al.* 2014). Até o momento, esta epidemia causou mais de 1 milhão de casos suspeitos em 45 países das Américas (PAHO 2014), com o potencial de se espalhar ainda mais pelo resto do continente (PAHO 2017).

Sem uma vacina ou tratamento específico disponível, a única estratégia para o controle de surtos de chikungunya continua sendo a supressão das populações de vetores e o uso de proteções individuais, como repelentes. O reforço da vigilância epidemiológica deve ser priorizado em

função da extensa distribuição de *Ae.aegypti* e *Ae. albopictus* no Brasil, uma vez que, ambas as espécies apresentam alta competência vetorial para CHIKV (Vega-Rúa *et al.* 2014). Por possuir sintomas semelhantes ao de outras arboviroses e com a maioria dos pacientes vivendo em áreas de alta incidência epidemiológica de dengue, muitas falhas no diagnóstico do CHIKV foram constatadas (Daumas *et al.* 2013). A eficácia dos programas de monitoramento epidemiológico depende do diagnóstico rápido e efetivo do vírus em regiões com alta incidência do vetor e população suscetível. Ações coordenadas para melhorar o reconhecimento da doença, disponibilizando testes diagnósticos rápidos e de fácil validação, comunicação e notificação dos casos de maneira integrada entre os laboratórios das redes de saúde privadas e das redes de saúde públicas, partilhamento de informações atualizadas e incentivo a programas sócio-educativos que possam mobilizar a população podem minimizar o impacto do CHIKV nos próximos anos. Além disso, pesquisas voltadas para a compreensão de fatores ecológicos e características biológicas do vetor e do vírus podem auxiliar no esclarecimento da dinâmica de transmissão e prevenir futuros surtos.

1.3.4. Zika

O vírus Zika (ZIKV) é um arbovírus emergente transmitido por espécies de mosquitos do gênero *Aedes,* principalmente *Ae.aegypti*, que é considerado responsável pelos atuais surtos urbanos da doença (Smithburn 1954; Pond 1963; Haddow *et al.* 1964; Marchette *et al.* 1969; Monlun *et al*. 1992; WHO 1999; Foy *et al.* 2011b; Faye *et al*. 2008; Musso *et al*. 2014; Marcondes *et al*. 2015; Kindhauser *et al.* 2016; Petersen *et al*. 2016; Wang *et al*. 2017). Na África, ZIKV possui um ciclo de transmissão zoótico envolvendo PNHs e espécies de mosquitos *Aedes* silvestres (Marchette *et al*. 1969; Diallo *et al*. 2014). Pertencente ao gênero *Flavivirus*, família *Flaviviridae,* o ZIKV foi inicialmente isolado em amostras coletadas de macacos *rhesus* na floresta de Zika, Uganda, no ano de 1947 (Dick *et al*. 1952; Dick 1953). O ZIKV possui duas linhagens: africana e asiática, e ambas as linhagens se propagaram no Pacífico e nas Américas (Lanciotti *et al*. 2015).

Após a primeira infecção humana por ZIKV em 1953, apenas casos esporádicos foram relatados no Sudeste Asiático e na África subsaariana (MacNamara 1954; Lanciotti *et al*. 2008; Hayes 2009; Reynolds 2017). Em 2007, um surto ocorreu nas ilhas de Yap, nos Estados Federados da Micronésia, com uma estimativa de 5.000 infecções em uma população média de 6.700 habitantes (Duffy *et al*. 2009). Outro surto ocorreu entre 2013–2014 na Polinésia Francesa, afetando 32.000 pessoas. A infecção pelo ZIKV foi associada à síndrome de Guillain-Barré

(Oehler *et al.* 2009; Ioos *et al.* 2014; Cao-Lormeau 2014). A transmissão autóctone do ZIKV aos seres humanos foi relatada pela primeira vez na América latina em 2015 (Anluca *et al.* 2015; WHO 2015). Em 2016, o ZIKV foi declarado uma emergência de saúde pública internacional devido ao aumento da incidência de síndrome de Guillain-Barré e casos de microcefalia em neonatos nas Américas (Oehler *et al*. 2014; Broutet *et al.* 2016; Johansson *et al*. 2016; dos Santos *et al* 2016; Martines *et al* 2016; WHO 2016).

A distribuição geográfica do ZIKV tem se ampliado constantemente desde que foi observado o primeiro surto da doença (Figura 1.6). Esta rápida disseminação pode indicar que o vírus está seguindo um padrão de expansão global semelhante ao observado para a dengue e chikungunya (Musso *et al*. 2015a). Atualmente, ocorre transmissão autóctone do vírus em 48 países da América. Globalmente, entre 1 de janeiro de 2007 e 9 de março de 2016, um total de 55 países comunicaram transmissão autóctone ou indicação de transmissão do ZIKV. Cinco desses 55 países informaram que o surto da doença foi neutralizado. Além disso, três países relataram infecção adquirida localmente na ausência de quaisquer mosquitos vetores conhecidos, provavelmente por transmissão sexual (WHO 2016). Atualmente, o Brasil é o país com o maior número de casos de ZIKV nas Américas (PAHO 2017).

O quadro clínico clássico da infecção por ZIKV assemelha-se ao da dengue e chikungunya, podendo manifestar febre, dor de cabeça, artralgia, mialgia, conjutivite e erupção cutânea maculopapular, um complexo de sintomas que dificulta o diagnóstico diferencial (Zanluca *et al*. 2015; Dar *et al*. 2016; Gatherer e Kohl 2016; Donald *et al*. 2016). Além disso, a maioria dos pacientes não apresenta ou apresenta apenas sintomas leves a moderados no ínicio da infecção; assim, geralmente não ficam doentes o suficiente para procurar atendimento médico o que faz com que o vírus seja potencialmente subnotificado pelas redes de saúde. Apesar de na maioria dos casos os acometidos apresentarem sintomas leves, autoridades de saúde confirmaram o surgimento de patologias neurológicas graves como síndrome de Guillain-Barré e microcefalia, simultaneamente com a infecção por ZIKV (Garcia *et al*. 2016; Donald *et al*. 2016, Parra *et al*. 2016, Frieden *et al*. 2016, Marrs *et al*. 2016). Síndrome de Guillain-Barré é uma doença neurodegenerativa caracterizada pelo déficit do densenvolvimento sensório-motor devido a resposta imune do indíviduo, ocorrendo tipicamente após infecções virais ou bacterianas menores (dos Santos *et al*. 2016). Dos 42 casos brasileiros de síndrome de Guillain-Barré, 62% apresentaram sintomas comuns com a infecção pelo ZIKV (WHO 2016). Infecção por ZIKV também foi relacionada aos casos de microcefalia observados no Brasil (Brasil 2015; Schuler *et al*. 2016; Marrs *et al.* 2016). Por essa razão, a OMS e outras organizações de saúde aconselharam

aos habitantes das regiões endêmicas, particularmente as gestantes, que observassem os sintomas de infecção por ZIKV e se resguardassem contra possíveis picadas de mosquitos.

Globalmente, muitos países estão lutando especificamente para lidar com a infecção pelo ZIKV (Walker *et al*. 2016; Hennigan 2016; Franca *et al*. 2016; Septfons *et al*. 2016; Barreto *et al*. 2016; Lessler *et al*. 2016; Jang *et al*. 2016). O surgimento do ZIKV no Brasil com o envolvimento de um grande número de casos mostra que as infecções arbovirais podem facilmente atravessar fronteiras internacionais e ter impacto em países que não têm preparação ou programas para lidar com extensos surtos. Além disso, a provável relação entre ZIKV e microcefalia congênita em pacientes representa um grande ônus para o sistema nacional de saúde e os países afetados devem se mobilizar para apoiar as crianças e suas famílias. Apesar de existirem vário grupos de pesquisa no Brasil estudando a relação do ZIKV com as complicações neurológicas ocasionadas pela infecção, existe uma unificação entre os grupos afim de se compreender em mais detalhes a dinâmica de transmissão, as consequencias clínicas e as possibilidades de tratamento desta infecção flaviviral. Avaliar o impacto e a epidemiologia do ZIKV é urgentemente necessário para melhorar a compreensão da história natural da infecção pelo ZIKV e estabelecer medidas eficazes de controle e prevenção de surtos.

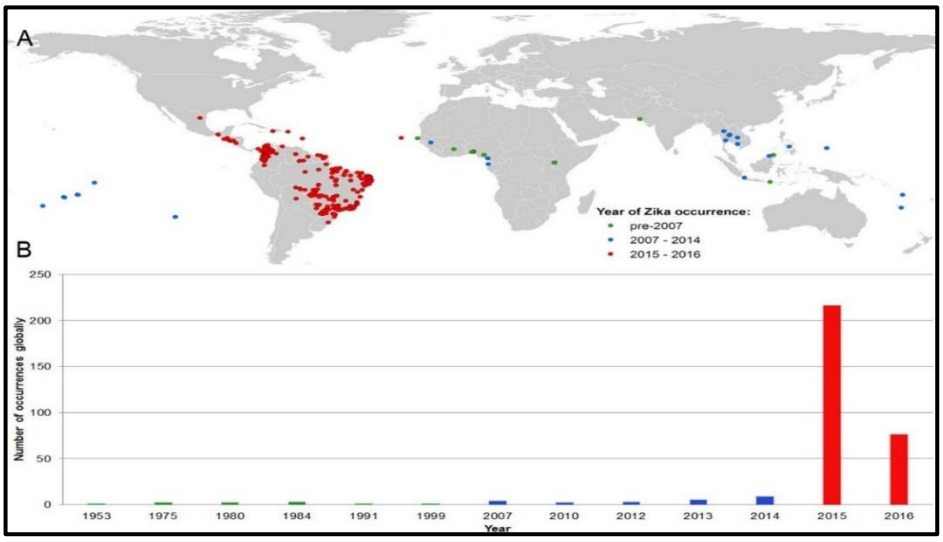

Figura 1.6. **(A)** Mapa mostrando a distribuição do conjunto final de 323 locais com ocorrência de ZIKV inseridos no procedimento de modelagem de árvore de regressão de conjunto. Os locais são classificados por ano de ocorrência para mostrar aqueles que ocorreram **(i)** antes do surto de 2007 nos Estados Federados da Micronésia; **(ii)** entre 2007 e 2014; e **(iii)** durante o surto de 2015–2016; **(B)** o número total de locais que relatam a ocorrência sintomática de ZIKV em humanos globalmente ao longo do tempo. DOI: 10.7554 / eLife.15272.003

1.4. Aspectos Fisiológicos, Morfológicos e Nutrição Larval

As larvas de *Ae.aegypti* são estritamente aquáticas e seu habitat natural geralmente é encontrado em locais com volume de água raso e em ambientes lênticos (com pouco ou nenhum movimento de água) – particularmente piscinas rasas, margens de riachos protegidas, pantânos com água limpa, buracos de árvores repletos de água e recipientes criados pelo homem (Laird 1988). Todas as larvas aquáticas devem possuir mecanismos de adaptação para alcançar o equilíbrio iônico de acordo com seu ecótopo natural, abrangendo fontes de água doce ou salgada. Além dos habituais órgãos de regulação de íons, as larvas de mosquito possuem quatro papilas anais que são responsáveis pelo equilíbrio osmótico da larva e estão localizadas na extremidade inferior do corpo. Essas estruturas são capazes de absorver íons a partir de uma solução bastante diluída. No momento da eclosão, a larva jovem encontra-se plenamenre adaptada ao ambiente aquático e apta para respirar o oxigênio atmosférico e usar as partículas em suspensão aquosa para ingestão de alimentos (Christophers 1960; Consoli-Lourenço 1994).

A aparência externa das larvas apresenta aspecto vermiforme e coloração esbranquiçada. Possuem o corpo dividido em cabeça, tórax e abdômen; os dois primeiros segmentos são mais esféricos, enquanto o abdômen tem o formato alongado e divide-se em nove tagmas semicilíndricos (Figura 1.7). O último segmento abdominal é o lobo anal. Próximo a essa estrutura, encontra-se o sifão respiratório, um órgão cilíndrico responsável pelas trocas gasosas entre o ambiente aquático e a superfície. O corpo da larva tem aproximadamente 222 pares de cerdas, alocadas de maneira simétrica. Oposto aos adultos que possuem um aparato bucal adequado para punção, larvas de mosquito tem um aparelho bucal compatível com o hábito alimentar mastigador-raspador (Harbach e Peyton 1993; Consoli e Lourenço-de-Olveira 1994).

Figura 1.7. Larvas de *Ae.aegypti* nos estádios L3 e L4. Fonte: Max Mallman.

As larvas de *Ae.aegypti* utilizam material particulado como principal fonte alimentar, compreendendo microrganismos como bactérias, fungos, protozoários, rotíferos, diatomáceas, algas e partículas de detritos que são em grande parte derivados de tecidos vegetais deteriorados ou pólen (Ameen e Iversen 1978; Walker *et al*. 1988; Merrit *et al*. 1992; Timmermann e Briegel 1996; Forattini 2002; Clements 2000; Ye-Ebiyo 2003; Kivuyo *et al*. 2014; Asmare *et al*. 2017). O modo de filtrar essas partículas naturais difere entre as espécies de larvas de mosquito (Merrit *et al*. 1992; Clements 2000). Larvas de *Ae.aegypti* costumam ser tanto nadadoras plantônicas quanto mergulhadoras, produzindo um fluxo natatório capaz de criar correntes na coluna d'água que fluem em direção à cabeça (Clements 2000). O movimento das escovas orais presas à mandíbula da larva faz com que as partículas de diferentes tamanhos sejam selecionadas, coletadas e ingeridas (Merrit *et al*. 1992; Figura 1.8).

Figura 1.8. Aparelho bucal com cerdas de larvas *de Ae.aegypti*. Fonte: Max Mallmann.

A nutrição durante a fase larval é determinante para o crescimento e o desenvolvimento dos mosquitos adultos (Gimnig *et al*. 2002; Rivera-Pérez *et al*. 2017). As necessidades nutricionais dos mosquitos podem ser divididas em duas principais categorias: os nutrientes energéticos ou macronutrientes, incluindo carboidratos, ácidos graxos e alguns aminoácidos (Foster 1995; Arrese e Soulages 2010; Canavoso *et al*. 2011) e os nutrientes não energéticos (NEN) ou micronutrientes que incluem vitaminas, sais, metais e esteróis. Os micronutrientes são necessários em quantidades reduzidas em comparação ao macronutrientes, mas são indispensáveis para manutenção das funções celulares (Friend e Dadd 1982). Muitos aminoácidos também podem ser considerados micronutrientes em razão de não serem usados como principal

fonte de energia, mas ainda assim, eles são indispensáveis para o equilíbrio nutricional (Dadd 1977; Friend e Dadd 1982).

Os componentes nutricionais requeridos por larvas de *Ae.aegypti* já foram explorados por outros pesquisadores anteriormente (Hinman 1930; Trager 1935b; Golberg *et al*. 1945). Alguns autores citam como essenciais para o desenvolvimento das larvas 10 aminoácidos: arginina, isoleucina, leucina, lisina, fenilalanina, metionina, treonina, triptofano, valina e histidina. Além dos aminoácidos, existem 9 vitaminas essenciais para nutrição larval: tiamina, riboflavina, ácido pantotênico, ácido nicotínico, pirodoxina, biotina, ácido fólico, vitamina B12 e cloreto de colina (Lea e Delong 1958; Singh e Brown 1957; Clements 1963).

Carboidratos, incluído açúcares simples, são macronutrientes necessários para o crescimento e desenvolvimento das larvas de mosquitos. Recursos nutricionais com baixos níveis de carboidratos podem retardar o desenvolvimento larval (Sneller e Dadd 1977). Estudos em *Manduca. sexta* relacionam a ingestão de carboidratos com o surgimento das pupas e reforçam a necessidade dos açúcares e proteínas na dieta para o pleno crescimento de insetos adultos (MacWhinnie *et al*. 2005). O papel dos carboidratos no desenvolvimento de larvas de *Ae.aegypti* está diretamente relacionado à pupação e há uma relação inversa entre os níveis de trealose na hemolinfa e os níveis de hormônio juvenil (JH) utilizados em processos neuroendócrinos (Jones *et al*. 1981; Chambers e Klowden 1990; Telang *et al*. 2007).

Assim como carboidratos, a ingestão de alimentos proteináceos por estágios imaturos é essencial para o crescimento, sobrevivência, reserva energética e principalmente para a produção de ovos pelas fêmeas adultas (Chan *et al*. 1990). Além da influência das proteínas e carboidratos no desenvolvimento larval, estudos prévios relataram que existe um limiar crítico de glicogênio que atua como estimulante da metamorfose em insetos. O processo de metamorfose irá ocorrer a partir de uma queda drástica do JH e no aumento concomitante da ecdisona, hormômio desencadeador do processo de muda. (Chambers e Klowden 1990; Timmermann e Briegel 1999). O tempo de liberação de ecdisteróides não é crítico para iniciar a muda larva-pupa em larvas de *Ae.aegypti*, no entanto tanto o nível de ecdiesteróide quanto o estado nutricional do quarto instar larval são fatores determinantes no início da metamorfose (Telang *et al*. 2007). Estudos anteriores indicaram que as reservas energéticas das larvas (proteínas, lipídios e glicogênio) são importantes para a produção de ovos e a regulação endócrina do desenvolvimento de ovos em *Ae.aegypti* e *Ochlerotatus atropalpus* (Telang *et al*. 2006). Altos níveis de glicogênio e proteína ao exceder um limiar estabelecido no sistema nervoso do inseto são capazes de ativar a secreção de ecdisteróides ovarianos e inibir a biossíntese de JH pela“*corpora allata”*. Reunidos, esses processos irão permitir que ocorra a vitelogênese e a produção de ovos. Sem níveis limiares

suficientes, a "*corpora allata"* irá aumentar os níveis de JH, diminuir a produção de ecdisteróides ovarianos e, como conseqüência, a maturação do ovo pode ser retardada (Telang *et al*. 2006).

Os lipídios influenciam na pupação e na regulação endócrina do desenvolvimento dos ovos em mosquitos fêmeas autógenas e anautógenas (Briegel 1990; Briegel *et al*. 2002; Foster 1995; Ziegler e Ibrahim 2001; Zhou *et al*. 2004; Telang *et al*. 2006). Sob condições nutricionais favoráveis, os lipídios começam a se acumular após o glicogênio ter atingido um platô (Van Handel 1984). Ambientes de estresse nutricional ocasionam reduções drásticas nos níveis de lipídios, proteínas e carboidratos no corpo dos insetos (Briegel 1990). Lípidios como o colestorol, a cefalina e a lecitina são especialmente importantes para larvas de *Ae.aegypti*, sem os quais não conseguem ultrapassar o terceiro ínstar larval (Singh e Brown 1957).

Larvas de mosquito bem nutridas se tornam adultos mais saudáveis (Zeller e Koella 2016). A restrição alimentar na fase imatura pode influenciar severamente diversos parâmetros da história de vida como a observação de um período prolongado de desenvolvimento (Tun-Lin *et al*. 2000; Arrivillaga e Barrera 2004; Dominic *et al*. 2005; Vantaux *et al*. 2016), com larvas que levam mais tempo para atingir a pupação (Telang *et al*. 2007; Levi *et al*. 2013; Banerjee *et al*. 2015). Uma fase larval prolongada é geralmente associada a um risco aumentado de mortalidade em consequência de predação, instabilidade no local de reprodução e/ou interferência humana (Padmanabha *et al*. 2011). Aspectos como emergência dos adultos (Okech *et al*. 2007), maturidade sexual, fecundidade, sobrevivência (Dominic *et al*. 1996; Reiskind e Lounibos 2009; Foster *et al*. 2012; Alto *et al*. 2012; Takken *et al*. 2013) e o tamanho dos adultos (Grimstad e Walker 1991; Foster *et al*. 2012) são drasticamente alterados. Adultos que emergem a partir de larvas com reservas nutricionais baixas são menores (Lehmann *et al.* 2006) e requerem mais sangue/repastos para produzir ovos (Briegel 1990), o que pode provocar um aumento da sua capacidade vetorial (Muturi *et al*. 2011).

A contribuição da microbiota intestinal dos insetos na ecologia nutricional é bastante relevante devido à sua impressionante capacidade biossintética e degradativa (Douglas 2009; Kukutla *et al.* 2014). Estima-se que 10% de todos os insetos utilizem dietas nutricionalmente pobres ou desequilibradas e dependam da microbiota residente para assimilação de nutrientes (Chapman *et al*. 2013). A microbiota dos insetos desempenha um papel importante na síntese de componentes dietéticos como vitaminas, aminoácidos essenciais, esteroides e atuam no metabolismo de carboidratos, promovendo o crescimento e o desenvolvimento dos insetos através da via insulínica (Storelli *et al*. 2011; Shin *et al*. 2011; Douglas 2014). Além da nutrição, os simbiontes auxiliam na fixação de nitrogênio, comportamento, reprodução, desenvolvimento e aumentam ou suprimem infecções por patógenos (Dillon e Dillon 2004; Hegde *et al*. 2015).

Aspectos como digestão, processamento, absorção e desintoxicação de dietas generalistas são o resultado de interações refinadas entre os simbiontes e a presença de importantes enzimas digestivas nos insetos. Apesar de enzimas digestivas já terem sido descritas em *Ae.aegypti* (Frank *et al*. 1952, Geering e Freyvogel 1975; Marinotti e James 1990; Souza *et al*. 2016) pouco se conhece sobre como as diferentes dietas podem influenciar o desenvolvimento da fisiologia intestinal larval em seu habitat natural e quais são as principais enzimas envolvidas no processamento desses nutrientes.

1.5. Digestão em Insetos

A digestão em insetos ocorre através da conversão de macromoléculas complexas dos alimentos em moléculas menores e mais simples, as quais são absorvidas pelas células epiteliais do intestino para nutrição dos insetos. O processo digestório é altamente eficiente, sendo realizado principalmente por enzimas intrínsecas, que co-evoluiram de acordo com a dieta ingerida pelo inseto e com as condições físico-químicas (principalmente o valor do pH luminal) encontradas nos diferentes compartimentos intestinais do inseto (Terra 1988; Terra 1990; Chapman e Hall 1996). Em algumas espécies, no entanto, materiais refratários, especialmente os de origem vegetal, não são digeridos adequadamente pelas enzimas intrínsecas, sendo assim necessário que essas moléculas sejam quimicamente processadas por enzimas secretadas pela microbiota intestinal dos insetos (Terra e Ferreira 2005; Douglas 2009).

Nos insetos em geral, a digestão e a absorção dos alimentos ocorrem preferencialmente no trato digestório. Este órgão é dividido em três regiões principais: intestino anterior - **IA** (ou estomodeu), de origem ectodérmica; intestino médio - **IM** (ou mesêntero), de origem endodérmica; e intestino posterior - **IP** (ou proctodeu), novamente de origem ectodérmica (Chapman 1985; Terra e Ferreira 1988 e 2005; Gulan 2010). Normalmente, o trato digestório é um tubo contínuo que vai da cavidade oral até o ânus, apresentando diferenciações fisiológicas e morfológicas que variam de acordo com a espécie e a dieta consumida (Engel e Moran 2013). Várias evaginações são encontradas no intestino dos insetos, incluindo divertículos no **IA** de alguns grupos, cecos gástricos no **IM** e túbulos de Malpighi na junção entre o **IM** e o **IP**. A conexão entre o **IM** e o **IP** é ocluída em alguns insetos que se alimentam de dieta mais fluida contendo pouco ou nenhum material residual sólido. Este é o caso de algumas espécies de Heteroptera fitofágos, onde a oclusão ocorre em diferentes espaços do **IM**; larvas de Neuroptera que digerem suas presas extra-oralmente e larvas de Hymenoptera sociais, que não possuem o hábito de conspurcar o ninho, depositando os excrementos em forma de bolo fecal durante a pupação (Chapman *et al*. 2013, Hakim *et al*. 2010).

Diferentemente da parede intestinal encontrada em vertebrados, onde pode-se observar a presença de multicamadas celulares, o trato digestório dos insetos é constituído por uma única camada de células epiteliais, delimitadas por uma membrana (lâmina) basal e envolvida por tecido muscular longitudinal circular (Ribeiro *et al*. 1990). A maioria das células intestinais são altas e colunares e podem ser conhecidas como enterócitos ou células principais. Essas células possuem uma membrana do lado luminal com microvilosidades que podem aumentar a magnitude da área da superfície celular e assim, potencializar a secreção de enzimas digestivas e a absorção de nutrientes (Klowden 2007). Outros tipos de células prevalentes no epitélio do **IM** são as células enteroendócrinas, que secretam hormônios capazes de regular diversas funções no **IM** e na síntese de células-tronco intestinais (precursora das células intestinais diferenciadas). A disposição da membrana basal varia de acordo com o tipo de alimentação dos insetos. Insetos que se alimentam de recursos fluidos possuem um arranjo muscular irregular na membrana basal, enquanto insetos que ingerem dietas sólidas, possuem camadas musculares mais organizadas e conectadas ao tecido conjuntivo (Ribeiro *et al*. 1990; Chapman e Hall 1996).

De origem ectodérmica, as células epiteliais do **IA** e **IP** secretam constantemente uma camada cuticular não esclerotizada composta principalmente por quitina, um polissacarídeo que possui uma cadeia de polímeros alongada de N-acetilglicosamina (Terra e Ferreira 2005). Diferentemente dos outros compartimentos, o **IM** não é recoberto por cutícula, mas por uma camada permeável não celular conhecida como membrana ou matriz peritrófica - **MP** (Peters 1992; Tellan *et al*. 1999; Klowden 2007). A **MP** atua como uma barreira física, isolando as células epiteliais do **IM** do alimento ingerido. Desta forma, pode-se proteger a parede intestinal de possíveis danos físicos causados pelo bolo alimentar, incluindo partículas de alimentos abrasivas, patógenos e certas toxinas. Além disso, a **MP** separa o lúmen intestinal em dois compartimentos: o espaço ecto-peritrófico (entre a **MP** e o epitélio do **IM**) e espaço endo-peritrófico (lúmen do intestino, no interior da **MP**), permitindo a compartimentalização das atividades enzimáticas e a geração de um fluxo contracorrente de fluídos, aumentando a eficiência da absorção de nutrientes e o aproveitamento máximo das enzimas (Terra 2001; Barbehenn 2001; Terra e Ferreira 2005; Hegedus *et al*. 2009).

A composição e o modo de formação da **MP** pode ser classificada de acordo com seu local de formação no inseto. A disposição da **MP** é geralmente estrututurada por um gel composto por proteínas e carboidratos, como as peritrofinas e proteoglicanos, sobre um esqueleto de microfibrilas de quitina. A **MP** possui poros que atuam como canais aquosos e cruzam o gel. Essa estrutura é permeável a íons inorgânicos, pequenas moléculas orgânicas como açúcares, aminoácidos e etc., peptídeos e pequenas proteínas, mas é uma barreira efetiva evitando a

passagem de agregados lipídicos, proteínas de alta massa molecular e polissacarídeos (Terra e Ferreira 2005; Klowden 2007; Hegedus *et al*. 2009). A **MP** pode ser classificada como do Tipo I, quando for produzida por células epiteliais encontradas em todo o **IM** e como **MP** doTipo II, quando for produzida por células de um tecido especializado chamado cardia, que se localiza no **IA** (Tellam *et al*. 1999; Hegedus *et al*. 2009). A **MP** Tipo I é encontrada nas ordens Coleoptera, Blattodea, Ephemeroptera, Hymenoptera, Odonata, Orthoptera, Phasmida, larvas de Lepidoptera e Dípteros adultos. (Hegedus *et al*. 2009; Terra e Ferreira 2005; Shao e Jacobs 2001; Chapman *et al*. 2013). A **MP** do Tipo II é característica de Dermaptera, Isoptera, alguns Lepidoptera e larvas de Diptera. Larvas de *Ae.aegypti* produzem **MP** do tipo II, apresentando camadas granulares externas e internas que delimitam as microfibrilas de quitina (Hegedus *et al*. 2009; Chapman et al 2013). A produção da **MP** não é sincronizada no **IM,** mas, ocorre através da união entre as microfibrilas de quitina que formam estruturas semelhantes a grades. Alguns insetos aparentemente não produzem **MP,** encontrando-se ausente em mosquitos adultos, exceto em fêmeas após a ingestão de sangue e entre os Lepidoptera adultos fitófagos (Chapman *et al*. 2013). Insetos das ordens Hemiptera e Thysanoptera não possuem **MP** convencional, mas sim, uma camada de lipoproteínas chamada membrana perimicrovilar, que se encontra externa às células do **IM** (Terra 1988; Del Bene *et al.* 1991; Silva *et al.* 2004).

1.5.1. Funcionalidade dos Compartimentos e Etapas da digestão em Insetos

A digestão em insetos ocorre através de um sofisticado sistema de válvulas que irão controlar a entrada dos alimentos e a saída do produto final da digestão. O **IA** possui quatro principais regiões: faringe, esôfago, divertículo e proventrículo. A faringe está relacionada com a ingestão e a passagem dos alimentos através de uma musculatura bem desenvolvida, o esôfago é geralmente um tubo simples que conecta a faringe ao divertículo. O divertículo é um órgão de armazenamento que, na maioria dos insetos, atua como uma parte extensível do intestino logo após o esôfago. O proventrículo geralmente está relacionado à maceração dos alimentos e ao seu transporte até o lúmen intestinal através de uma válvula simples na extremidade proximal do **IM**. O **IM**, também conhecido como ventrículo, é uma estrutura tubular com células envolvidas na produção, secreção de enzimas e absorção de nutrientes. O fluido contendo as moléculas de alimento parcialmente digeridas é transportado do **IM** em direção ao **IP** através do espaço endoperitrófico. A ocorrência de um contra-fluxo circulatório entre os espaços endo e ectoperitróficos, permite que a eficiência da digestão seja maximizada e as enzimas digestivas sejam retiradas do produto de digestão antes que cheguem ao **IP**. O **IP** geralmente diferencia-se em três regiões: o piloro, íleo e reto. O piloro é a região de entrada dos túbulos de Malpighi no

intestino e onde os restos da digestão do **IM** e as secreções dos túbulos de Malpighi se encontram. Em muitos insetos, o piloro pode atuar como um esfíncter, controlando a passagem de alimentos do **IM** para o **IP** e impedindo o refluxo do conteúdo do **IP** de volta para o **IM**. O íleo é um tubo estreito entre o piloro e o reto, a parte distal do íleo também pode ser conhecida como cólon. As almofadas retais são responsáveis pela maior parte da absorção de moléculas de água, sais minerais e outras subtâncias importantes antes que as fezes sejam expelidas (Terra e Ferreira 1996; Bolognesi *et al*. 2001; Gullan 2010; Hakim *et al*. 2010; Terra e Ferreira 2005, 2012; Figura 1.9).

A digestão em insetos ocorre principalmente no **IM** e é realizada em três etapas distintas: digestão inicial, digestão intermediária e digestão final (Figura 1.10). A digestão inicial ocorre no espaço endoperitrófico logo após a ingestão de polímeros complexos. Após a passagem pelos poros encontrados na superfície da **MP**, despolimerases conseguem transformar quimicamente as macromoléculas ingeridas em moléculas menores, como oligômeros. Uma vez processados, os oligômeros atravessam a **MP** e adentram no espaço ectoperitrófico. Na digestão intermediária, um conjunto de enzimas digestivas especializadas, através das suas atividades hidrolíticas, clivam esses oligômeros em moléculas mais simples, como dímeros ou monômeros. A maioria dos insetos tem um conjunto semelhante de enzimas que digerem proteínas, carboidratos e lipídios no **IM**. No entanto, as enzimas produzidas irão refletir o tipo de alimento ingerido por cada espécie e estádio naquele momento. Na digestão final, os monômeros e dímeros serão clivados por enzimas integrais presentes na membrana apical das células epiteliais do **IM** ou por enzimas fixadas ao glicocálix. Assim, a maioria dos produtos finais da digestão como açúcares, aminoácidos, lipídios e etc serão absorvidos pela membrana apical dos enterócitos (Terra e Ferreira 2005, 2012). Após a absorção e captação dos compostos pelas células no lúmen do intestino, estes atravessam a parede do intestino para o hemocele. Em nível celular, a maior parte da absorção é transcelular, o que significa que o composto é transportado através da membrana celular apical das células epiteliais do intestino, transita pela célula e é exportado para a membrana basal. O transporte paracelular, isto é, entre células do epitélio intestinal, é geralmente incomum (Chapman *et al*. 2013). Para muitos insetos, nem toda a atividade digestória ocorre dentro do lúmen intestinal. O alimento pode ser parcialmente ou completamente digerido antes mesmo de ser ingerido, através de enzimas digestivas secretadas na saliva ou regurgitadas do **IM** diretamente no alimento. A digestão antes da ingestão é denominada digestão extra-oral ou digestão extraintestinal (Terra e Ferreira 1994; Chapman *et al*. 2013).

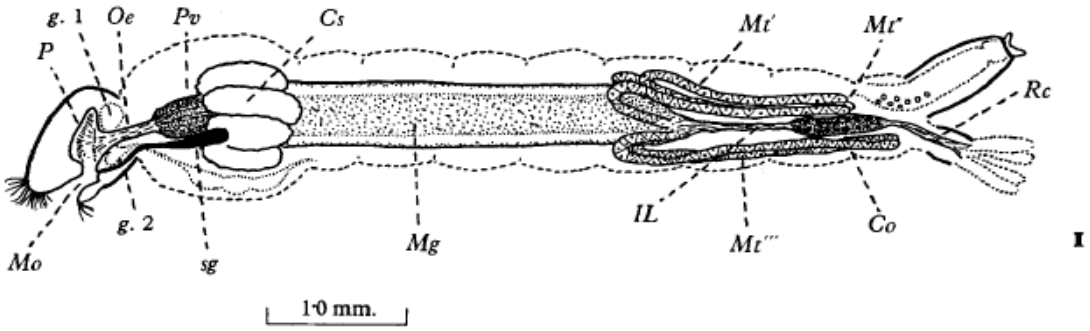

Figura 1.9. Vista lateral da anatomia geral do intestino de larva de culícideo. Legenda: *P*. Faringe; *g. 1*, gânglio supra-esofágico; *Oe*, esôfago; *Pv*, proventrículo; *Cs*, cecos gástricos; *Mt'*, Túbulo de Malpighi (dorsal); *Mt''*, Túbulo de Malpighi (dorso-lateral); *Mo*, cavidade oral; *g. 2*, gânglio subesôfágico; *sg*, glândula salivar; *Mg*, intestino médio; *IL*, íleo; *Mt'''*, (ventro-lateral); *Co*, colon; *Rc*, reto. Fonte: Christophers; 1960.

O trato digestório é um dos órgãos mais imunologicamente ativos do inseto, juntamente com o corpo gorduroso (Chapman *et al*. 2013). Na maioria dos insetos, o trato digestório é a principal superfície de contanto com o ambiente exterior e desempenha um papel central na resposta a muitos patógenos potenciais que entram em contato com os insetos através da ingestão ou através do desequilíbrio na comunidade de microrganismos residentes (Bragato 2010). A resposta imunológica do trato digestório é importante tanto para proteção contra patógenos quanto para o manejo da microbiota residente (Gillespie *et al*. 1997; Royet 2004; Strand 2008). Duas importantes respostas imunológicas complementares são induzidas no trato digestório dos insetos: a produção de espécies reativas de oxigênio (ROS) e a produção de peptídeos antimicrobianos (PAMs).

As ROS são produzidas por uma oxidase dupla (DUOX) a partir de duas enzimas: NADPH oxidase, que medeia a redução de oxigênio dependente de NADPH para gerar íons superóxido que podem se dismutar espontaneamente para formar peróxido de hidrogênio (H_2O_2); e a atividade de peroxidase que combina peróxido de hidrogênio com íons cloreto para formar ácido hipocloroso (HClO). O HClO é um microbicida muito potente e costuma até ser utilizado como alvejante em ambientes domésticos. Existem evidências genéticas de que a atividade da peroxidase do DUOX é absolutamente necessária para proteger o intestino do inseto contra a invasão microbiana (Thannickal e Fanburg 2000; Ha *et al*. 2005; Munks *et al*. 2005; Molina *et al*. 2008; Bonekamp *et al*. 2009).

Os PAMs são peptídios anfipáticos, que atuam na região membranar e matam microorganismos por permeabilização ou através de alvos citoplasmáticos, como inibição de síntese de DNA, RNA ou proteínas (Otvos 2000; Bulet e Stocklin 2005). As células do intestino

dos insetos secretam PAMs, que são ativos principalmente contra bactérias Gram-negativas. A resposta imune local irá ser mediada, entre outras, pela via da imunodeficiência (IMD) através do reconhecimento de um dos principais constituintes da parede celular bacteriana, o peptidoglicano. A quantidade e a composição dos PAMs podem atuar na regulação da composição microbiana residente (Imler e Bulet 2005; Li *et al*. 2006; Lemaitre e Hoffmann 2007).

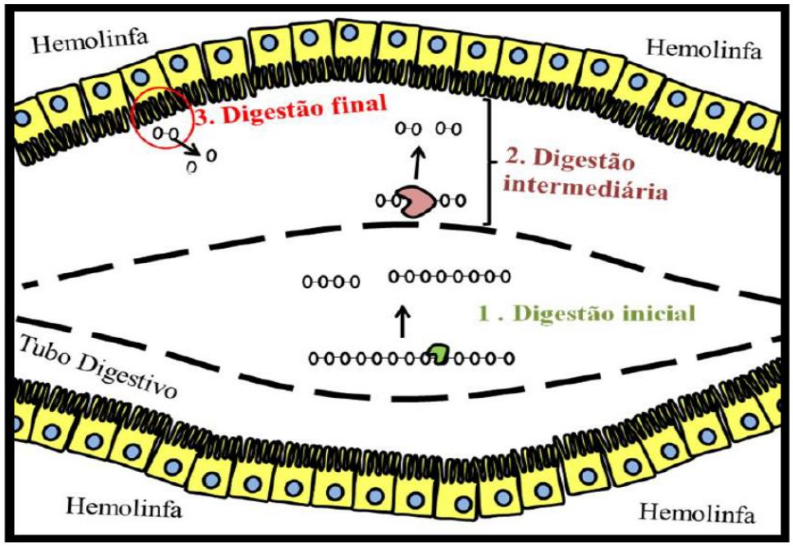

Figura 1.10. Representação esquemática das três fases de digestão em insetos. Enzimas de digestão inicial (despolimerases) estão representadas em verde, enzimas de digestão intermediária em rosa, carboidratos (substrato) estão representados pelos círculos interligados e a MP pela linha pontilhada. Fonte: Caroline da Silva Moraes [Tese 2012].

1.5.2. Enzimas Digestivas em Insetos

A atividade enzimática no **IM** é um fator crucial que determina a disponibilidade de nutrientes para o inseto e, portanto, é rigidamente regulada de acordo com a composição da dieta e as suas necessidades nutricionais. Algumas enzimas digestivas são sintetizadas constitutivamente, enquanto outras são produzidas em resposta à presença de substrato no **IM** (um processo conhecido como regulação pradial), ou sob o controle de hormônios. O padrão de produção de enzimas varia entre espécies de insetos (Terra e Ferreira 1996 e 2012). Todos os insetos possuem um conjunto de enzimas digestivas intrínsecas conhecidas como hidrolases. Dentre essas hidrolases, destacam-se três categorias: peptidases, glicosidases e lipases (Terra e Ferreira 1994 e 1996).

A hidrólise de proteínas em insetos é realizada por peptidases (EC 3.4). Endopeptidases são responsáveis por clivar ligações peptídicas nas proteínas, e as exopeptidases, irão remover os aminoácidos terminais nessas moléculas. Dentro destas categorias gerais, as enzimas são

classificadas de acordo com a natureza dos seus sítios ativos e os locais nos quais clivam as moléculas de proteína. Três tipos principais de endopeptidases foram demonstradas em insetos: serino proteases (tripsina e quimotripsina), que possuem serina no sítio ativo; cisteína (tiol) proteases, incluindo as famílias B e L de catepsinas, com resíduos de cisteína no sítio ativo; e proteases aspárticas (carboxílicas) com resíduos de aspartato nos sítios ativos (catepsina D). As exopeptidases são em geral metaloproteases e se dividem em duas categorias: as carboxipeptidases que atacam os peptídeos da extremidade –COOH e as aminopeptidases que atacam a cadeia a partir da extremidade –NH2 (Terra e Ferreira 1994 e 1996). Muitos insetos têm múltiplas proteases. Larvas de Lepidoptera e muitos Diptera tendem a ter serino proteases, especialmente tripsinas e quimotripsinas, que possuem um elevado pH ótimo. Trabalhos prévios com mosquitos *Ae.aegypti*, reportaram a expressão de três genes de tripsina no **IM** dos mosquitos (Isoe et al 2009) e outras sete serino proteases; onde quatro genes são constitutivamente expressas e três são transcricionalmente induzidos com a alimentação sanguínea (Brackney et al. 2010).

Em insetos, as enzimas envolvidas na hidrólise de carboidratos são classificadas como glicosidases (EC 3.2.1.3). Polissacarídeos e dissacarídeos são geralmente digeridos e posteriormente absorvidos como monossacarídeos, em forma de glicose, frutose e etc. Amido e glicogênio, os principais polissacarídeos de armazenamento de plantas e animais, respectivamente, são digeridos nos insetos por amilases que hidrolisam ligações α-1,4-glicosídicas. Pode haver endo- e exo-amilases separadas, atuando no amido nas cadeias internas ou terminalmente. Outras glicosidases comuns são as alfa-glicosidases e beta-glicosidases, que hidrolisam dissacarídeos e oligossacarídeos específicos com ligações α e β respectivamente. Uma fonte potencial muito abundante de carboidrato para insetos que se alimentam de plantas é a celulose (polímero de glicose com ligações beta-1,4). A celulose é degradada pela ação combinada de três conjuntos de enzimas: endoglucanases (celulases), que irão clivar as ligações entre os resíduos de glicose dentro da cadeia; exoglucanases, que irão clivar as ligações próximas das extremidades da molécula de celulose (celobiohidrolases); e as beta-glicosidases, que hidrolisam a celobiose (Terra e Ferreira 1994 e 1996). Enzimas com atividade de α-glicosidase provenientes das glândulas salivares de *Ae.aegypti* adultos já foram descritas e, de acordo com os autores, correlacionaram-se com proteínas codificadas pelo gene Maltase-like I (Marinotti e James 1990). A capacidade de degradação da celulase pode ser observada nos insetos através da secreção de enzimas digestivas intrínsecas; fermentação por simbiontes microbianos residentes e enzimas ingeridas juntamente com o próprio alimento. Outro grupo de carboidrases que desempenham um papel significativo na digestão dos insetos são as beta-1,3-glucanases. Essas enzimas são abundantes no trato digestório de vários insetos herbívoros e detritívoros, incluindo

Collembola, Trichoptera, Blattodea, Orthoptera, Coleoptera e Diptera. As Beta-1,3-glucanases contribuem para a degradação das hemiceluloses nas paredes celulares das plantas e também para as Beta-1,3 e Beta-1,6-glucanas na parede celular de fungos. Elas são, portanto, potencialmente importantes na liberação do conteúdo de células vegetais e fúngicas para acesso por outras enzimas digestivas, bem como na degradação de polímeros de carboidratos. Em larvas de *Ae.aegypti*, beta-1,3-glucanases intestinais foram caracterizadas e estão diretamente envolvidas na digestão da parede de leveduras (Souza *et al*. 2016). Assim como a celulose, estas enzimas estão aparentemente ausentes em vertebrados, tornando-se significativos alvos de estudo.

As enzimas mediadoras da digestão lipídica de insetos são lipases (EC 3.1.1.3), fosfolipases (E.C.3.1.1.4) e esterases (EC 3.1.1.2). Estas enzimas clivam ligações carboxil-éster em triacilgliceróis (TAGs), diacilgliceróis (DAGs), galactolípidos e fosfolípidos. As enzimas que hidrolisam as ligações éster compreendem: lipases, esterases e fosfolipases A e B (grupo éster carboxílico hidrolases); fosfatases (grupo monoester hidrolases fosfóricas); fosfolipases C e D (grupo diéster hidrolases fosfóricas). A lipólise irá produzir ácidos graxos livres, glicerol, acilgliceróis parciais e lisofosfolipídeos (Terra e Ferreira 1994 e 1996). Os genes das lipases em insetos se diversificaram independentemente entre e dentro de diferentes ordens de insetos. Lipases múltiplas já foram implicadas na digestão lipídica por alguns Dipteros, incluindo o mosquito *Anopheles gambiae*, bem como em Lepidoptera, Coleoptera e Hemiptera. Em *Ae.aegypti*, esterases responsáveis pela clivagem de triglicerídeos foram identificadas no **IM** em mosquitos alimentados com açúcar e sangue (Geering e Freyvogel 1975).

1.6. Importância dos Estudos de Nova Geração em Insetos Vetores

O número de estudos genômicos aumentou exponencialmente no campo da entomologia nos últimos dez anos. O aumento do número de insetos com o genoma e transcriptomas sequenciados é resultado do advento de novas tecnologias e a substancial diminuição dos custos de sequenciamento. A disponibilidade de múltiplos genomas de insetos criou um excelente potencial para genômica comparativa entre espécies que pode levar a uma riqueza maior de informações sobre a biologia desses insetos (Severson e Behura 2012). Atualmente, os genomas de pelo menos 138 insetos foram sequenciados e depositados em bancos de dados públicos, como o banco de dados do genoma do NCBI (NCBI 2015), FlyBase (dos Santos *et al*. 2015), i5k Workspace@NAL (Poelchau *et al*. 2015), VectorBase (Giraldo-Calderon *et al*. 2015), SilkDB Base (Duan *et al*. 2010), ButterflyBase (Papanicolaou *et al*. 2008), BeetleBase (Kim *et al*. 2010), MonarchBase (Zhan *et al*. 2013), AphidBase (Legeai *et al*. 2010), NasoniaBase, BeeBase e Portal Ant Genomes (Munoz-Torres *et al*. 2011), Hessian FlyBase e ManducaBase (http:

//www.agripestbase.org), ChiloDB (Yin et al 2014), DBM-DB (Tang *et al*. 2014), KAIKObase (Shimomura *et al*. 2009) e KONAGAbase (Jouraku *et al*. 2013).

Historicamente, estratégias de controle de vetores têm se baseado no conhecimento biológico sobre as espécies para reduzir o tamanho de suas populações e limitar o contato humano (Walker e Lynch 2007). Atualmente, iniciativas de controle de vetores também tem sido influenciadas pelo uso de abordagens de modelagem computacional cada vez mais sofisticadas, assim como pela expansão da riqueza de informações e teconologias de edição genética. O uso de sequenciamento de próxima geração (NGS) e outras técnicas moleculares como a reação em cadeia da polimerase (PCR) e a cariotipagem molecular permitem que ocorra um conhecimento sistemático e epidemiológico mais detalhado de insetos vetores. A utilização desses dados acoplados a avanços em modelagem computacional e tecnologias mais aprimoradas de edição genética, podem conduzir a estimativas mais precisas do risco de doença infecciosas transmitidas por vetores (Chiyaka *et al*. 2013), bem como utilizar ferramentas de modificação de genomas para reduzir a competência ou o tamanho da população (Nguyen *et al*. 2015, Winskill *et al*. 2015).

A análise da expressão genômica de todos os genes anotados revela muitos aspectos importantes da biologia dos insetos, incluindo o compartilhamento ou a especialização de muitas funções gênicas entre os estágios de desenvolvimento de ovos e pupas e entre os estágios ativos de larvas e adultos (Arbeitman *et al*. 2002). Agora é muito factível que pesquisadores individuais obtenham não apenas sequências genômicas completas para o táxon de interesse, mas também informações genômicas específicas para indivíduos dentro desses táxons. Além do sequenciamento genômico do DNA, o sequenciamento massivo paralelo de moléculas de RNA (RNA-seq) também aumentou significativamente as análises genômicas dada a abundância do volume de transcritos altamente quantitativos, bem como da riqueza de informações de sequência, isoforma e expressão para a grande maioria dos genes codificados em uma espécie de vetor. Essa técnica é importante porque o RNA-seq captura em grande parte apenas transcritos que serão expressos, formando um conjunto informativo de sequências de RNA que pode ser gerado de forma acessível e analisado eficientemente, mesmo na ausência de um genoma montado. Conjuntos de sequências formadas *de novo* a partir de transcriptomas de insetos derivados de RNA-seq podem fornecer uma riqueza de informações genéticas e filogenéticas passíveis de poderosas análises evolutivas moleculares e perfis de expressão de genes quantitativos (Martin e Wang 2011).

A maioria das pesquisas publicadas com a tecnologia NGS continua focada em mosquitos (Diptera: *Culicidae*), que são os responsáveis pela transmissão da maioria das doenças vetoriais mais prevalentes em todo o mundo (Nene *et al*. 2007; Arensburger *et al*. 2010; Marinotti *et al*.

2013; Neafsey *et al*. 2015; Jiang *et al*. 2014; Fontaine *et al*. 2015; Chen *et al*. 2015). No entanto, as tecnologias NGS também devem ser mais exploradas em questões relativas a outros vetores importantes, como triatomíneos e carrapatos. Sob uma ampla ótica de abordagens, a tecnologia NGS têm sido usada para examinar fatores relacionados a tamanhos populacionais de vetores (como a biologia reprodutiva e resistência a inseticidas) (Hall *et al*. 2015; Shawn *et al*. 2015) a capacidade de uma espécie resistir à infecção (competência vetorial) (Mitri e Vernick 2012; Massad e Coutinho 2012) e a freqüência do contato humano-vetor (busca do hospedeiro) (Leal *et al*. 2013; McBride *et al*. 2014).

As tecnologias genômicas permitem que questões biológicas complexas que estão refletidas no código genético sejam respondidas e, portanto, oferecem recursos valiosos para aumentar nossa compreensão dos vetores de doenças (Rinker *et al*. 2016). Desde a sequência genômica básica, os polimorfismos de nucleotídeos até os perfis de expressão de RNA, as tecnologias de sequenciamento NGS podem ser aproveitadas para investigar uma ampla gama de perguntas sobre a organização, função e histórias evolutivas de genomas vetoriais. A expertise adquirida por ter acesso a famílias gênicas completas pode criar novas e mais eficientes estratégias de controle de vetores de maneira mais eficiente que os estudos clássicos analisando gene a gene.

1.7. Estrutura da Tese

Os principais interesses de pesquisa dessa tese concentraram-se na investigação da fisiologia intestinal das larvas de *Ae.aegypti*. A compreensão de aspectos moleculares, bioquímicos e fisiológicos associados a digestão larval podem impactar o conhecimento da biologia do vetor adquirido até aqui e contribuir para que novas estratégias de controle sejam reveladas.

A tese está dividida em quatro capítulos de resultados que são apresentados sob a forma de artigos científicos. No primeiro trabalho, introduzimos a busca por genes que codifiquem proteínas pertencentes à família 16 das glicosídeo hidrolases no genoma de *Ae.aegypti*. De acordo com a literatura, enzimas pertencentes a família GH16 estão envolvidas na digestão de diversas ordens de insetos com hábitos alimentares detritívoros. Larvas de *Ae.aegypti* possuem cinco genes codificantes para beta-1,3-glucanases e um gene que codifica uma proteína ligante de beta-1,3-glucana, essas proteínas ligantes estão envolvidas em mecanismos de defesa contra fungos e bactérias. A função de alguns dos genes identificados foi avaliada através da técnica de RNAi, onde foi possível verificar a presença da principapal beta-1-3-glucanase intestinal em larvas de *Ae.aegypti*.

No segundo trabalho, nosso objetivo foi associar a presença das beta-1-3-glucanases em larvas de *Ae.aegypti* à digestão de leveduras. Realizamos uma série de experimentos bioquímicos e fisiológicos utilizando *S. cerevisae* como única fonte de nutrição larval e observamos que larvas de *Ae.aegypti* conseguem se desenvolver completamente em uma dieta exclusiva de leveduras e beta-1,3-glucanase parece ser essencial para a atividade lítica de leveduras em larvas de *Ae.aegypti*.

No terceiro trabalho, cepas de bactérias, leveduras e microalgas foram utilizadas como única fonte nutricional das larvas. Avaliamos o desenvolvimento larval de maneira detalhada, observando parâmetros biológicos como: tempo total de desenvolvimento larval, taxa de emergência, "*sex ratio*", tamanho e sobrevivência dos adultos. Análises da qualidade nutricional entre as diferentes dietas oferecidas e os valores de reserva energética estocados por esses insetos durante a fase larval também foram examinados. Apesar de larvas de mosquito alimentarem-se principalmente dos microrganismos disponíveis no ecótopo aquático, estudos sobre a biologia do desenvolvimento desses vetores sob essas condições fisiológicas são escassos. Com esse trabalho, elucidamos aspectos nutricionais que ainda não haviam sido descritos e destacamos a natureza versátil do *Ae.aegypti* tão bem adaptado a diversas situações de estresse nutricional.

No quarto trabalho, nossa pesquisa voltou-se para a investigação da compartimentalização intestinal de larvas de *Ae.aegypti* a nível transcricional. Além dos experimentos bioquímicos e fisológicos, nosso grupo tem grande interesse em compreender como ocorre a distribuição dos transcritos entre os compartimentos intestinais e assim, associar esses transcritos às funções fisiológicas dessas estruturas já previamente descritas. A indicação de que a compartimentalização fisiológica observada no trato digestório dos insetos possui base transcricional é algo inédito em larvas de *Ae.aegypti*. Estudos moleculares da digestão em insetos, podem proporcionar subsídios para um melhor conhecimento do vetor.

2. Objetivos

2.1. Objetivo Geral

Avaliar aspectos moleculares, fisiológicos e nutricionais na digestão de larvas de *Ae.aegypti*.

2.2. Objetivos específicos

1. Caracterizar bioquimicamente beta-1,3-glucanases digestivas;
2. Detalhar aspectos fisiológicos e bioquímicos da digestão de leveduras;
3. Determinar parâmetros fisiológicos sob dietas com microrganismos específicos;
4. Caracterizar o transcriptoma dos compartimentos intestinais.

3. Resultados e Discussão

Trabalho 1: Biochemical and functional characterization of Glycoside Hydrolase Family 16 genes in *Aedes aegypti* larvae: identification of the major digestive Beta-1, 3-glucanase. Raquel Santos Souza, Maiara do Valle Faria Gama, Renata Schamma, José Bento Pereira Lima, Hector Manuel Diaz-Albiter, Fernando Ariel Genta. **(Submetido para as Memórias do Instituto Oswaldo Cruz)**

Trabalho 2: Digestion of Yeasts and Beta-1, 3-Glucanases in Mosquito Larvae: Physiological and Biochemical Considerations. Raquel Santos Souza, Hector Manuel Diaz-Albiter, Vivian Maureen Dillon, Rod J. Dillon, Fernando Ariel Genta. **(PLoS One 2016; 11: e0151403 DOI: 10.1371/journal.pone.0151403)**

Trabalho 3: Microorganism-based larval diets affect mosquito development, size and nutritional reserve in the yellow fever mosquito *Aedes aegypti* (Diptera: Culicidae). Raquel Santos Souza, Flávia Vírginio, Lincoln Suesdek, José Bonomi Baruffi, Fernando Ariel Genta. **(Submetido para Frontiers)**

Trabalho 4: Transcriptome Sequencing based analysis of compartmentalization in the midgut of Aedes aegypti larvae. Raquel Santos Souza, Caroline da Silva Moraes, Maiara do Valle Faria Gama, Fábio Faria da Mota, Christiane Cardoso, Clélia Ferreira, Walter Terra, Fernando Ariel Genta. **(Submetido para Frontiers)**

ARTIGO 1

Artigo 1: Biochemical and functional characterization of Glycoside Hydrolase Family 16 genes in *Aedes aegypti* larvae: identification of the major digestive Beta-1, 3-glucanase

Autores: Raquel Santos Souza, Maiara do Valle Faria Gama, Renata Schamma, José Bento Pereira Lima, Hector Manuel Diaz-Albiter, Fernando Ariel Genta.

Biochemical and functional characterization of Glycoside Hydrolase Family 16 genes in Aedes aegypti larvae: identification of the major digestive Beta-1,3-glucanase

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Author contribution statement

RSS, HMD, RS, JBRL, and MVFG performed the experiments and analyzed the data. RSS and FAG conceived the study and wrote the manuscript. All authors read and approved the final manuscript.

Keywords

Aedes aegypti, Beta-1,3-glucanase, Knock-down, Digestion, Immunity, Glycoside hydrolase family 16, GHF16

Abstract

Word count: 259

Insect Beta-1,3-glucanases belong to Glycoside Hydrolase Family 16 (GHF16) and are involved in digestion of detritus and plant hemicellulose. In this work, we investigated the role of GHF16 genes in A.aegypti larvae, due to their detritivore diet. The genome of A.aegypti contains six genes coding for GHF16 proteins (Aae GH16.1 - Aae GH16.6), containing 2-6 exons. Phylogenetic analysis suggests that Aae GH16.1, 2, 3, 5 and 6, which contain the GHF16 conserved catalytic residues, are related to other insect glucanases. These genes suffered duplications in the genomes of Nematocera. Aae GH16.4 is related to Beta-1,3-glucan binding proteins, which have no catalytic activity and are involved in the activation of innate immune responses. Additionally, A. aegypti larvae contain significant Beta-1,3-glucanase activities in the head, gut and rest of body. These activities have optimum pH about 5-6 and molecular masses between 41 and 150 kDa. All GHF16 genes above showed different levels of expression in the larval head, gut or rest of body. Knock-down of AeGH16.5 resulted in survival and pupation rates lower than controls (dsGFP and water treated). However, under stress conditions, severe mortalities were observed in AeGH16.1 and AeGH16.6 knocked-down larvae. Enzymatic assays of Beta-1,3-glucanase in AeGH16.5 silenced larvae exhibited lower activity in the gut and no change in the rest of body. Chromatographic activity profiles from gut samples after GH16.5 silencing showed suppression of enzymatic activity, suggesting that this gene codes for the digestive larval Beta-1,3-glucanase of A. aegypti. This gene and enzyme are attractive targets for new control strategies, based on the impairment of normal gut physiology.

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Ethics statements

(Authors are required to state the ethical considerations of their study in the manuscript, including for cases where the study was exempt from ethical approval procedures)

Does the study presented in the manuscript involve human or animal subjects: No

Biochemical and functional characterization of Glycoside Hydrolase Family 16 genes in *Aedes aegypti* larvae: identification of the major digestive Beta-1, 3-glucanase

Running title: GHF16 Beta-1, 3-glucanases in mosquito larvae

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ABSTRACT

Insect Beta-1,3-glucanases belong to Glycoside Hydrolase Family 16 (GHF16) and are involved in digestion of detritus and plant hemicellulose. In this work, we investigated the role of GHF16 genes in *A.aegypti* larvae, due to their detritivore diet. The genome of *A.aegypti* contains six genes coding for GHF16 proteins (Aae GH16.1 - Aae GH16.6), containing 2-6 exons. Phylogenetic analysis suggests that Aae GH16.1, 2, 3, 5 and 6, which contain the GHF16 conserved catalytic residues, are related to other insect glucanases. These genes suffered duplications in the genomes of Nematocera. Aae GH16.4 is related to Beta-1,3-glucan binding proteins, which have no catalytic activity and are involved in the activation of innate immune responses. Additionally, *A. aegypti* larvae contain significant Beta-1,3-glucanase activities in the head, gut and rest of body. These activities have optimum pH about 5-6 and molecular masses between 41 and 150 kDa. All GHF16 genes above showed different levels of expression in the larval head, gut or rest of body. Knock-down of AeGH16.5 resulted in survival and pupation rates lower than controls (dsGFP and water treated). However, under stress conditions, severe mortalities were observed in AeGH16.1 and AeGH16.6 knocked-down larvae. Enzymatic assays of Beta-1,3-glucanase in AeGH16.5 silenced larvae exhibited lower activity in the gut and no change in the rest of body. Chromatographic activity profiles from gut samples after GH16.5 silencing showed suppression of enzymatic activity, suggesting that this gene codes for the digestive larval Beta-1,3-glucanase of *A. aegypti*. This gene and enzyme are attractive targets for new control strategies, based on the impairment of normal gut physiology.

KEYWORDS: *Aedes aegypti*, beta-1,3-glucanase, knock-down, digestion, immunity, Glycoside Hydrolase Family 16, GHF16

INTRODUCTION

Culicides are important mosquitoes in the epidemiology of vector-borne diseases. They were associated with the spread of several infectious diseases since the beginning of the XX century (Ross 1911). *Aedes aegypti* is the main vector of Dengue, Urban Yellow Fever, Chikungunya and Zika viruses in humans (Rodhain and Rosen 1997, Jentes et al. 2010, Leparc 2014, Freitas et al. 2016). *A. aegypti* maintains a strong association with humans, breeding in virtually any container that holds enough water for larval/pupal development (Nelson et al. 1976). These containers are widely available in most developing countries, where water distribution and sanitary conditions are defective (Consoli 1994). Due to a strong dietary preference for human blood, *A. aegypti* is capable of completing the entire life cycle within human dwellings (Edman et al. 1992). In this respect, *A.aegypti* is a vector of enormous medical importance and probably will continue to be for the next years to come (Weaver 2010).

The dengue virus has become the most important arboviral pathogen in recent years, due to its increasing incidence in the tropics and subtropics, as well as its high mortality and morbidity (Murray 2013). It resembles malaria in geographic distribution and could be more meaningful regarding morbidity and economic impact (Gubler DJ 2002 and 2012, Guzman et al. 2010 and Bath et al. 2013). The WHO estimates dengue virus reaching 390 million people per year and 3.9 billion people are living at risk in 128 countries around the world (Brady et al. 2012, WHO 2017). Yellow fever affects around 200,000 people a year, and Chikungunya virus during one outbreak infected more than 1.5 million people in India. Recently, the Zika virus infection has been recognized as a Public Health Emergency of International Concern, due to the related number of newborn infants with microcephaly and high incidence of Guillain-Barré syndrome cases (Vainio and Cutts 1998, Malavankar et al 2008 and WHO 2017). These undesired outcomes increase the need for the creation of new methods to block the development of the mosquito (WHO 2013). Current strategies for facing these infectious diseases rely almost exclusively on vector control efforts (Maciel-de-Freitas et al 2012). There are no drug treatments, and the Dengue vaccine licensed in 2015 was not completely safe and efficient to protect against hospitalization due to dengue and severe dengue in all ages groups (WHO 2017).

Most studies about *A. aegypti* have focused in the physiology of female adults and the knowledge about larval behavior is poor (Clements 2000, Chen et al. 2008, Borovsky 2003, Douglas 2014, Oviedo et al. 2008 and Venancio et al. 2009). The occurrence of *A. aegypti*-transmitted diseases is majorly determined by the presence of larval breeding sites (Kay 1999). Thus, information on larval physiology and biochemistry may expand biological knowledge and result in new insights for vector control (Coutinho-Abreu 2010).

In insects, digestion and absorption of its products occur mainly in the digestive tract (Terra e Ferreira 2005). The gut is a major surface where exchanges with the external environment take place, being a strategic topic of investigation for the screening of targets for insect control. *A. aegypti* larvae had a detritivore feeding mode, filtering solid particles from liquid media and scraping organic material from surfaces. Among the particles ingested by mosquito larvae several microorganisms, such as bacteria, fungi, algae, protozoa, and rotifers have been found inside the gut (Walker et al. 1988, Merrit et al. 1990, HO BC et al. 1992, Avissar et al. 1994 and Muniaraj et al 2012), but the mechanisms used by larvae for the breakdown of these nutritional sources remain unexplored.

Beta-1,3-glucans are polysaccharides that are abundant. They are produced by many organisms such as algae, higher plants, and fungi. Beta-1-3-glucanases are enzymes capable of hydrolyzing beta-1,3 bonds present in beta-1,3-glucans. Beta-1,3-glucanases play an important role in the digestion of detritorous or grass-eating insects (Terra and Ferreira 1994), and they have been found in almost all insect groups (Genta et al. 2003, Genta et al. 2009, Genta et al. 2007, Erthal Jr. et al. 2007, Pauchet et al. 2009, Bragatto et al. 2010, Lucena et al. 2011, Moraes et al. 2012 and Souza et al. 2016).

In addition to their digestive role, in some Beta-1-3-glucanases such as in the lepidopteran *Helicoverpa armigera* and different termite species, a role related to the insect's immune defense has been described (Bulmer and Crozier 2006, Pauchet et al. 2009). Insects express several pattern recognition receptors like beta-glucan recognition proteins (βGRPs), beta-glucan binding proteins (GBP), and gram-negative bacteria binding proteins (GNBP), responsible for triggering the innate immune response (Ochiai 1988, 2000). These receptors are homologs to some Beta-1,3-glucanases, but without catalytic

activity (Bragatto et al. 2010 and Hughes 2012). Both insect Beta-1,3-glucanases and Beta-glucan-binding proteins structurally belong to family 16 of glycoside hydrolases (GHF16) (Genta et al 2009, Bragatto et al 2010).

Our current understanding of the intestinal physiology of mosquito larvae is highly incomplete. This issue becomes more relevant when we consider the great potential of larval stages as targets for vector control. In this study, we identified coding sequences for GHF 16 in the genome of *A. aegypti*, compared the expression of these GHF 16 genes in larval tissues and evaluated the physiological role of some GHF16 by knockdown experiments. We showed that beta-1,3-glucanases are likely to be involved in digestion and recognition of invading microorganisms in *A. aegypti* larvae. Besides that, we were able to identify the gene that is responsible for coding the major larval gut beta-1,3-glucanase. This enzyme might be an interesting target for inhibition studies and the development of a new generation of larvicides, as beta-1,3-glucanases are absent in humans are seem to be essential for mosquito larval physiology.

MATERIALS AND METHODS

Insects rearing and maintenance

Aedes aegypti eggs (Rockefeller strain) were obtained from the colony of the Laboratory of Physiology and Control or Arthropod Vectors (LAFICAVE/IOC-FIOCRUZ; Dr. José Bento Pereira Lima). Insects were reared until adult stage at 26 ± 2°C and 70 ± 10% relative humidity with a 12-h light/12-h dark cycle. For the obtention of synchronized larvae, hatching was induced by adding 100 mL of distilled water into 200 mL plastic cups containing eggs, and then incubating at 28ºC for 30 minutes. After incubation, groups of first instar larvae (n=80) were transferred to plastic bowls containing 100 mL of dechlorinated water plus 0.1g cat food (Whiskas®, Purina, Brazil), and kept at 26±1°C until adult stage. The food was added only once at the beginning of each experiment.

Identification of GHF 16 sequences in the genome of *Aedes aegypti*

Genes belonging to the glycoside hydrolase family 16 (GHF16) (defined as described in the CAZy database) in *Aedes aegypti* genome were characterized using FAT software (Seabra-Junior et al., 2011), which integrates HMMER (http://hmmer.janelia.org/) and BLAST+ tools (Camacho et al., 2009) to filter the initial dataset and perform automatic annotation. The filter step used the HMG-box conserved domain (Pfam code PF00722) to identify and extract only proteins containing such a domain in the *A. aegypti* dataset (VectorBase, http://www.vectorbase.org, *Aedes aegypti* Liverpool, AaegL1.3). The annotation step compared the filtered proteins for similarity with proteins and conserved domains databases using BLAST with nr and Swiss-Prot uniprot databases. All results were manually inspected.

Bioinformatic and phylogenetic analysis of GHF16 sequences

Alignment of the homologous sequences coding for GHF16 proteins from *A. aegypti* and the generation of consensus sequences were performed using the algorithm CLUSTAL (Higgins 1994; http://www.ch.embnet.org/software/ClustalW.html) and the software BIOEDIT (Hall 1999; http: //www.mbio .ncsu.edu / bioedit / bioedit.htm). The sequences obtained were analyzed by the algorithms BLAST (Altschul et al 1990, http://blast.ncbi.nlm.nih.gov/Blast.cgi), signal IP (Dyrløv et al 2004; .dtu.dk / services / SignalP /), NETOGlyc 4.0 (Julenius et al 2005; http://www.cbs.dtu.dk/services/NetOGlyc/), NETNGlyc 1.0 (http: //www.cbs.dtu .dk / services / NetNGlyc /) and ProtParam (Gasteiger et al 2003, http://web.expasy.org/protparam/). Trees were generated using MEGA5.05 (Tamura et al 2011). Bootstrap values were set at 1000 replications.

RNA extraction and cDNA synthesis

Total RNA was extracted from 50 whole larvae in the fourth instar stage and the tissues: head, digestive tract and the rest of the body using the TRI® reagent (SIGMA # T9424) according to the manufacturer's instructions. After extraction, the RNA was quantified using a Nanodrop® (NanoDrop Technologies, Wilmington, USA). To obtain the cDNA, the obtained RNA samples were treated with the DNase of the TURBODNAse FREE kit (Turbo DNA-free^{TM}, Ambion, AM1907), and were subsequently submitted to the reverse transcription reaction (RT), performed according to the protocol of the kit manufacturer SUPERSCRIPT III (Superscript III First-Strand kit, Invitrogen, Cat.no. 18080-051) with an oligo dT (18) primer (PRODIMOL Technology). The material after the RT reaction was treated with RNase H and the cDNA was quantified in Nanodrop again. For further analysis, larval and tissue cDNA samples were normalized to the concentration of 50 ng / μL.

PCR and semi-quantitative RT-PCR

For the amplifications of DNA fragments corresponding to GH16 sequences, specific oligonucleotides were designed (Table S1). PCR reactions were performed with the GoTaq® DNA Polymerase kit (Promega) and the constitutive gene RP49 as a control

(Gentile et al 2005). Each reaction (20 μL) contained buffer 1X, dNTP (0.2 mM), $MgCl_2$ (1,5 mM), oligonucleotides (10 μM each), Taq DNA polymerase (0, 025 U) and 1 μL of cDNA or genomic DNA (50 ng/μL). Each reaction consisted in a varied number of cycles with intervals of 1 minute at 94°C (denaturation), 30 seconds at 55°C (annealing) and 1.5 minutes at 72°C (extension). Different numbers of cycles were performed in each experiment, with 40 cycles for the initial experiments, and a range from 24 to 40 cycles for the semi-quantitative determination of relative expressions, and for the confirmation of the silencing of the genes after larval feeding with dsRNA.

Electrophoresis and densitometric analysis

PCR and RT-PCR products were subjected to agarose gel electrophoresis with a final concentration of 1% (w/v) in TBE buffer. After electrophoresis, the material was evidenced with ethidium bromide solution (0.5 μg/mL) and visualized in a UV light transilluminator (312 nm). Gels were photographed (E-Gel Image, Life Technologies, USA) and analyzed with the ImageJ program (Sheffield 2007), generating semi-quantitative profiles of gene expression based on the intensity of bands developed with UV light, subtracting the background from each lane.

Preparation of dsRNA

Specific primers were designed for the synthesis of dsRNA (Table S2). We used QIAquick PCR Purification Kit or QIAquick Gel Extraction Kit (QIAGEN, USA) to purify PCR products. For the *in vitro* transcription and purification of dsRNA, we used the MEGAscript® RNAi Kit (Ambion, Life Technologies, USA). Purified dsRNA was quantified using Nanodrop®.

Feeding with dsRNA and measurement of biological parameters

We investigated if feeding larvae of *A.aegypti* with dsRNA coding for different GHF16 gene sequences could have an impact in the development of fourth instar larvae. In all experiments, larvae in the L2 larval stage were immersed in solutions containing 0.5 μg/μL dsRNA (prepared as above) plus 2.5% (w/v) bromophenol blue according to Singh

et al. (2013). Sixty insects were kept in this solution for 2 hours at room temperature. After this, 20 insects were selected according to the intensity of the blue color in their digestive tract. Those with gut contents with intense blue color were then transferred to individual pots and maintained until adulthood. After dsRNA treatment, survival and pupation were followed in experiments where 20 larvae were kept in containers containing 100 mL of filtered water and 0.001 mg of cat food (Whiskas® - Masterfoods Brasil Alimentos Ltda). In experiments under stress conditions, in each group after dsRNA treatment, 120 larvae were kept in microtubes containing 1 mL of filtered water and no nutritional source during two days. Larval and pupal mortality, pupation and emergence were monitored and recorded daily. Pupation and emergence data were plotted and compared using the Log-rank (Mantel-Cox) test. Mortality and weights were expressed as means ± SEM, and non-transformed data were compared by ANOVA or pairwise t-tests.

Preparation of samples for enzymatic assays

Fifty larvae were immobilized by placing them on the ice, after which they were dissected in cold 0.9% (w/v) NaCl. During dissection of each larva, we separated the head and the entire gut, and the remaining tissues were assembled and named as ¨rest of body¨ samples. 10 Heads and rest of bodies were homogenized in MilliQ water with the aid of a microtube pestle (Model Z 35, 997-1, Sigma, USA), using the proportion of 100 µL of water for ten insects. Ten guts were homogenized in 100 µL of cold MilliQ water containing 2.5 µL of 20 mM phenylmethylsulfonyl fluoride (PMSF), 20 µM Pepstatin A and 20 µM trans-epoxysuccinyl-L-leucyl amino (4-guanidino)butane (E-64). All samples were centrifuged for 10 min at 14,000-x *g* at 4°C. Both pellets and soluble fractions were stored at -20°C until used as enzyme source for enzymatic assays.

Enzymatic assays and effect of pH

Beta-1,3-glucanase activity in *A. aegypti* larvae was determined by measuring the release of reducing groups from 0.25% (w/v) laminarin from *Laminaria digitata*, (SIGMA Cat. no. L9634) in a thermocycler with a modified bicinchoninic acid reagent (Lucena et al.2013). The influence of pH in the enzymatic activity was studied using the buffers:

sodium citrate (pH 3-7, 200 mM), EPPS (pH 7-9, 200 mM), AMPSO (pH 9-10, 200 mM) and CAPS (pH 10-11, 200 mM) with overlapping pH values to rule out possible inhibition by the buffering species. All assays used the buffers at a final concentration of 83 mM and were performed at 30ºC under conditions such that activity was proportional to protein concentration and time. Controls without enzyme or substrate were included. One unit of enzyme (U) is defined as the amount that hydrolyzes 1 µmol of glycosidic bonds per min, using a glucose standard curve in the same conditions. Comparisons between means of two independent groups were made with a pairwise t-test. Results are expressed as the group mean ± SEM.

Chromatography and determination of molecular masses by gel filtration

Samples containing 50 guts, 50 heads and 50 rest of bodies were homogenized in 100 µL of 10 mM Phenylthiourea (PTU) and 600 µL of 50mM citrate buffer pH 7.0 containing 150 mM NaCl. Additionally, gut samples also were homogenized in 10 µL of PMSF, (20 µM), 10 µL of Pepstatin A (20 µM) and 10 µL of E-64 (20 µM). The samples were then centrifuged for 10 min at 10.000g at 4ºC and the soluble fractions were collected. Samples with 500 µL from each of the soluble fractions obtained from tissues were applied into an HR 10/10 Superdex 200 column (GE Healthcare Biosciences) equilibrated with 50 mM citrate buffer pH 7.0 containing 150 mM NaCl. Proteins were eluted with the same buffer (30 mL), with a flow of 0.5 mL/min, and fractions of 0.5 mL were collected and assayed for enzymatic activity. The assays were performed with 35 µL of each fraction and 25 µL of laminarin 0.25% (w/v) in deionized water (Millipore, USA). Molecular mass standards used were: aprotinin (6.5 kDa), cytochrome C (12.4 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), amylase (200 kDa) and blue dextran (2000 kDa). Molecular masses of eluted activities were calculated using the correlation between K_{av} and log10 of molecular mass (Bonner, 2007).

Statistical analysis

Linear regressions were performed using Microsoft Excel (Microsoft). Statistical comparisons were made using GraphPad Prism software (version 7.0, GraphPad Software Inc.). Significance was considered when $p<0.05$.

RESULTS

Six sequences of genes coding for proteins belonging to the family 16 of glycoside hydrolases (GHF16) were found in the *A.aegypti* genome. They were named AaeGH16.1, AaeGH16.2, AaeGH16.3, AaeGH16.4, AaeGH16.5 and AaeGH16.6 (GenBank codes: EAT44802.1, EAT44801.1, EAT41280.1, EAT40654.2, EAT40654.2, EAT38986.1). These genes contain 2 to 6 exons (Figure 1).

Figure 1. Schematic diagram of exons and introns in the genes coding for glycoside hydrolases of Family 16 in the *A. aegypti* genome.

We performed the alignment of the amino acid sequences of each predicted protein with the homologous sequences found in different databases. We also analyzed aspects such as the presence of peptide signals, glycosylation sites, and conserved catalytic residues. Of the six *A.aegypti* GH16 sequences, five (AaeGH16.1, AaeGH16.2, AaeGH16.3, AaeGH16.5 and AaeGH16.6) showed putative signal peptides and the typical conserved catalytic glutamate residues of this GH family, that are included in the consensus region SGE(I/V)DL(M/L)ES(R/K). The only protein in this group of GHF16 sequences that did not show a putative signal peptide was AaeGH16.4. AaeGH16.4 also do not present the conserved catalytic glutamate residues (Figure 2).

Figure 2. Alignment (Clustal X program) of the amino acid sequences of members of the family 16 of glycoside hydrolases in the *A. aegypti* genome. Hypothetical glycosylation sites are in bold and underlined, residues with similar properties are in gray and fully conserved residues in black. Putative signal peptides are boxed. The catalytic glutamate residues are marked by a circle.

These six GHF16 genes encode proteins with distinct predicted molecular masses and isoelectric points, ranging from 39 to 57 kDa, and from 4.9 to 9.8, respectively. They also present a varying number of putative N- and O- glycosylated residues, ranging from 0 to 5 and 0 to 4, respectively. All sequences have only one conserved GH16 domain (Table 1).

Table 1. Summary of the gene sequence characteristics of the glycoside hydrolases of family 16 that are present in *A. aegypti* genome. CDD (Conserved Domain Database), CDS (protein coding sequence), ND (unidentified).

We also constructed phylogenetic trees using a Neighbor-Joining algorithm to have a better understanding of the relationships among the GH16 genes from A. aegypti and other insect sequences of this protein family (Figure 3). A second aim was to locate this protein in two well-known functional groups in GHF16, namely β-glucanases and β-glucan binding proteins. Analysis of GH16 sequences from several insect orders revealed two major clades: one clade includes sequences that lack the catalytic glutamates and another clade including sequences containing the conserved catalytic residues (Figure 3). The same analysis indicates a monophyletic group containing all putative Beta-1,3-glucanases that bear the conserved catalytic residues. Some of these sequences were annotated in the databases as Gram-Negative Binding Proteins (GNBPs) or glucan binding/recognition proteins (GBPs or GRPs), as is the case of many putative enzymes of the genus *Anopheles*. The cladogram also shows a paraphyletic group of putative beta-glucan binding protein sequences lacking the catalytic residues. The arrangement of the sequences in the clades also suggests the occurrence of an expansion in the genes of Beta-1,3-glucanases in the Nematocera dipterans. Sequences from *A. aegypti* containing the catalytic residues (AaeGH16.1, 2, 3, 5 and 6) grouped in branches with sequences of other Nematoceran dipterans, suggesting the diversification of these genes in the ancestor of the suborder.

Figure 3. Cladogram of selected protein sequences of insect Beta-1, 3-glucanases, and Beta-1,3-glucan binding proteins. Branches are statistically supported by bootstrap analysis (cutoff 70%). The blue branches discriminate the monophyletic sequences of the insects that have the conserved catalytic glutamates; the green branches discriminate the paraphyletic group of sequences that do not have the conserved catalytic residues. The bootstrap values were obtained from the analysis of 10000 replicates, using the Neighbor-Joining algorithm (MEGA software 5.05). Consensus phylogenetic tree used sequences of: Anopheles gambiae (AGAP002798-PA, AGAP002799-PA, AGAP002796-PA, AGAP006761-PA, AGAP012409-PA), Anopheles christyi (ACHR004102-RA, ACHR005689, ACHR008721-RA,

ACHR001881-RA, ACHR009179-RA), Anopheles darlingi (ADAR007290-PA, ADAR007286-PA, ADAR006526-PA, ADAR009199-PA), Anopheles dirus (ADIR003516-RA, ADIR010616-RA, ADIR003518-RA, ADIR003625-RA, ADIR000553-RA) Anopheles epiroticus (AEPI010194-RA, AEPI009256-RA, AEPI005496-RA, AEPI002293-RA), Anopheles funestus (AFUN006014-RA, AFUN009437-RA, AFUN006016-RA, AFUN002755-RA, AFUN004083-RA), Anopheles minimus (AMIN004837-RA, AMIN003902-RA,AMIN003903-RA,AMIN003900-RA,AMIN010081-RA,AMIN008919-RA), Anopheles quadriannulatus (AQUA008516-RA, AQUA009400-RA, AQUA009402-RA,AQUA003848-RA,AQUA014348-RA), Anopheles stephensi (ASTE003966-RA, ASTE009324-RA ,ASTE009326-RA,ASTE010371-RA, ASTE004573-RA),Culex quinquefasciatus (XM_001845911.1,X M_001845228.1, XM_001845913.1, XM_001845759.1, JF907421.1, XM_002135149.1, XM_001845915.1, XM_001847484.1, XM_001847484.1, XM_001845910.1,XM_001845757.1,XM_001864211.1,XM_001845229.1), Phlebotomus papatasi (PPATMP000880-PA, PPATMP002587-PA, PPATMP002588-PA, PPATMP010440-PA), Rhodnius prolixus (RPRC011769-PA, RPRC003210-PA,ABU96697.1),Simulium vittatum (EU930267.1),Anopheles arabiensis (ACN38171.1,CAO83421.1),Anopheles bwambae (ABU80038.1), Anopheles melas (ABU80011.1), Anopheles merus (ABU80005.1,AAZ08489.1, AAZ08502.1),Ochlerotatus triseriatus (ACU30929.1), Phlebotomus perniciosus (ADH94599.1). The code of the other sequences can be seen in Table S3.

Because several members of GHF16 have beta-1,3-glucanase activity, and because previous work has demonstrated this activity in the head, gut and rest of body of *A. aegypti* larvae (Souza et al., 2016), we decided to further characterize the beta-1,3-glucanase activities in the tissues of this insect. Initially, we compared the molecular properties of these activities among them and with the properties expected from the coding sequences of *A. aegypti* GHF16 genes. Initially, we determined the effect of pH on the Beta-1,3-glucanases activities obtained from each tissue. *Aedes aegypti* beta-1,3-glucanases extracted from head, gut or rest of body presented maximum activities between pH 6-9, 5-9 and 5-10, respectively (Figure 4).

Figure 4. Effect of pH on Beta-1,3-glucanase activities obtained from the head (A), digestive tract (B) and rest of the body (C) of *A. aegypti* fourth instar larvae. Activity was determined using laminarin as a substrate and buffers: sodium citrate (pH 3-7), EPPS (pH 7-9), AMPSO (pH 9-10) and CAPS (pH 10-11).

We decided to submit the soluble fractions from the larval gut, head or rest of body to gel filtration chromatography, in order to compare the presence and molecular masses of beta-1,3-glucanase isoforms in those tissues. The results are presented in figure 5. The beta-1,3-glucanase activities from all tissues of *A. aegypti* larvae were eluted as one single peak (Figure 5), but with quite different molecular masses when we compare the tissues to each other. Molecular masses observed for beta-glucanases from head, gut and rest o body were respectively 142, 41 and 150 kDa (Figure 5).

Figure 5. Activity against laminarin in fractions obtained after gel filtration chromatography (Superdex 200 - AKTA FPLC) of soluble fractions obtained from the (A) head, (B) digestive tract and (C) rest of the body of Aedes aegypti larvae.

We decided to follow the expression of GHF16 coding transcripts in the head, gut, and the rest of the body of *A. aegypti* larvae, trying to correlate the expression of some particular gene to the beta-1,3-glucanase activities characterized above. Using specific oligonucleotides (Table S1), we were able to amplify fragments with the expected size both from genomic DNA (PCR, Figure 6) and cDNA (RT-PCR, Figure 7). Amplification from genomic PCR confirmed the presence and structure of the six GHF16 genes in the genome of *A. aegypti*. Amplification from cDNA obtained from entire larvae (Figure 7A), heads (Figure 7B), guts (Figure 7C) and rest of bodies (Figure 7D) showed that all larval tissues express the six GHF16 genes at different levels.

Figure 6. PCR amplification of fragments of genes encoding GHF16 proteins from the genomic DNA of *Aedes aegypti*. Lane 1 - 1Kb molecular standard PROMEGA # 100787-018; Lane 2 - RP49 - ribosomal constitutive gene; Lanes 3 -8: AeGH16.1,

AeGH16.2, AeGH16.3, AeGH16.4, AeGH16.5 and AeGH16.6, respectively. Genomic DNA was extracted from larvae, and specific primers were used for each gene. After 40 cycles of amplification, the PCR products were analyzed on agarose gel and developed with ethidium bromide.

Figure 7. Amplification of fragments of the transcripts encoding GHF16 proteins from the cDNA obtained from (A) Entire fourth instar larvae, (B) heads, (C) digestive tracts and (D) rest of bodies of *A. aegypti* larvae. Lane 1 - Molecular standard of 100pb PROMEGA # 15628-019; Lanes 2 – 7 - AeGH16.1, AeGH16.2, AeGH16.3, AeGH16.4, AeGH16.5 and AeGH16.6, respectively. Lane 8 - RP49 - constitutive ribosomal gene. RNA was extracted from the larvae and tissues, the cDNA generated from that RNA by RT was used for PCR reactions with specific primers. After 40 cycles of amplification, the PCR products were analyzed on agarose gel and developed with ethidium bromide.

To have a more defined picture of the specificity of the expression levels of these genes in different tissues and the whole larvae, we performed RT-PCR reactions using increasing numbers of cycles for each gene and sample. In this way we obtained saturation curves of the amplified products, allowing us to locate higher levels of expression of one or more genes to the larval stage or specific tissues of the larvae (Figures 8A-D). Whole larvae seem to have higher expression of AaeGH16.4, AeGH16.5, and AaeGH16.6. This is particularly visible after 27 cycles of RT-PCR amplification (Figure 8A). In the head of the larvae, the most expressed genes are AeGH16.1 and AaeGH16.4, a clear picture visible after 27 cycles (Figure 8B). In the gut, AaeGH16.5 and AeGH16.6 are the most expressed genes. This is observed after 24 cycles of amplification (Figure 8C). In the rest of the body, AaeGH16.1 and AeGH16.4 are the most expressed genes (27 cycles; Figure 8D). The genes AaeGH16.2 and AaeGH16.3 appear to have low expression levels in *A. aegypti* larvae in general.

Figure 8. Relative expressions of the genes encoding GHF 16 proteins in fourth instar larvae of *A. aegypti*, measured in series of semi-quantitative RT-PCR reactions with increasing number of cycles (24-40). Numbers are relative expressions normalized using the gene RP49 as a constitutive control. cDNA samples were prepared as described in Methods sections from (A) whole L4 larvae, (B) heads,

(C) guts and (D) rest of bodies. 1-6 correspond to the relative expression levels of AaeGH16.1-6, respectively.

To have a better understanding of the physiological role of the genes with higher expression in the larval tissues (AaeGH16.1, AaeGH16.4, AaeGH16.5, and AaeGH16.6), we fed third instar larvae of *A. aegypti* with dsRNA specific for each gene, checking for RNAi knock-down effects and their resulting phenotypes. As controls, we used larvae fed only with water and larvae fed with dsRNA coding a GFP sequence. We evaluated the survival of the larvae until the fifth day after ingestion of dsRNA. Survival curve data indicate that knockout of the gene AeGH16.5 is associated with increased mortality, followed by AeGH16.6, AeGH16.4, and AeGH16.1 genes, compared to the GFP and water control groups (Figure 9).

Figure 9. Survival curve of *A. aegypti* fourth instar larvae after feeding with dsRNA. dsGH1, dsGH4, dsGH5, dsGH6 correspond to larvae fed with dsRNA coding respectively for the genes AaeGH16.1, AaeGH16.4, AaeGH16.5, and AaeGH16.6. GFP and H2O correspond to the control groups fed with dsGFP or water only. Comparison of survival curves *vs* the control treated with dsGFP used the Log-rank test (*- $p < 0,05$; **- $p < 0.01$; *- $p < 0.001$).**

The next parameter evaluated after treatment with dsRNA was pupation of larvae. The group treated with dsRNA coding for AaeGH16.4 was the only one that presented pupation similar to the controls (Figure 10). Contrastingly, all other treated groups showed lower pupation rates, especially the larvae treated with dsRNA coding for the gene AeGH16.5, which had the lowest proportion of pupae produced during the experiment (Figure 10). Larvae treated with dsRNA coding for the genes AeGH16.1 and AeGH16.6 showed intermediate results (Figure 10). No changes were observed in the external appearance or general behavior of the larvae in any of the experimental groups.

Figure 10. Percentage of *A.aegypti* fourth instar larvae and pupae in after treatment with dsRNA and following for 12 days in control diet (cat food). GH1/GH4/GH5/GH6/GFP/H2O correspond to larvae exposed to dsRNA coding for AaeGH16.1, AaeGH16.4, AaeGH16.5, AaeGH16.6, GFP, and water respectively. Comparison of groups *vs* the control treated with dsGFP used the Chi-square test (*- $p < 0,05$; **- $p < 0.01$).

Mosquito larvae go through enormous biotic and abiotic challenges that affect their development and survival. Once we detected moderate phenotypes both in larval survival (Figures 9) and pupation (Figure 10), we decided to repeat these experiments under stress conditions. We monitored larvae after the treatment of larvae with dsRNA coding for genes whose knockdown resulted in higher mortality in the larvae and a longer pupation delay (AeGH16.1, AeGH16. 5 and AeGH16.6). We isolated a larger number of larvae and restricted them in much smaller compartments and, after treatment with dsRNA, no food was added for two days. These unfavorable conditions of high density and food restriction resulted in the death of all larvae after treatment with dsRNA coding for the genes AaeGH16.1 and AaeGH16.6 (Figure 11). Larvae treated with water only showed 100% survival, and groups treated with dsRNA for GFP or AaeGH16.5 showed intermediate but similar rates of survival, around 30-50% (Figure 11).

Figure 11. Mortality and survival of *A. aegypti* fourth instar larvae treated with dsRNA under conditions of high density and food restriction. GH1, GH5, and GH6 correspond to insects treated with dsRNA coding for AaeGH16.1, AaeGH16.5, and AaeGH16.6, respectively. GFP and H2O correspond to controls treated with dsRNA coding for GFP and water, respectively. Comparison of groups *vs* the control treated with dsGFP used the Fisher´s exact test (- $p < 0.01$; ****- $p < 0.0001$).**

Despite the observation of phenotypes described above, we decided to confirm the knockdown effect of genes AeGH16.1, AeGH16.4, AeGH16.5, and AeGH16.6 after feeding larvae with dsRNA. For that, five days after treatment, we isolated the total RNA

from whole larvae, gut and rest of bodies, and analyzed the expression levels of each transcript. We were able to observe a significant silencing effect on whole larvae for the AeGH16.4 gene, in the digestive tract only for the AeGH16.5 gene and in the rest of the body only in the AeGH16.6 gene (Figure 12).

Figure 12. Levels of expression of the genes AeGH16.4 (GH4), AeGH16.5 (GH5), and AeGH16.6 (GH6) in *A. aegypti* fourth instar larvae after treatment with dsRNA. (A) Relative expression of the gene AeGH16.4 in whole larvae. (B) Relative expression of the gene AeGH16.5 in the gut. (C) Relative expression of the gene AeGH16.6 in the rest of the body. Expression levels were normalized using the ribosomal RP gene 49 as the constitutive marker. We used 24 cycles for AeGH16.4 and 27 cycles for AeGH16.5 and AeGH16.6 in the RT-PCR reactions. See Material and Methods for details.

To further investigate the physiological and biochemical role of GHF16 genes in *A. aegypti*, we decided to assay the Beta-1,3-glucanase activity in larvae silenced for the genes AaeGH16.5 and AaeGH16.6. Several insect GHF16 genes are digestive beta-1,3-glucanases, and AaeGH16.5 and AaeGH16.6 showed their highest relative expression in the gut of larvae (Figure 8). We observed a significant decrease in the beta-1,3-glucanase activity in the gut soluble fraction of larvae treated with dsRNA coding for AeGH16.5 gene, with no changes in the gut activity of larvae treated with dsRNA coding for AaeGH16.6 or GFP (Figure 13A) when compared to controls treated with water only. No changes in the soluble beta-1,3-glucanase activity of the rest of body were observed after any of the treatments above (Figure 13B).

Figure 13. Beta-1,3-glucanase activity (laminarin as substrate) in the soluble fractions of the digestive tract (A) and rest of the body (B) of fourth instar larvae of *A. aegypti* treated with water (H2O) or dsRNA coding for GFP, AaeGH16.5 (dsGH5), and AaeGH16.6 (dsGH6). Comparison of groups *vs.* the control treated with dsGFP used the t-test (*- $p < 0, 05$).

We submitted the soluble fractions of the gut from larvae treated with dsRNA to gel filtration chromatographies, to verify if some change in the molecular mass of the major isoform had occurred after treatment. Gut samples from the insects fed with dsRNA coding for AeGH16.5 showed a significantly lower activity peak in the chromatographic profile when compared to the other groups (Figure 14).

Figure 14. Beta-1,3-glucanase activity (laminarin substrate) after gel filtration chromatography (Superdex 200 column / AKTA-FPLC) of the soluble fraction of the gut from L4 larvae of *A. aegypti* after ingestion of (A) water, (B) dsRNA coding for GFP, (C) dsRNA coding for AaeGH16.5 and (D) dsRNA coding for AaeGH16.6. The data presented is the absorbance increase (ΔAbs) relative to the baseline of the chromatographic profile after incubation with laminarin. The experiment was performed independently twice.

DISCUSSION

Intestinal beta-1,3-glucanase activities were already described in cockroaches (Genta et al 2003), termites (Lucena et al 2011), grasshoppers (Genta et al 2007), beetles (Genta et al 2009), moth larvae (Pauchet et al 2009, Bragatto et al 2010), sandfly larvae (Moraes et al 2012; 2014), and recently in *A.aegypti* larvae (Souza et al 2016). These enzymes belong mainly to the family 16 of glycoside hydrolases (Davies and Henrissat 1995, Coutinho and Henrissat 1999). The search in the genome of *A. aegypti* revealed six sequences with shared characteristics among members of the family 16 of glycoside hydrolases (Table 1 and Figure 1), such as the PFAM-00722 domain (Bateman et al., 2004). Five of the six *A. aegypti* sequences (AeGH16.1, AeGH16.2, AeGH16.3, AeGH16.5 and AeGH16.6) had conserved glutamate residues within the consensus region SGE(I/V)DL(M/L)ES(R/K), acting as donors and proton acceptors for the enzymatic activity of Beta-1,3-glucanases (Hahn et al 1995), while such residues were absent in the AeGH16.4 sequence (Figure 2).

The active site glutamate residues are essential for the catalytic activity of Beta-1,3-glucanases (Zhang et al. 2003), and are conserved in all glucanases subfamilies but not in Beta-1,-3-glucan binding proteins (βLP) or beta-1,-3-glucan recognizing proteins (βRP). Thus, it is likely that *A. aegypti* has five Beta-1,3-glucanases with conserved catalytic regions and that AeGH16.4 would be a βLP or βRP, having no signal peptide sequence or conserved catalytic residues. The functional divergence between glucanases and βLP / βRP is known to be involved in the loss of the catalytic activity of Beta-1,3-glucanase and the addition of an N-terminal region or a carbohydrate recognition domain (Zhang et al. 2003; Pauchet et al. 2009). Since they are homologous proteins, some authors suggest that Beta-1,3-glucanases and βLP / βRP originated from the duplication of an ancestral Beta-1,3-glucanase gene originating in the predecessor of Hexapoda, and that βLP / βRP had lost their catalytic activities but maintained the characteristic of recognition and binding to polysaccharides such as Beta-1,3-glucans (Bragatto et al. 2010, Hughes 2012).

Previous sequence comparisons and phylogenetic analysis supported an evolutionary relationship between Beta-1,3-glucanases and Beta-glucan binding proteins belonging to GHF16 (Pauchet et al. 2009, Bragatto et al. 2010, Hughes 2012). Consistently, two main

clades were found in our analysis (Figure 3). (1) A monophyletic clade assembling the sequences with conserved catalytic residues (β-1-3-glucanases) and (2) a paraphyletic clade, which did not show catalytic residues (βLP). It has been proposed that the animal Beta-1,3 glucanase ancestral gene suffer a duplication. Thus, insects should bear at least two copies of genes from GHF16 (Bragatto et al. 2010). Our results show that many additional Nematoceran dipteran sequences are clustered into monophyletic sub-branches of Beta-1,3-glucanases, suggesting that several duplication events might have occurred in the Beta-1,3-glucanase gene family present in the Culicidae ancestor, resulting in at least five beta-glucanase paralog genes in the genomes of *Culex*, *Aedes*, and *Anopheles*.

The description of the beta-1,3-glucanase activity in Culicidae larvae is recent (Souza et al. 2016). Beta-1,3-glucanase seems to be an important enzyme for larval nutrition in *A. aegypti* and might be an interesting target for inhibition, as mammals lack this enzyme (CAZY, www.cazy.org). Beta-1,3-glucanase might be an essential enzyme for mosquito larvae feeding on fungi, as mechanical disruption of cells in insect digestion is negligible, and chemical breakdown of cell wall polysaccharides in necessary to permit access to intracellular nutrient sources as proteins, glycogen and nucleic acids (Terra and Ferreira 1994, 2005).

The optimum pH of *A.aegypti* larvae beta-1,3-glucanases is similar to the observed in other insects as *Periplaneta americana* (pHo=6; Genta et al 2003), *Tenebrio molitor* (pHo=6; Genta et al., 2009), *Abracris Flavolineata* (pHo=6; Genta et al., 2007), and *Lutzomia longipalpis* (pHo=6-8; Moraes et al., 2012). Although the maximum activity range of the enzymes in the different tissues is similar, the comparison suggests the presence of different Beta-1,3-glucanases among the tissues of the larvae (Figure 4). Coherently, beta-1,3-glucanases obtained from each tissue presented different molecular masses (Figure 5). The molecular mass of beta-1,3-glucanases recovered from head and rest of body were strikingly different from the observed for the enzyme from the gut, which showed a molecular mass similar to the measured for another insect intestinal Beta-1,3-glucanases (Genta et al. 2003, 2007, 2009, Bragatto et al. 2010).

The luminal pH of *A. aegypti* midgut is buffered around 7, 11, 8 and 7 in the gastric caeca, anterior midgut, posterior midgut, and hindgut, respectively (Linser et al., 2009). It is interesting to notice that gut and head beta-1,3-glucanases of this insect retain significant

activities even at highly basic pH values like 10, with 80 and 50% of maximal activity, respectively. In this way, these enzymes are probably active in the anterior compartments of the midgut, where initial digestion of the ingested fungal cells might take place. A recent report suggests that ingested yeast cells are killed quickly, probably in the initial portions of the larval midgut (Souza et al., 2016).

Beside the biochemical characterization, new approaches as molecular biology studies are required to understand better the role of beta-1,3-glucanases in *A. aegypti* larvae. Initially, we found the expected size of amplified fragments in PCR reactions using both genomic DNA and cDNA from larvae (Figures 6 and 7), confirming the presence and structure of several GHF16 genes in the genome of *A. aegypti*. The expression level of GHF16 genes was analyzed in entire *A. aegypti* larvae, head, gut and rest of body. All six genes were expressed at different levels in larvae and the tissues (Figure 7). To date, gene expression studies in *A. aegypti* larvae are limited, but in general, they show that genes involved in the developmental programs of this mosquito are highly stage-specific (Harker et al. 2013). There is a summary of the general expression of mosquito genes in the Vector Base database (https://www.vectorbase.org/), but the data are focused mostly in pupae and adults.

Experiments using the RNAi technology have greatly enhanced knowledge about gene functions and, because of their specificity, the RNAi technique also offers excellent potential for pest control strategies (Baum et al. 2007, Price and Gatehouse 2008, Huvenne and Smagghe 2010, Singh et al. 2013). To investigate the roles of GHF16 genes in *A. aegypti* larvae, we selected four of the six genes found for knock-down, based on the expressions of these genes in entire larvae or larval tissues (AaeGH16.1, AaeGH16.4, AaeGH16.5, and AaeGH16.6; Figure 8). Silencing of AeGH16.5 resulted in the highest observed mortality rate, followed by AaGH16.6, AaGH16.1, and AaGH16.4 (Figure 9). Accordingly, AaeGH16.5 knockdown resulted in the lowest pupation rate, followed by AaeGH16.6 (Figure 10). Mortality and the smaller pupation rate in the larvae fed with the dsRNA targeting the AeGH16.5 gene suggest a digestive function since the development of the larvae seems to be impaired.

It has been shown that nutritional stress in mosquito larvae leads to the emergence of adults with immune deficiency (Telang 2012). To test a possible phenotype related to the

immune function of these genes, we kept the larvae under stress conditions for five days after gene silencing. The larvae were kept in small containers with high population density and were not fed for two days. We observed that these conditions resulted in 100% mortality in larvae knocked-down for the genes AeGH16.1 and AeGH16.6. The AaeGH16.5 knockdown also resulted in high mortality, but at similar rates than the dsGFP control (Figure 11). It is possible that the genes AeGH16.1 and AeGH16.6 exert an immune function in the larvae and that the adverse conditions in which the larvae remained in five days have challenged this system culminating in their death.

We were able to obtain transcriptional knock-down results in whole larvae for the AeGH16.4 gene, and in gut and rest of the body for the genes AaeGH16.5 and AaeGH16.6, respectively (Figure 12). Although these two last genes are more expressed in the gut, AeGH16.6 has been knocked-down only in the rest of the body. The reason for this tissue specificity in silencing is still not clear, but this lack of response in some tissues must be considered when interpreting the phenotypes for each gene.

It was not possible to obtain an effective knock-down of the gene AeGH16.1. One of the generally limiting factors for the RNAi technique is the internalization of the dsRNA by cells. Techniques such as soaking, feeding or injection of dsRNA require the absorption of dsRNA molecules by the cells, which may or may not occur. In this work, we believe that the knock-down effect due to ingestion of dsRNA can spread from the gut to other tissues of the body, as described in other studies (Zangh et al. 2010, Singh et al. 2013). Considering that, it is also not clear to us why the gene AaeGH16.1 was not silenced in the conditions tested. It is noteworthy that even without a significative knockdown we were able to detect important phenotypes in the larvae treated with dsRNA targeting this gene. It is possible that even a small or temporary knockdown, not detected by semi-quantitative RT-PCR, resulted in physiological impairment, resulting in larval mortality (Figure 9), delay in pupation (Figure 10) and inability to cope with nutritional stress (Figure 11).

The knockdown experiments of all the GHF16 genes tested here resulted in moderate phenotype under normal conditions and severe phenotype under stressful conditions. In insects, most of the RNAi experiments using the soaking technique were performed on cell lines. This technique appears to present a less potent knockdown than microinjections

directly into the hemocoel, due to barriers such as the insect cuticle. However, it appears to be much more applicable and effective in cell cultures. Thus, it is possible that the observed knockdown for some GHF16 genes in *A. aegypti* larvae has not reached its full potential. Nevertheless, our experiments strongly suggest that it is possible to use the soaking technique for functional screenings on whole insects efficiently.

Beta-1,3-glucanase enzymatic assays were also performed on tissue samples obtained from silenced *A.aegypti* larvae. Gut and rest of the body samples were evaluated in larvae silenced for the genes AaeGH16.5 and AeGH16.6. In the assays of guts of larvae treated with dsRNA for AaeGH16.5, the beta-1,3-glucanase activity suffered a considerable decrease; however, there were no changes in the activity found in the rest of the body (Figure 13). Gel filtration chromatography showed a significantly lower peak of Beta-1,3-glucanase for the gut of larvae treated with dsRNA for AeGH16.5 when compared to larvae treated with dsRNA for AeGH16.6 or the control groups (Figure 14). These data strongly suggest that AeGH16.5 code for the major digestive beta-1,3-glucanase of *A. aegypti* larvae. Due to the small size of *A. aegypti* larvae and small amounts of protein in gut samples from this insect, the identification of digestive enzymes using the traditional techniques of purification and characterization are strongly hindered. In this way, the use of an efficient knockdown strategy was extremely important for the identification of the major gut beta-1,3-glucanase of this insect as coded by the gene AaeGH16.5.

One putative role of the AaeGH16.5 protein in the gut of *A. aegypti* larvae is the digestion of ingested fungal cells. The dependence on beta-1,3-glucanase activity for the breakdown of yeast cells in *A. aegypti* was already established using lytic assays and competition with laminarin, a canonical substrate for this enzyme (Souza et al., 2016). In other insects, gut beta-1,3-glucanases were already implicated in digestion of fungal cells (Genta et al., 2003; Genta et al., 2009; Lucena et al., 2011; Moraes et al., 2012; Moraes et al., 2014) or plant hemicelluloses (Genta et al., 2007; Bragatto et al.. 2010). In some insects, the gut beta-1,3-glucanase activity was correlated to the innate immune defense against pathogens at the mucosa level (Pauchet et al., 2009; Bulmer et al., 2009). Despite that, when we consider the complexity of the diet of *A. aegypti* larvae, it is not possible to discard a role in the digestion of plant hemicelluloses, as the relative contribution of ingested plant or fungal cells is unknown. Independently of the substrate that is recognized by this enzyme, it is clear from the phenotypes of larval death and pupation

arrest, observed after knockdown of AaeGH16.5, that the nutrient acquisition which results from its action in critical for larvae development and pupation. In this respect, it is important to consider that a deficiency in fungal or plant cell wall degradation may impair the absorption of intracellular nutrients as proteins or nucleic acids, resulting in severe nutritional restraints, like insects, in general, do not disrupt cells by mechanical breakdown (Terra and Ferreira, 2005).

Regarding the other genes studied, it is noteworthy that the properties of the amino acid sequences coded by AaeGH16.1, AaeGH16. 2, AaeGH16.3 and AeGH16.6 suggest that these proteins are secreted beta-1,3-glucanases, having catalytic activity and being probably secreted to the extracellular space. These sequences contain the conserved catalytic glutamates which are essential for the hydrolytic mechanism of GHF16, and a putative signal peptide, suggesting secretion via the canonical exocytic route. Interestingly, AaeGH16.6 is also expressed preferentially in the gut of larvae, in a pattern that resembles AaeGH16.5. Additionally, knockdown of AaeGH16.6 resulted in larval mortality and arrest of pupation. However, no change in the total beta-1,3-glucanase activity in the gut or the body was observed after treatment with dsRNA targeting this gene, and the chromatographic profile of secreted intestinal beta-1,3-glucanase activity was not changed either. It is possible that AaeGH16.6 codes for an enzyme that is insoluble, unstable, associated with a different tissue than the gut (e.g., hemocytes), or acts against other substrates than laminarin. Activities against the most varied substrates, like agar, carrageenan, xyloglucan or hyaluronic acid, have been described in GHF16, totalizing 14 different activities (Lombard et al., 2014). From these, only two, endo-1,3-β-glucanase (EC 3.2.1.39) and endo-1,3(4)-β-glucanase (EC 3.2.1.6) would show significant activity against laminarin. In this respect, the activity of the protein coded by AaeGH16.6 and its exact role in the gut physiology of the larvae needs further investigation.

The genes AaeGH16.2 and AaeGH16.3 are not preferentially expressed in the larval stage when compared to the other GHF16 of A. aegypti. It is possible that these genes exert their role in the pupae or adults, and investigation about the presence of beta-1,3-glucanases activity in these stages might clarify their role. It is interesting to observe that the protein coded by the gene AaeGH16.4 contains neither the catalytic glutamates nor the putative signal peptide observed in the other members of GHF16. These features

suggest that AaeGH16.4 codes for a beta-glucan binding protein and this is coherent with the observation that the knockdown of this gene does not result in larval mortality or pupal arrest in regular conditions, as these insects are not being challenged with pathogens. Besides that, it is more expressed in the head and in the rest of body of the larvae, which is consistent with participation in the innate immune response.

The data gathered here also suggest an immune role for the gene AaeGH16.1. This gene is expressed preferentially in the head and rest of body of larvae, and its sequence codes for the conserved catalytic glutamates and a putative signal peptide. Silencing of AaeGH16.1 resulted in moderate larval mortality and pupation arrest, and in pronounced mortality in stress conditions. Beta-1,3-glucanases were already reported for the head and rest of body of *A. aegypti* larvae (Souza et al., 2016), so it is possible that AaeGH16.1 is the gene responsible for the expression of this activities. Interestingly, the molecular masses observed for beta-1,3-glucanases in samples from the tissues above exceed the predicted molecular mass of AaeGH16.1 in 100 kDa, suggesting that in this case the AaeGH16.1 protein might be associated to other proteins from the innate immune cascade, as serine proteases or prophenoloxidases. Binding to other proteins in a macromolecular complex is a standard feature of insect beta-1,3-glucan binding proteins (Cerenius et al., 2010; Baxter et al., 2017).

The gene AaeGH16.4 seems to code a beta-glucan binding protein associated with the innate immune cascade. Its function may be inferred from the lack of the conserved catalytic residues of GHF16 and the lack a putative signal peptide. The expression pattern of AaeGH16.4 also suggests a systemic role not related to the digestive system, as it is preferentially expressed in the head and rest of body of larvae. Besides that, knockdown of AaeGH16.4 resulted in no detectable phenotype in control conditions, suggesting that this protein is involved in the immune response and not in nutrient acquisition. However further experiments need to be performed to prove this hypothesis, especially using challenges with recognized pathogens and more biochemical assays.

The presence of several GHF16 sequences in the genome of *A. aegypti*, *C. quinquefasciatus*, and *A. gambiae* strongly suggests that this gene family suffered duplication and diversification during the evolutionary establishment of the Culicidae

ancestor. That gene expansion might be related to the adaptation of the larvae for the aquatic environment, with a higher exposition to pathogens. However, it is not clear how catalytically active beta-1,3-glucanases might participate in the insect immune response, as the canonical role described for GHF16 proteins in the immune cascade is the recognition of Pathogen Associated Molecular Patterns (PAMPs). The presence of conserved catalytical residues and enzymatic activity contrast with a binding role, as glucanases tend to dissociate from their substrates after the hydrolytic cleavage of glycosidic bonds. In some cases, a processive mode of action and a secondary non-catalytical binding site have been described, resulting in a more stable association with the recognized polysaccharide (Genta et al., 2007).

Another aspect that should be considered is the possibility to explore GHF16 proteins as targets for inhibition and control of mosquito larvae. Proteinaceous inhibitors of beta-1,3-glucanases have been described in marine algae (Ermanova et al., 2001). Besides that, GHF16 proteins are absent in mammals (Lombard et al., 2014), indicating that inhibitors of beta-1,3-glucanases might not have a binding target in humans, with consequent low toxicity. Inhibition of insect beta-1,3-glucanases has been explored with drastic effects in survival (Bulmer et al., 2009). However, more detailed studies about the structure, specificity, and function of *A. aegypti* GHF16 proteins are necessary for the development and validation of this strategy. The study of this protein family in mosquitoes may reveal a new aspect of insect-pathogen relationships and the development of new targets for the control of insect vectors.

CONCLUSIONS

The genome of *A. aegypti* has six genes encoding GHF 16 proteins. Comparative sequence analysis, gene expression, and functional studies of these genes allowed us to identify AaeGH16.5 as the gene coding for the major gut beta-1,3-glucanase in the larvae of *A. aegypti*. Besides that, AaeGH16.1, AaeGH16.4, and AaeGH16.6 seem to be related to the innate immune response. These findings may improve our understanding of physiology and evolution of Culicidae larvae, as well as potentiate the development of new strategies for vector control.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHOR'S CONTRIBUTIONS

RSS, HMD, RS, JBRL, and MVFG performed the experiments and analyzed the data. RSS and FAG conceived the study and wrote the manuscript. All authors read and approved the final manuscript.

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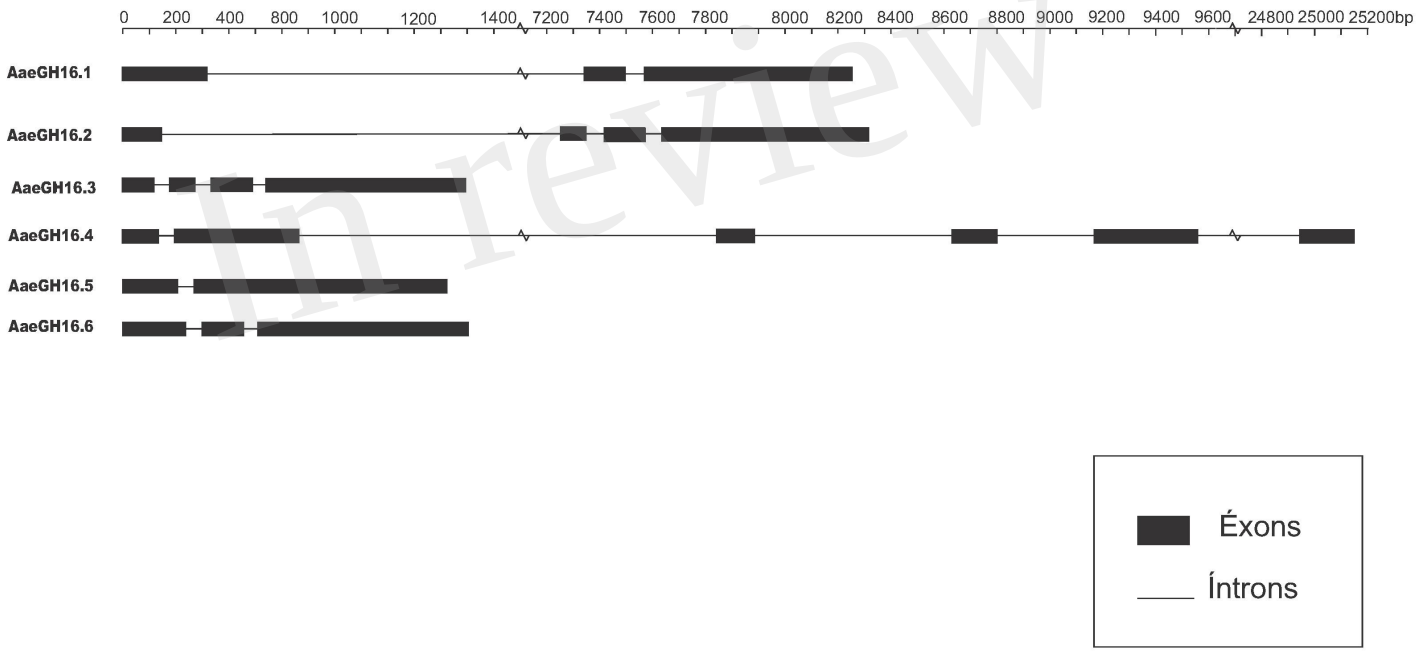

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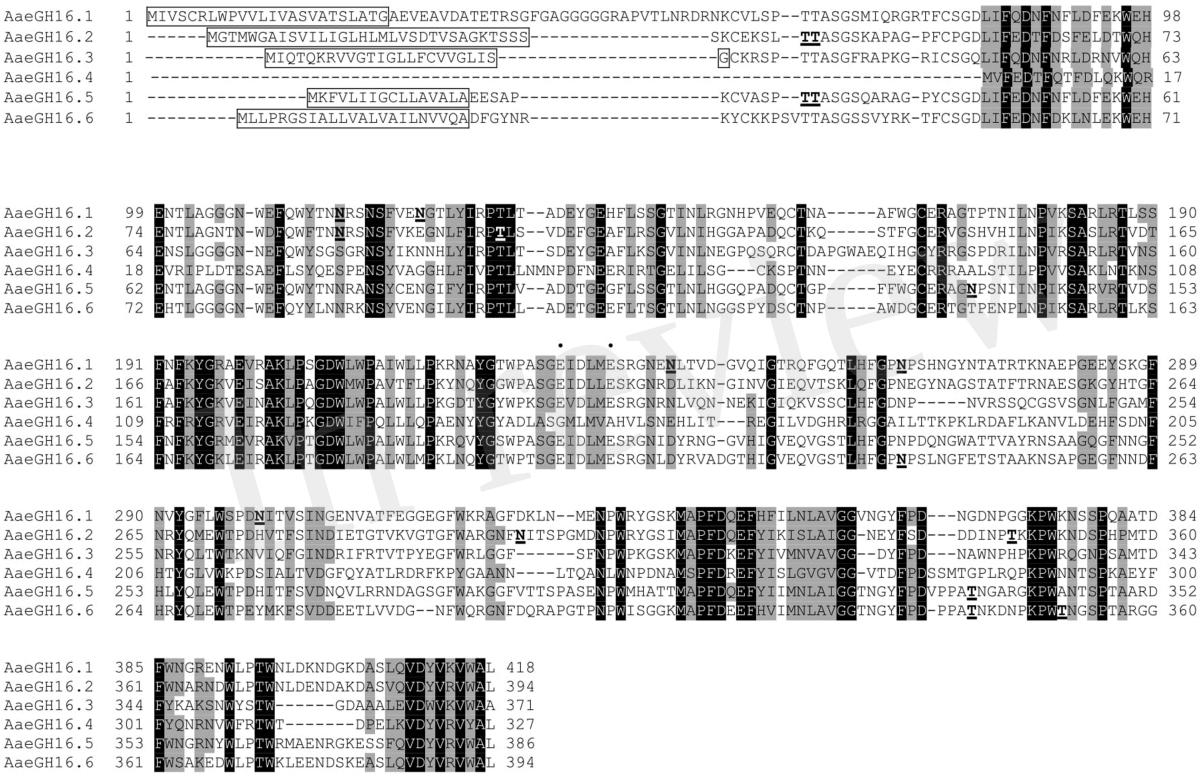

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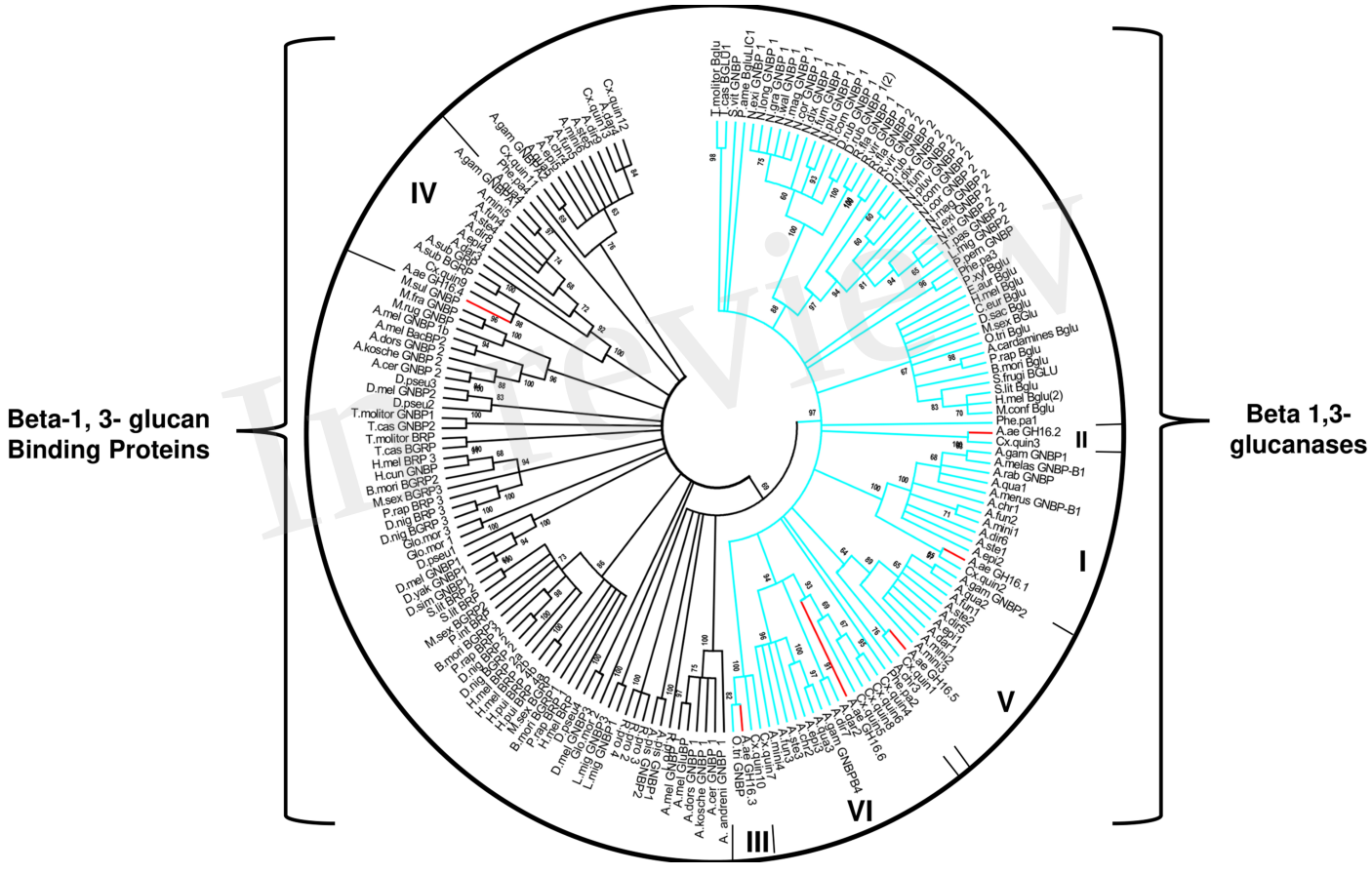

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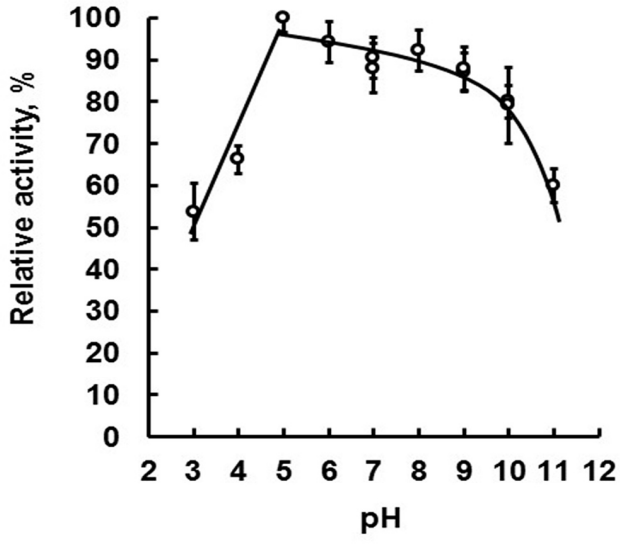

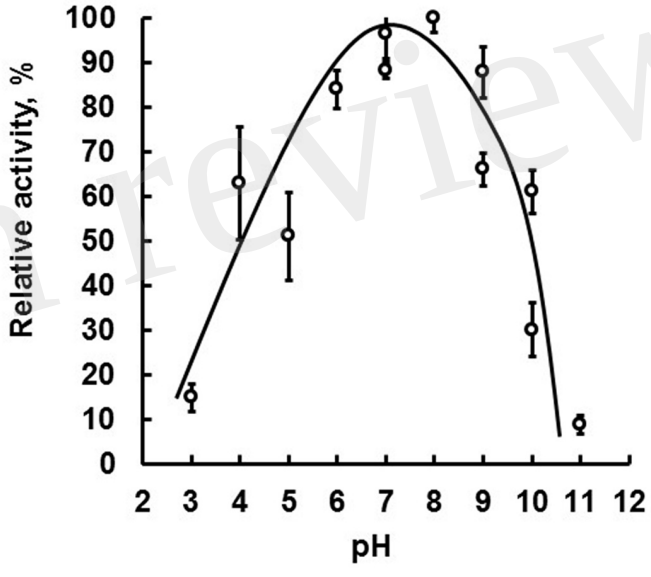

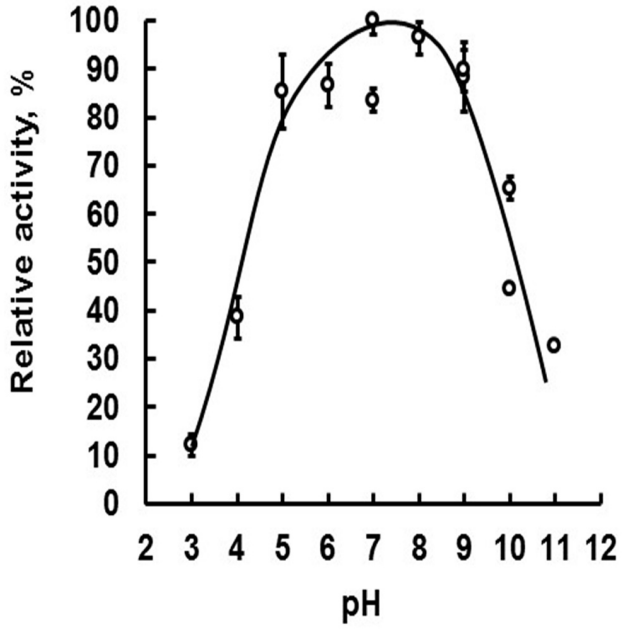

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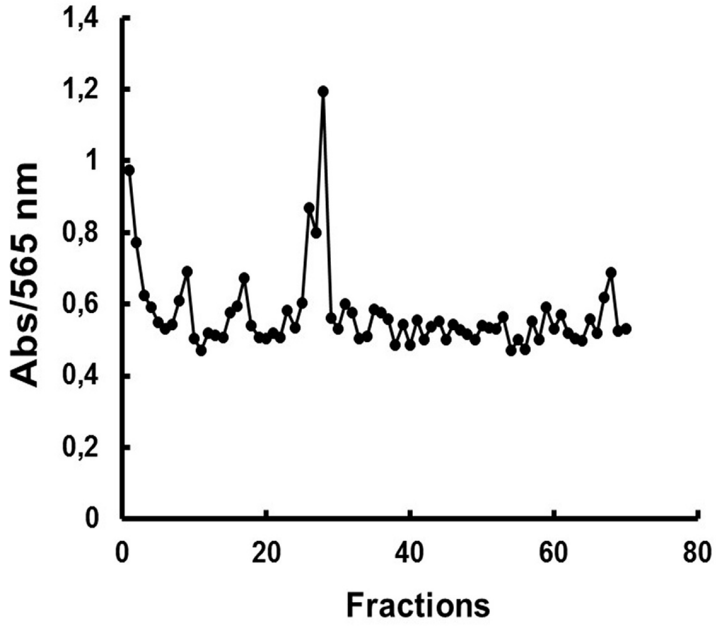

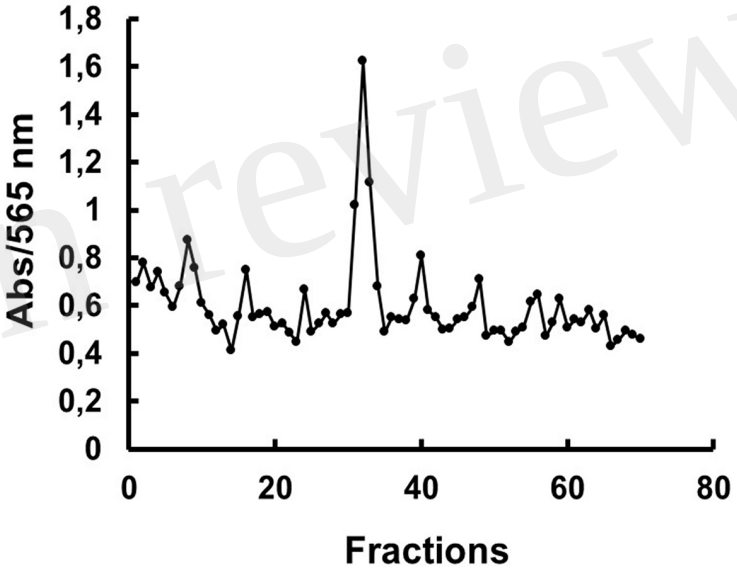

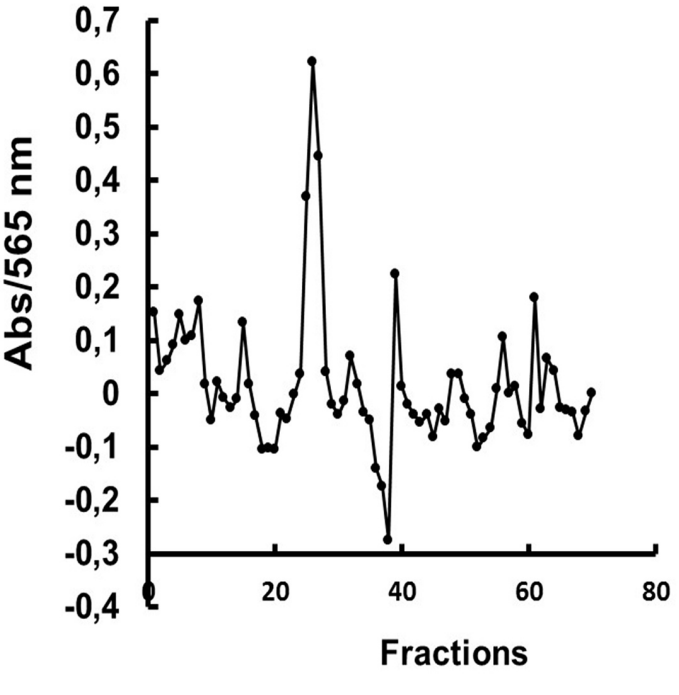

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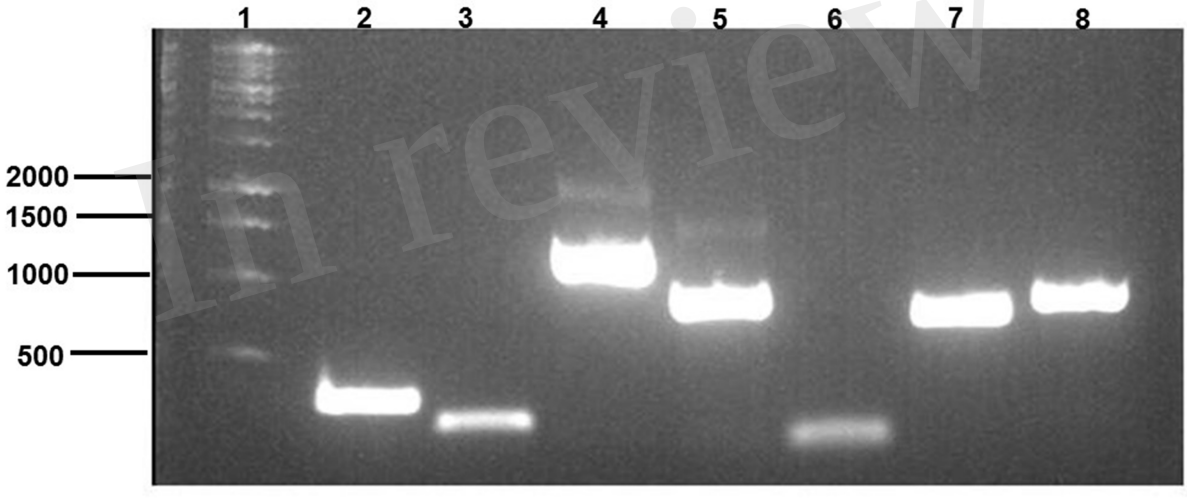

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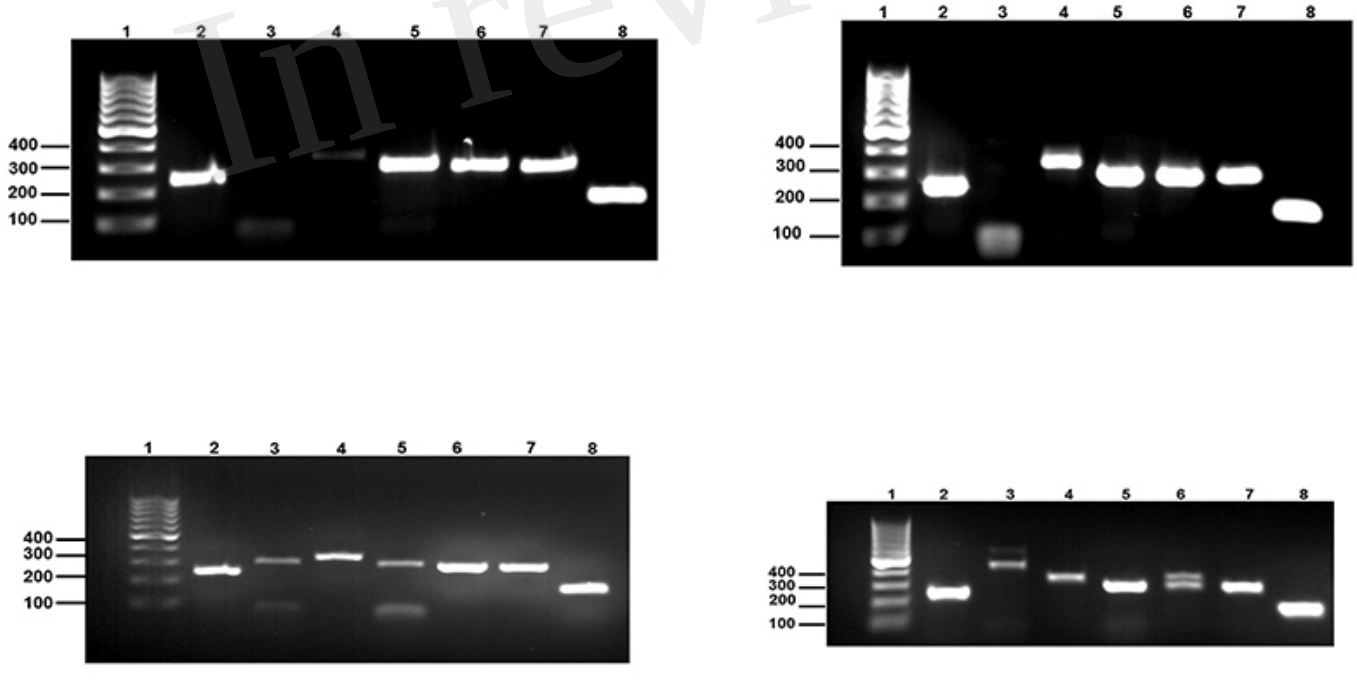

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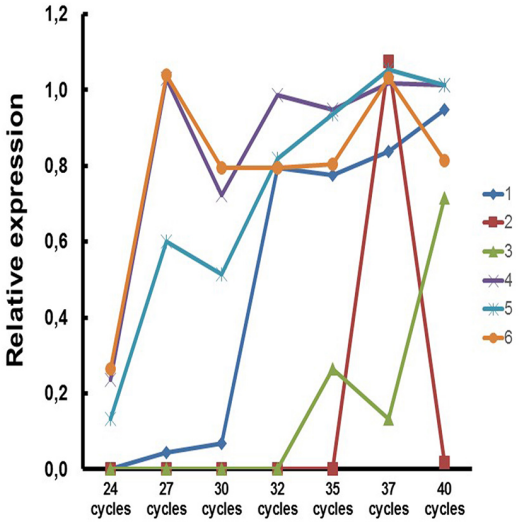

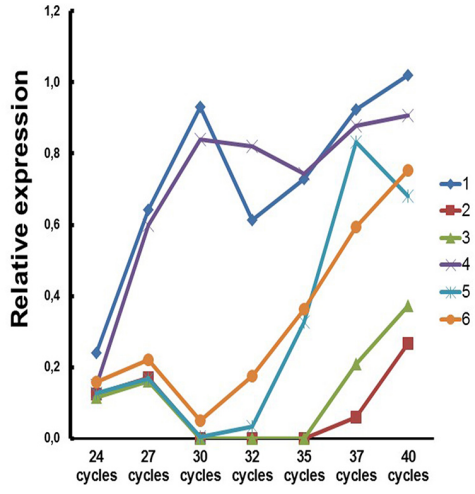

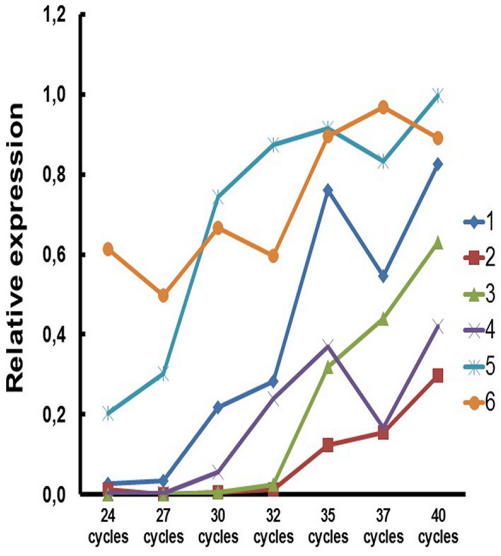

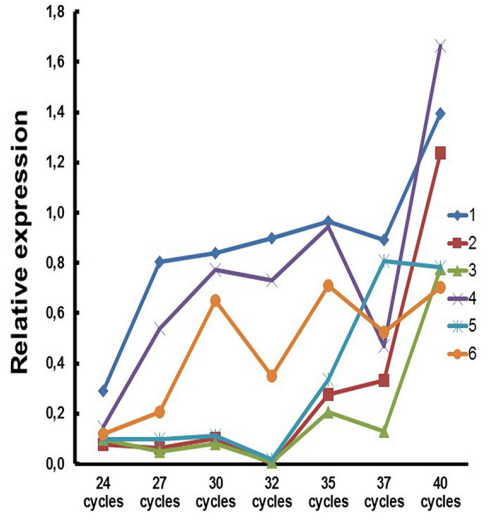

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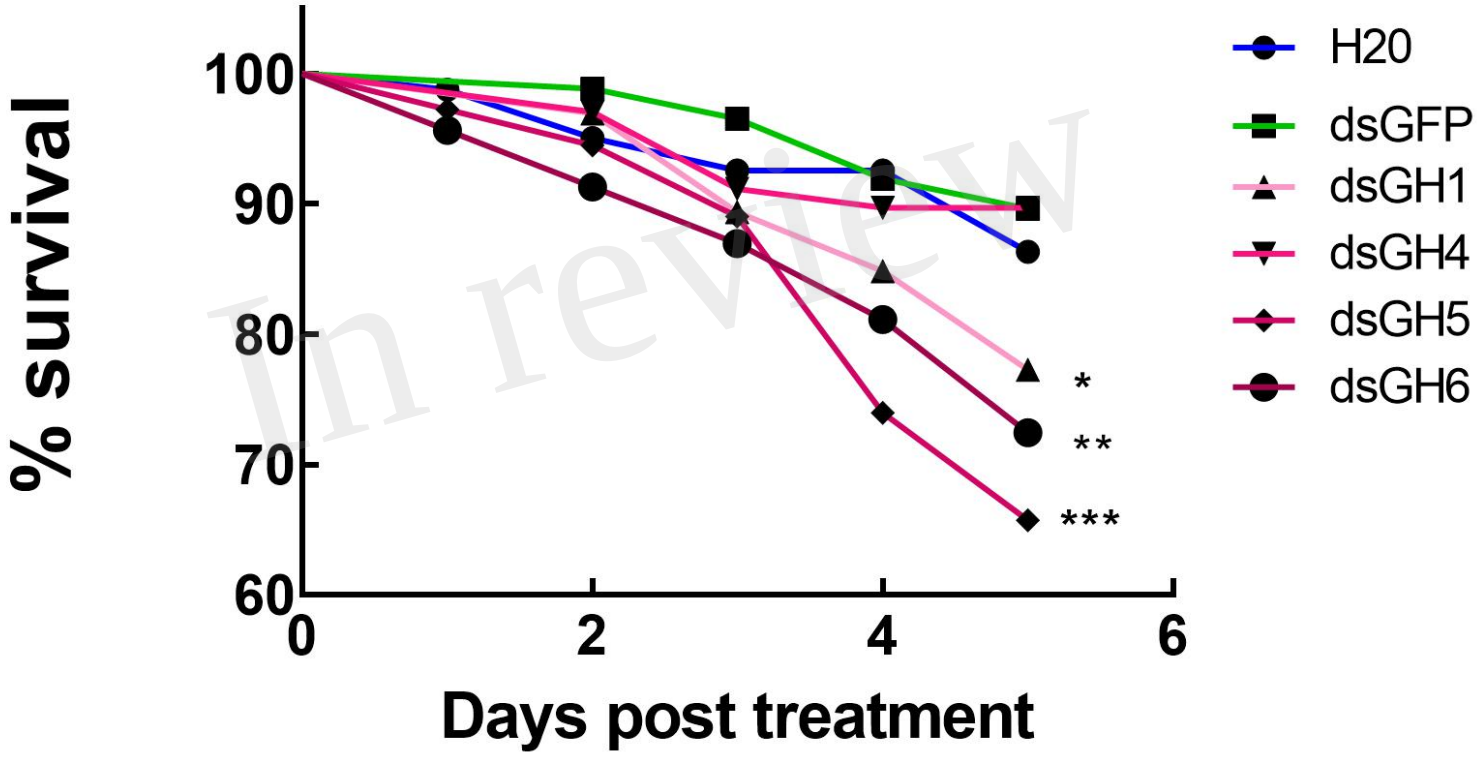

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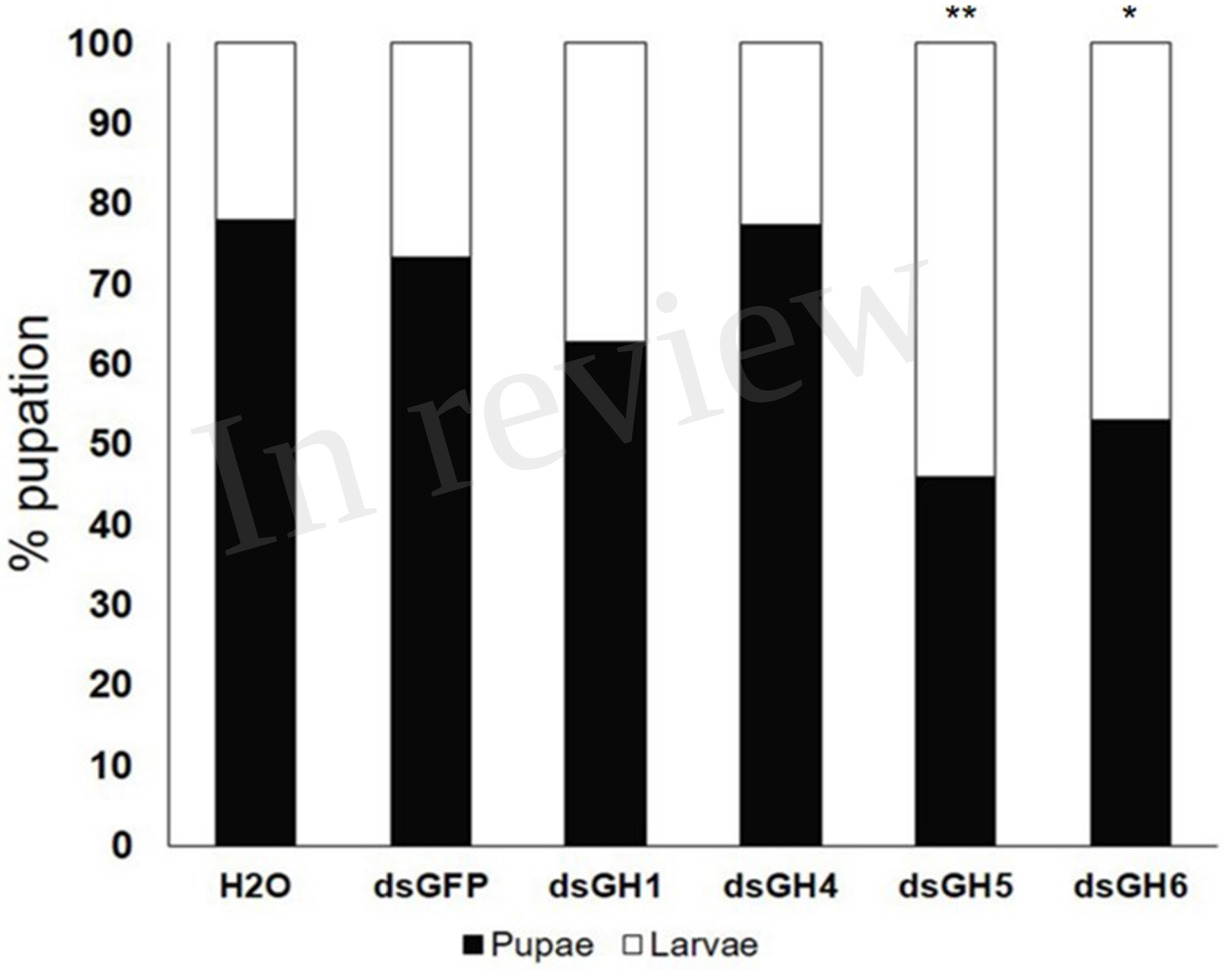

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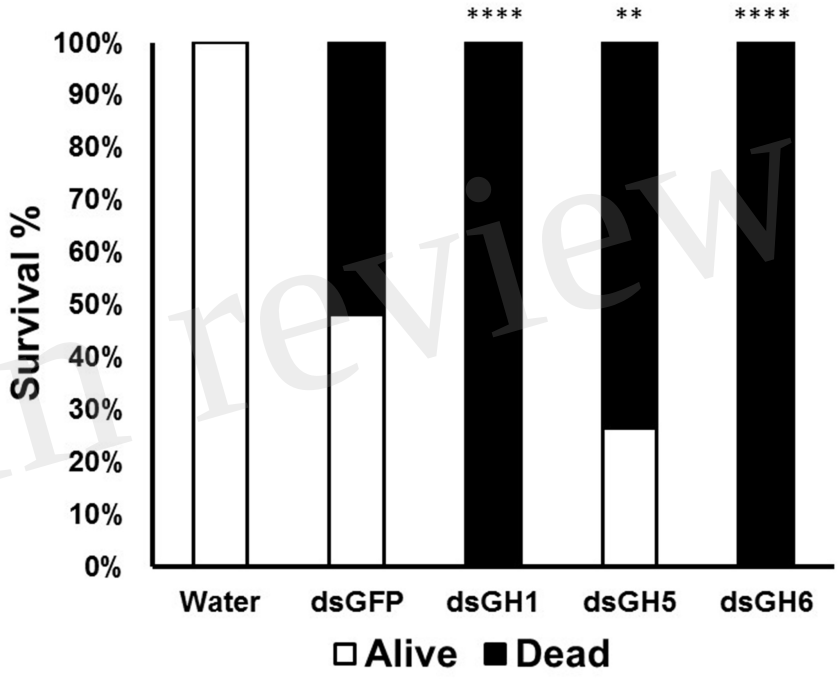

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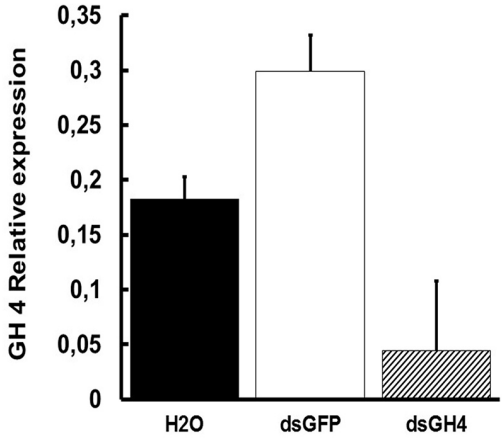

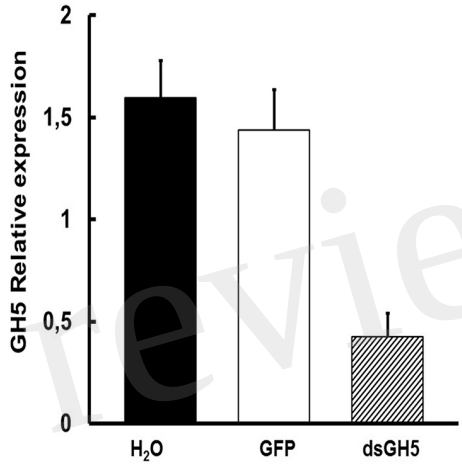

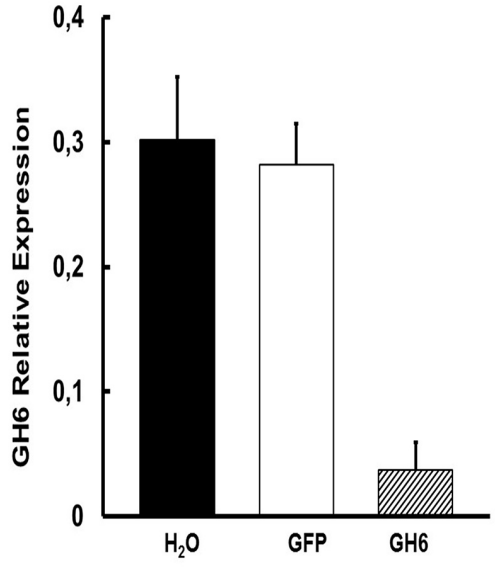

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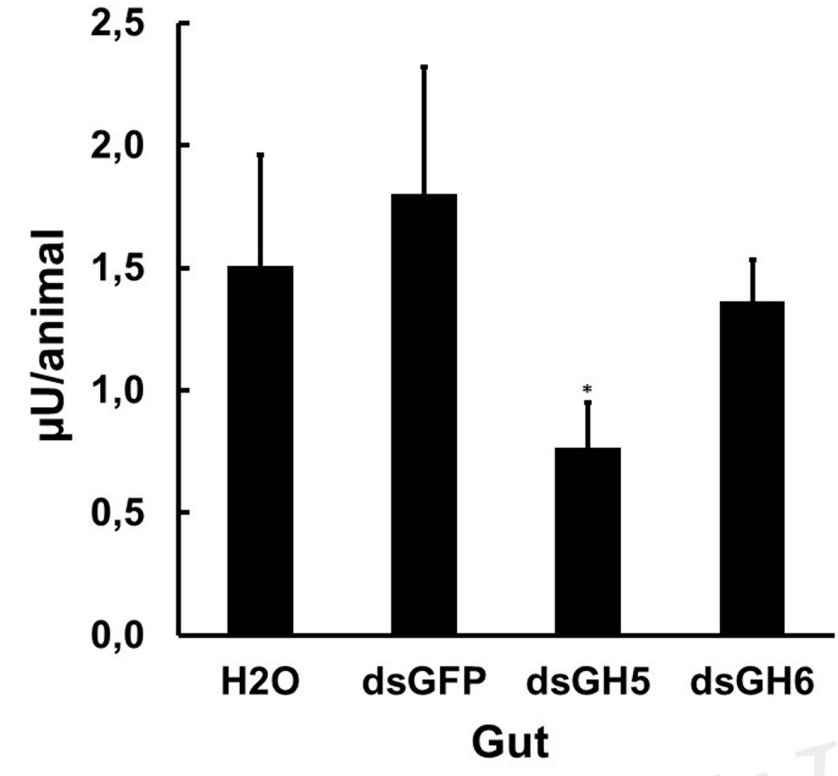

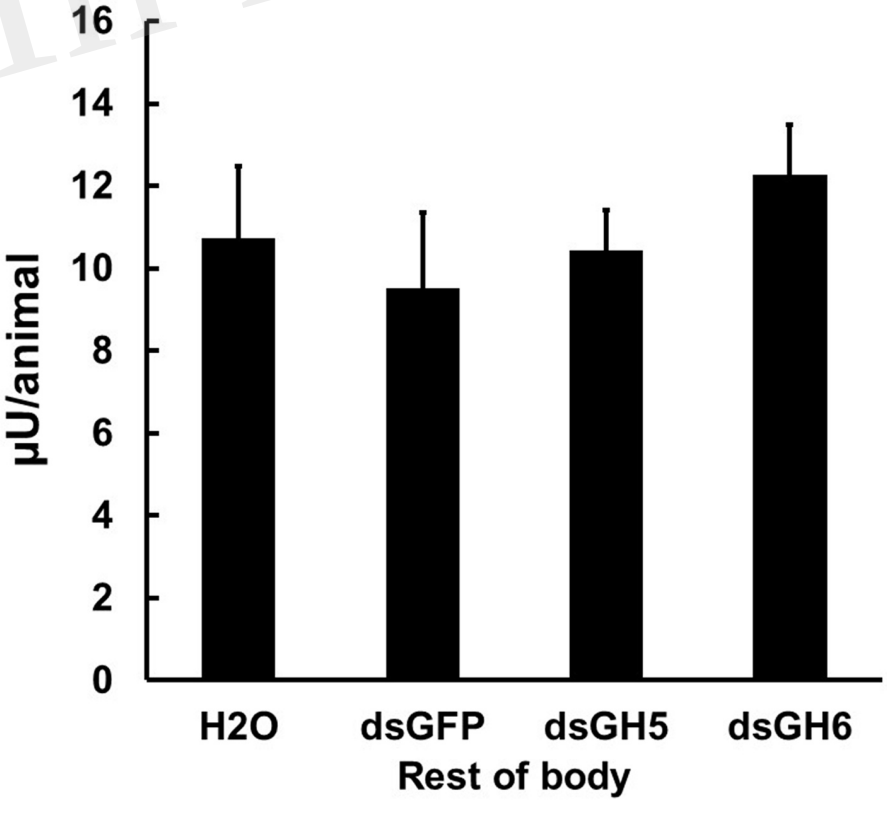

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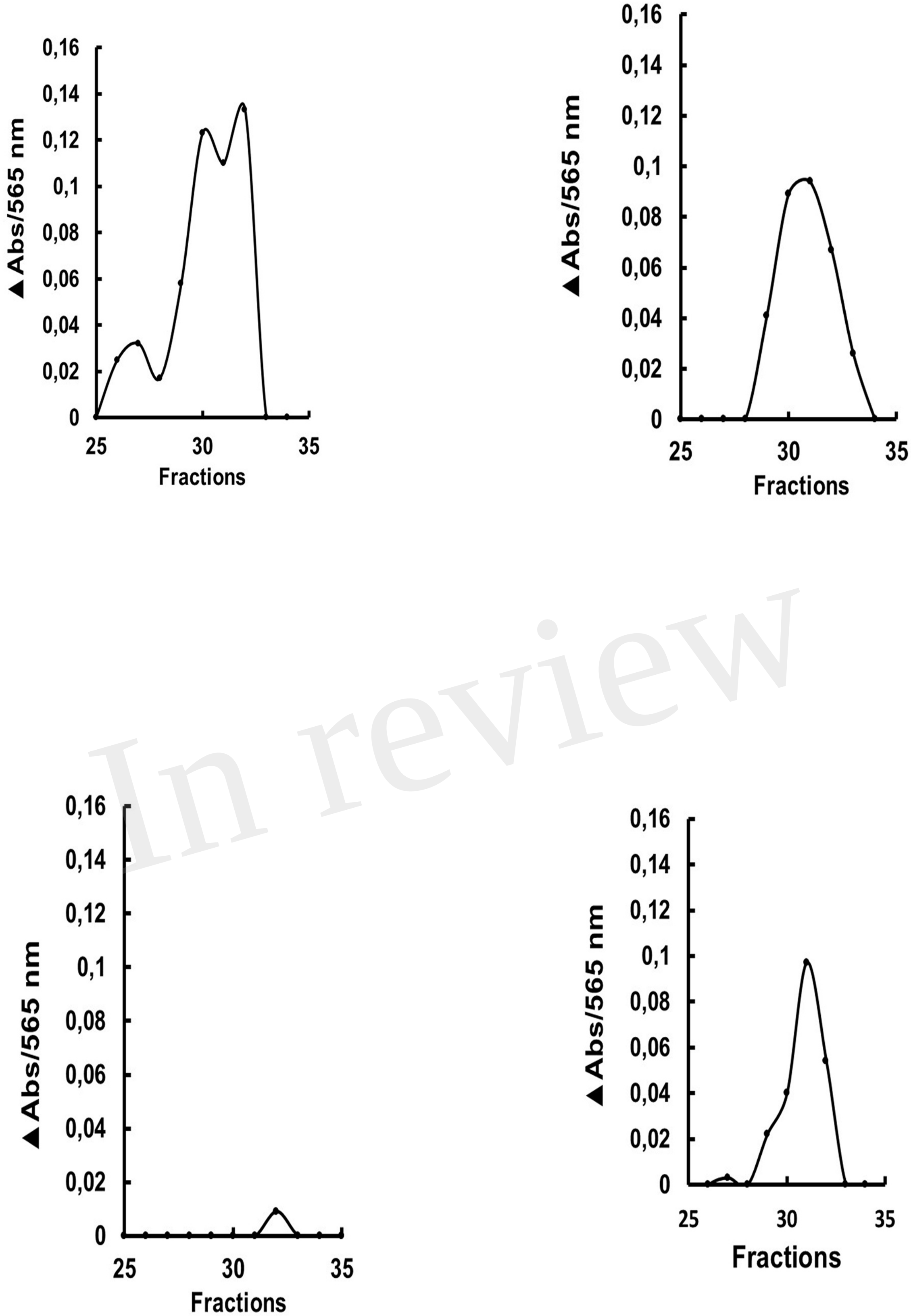

Artigo 2

Digestion of Yeasts and Beta-1, 3-Glucanases in Mosquito Larvae: Physiological and Biochemical Considerations

Autores: Raquel Santos Souza, Hector Manuel Diaz-Albiter, Vivian Maureen Dillon, Rod J. Dillon, Fernando Ariel Genta. PLoS One 2016; 11: e0151403 DOI: 10.1371/journal.pone.0151403.

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RESEARCH ARTICLE

Digestion of Yeasts and Beta-1,3-Glucanases in Mosquito Larvae: Physiological and Biochemical Considerations

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Abstract

Aedes aegypti larvae ingest several kinds of microorganisms. In spite of studies regarding mosquito digestion, little is known about the nutritional utilization of ingested cells by larvae. We investigated the effects of using yeasts as the sole nutrient source for *A. aegypti* larvae. We also assessed the role of beta-1,3-glucanases in digestion of live yeast cells. Beta-1,3-glucanases are enzymes which hydrolyze the cell wall beta-1,3-glucan polyssacharide. Larvae were fed with cat food (controls), live or autoclaved *Saccharomyces cerevisiae* cells and larval weight, time for pupation and adult emergence, larval and pupal mortality were measured. The presence of *S. cerevisiae* cells inside the larval gut was demonstrated by light microscopy. Beta-1,3-glucanase was measured in dissected larval samples. Viability assays were performed with live yeast cells and larval gut homogenates, with or without addition of competing beta-1,3-glucan. *A. aegypti* larvae fed with yeast cells were heavier at the 4th instar and showed complete development with normal mortality rates. Yeast cells were efficiently ingested by larvae and quickly killed (10% death in 2h, 100% in 48h). Larvae showed beta-1,3-glucanase in head, gut and rest of body. Gut beta-1,3-glucanase was not derived from ingested yeast cells. Gut and rest of body activity was not affected by the yeast diet, but head homogenates showed a lower activity in animals fed with autoclaved *S. cerevisiae* cells. The enzymatic lysis of live *S. cerevisiae* cells was demonstrated using gut homogenates, and this activity was abolished when excess beta-1,3-glucan was added to assays. These results show that live yeast cells are efficiently ingested and hydrolyzed by *A. aegypti* larvae, which are able to fully-develop on a diet based exclusively on these organisms. Beta-1,3-glucanase seems to be essential for yeast lytic activity of *A. aegypti* larvae, which possess significant amounts of these enzyme in all parts investigated.

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Introduction

Aedes aegypti, among other species of the genera *Aedes*, is the main vector of several pathogens-like Dengue, Urban Yellow Fever, Chikungunya, West Nile and Zika viruses, whose endemic areas include 40% of human populations worldwide (2.5 billion people) [1, 2]. In spite of being considered diseases restricted to tropical countries, recent global warming has increased concerns about their spread to regions with temperate climate [3], including reports of West Nile virus in Europe, Asia, North America and Australia [4].

Current main strategies for fighting these diseases rely on vector control, as there are no vaccines commercially available. Historically, the control of mosquitoes has been done with chemical insecticides, which are losing their potential effectiveness due to appearance of resistant populations [5]. New strategies for control of vector populations as transgenic mosquitoes, transfection of insects with *Wolbachia* or paratransgenesis have been proposed and are currently under evaluation [6–8]. Interestingly, some of these strategies depend on rearing massive amounts of insects and, therefore, mosquito nutrition has become a strategic point of investigation.

The haematophagic bevaviour of adult female *A. aegypti* and the fact that the initial site for development and transmission of pathogens by this insect is the intestine, had led to several studies of its digestive physiology [9–12]. Comprehensibly, those studies have focused the physiology of female adults, and larval digestion is known to a lesser extent [9, 13–14].

Interestingly, burden of *A. aegypti*-transmitted diseases is primarily determined by the occurrence of larval breeding sites [15]. Thus, knowledge of larval physiology and biochemistry can result in new insights for vector control. *A. aegypti* larvae are considered as detritivores, ingesting solid particles from liquid media and scraping solid material from surfaces. Among the particles ingested by mosquito larvae several microorganisms, such as bacteria, fungi, protozoa and rotifers have been found [16–21], but the mechanisms used by larvae for breakdown of these nutritional sources remain largely unknown.

Recent understanding of the importance of gut microbiota in several aspects of insect physiology [22] resulted in more detailed investigations of the role of bacteria in development and vectorial capacity of mosquitoes. For example, it was demonstrated the dependence of *Aedes aegypti*, *Anopheles gambiae* and *Georgecraigius atropalus* larvae on gut bacteria for full development [23]. In spite of that, the exact mechanisms of interaction between these organisms was not fully investigated, as beneficial effects of ingested bacteria might be of nutritional, immunological or even endocrinological nature. In this respect, a deep understanding of interactions between specific microorganisms and mosquito larvae is still lacking.

The main objective of this work was to investigate physiological consequences of yeast ingestion by *A. aegypti* larvae, using *Saccharomyces cerevisiae* as model nutrient source. Yeasts are a more defined food source, antibiotic free and less likely to transmit pathogens to the insects than the standard cat or animal food which is used to raise larvae in regular mosquito colonies [24–26]. We discovered that *A. aegypti* larvae could nourish exclusively from live *S. cerevisiae* cells, revealing that this insect bears mechanisms for yeast cell wall breakdown and full acquisition of nutrients from this microorganism. Accordingly, we showed *in vitro* that larval gut homogenates have lytic activity against live *S. cerevisiae* cells.

Beta-1,3-glucanases hydrolyse glicosidic bonds in beta-1,3-glucans, which are the major polysaccharide component of the yeast cell wall. We investigated the effects of a *S. cerevisiae* exclusive diet on larval beta-1,3-glucanase activity, and competition experiments revealed that this enzyme is crucial for the larval lytic activity against this microorganism. These findings, besides unravelling new basic physiological aspects of culicid larvae, could help in the establishment of better defined, pathogen free artificial diets for large-scale mosquito larvae rearing in future.

Materials and Methods

Insects rearing and maintenance

Aedes aegypti eggs from the Rockfeller strain were obtained from the colony of the Laboratory of Physiology and Control or Arthropod Vectors (LAFICAVE/IOC-FIOCRUZ; Dr Denise Valle and Dr José Bento Pereira Lima). Insects were reared until adult stage in the Laboratory of Insect Biochemistry and Physiology (LABFISI, IOC/FIOCRUZ) at 27±2°C and 70±10% relative humidity with a 12-h light/12-h dark cycle. To obtain synchronized developing larvae, hatching was induced by adding 100 mL of distilled water into 200 mL plastic cups containing eggs and then incubating at 28°C for 30 minutes. After incubation, first instar larvae (n = 80) were transferred together to plastic bowls containing 100 mL of dechlorinated water and 0.1 g of cat food (Whiskas®, Purina, Brazil) and kept at 26±1°C until adult stage. The food was added only once in the beginning of each experiment. Larvae which received cat food are considered the control group.

Saccharomyces cerevisiae S14 was kindly donated by Professor Pedro Soares de Araújo (Chemistry Institute, University of São Paulo, Brazil). For feeding experiments with live *S. cerevisiae*, a single colony was transferred into 3–5 mL of liquid Sabouraud medium [27] and incubated overnight at 30°C under shaking at 100 rpm. After overnight incubation, 100 uL of culture were subpassaged into 50 mL of Sabouraud medium and incubated overnight at 30°C under shaking at 100 rpm. 50 mL of cultures were then centrifuged (7,500 x *g*, 30 min, 4°C) and the supernatant was discarded. All cells were then suspended in water and released into larval cups. A similar experiment was performed autoclaving the cells (120°C, 20 min, 1.5 atm) before larval feeding.

Biological parameters

Initially, we investigated if a yeast diet could have an impact in development of fourth instar larvae of *Aedes aegypti*. With this objective, recently molted 4th instar larvae were fed on live *Saccharomyces cerevisiae* cells until the prepupae phase. To investigate if *A. aegypti* could fully-develop when feeding exclusively on cells of this yeast species, recently hatched first instar larvae were transferred to a bowl containing yeasts as the sole food source. Larval and pupal mortality, pupation and emergence were monitored and recorded daily. Fourth instar larvae, pupae, and male and female adults were weighed individually or in pools of 10 individuals each. Pupation and emergence data were plotted and compared by the Log-rank (Mantel-Cox) Test. Mortality and weights were expressed as means ± SEM and non-transformed data were compared by ANOVA or pairwise t-tests.

Preparation of samples for enzymatic assays

Larvae were immobilized by placing them on ice, after which they were dissected in cold 0.9% (w/v) NaCl. Parts dissected in each larva were the head, gut and rest of body. Heads and rest of bodies were homogenized in MilliQ water with aid of a micro tube pestle (Model Z 35, 997–1, Sigma, USA), using a ratio of 100 μL of water per 10 insects. Guts were homogenized in cold MilliQ water containing 20 mM phenylmethylsulfonyl fluoride (PMSF), 20 μM Pepstatin A and 20 μM trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64). All samples were centrifuged for 10 min at 14,000 x *g* at 4°C. Both pellets and soluble fractions were stored at -20°C until used as enzyme source for enzymatic assays.

Yeast viability assays

To test if larval gut contents have some influence in live yeast cells, we performedassays incubating these two materials mixed and followed yeast viability. Gut soluble fraction was

prepared as above and filtered through a 0.45 μm PVDF syringe filter (Millipore Code. JBR6 103 14 Lot. B2MN40511) and then incubated at 30°C with 10 colony-forming units (CFUs)/μL of live *S. cerevisiae* cells in 10 mM citrate-sodium buffer pH 7.0.

After different time points, assays were sampled and aliquots were plated onto solid Sabouraud medium (1% w/v yeast extract, 1% w/v peptone, 1% w/v dextrose, 2% w/v Agar). After overnight incubation at 30°C, colonies were counted. Cell stability under assay conditions was confirmed by using controls without enzyme.

Enzymatic assays and protein quantitation

β-1,3-glucanase activity in *Aedes aegypti* larvae was determined by measuring the release of reducing groups from 0.25% (w/v) laminarin from *Laminaria digitata*, (SIGMA Cat. no. L9634) in a thermocycler with a modified bicinchoninic acid reagent according to ref. [28]. All assays were performed at 30°C under conditions such that activity was proportional to protein concentration and time. Controls without enzyme or without substrate were included. One unit of enzyme (U) is defined as the amount that hydrolyses 1 μmol of substrate (or bonds)/min. Protein concentration was determined according to [29] using ovalbumin as a standard.

To test if feeding with yeasts could change beta-1,3-glucanase expression, we compared activities in all parts of *A. aegypti* 4^{th} instar larvae reared on live or autoclaved *S. cerevisiae* cells with levels found in larvae fed on cat food. Comparisons between means of two independent groups were done with a pairwise t test. Results are expressed as the group mean ± SEM.

Yeast cell counts

To confirm that *A. aegypti* larvae are actively ingesting live *S. cerevisiae* cells, and not merely filtrating released molecules from broken or dead cells, we decided to check the integrity and viability of the yeasts in our experimental conditions. During the preparation of the experimental diets, the yeasts, after growing on liquid Sabouraud media, are centrifuged and ressuspended in water. We decided to count viable cells using Trypan Blue staining and by light microscopy after these treatments to check viability as below.

S. cerevisiae cultures (45 mL) were prepared in Sabouraud liquid media as described previously and then centrifuged (7,500 x *g*, 40 min, 4°C). The supernatant was discarded and cells were resuspended in the same volume of Sabouraud liquid media or water. Ten microliter aliquots of each suspension were withdrawn and then combined with 90 μL of PBS. These samples were mixed with 100 μL of a 0.4% (w/v) Trypan Blue solution in PBS and then 15 μL were loaded on a Neubauer chamber (hemocytometer), where dead and live cells were counted in a light microscope (400 x magnification). In one experiment, yeast cells ressuspended in water were kept at 26°C for 24 hours before staining and counting.

For counting yeast cells ingested by *A. aegypti* larvae, insects were raised on cat food as described previously until they reached the fourth larval instar. Then larvae were transferred to a bowl with *S. cerevisiae* cells as food source as described, and after different time points larvae were withdrawn from the pots and dissected. Entire guts were homogenized in 100 μL PBS, combined with Trypan Blue and then live and dead yeast cells were counted as above.

Food protein and sugar contents

For quantitations in *S. cerevisiae*, cells were grown in Sabouraud liquid media as described and 45 mL of culture were centrifuged (7,500 x *g*, 30 min, 4°C). Supernatant was discarded and cells were ressuspended in 5 mL of water. Ten microliter aliquots were withdrawn for protein and sugar measurements. For quantitations in cat food samples, 0.1 g of cat food was homogeneized in 1 mL water and 20 μL aliquots were withdrawn for measurements. Proteins were determined

with the bicinchoninic acid method [30] and total sugars were measured with the phenol-sulfuric method [31]. Due to the presence of insoluble material, cat food samples submitted to reaction with BCA were centrifuged (quick spin) before absorbance readings.

Statistical analysis

Linear regressions were performed using Microsoft Excel (Microsoft). Statistical comparisons were made using GraphPad Prism software (version 5.0, GraphPad Software Inc.). Significance was considered when $p<0.05$.

Results

A. aegypti 4th instar larvae fed on live *Saccharomyces cerevisiae* cells reached the end of the larval stage with significantly higher weights when compared to controls ($p < 0.05$, unpaired t-test, n = 3, Table 1). *A. aegypti* raised from eggs on live *S. cerevisiae* cells resulted in larvae heavier than controls ($p < 0.05$, unpaired t-test, n = 6, Table 1).However, pupae and female adults derived from these larvae had similar weights when compared to controls ($p > 0.05$, unpaired t-test, n = 6, Table 1). Yeast fed male adults had weights significantly higher than controls ($p < 0.01$, unpaired t-test, n = 6, Table 1). We observed a small but significant delay in both pupation and adult emergence ($p < 0.05$, Log-rank (Mantel-Cox) Test, n = 320, Fig 1), but no significant changes in larval or pupal mortality ($p > 0.05$, unpaired t-test, n = 6, Table 1).

Viable cell counts revealed that ressuspension in water does not affect the number or viability of yeast cells ($p > 0.05$, unpaired t-test, n = 9, Fig 2A). Yeast cells remain viable even after being incubated in water for 24 hours ($p > 0.05$, unpaired t-test, n = 9, Fig 2A), which suggests that larvae have been exposed to live cells throughout our experiments.

Counting of yeast cells inside the gut of 4th instar larvae which were exposed to *S. cerevisiae* diets revealed that the insects have ingested a significant amount of cells already at the first time point analysed (2 hours; Fig 2B). During 48 hours of exposure of larvae to the yeast diet, the total number of ingested cells does not dramatically change. However, a significant decrease

Table 1. Biological life parameters of *Aedes aegypti* raised on different diets. Cat food—insects fed on control diet. Yeast—insects fed on live *S. cerevisiae* cells. Weights are presented in mg and mortalities as percentages.

Parameter	Cat Food	Yeast
Larval body weight (1)	1.7 ± 0.1	3.3 ± 0.4 *
Larval body weight (2)	3.20 ± 0.05	5.5 ± 0.1 *
Pupal body weight (2)	6.6 ± 0.4	7.4 ± 0.5
Adult female body weight (2)	2.0 ± 0.2	2.1 ± 0.2
Adult male body weight (2)	0.9 ± 0.1	1.7 ± 0.2 **
Larval mortality (2)	7 ± 2	6 ± 1
Pupal mortality (2)	12 ± 4	17 ± 5

Insects were raised in groups from eggs on cat food and exposed to different diets only during the 4 th larval instar. Figures are means ± SEM of 3 experiments with 40 larvae each.

* $p < 0.05$

Insects were raised on different diets thoughout the entire larval development. Figures are mean ± SEM of 6 experiments with 80 insects each.

* $p < 0.05$

** $p < 0.01$.

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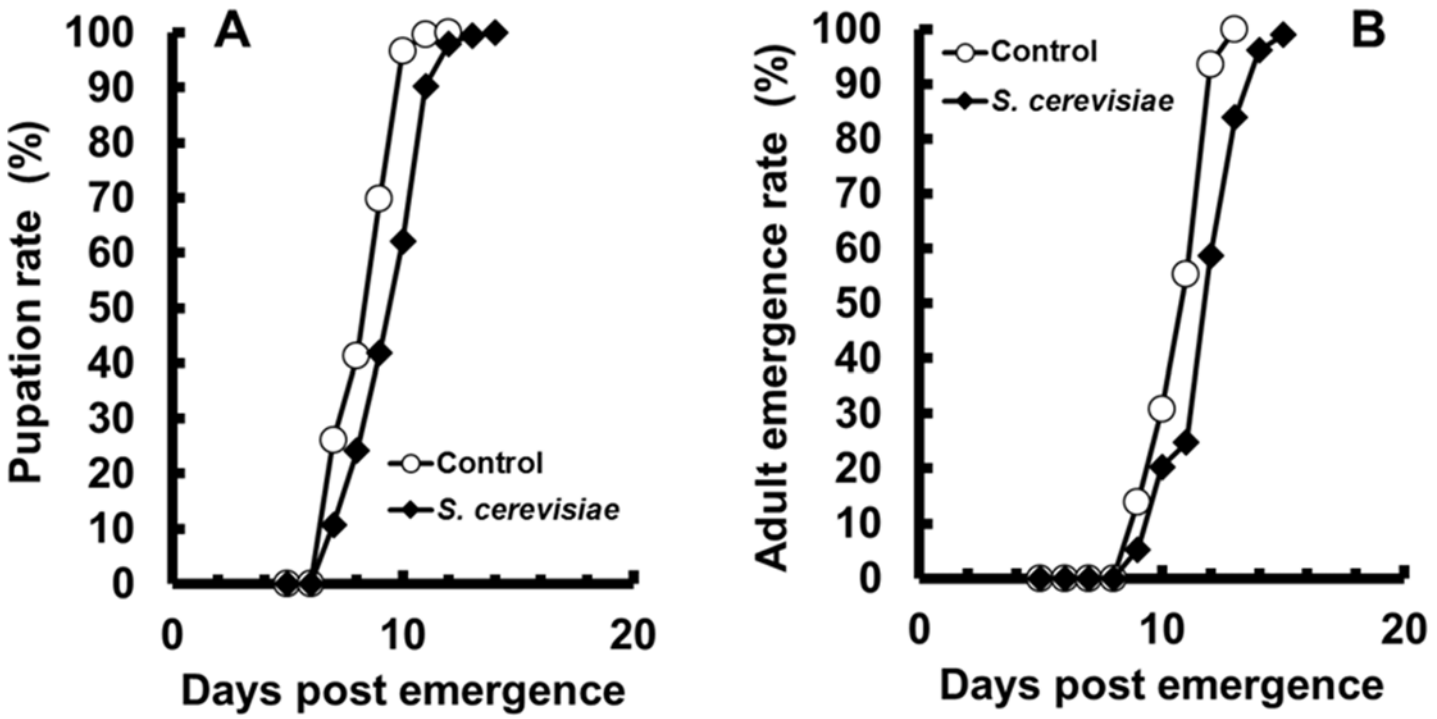

Fig 1. Life parameters of *Aedes aegypti* larvae fed since egg hatching exclusively with cat food or *Saccharomyces cerevisiae* live cells. Percentage of pupation (A) and percentage of emergence of adults (B). Figures are means of 4 experiments with 80 larvae each.

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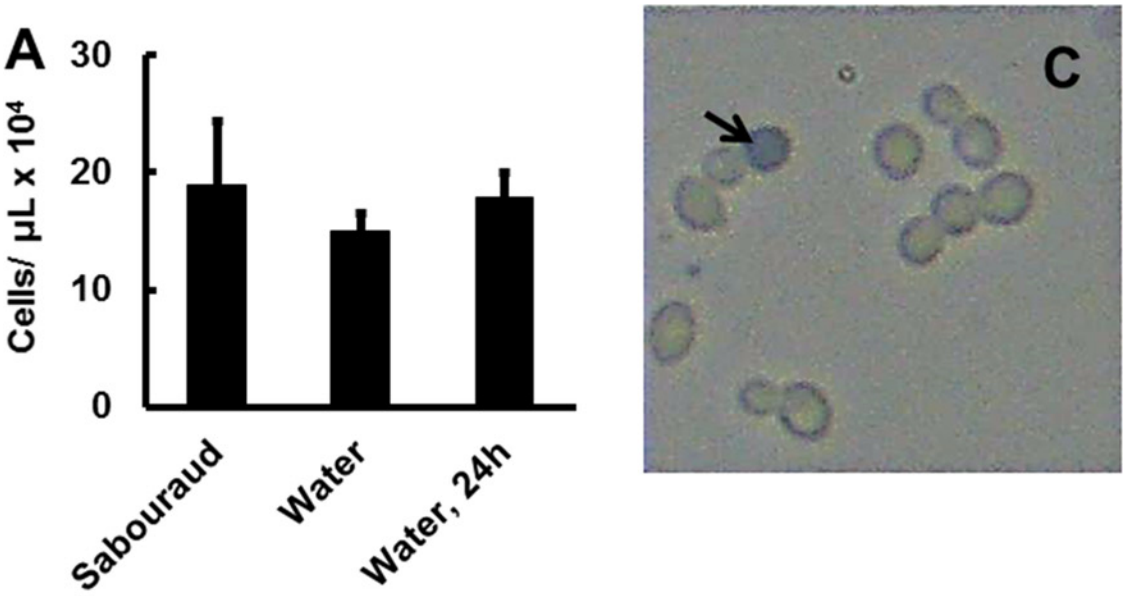
in viable cells occured after 24 hours, with an increase of dead cells (Fig 2B and 2C). At the same conditions, control insects maintained on cat food showed no yeasts inside the gut (Fig 2B). Taken together, these results clearly show that, in spite of some changes in development, *A. aegypti* can nourish and fully-develop from alive *S. cerevisiae* cells.

To have a better understanding of possible reasons for the observed changes in development when *A. aegypti* larvae are raised in live yeast cells, we compared the protein and sugar amounts in the yeast diet to the amounts present in the regular cat food which was given to controls. The yeast diet contains respectively 11.6 and 3.2 times more protein and sugar than the cat food, when we compare the amounts which were given to each group (Table 2).

Since *A. aegypti* larvae were able to develop solely on a live *S. cerevisiae* diet, we hypothesized that larvae were able to break down the macromolecules from this nutrient source. Because one of the main constituents of the yeast cell wall is beta-1,3-glucan [32], we decided to investigate if *A. aegypti* larvae produced beta-1,3-glucanase. Beta-1,3-glucanase activity was present in all parts of 4th instar larvae, with a prevalence in the rest of body and minor activities in the head and gut (Fig 3A). Surprisingly, specific activity (measured as μU/mg protein) in the head was ten times higher than in gut or rest of body (Fig 3B). Activity present in the suspension from containers used to raise the larvae was negligible (Fig 3A), suggesting that activity present in the gut is secreted at this organ and not acquired from food.

After finding significant beta-1,3-glucanase activities in all parts of *A. aegypti* 4th instar larvae, we verified whether these activities could be modified (elicited or inhibited) by a diet with live *S. cerevisiae* cells. Rearing of *A. aegypti* exclusively on live *S. cerevisiae* did not result in any significant changes in beta-1,3-glucanase levels in the soluble fraction of all samples tested when compared to controls fed with cat food ($p > 0.05$, unpaired t-test, n = 4, Fig 4A). We also measured the activity associated with the insoluble fraction of samples, which in the case of gut


A
30
20
10
0
Cells/ µL x 10⁴
Sabouraud
Water
Water, 24h
C

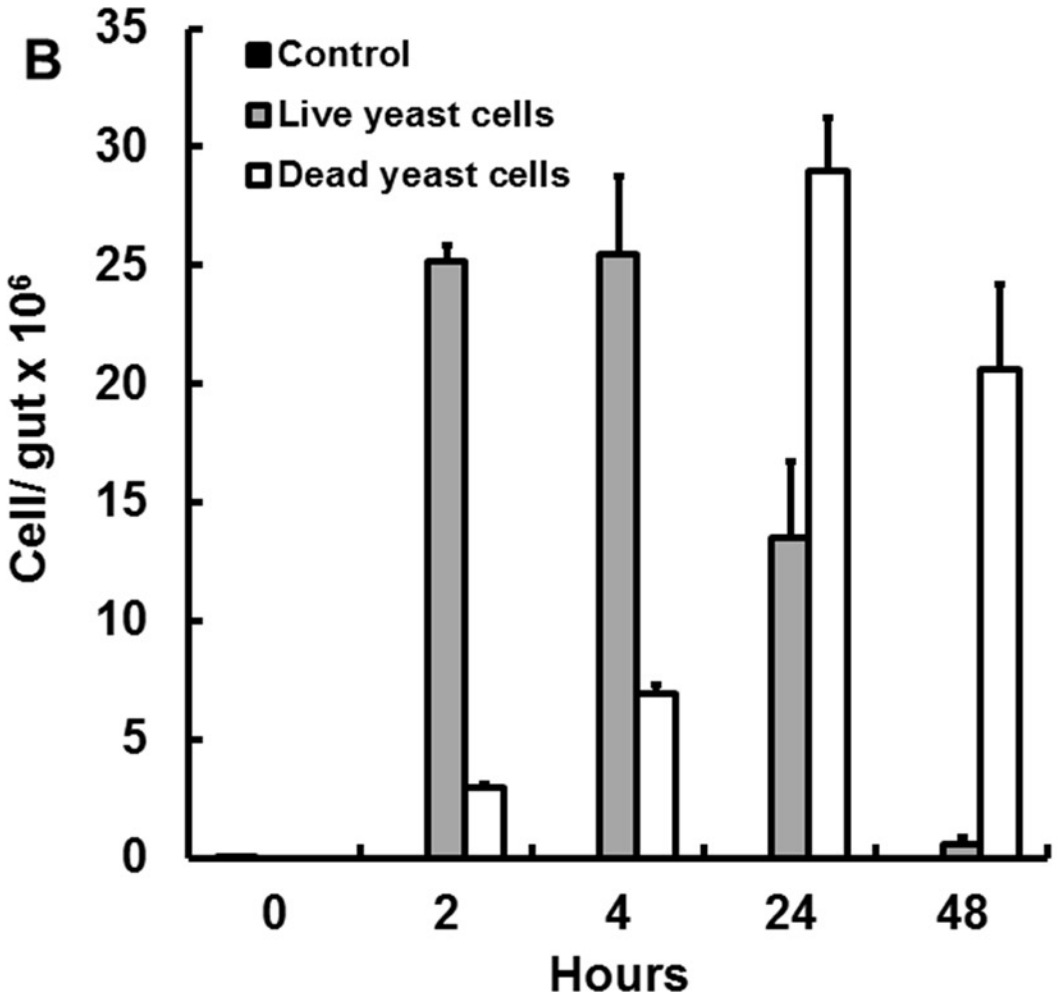

B
Control
Live yeast cells
Dead yeast cells
35
30
25
20
15
10
5
0
Cell/ gut x 10⁶
0
2
4
24
48
Hours

Fig 2. Cell counts during preparation of yeast-based diets and their ingestion by *Aedes aegypti* larvae. (A) Total counts of yeast cells after centrifugation of *Saccharomyces cerevisiae* liquid cultures and resuspension in media (Sabouraud), water and after keeping the resuspended cells in water for 24 hours. Figures are means ± SEM of 9 experiments each. (B) Time progression of yeast cells total counts in the gut of insects fed with cat food (Control), and live/dead yeast cell counts in the gut of insects fed with *S. cerevisiae* diet. Figures are means ± SEM of 5 samples with one larva each (C) Illustrative image of *S. cerevisiae* cells recovered from the gut of *A. aegyti* larvae fed with the yeast-based diet. The black arrow shows a dead yeast cell (Trypan Blue staining). See Material and Methods for details.

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putatively contains undigested *S. cerevisiae* cells and cell walls. The activities in the insoluble fraction of guts and heads were also not changed ($p > 0.05$, unpaired t-test, n = 4, Fig 4B), as well as the total activity at each tissue ($p > 0.05$, unpaired t-test, n = 4, soluble + insoluble fractions; Fig 4C).

Surface exposure of structural components of the cell wall could be an important factor in possible changes in beta-1,3-glucanase activity in larvae during development when feeding on yeast cells. Nevertheless, activities from insects fed on autoclaved *S. cerevisiae* did not differ from controls in all tissues, neither in the soluble fraction ($p > 0.05$, unpaired t-test, n = 4, Fig 5A), the insoluble fraction ($p > 0.05$, unpaired t-test, n = 4, Fig 5B) or in total amount ($p > 0.05$, unpaired t-test, n = 4, Fig 5C). The only remarkable exception on this pattern was the activity in the head, which was significantly lower in larvae fed on autoclaved yeasts compared with controls fed on cat food. This was observed in total as well as both soluble and insoluble fractions ($p < 0.05$, unpaired t-test, n = 4, Fig 5A–5C).

The presence of a constitutive beta-1,3-glucanase activity in the gut of *A. aegypti* larvae raised the question about the real importance of this enzyme in the breakdown of ingested yeast cell walls. These cells were stable under assay conditions (see controls, Fig 6), and incubation of these cells with gut soluble fraction from *A. aegypti* larvae resulted in rapid loss of viability ($p < 0.05$, unpaired t-test *vs* controls, n = 9, Fig 6). Addition of laminarin, a beta-1,3-glucan from *Laminaria digitata* and a commercial substrate for beta-1,3-glucanases, to the assay mixture prevented the effect of *A. aegypti* larval gut soluble fraction on live *S. cerevisiae*. cells ($p > 0.05$, unpaired t-test *vs* controls, n = 9, Fig 6).

Discussion

Mosquito larvae feed on particulate material, which can include plant debris, algae, protists, and fungal and bacterial cells. In fact, several works supported by evidence derived with microscopes showed the active ingestion of microorganisms by mosquito larvae [16–21]. Sometimes the identification of intact cells inside the larval gut is difficult due to their quick disruption, which seems to be the case for protists. Considering the observed speed of the effect of *A. aegypti* larval gut homogenates on *S. cerevisiae* cells (20% viability loss in 15 minutes), this could partially explain the poor record of yeast cells inside the gut of culicid larvae.

Culicidae larvae present different modes of feeding. Although classified as filter feeders, sometimes it is hard to distinguish passive ingestion from active selection of food components. In spite of that, yeast cells have already been described as part of the mosquito diet, but there is

Table 2. Nutritional parameters of the different diets tested for *Aedes aegypti* larvae. Cat food was used to raise insects in control conditions. Yeast cells (*Saccharomyces cerevisiae*) were grown in liquid Sabourad media and offered to larvae as described. Figures correspond to protein and sugar quantities which are present in the amounts of food used to raise A. aegypti adults from eggs. See Material and Methods for details.

Nutrients	Cat Food	Yeast
Protein (mg)	6.9 ± 0.8	81 ± 4
Total sugars (mg)	54 ± 10	170 ± 20

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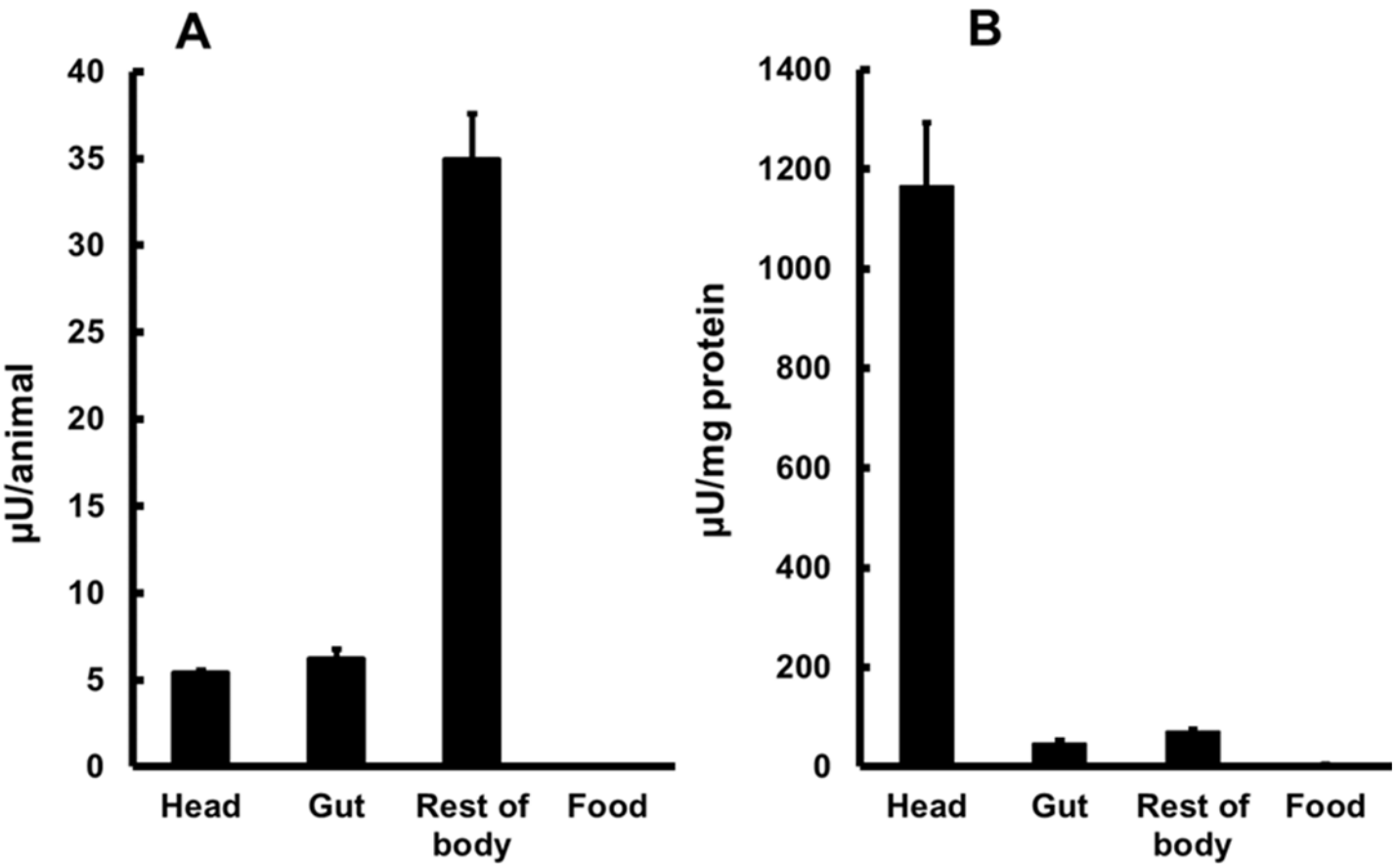

Fig 3. Beta-1,3-glucanase activity in head, gut, rest of body and food of *Aedes aegypti* larvae. (A) Activity per animal (µU/animal). (B) Specific activity (µU/mg protein). Insects were fed with cat food (Whiskas®). Figures are means ± SEM of 2 experiments with 3 samples obtained from 50 insects each.

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no evidence about the nutritional importance of these microorganisms in wild larvae. In fact, mosquito larvae seem to be strongly generalist, coping with extreme variations of microbial composition in nursing sites [9]. The data presented in this work showed that *A*. *aegypti* larvae was able to ingest yeast cells, but more experiments should be performed to assess the preference of larvae for *S*. *cerevisiae* over other dietary microbes.

Since *S*. *cerevisiae* sp. live cells were the only source of carbon and nitrogen for *A*. *aegypti* larvae in our experiments, it was expected that they produced enzymes capable of digesting the main polyssaccharides and proteins of this yeast. Digestive chitinases and trypsin-like proteases were already described in mosquito larvae [14, 33–34], but digestion of beta-1,3-glucan, the major yeast cell wall polyssaccharide [32], was never studied in Culicidae. Beta-1,3-glucanase activities were described in cockroaches, termites, grasshoppers, beetles, moth larvae and, recently, in sandfly larvae [33–41]. These are digestive enzymes involved in the breakdown of plant hemicellulose or fungal cell wall disruption. Some insect gut beta-1,3-glucanases have high lytic capacity against yeast cells, being in those cases, endo-beta-1,3-glucanases (E. C.3.2.1.39) [37].

Insects beta-1,3-glucanases are proteins belonging to glycoside hydrolase family 16 [36, 38]. In some insects as termites and moths, GHF16 proteins were incriminated in pathogen recogniton [42–43]. This dual physiological role is evident in the distribution of beta-1,3-glucanase activity in *A*. *aegypti* larvae. Gut activities seem to be constitutive, as it would be expected for a

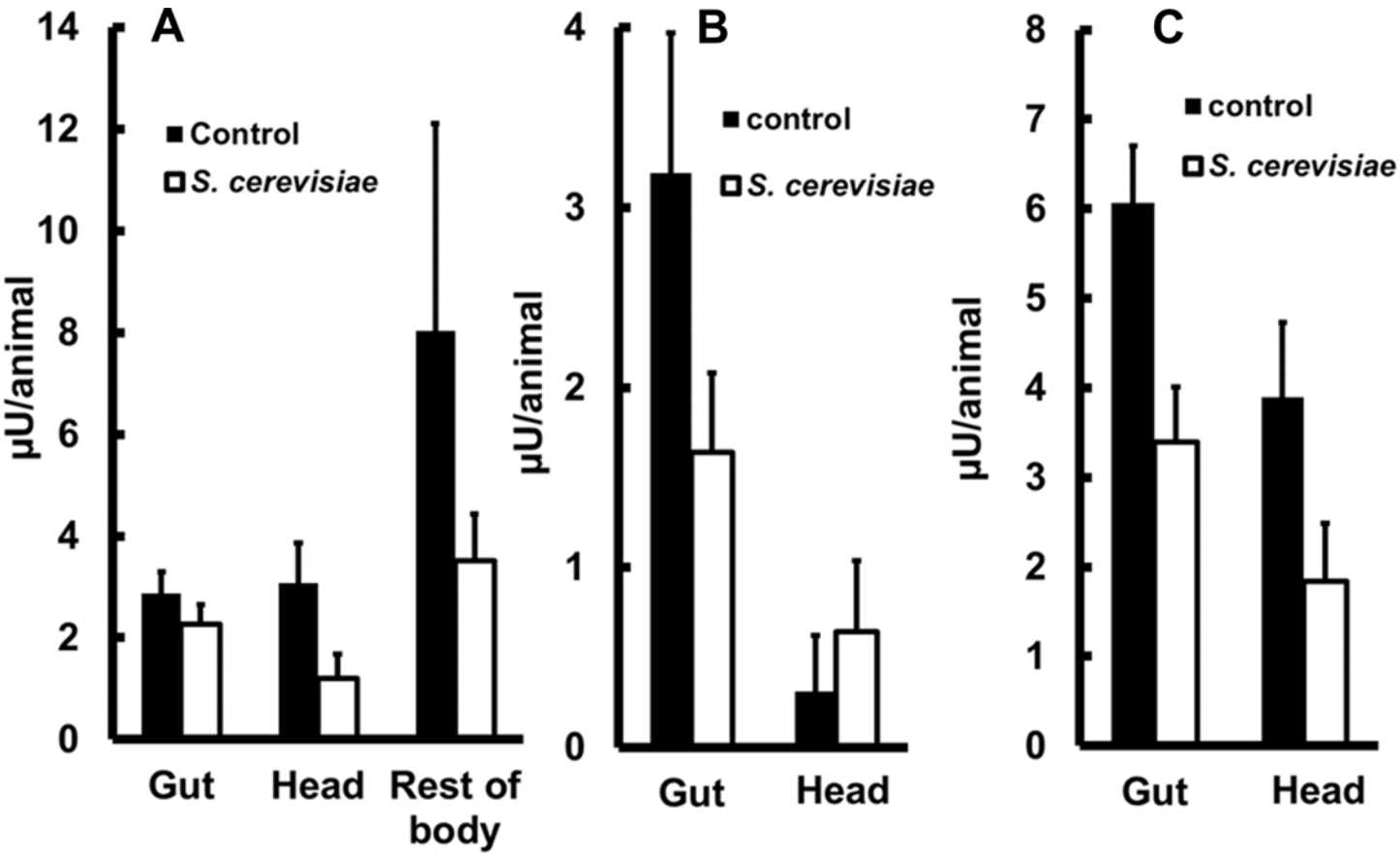

Fig 4. Beta-1,3-glucanase activity in heads, guts and rest of bodies of *Aedes aegypti* larvae fed with different diets. (A) Activities per insect (µU/animal) from soluble fractions of heads, guts and rest of bodies, (B) Activities per insect (µU/animal) from insoluble fractions of heads and guts and (C) Sum of activities in soluble and insoluble fractions of heads and guts. Larvae were fed since hatching exclusively with cat food (Whiskas®) or *Saccharomyces cerevisiae*. live cells. Figures are means ± SEM of 4 experiments with samples obtained from 50 insects each.

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digestive enzyme in holometabolan larvae [44–45]. Head beta-1,3-glucanase seems to be involved in sensing of microbes in ingested food, as autoclaved food resulted in ablation of this enzyme. A similar pattern of expression was observed for lysozymes in *Drosophila* larvae [46]. Activity in the rest of body is putatively involved in defense against pathogens, since digestion does not occur in these tissues and beta-1,3-glucans were never described as intermediate metabolites in animals. In this respect, *A. aegypti* larval beta-1,3-glucanases could be homologous to the beta-1,3-glucanases already described in other insects as beetles (gut, [37]) and moths (rest of body, [43]).

Notably, this is the first description of beta-1,3-glucanases in larvae of Culicidae. Beta-1,3-glucanase activity in sand fly *Lutzomyia longipalpis* larvae is putatively related with the active ingestion of fungal cells by this insect [40–41]. The presence of beta-1,3-glucanases in guts of *A. aegypti* larvae suggests that fungal and plant hemicelluloses could be regular components in their diet, as this enzyme has these structures as substrates in other insects. In this respect, *A. aegypti* larval gut beta-1,3-glucanase could be complementing the chitinase activity already described [33], which is putatively involved in digestion of fungi and other chitin-containing particles.

It is possible that chitinase and beta-1,3-glucanase have complementary roles in fungal cell disruption by mosquito larvae, but the observation that the presence of laminarin (commercial

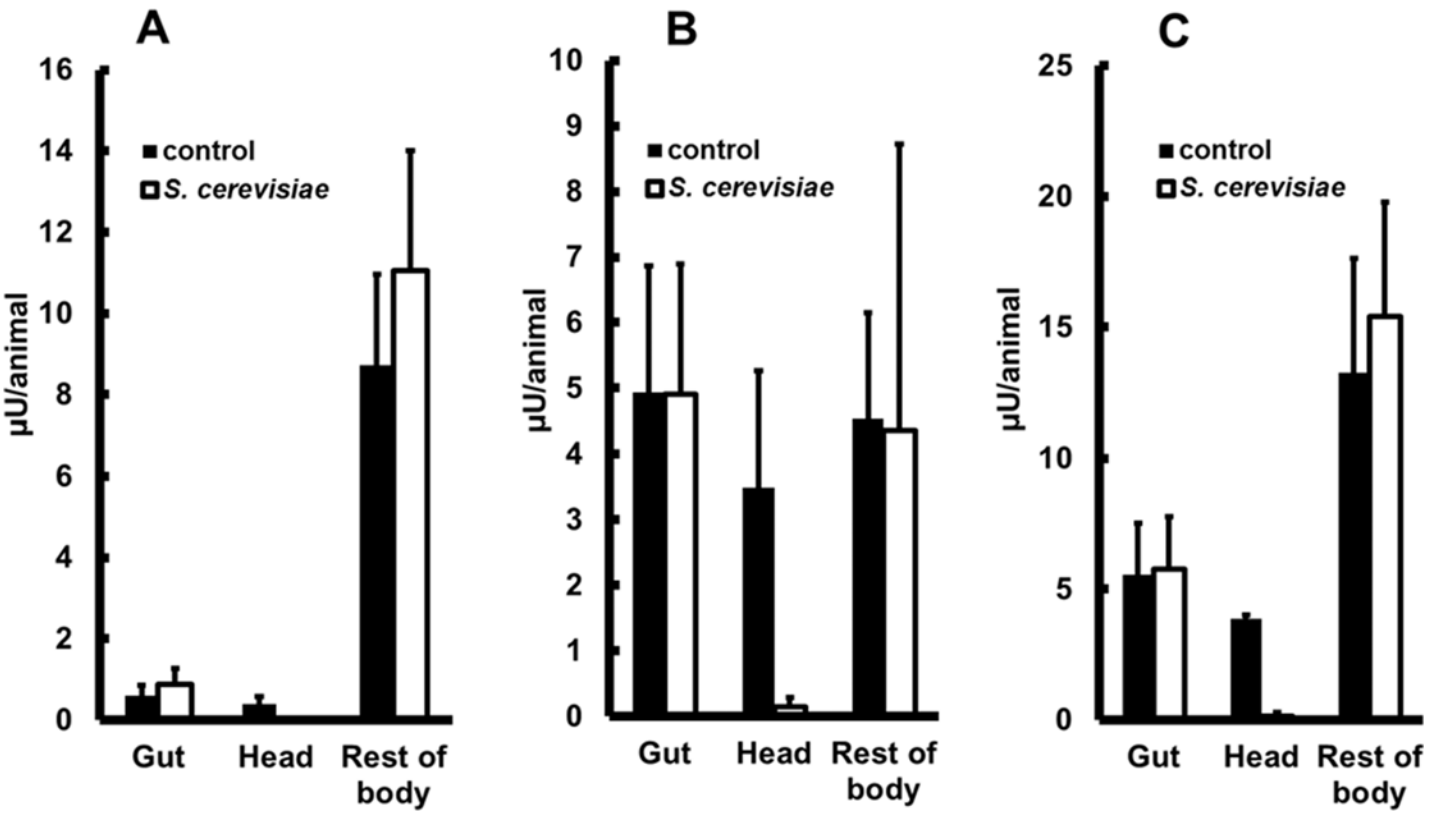

Fig 5. Beta-1,3-glucanase activity in heads, guts and rest of bodies of *Aedes aegypti* larvae fed on different diets. (A) Activities per insect (µU/animal) from soluble fractions of heads, guts and rest of bodies, (B) Activities per insect (µU/animal) from insoluble fractions of heads, guts and rest of bodies, (C) Total activities in heads, guts and rest of bodies (soluble plus insoluble fractions). Larvae were fed since egg hacthing exclusively with cat food (Whiskas®) or autoclaved *Saccharomyces cerevisiae* cells. Figures are means ± SEM of 4 experiments with samples obtained from 50 insects each.

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beta-1,3-glucan) in excess prevented lysis of live *S. cerevisiae* cells by gut homogenates suggest that beta-1,3-glucanase is essential for disruption of yeast cell walls. This evidence coincides with the predominance of beta-1,3-glucans in fungal cell walls and their structural role [32, 47]. In this respect, beta-1,3-glucanase might be an important enzyme for larval nutrition in *A. aegypti* larvae and an interesting target for inhibition, as mammals lack this enzyme (CAZY, www.cazy.org). Additionaly, beta-1,3-glucanase might be an essential enzyme for mosquito larvae feeding on fungi, as mechanical disruption of cells in insect digestion is negligible, and chemical break down of cell wall polysaccharides in necessary to permit access to intracellular nutrient sources as proteins, glycogen and nucleic acids [44–45].

Nevertheless, further molecular characterization of beta-1,3-glucanase activity in *A. aegypti* is required, because some insect beta-glycosidases also have activity against laminarin [48] and lytic activity against yeast cells was also reported for glycosidases [49]. However, it is unlikely that beta-glycosidase is the main responsible for lysis in *A. aegypti* larvae, because insect glycosidases have low binding affinity for laminarin, and in this case this substrate would constitute a poor competitor in the lytic assay.

Results shown here demonstrate that *A. aegypti* can complete development on a diet exclusively of *S. cerevisiae* cells. In this respect, these cells must contain all macro and micronutrients which are necessary for mosquito development. This is expected to a certain extent, as yeast extract (*S. cerevisiae*) had already been used as an exclusive food source to fully develop *A. aegypti* [49]. It is interesting to notice that using *S. cerevisiae* live cells we obtained a similar

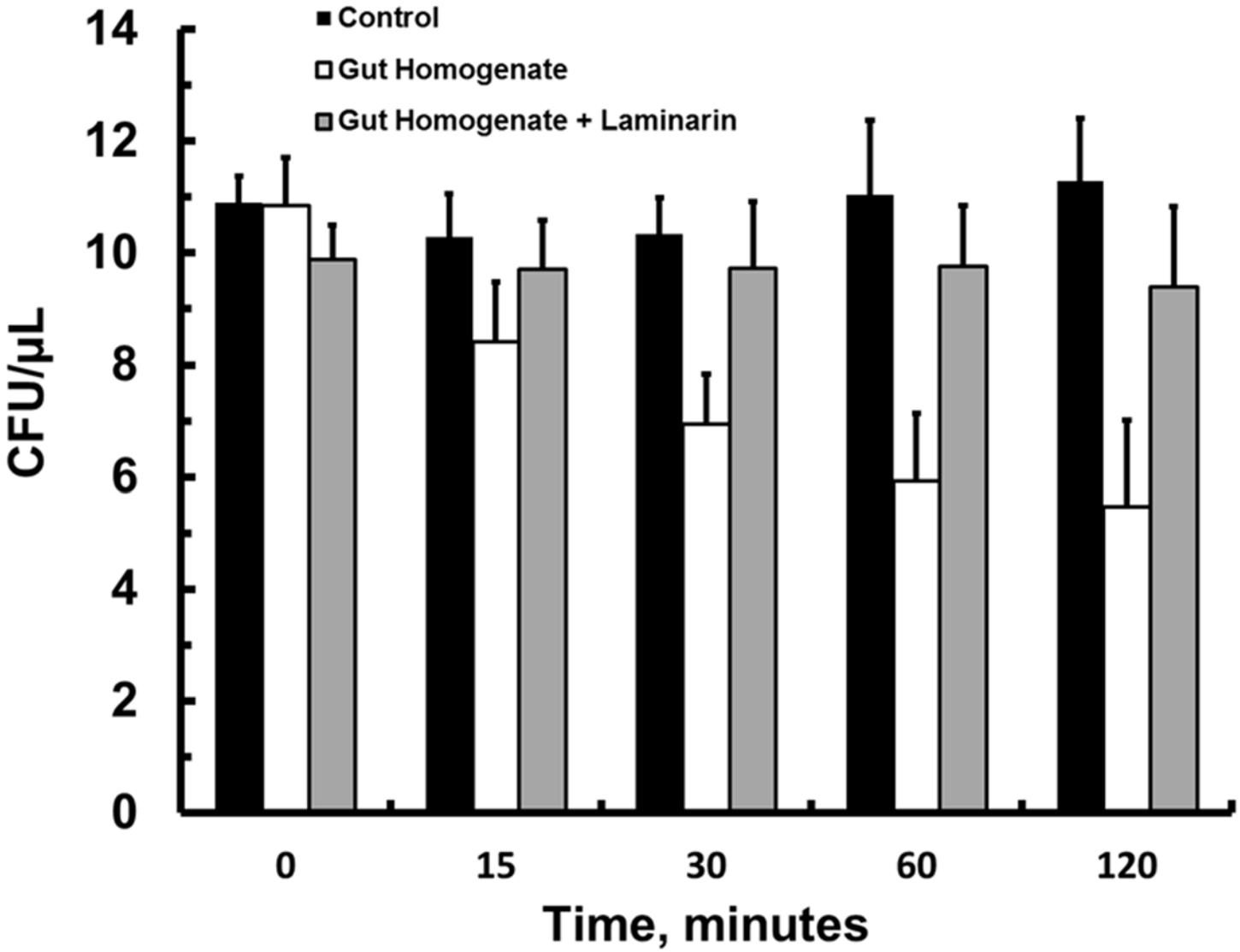

Fig 6. Lysis of *Saccharomyces cerevisiae* live cells in hypotonic media during incubation with soluble fraction of *Aedes aegypti* larvae guts. The cells were incubated in 10 mM citrate-sodium phosphate buffer pH 7. Controls—*Saccharomyces cerevisiae* cells (10 CFUs/uL) in buffer; Gut Homogenates—*S. cerevisiae* cells (10 CFUs/uL) incubated with the soluble fraction of *A. aegypti* larval midguts (0.1 animal/uL) Gut Homogenates + Laminarin—*S. cerevisiae* cells (10 CFUs/uL) incubated with the soluble fraction of *A. aegypti* larval midguts (0.1 animal/uL) plus laminarin (1.7%, w/v). Figures are means ± SEM of 3 experiments with 3 samples obtained from 10 insects each.

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delay in pupation when compared to controls (2 days), but higher percentages of adult emergence (80%) when compared to insects developed on yeast extract (58%) [50]. Our observation that the yeast-based diet has much higher protein and sugar contents than the regular cat food points to a possible deficiency in some essential micronutrient, but more studies are necessary to elucidate this issue.

In a very recent report, it was shown that *Culex pipiens* larvae are able to nourish from yeast cells of several species, including *S. cerevisiae* [51]. This fact suggests that the nutritional relation between mosquito larvae and yeasts is at least partly shared among Culicidae. It is likely that the lytic mechanism in *C. pipiens* involves the action of a β-1,3-glucanase as in *Aedes*, but this still needs to be confirmed. Interestingly, our data suggest that *S. cerevisiae* might be a potential probiotic for mosquito larvae, besides being a promising component for the

development of diets for larvae based on microorganisms. This might result in cheaper, pathogen free, and more reproducible diets for these insects, with an important impact in mass rearing which is necessary for the development of new vector control management strategies. Considering that *Drosophila* may also be reared on yeasts [52], there is an interesting nutritional parallel throughout the order Diptera. Diets containing yeast cells may be a starting point to novel strategies for intervention in the metabolism or genetics of mosquitoes. These new approaches might include knockout or mutant yeasts, yeast producing recombinant proteins, GFP tagged peptides or dsRNA.

Conclusions

Aedes aegypti larvae were able to ingest and break down live yeast cells (*S. cerevisiae*). Beta-1,3-glucanase activities were present in the head, gut and rest of body of these insects, being involved in yeast digestion (gut) and possible recognition of invading microorganisms (head and rest of body). Beta-1,3-glucanase in the gut and rest of body were not affected by yeast diets, but head activity is suppressed in insects fed on autoclaved cells, suggesting a role in sensing of food-borne microbes. *A. aegypti* larval gut beta-1,3-glucanase was essential for lysis of yeast cells, and might be a crucial enzyme when these insects feed solely on this nutrient source.

Supporting Information

S1 File. The file S1_File.xls includes raw data used for the calculations of biological parameters, enzyme activities, sugar and protein quantitatities and yeast cell counts, used in Figs 1–6 and Tables 1 and 2.
(XLSX)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: RJD VMD FAG. Performed the experiments: RSS HMD. Analyzed the data: RSS HMD. Contributed reagents/materials/analysis tools: FAG. Wrote the paper: FAG VMD RJD.

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Artigo 3

Microorganism-based larval diets affect mosquito development, size and nutritional reserve in the yellow fever mosquito *Aedes aegypti* (Diptera: Culicidae)

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Microorganism-based larval diets affect mosquito development, size and nutritional reserves in the yellow fever mosquito Aedes aegypti (Diptera: Culicidae)

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Author contribution statement

Performed the experiments - RSS, FV
Analyzed the data - RSS, FV
Conceived the study - all authors
Wrote and revised the manuscript - all authors

Keywords

Aedes (Ae.) aegypti, nutrition, Digestion, development, insect, Larvae - development, Larvae & pupae, Nutritional reserves, Microorgamisms

Abstract

Word count: 304

Background: Mosquito larvae feed on organic detritus from environment, particularly microorganisms comprising bacteria, protozoa and algae as well as crustaceans, plant debris and insect exuviae. Little attention has been paid to nutritional studies in A. aegypti larvae. Objectives: Here, we investigated the effects of yeast, bacteria and microalgae diets on larval development, pupation time, adult emergence, survivorship and lifespan. Material and Methods: All microorganisms used were ingested by larvae after 2h. Protein, carbohydrate, glycogen and lipid were analysed in single larvae to correlate energetic reserve accumulation by larva with the developmental rates and nutritional content observed. Results and Discussion: Immature stages develop in all diets, however larvae fed with bacteria and microalgae showed a severe delay on development rates, pupation time, adult emergence and low survivorship. Adult males eclosing earlier and had longer survive than females. Bacterial diets affect size and shape of adult wings. Asaia sp. and E. coli seem the best candidates to future studies using symbiont-based control. The diet quality was measured and presented different protein and carbohydrate amounts. Bacteria had the lowest protein and carbohydrate rates, S. cerevisiae and Pseudozyma sp had the highest carbohydrate amount and Chlorella sp. and A. platensis showed the highest protein content. In bacterial diet of E.coli and Asaia sp., as expected, the larvae had more nutrient reserves. Larvae fed with microalgae seem not able to process and storage these diets properly. Larvae showed to be able to process yeast cells and store their energetic components efficiently. Conclusion: Together, our results point that A. aegypti larvae show high plasticity to feed, being able into develop under different microorganism-based diets. The important role of A. aegypti in the spread of infectious diseases require exhaustive biology studies in order to undestand the vector phisiology and thus manage the larval natural breeding sites aiming a better mosquito control.

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Running Title: Physiological effects of Microorganism-based larval diets for *Aedes aegypti* larvae.

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ABSTRACT

Background: Mosquito larvae feed on organic detritus from environment, particularly microorganisms comprising bacteria, protozoa and algae as well as crustaceans, plant debris and insect exuviae. Little attention has been paid to nutritional studies in *A. aegypti* larvae. **Objectives:** Here, we investigated the effects of yeast, bacteria and microalgae diets on larval development, pupation time, adult emergence, survivorship and lifespan. **Material and Methods:** All microorganisms used were ingested by larvae after 2h. Protein, carbohydrate, glycogen and lipid were analysed in single larvae to correlate energetic reserve accumulation by larva with the developmental rates and nutritional content observed. **Results and Discussion:** Immature stages develop in all diets, however larvae fed with bacteria and microalgae showed a severe delay on development rates, pupation time, adult emergence and low survivorship. Adult males eclosing earlier and had longer survive than females. Bacterial diets affect size and shape of adult wings. *Asaia* sp. and *E. coli* seem the best candidates to future studies using symbiont-based control. The diet quality was measured and presented different protein and carbohydrate amounts. Bacteria had the lowest protein and carbohydrate rates, *S. cerevisiae* and *Pseudozyma* sp had the highest carbohydrate amount and *Chlorella* sp. and *A. platensis* showed the highest protein content. In bacterial diet of *E.coli* and *Asaia* sp., as expected, the larvae had more nutrient reserves. Larvae fed with microalgae seem not able to process and storage these diets properly. Larvae showed to be able to process yeast cells and store their energetic components efficiently. **Conclusion:** Together, our results point that *A. aegypti* larvae show high plasticity to feed, being able into develop under different microorganism-based diets. The important role of *A. aegypti* in the spread of infectious diseases require exhaustive biology studies in order to undestand the vector phisiology and thus manage the larval natural breeding sites aiming a better mosquito control.

Keywords: *Aedes aegypti*, microorganism, development, nutritional reserves

INTRODUCTION

Mosquitoes are medically the most significant group of insects due to their important role in the widespread of several human infectious diseases including malaria, dengue fever, encephalitis, yellow fever and filariasis (Weaver and Reisen 2010). The global magnitude of morbidity and mortality caused by arthropod borne diseases has been a public health emergency of international concern. Early stages of mosquito development are related to aquatic environments, thus understanding ecological factors involved in the aquatic habitats is essential in order to develop and/or improve effective strategies of mosquito control.

The biotic and abiotic environmental conditions experienced during the immature stage are determinant for the growth and development of mosquitoes. A considerable number of studies in the early 20th century devoted attention to investigating the food requirements of larvae in order to reduce or eliminate the nutritional supply of these insects in nature (Hinman 1930). Studies on holometabolous insects suggest that well-nourished larvae become healthier adults (Zeller and Koella 2016). The biomass accumulation of mosquitoes can be attributed to the efficiency of foraging by larvae and withstanding of starvation (Barrera 1996). Quantitative and qualitative aspects of larval nutrition exert immediate effects on immature survivorship and development rate, which can alter population dynamics of mosquitoes and determine adults life traits (Subra and Mouchet 1984, Gimnig et al 2002, Barrera et al 2006, Araújo et al 2012, Li et al 2014, Radchuk et al 2013, Kivuyo et al 2014).

Mosquito populations that develop in containers can be regulated by availability and amount of food resources in the aquatic habitat (Washburn 1995). Food deprivation can have carry-over several effects on mosquito life. A longer development time under conditions of food insufficiency has been observed before (Tun-Lin et al 2000, Arrivillanga and Barrera 2004, Dominic et al 2005, Vantaux et al 2016, Aznar et al 2018), with mosquito larva that take longer time to reach pupa stage (Telang et al 2007, Levi et al 2013, Banerjee et al 2015). An extended larval stage is generally associated with an increased risk of mortality as a consequence of predation, breeding site instability and/or human interference (Padmanabha et al 2011). Beyond development time, the amount of food influences characteristics such as: nutritional reserves (Van Handel and Day 1989, Briegel 1990, Gullan and Cranston 1999, Arrivillaga and Barrera 2004), adult emergence

(Okech et al 2007), body size (Grimstad and Walker 1991, Strickman and Kittayapong 2003, Jirakanjanakit et al 2007, Foster et al 2012, Aznar et al 2018), response to repellents and insecticides (Xue et al 1995, Xue and Bernard 1996), survival (Dominic et al 1996, Landry et al 1988, Sumanochitrapon et al 1998, Aznar et al 2018), sexual maturity, fecundity, egg production and longevity of the adult female (Briegel 1990, Nasci and Michell 1994, Sumanochitrapon et al 1998, Naksathit and Scott 1998, Reiskind and Lounibos 2009, Foster et al 2012, Alto et al 2012, Takken et al 2013). The vector competence also could be influenced by the available food resource. Adults that emerge from larvae with low nutritional reserve are smaller (Lehmann et al 2006) and require more blood feeds to produce eggs (Briegel 1990), which may lead to an increase in their vectorial capacity (Muturi et al 2011). Restricted larval food can extend the time for mosquitoes to become infectious (Shapiro et al 2016, Vantaux et al 2016), modulate microbiota (Linenberg et al 2016) and permissiveness to parasites (Linenberg et al 2016, Takken et al 2013), affecting immune traits (Suwanchaichinda and Paskewitz 1998, Telang et al 2012).

Previous studies reported that *A. aegypti* size is vulnerable to food amount and population density in immature stages (Jirakanjanakit et al 2007). To measure directly the mosquito body is not a satisfactory estimation of size, due the variation of three-dimensional structures, besides the variation in dryness of the abdomen. Weight can be another estimator of size, but it can be influenced and variated by the blood feeding, egg-production, etc. The estimation of the mosquito body size can be doing using the wing length, in a linear measurement, using the traditional morfometry (Carron 2007, Jirakanjanakit et al 2007, Siegel et al 1992, Lounibos 1994, Strickman e Kittayapong 2003, Lehmann et al. 2006). But the arising of the geometric morphometrics allowed the assess of the size sensible to various directions of change by means of the calculation of the centroid size (Bookstein 1991), besides the decomposition of the metric variations into size and shape and to assess shape changes.

Immature stages of culicides are generally undemanding and have a pliant food behavior, ingesting through different fedding modes (e.g filtering, suspension feeding, browsing, interfacial feeding) organic particles on water and almost everything available in the natural or artificial environments (Walker et al 1988, Merritt 1992, Clements 2000). Particulate microorganisms and organic debris are commonly the main nutritional source of mosquito larvae. Bacteria, viruses, protozoa, fungi (Timmermann and Briegel 1996, Forattini 2002) and algae (Merrit 1992, Kivuyo et al 2014) are some of the organisms that actively contribute to foraging and development during the larval stage. Bacteria seems the most abundant microorganisms present in the larval diet, and may even be the only nutritional source for insect growth and development (Merritt 1992). Pollen particles dispersed in the aquatic environment can also be used as food sources by immature forms (Ye-Ebiyo 2003, Kivuyo et al 2014, Asmare et al 2017).

The evolutionary success and extensive dispersal of mosquitoes may have been widely motivated by symbiotic relationships with microorganisms (Ricci et al 2011, Coon et al 2014). Insects harbor numerous symbiont microbial communities, which possibly supplant the number of cells in the insect itself (Gusmão et al 2010). Intracellular symbionts can occur in up to 70% of all insect species and the intestinal compartment concentrates most of these microorganisms (Gusmão et al 2010). The contribution of the intestinal microbiota of insects in nutritional ecology is quite relevant due to its impressive biosynthetic and degradative capacity (Douglas 2009, Kukutla at al 2014). The insect microbiota plays a important role in synthesis of vitamins and essential amino acids, steroids and carbohydrates metabolism and promoting the growth and development using the insulin pathway (Storelli et al 2011, Shin et al 2011, Douglas 2014). Besides nutrition, symbionts aid in nitrogen fixation, behavior, reproduction, development and enhance or suppress infections by pathogens (Dillon and Dillon 2004, Hegde et al 2015).

Aspects such as digestion, processing, absorption and detoxification of such generalist diets are result of refined interactions with symbionts and digestive enzymes (Fisk 1952, Geering 1975, Marinotti 1990, Ho et al 1992, Souza et al 2016). Yet, it is still unclear as the several microbial nutritional source may influence the physiology of larval mosquito and which are the main enzymes involved in the digestion of these nutrients.

In this study, we investigated *A. aegypt*i larval feeding using a range of microorganisms as nutritional source. Life parameters including development rates, survival, sex ratio, body size, ingestion rates, quantity and quality of food and nutritonal reserve accumulation were reported in this paper. The results suggest that microorganism-based diets can be supported by these insects in laboratorial conditions and aim to provide informations to laboratory breeding or studies for potencial biolarvicides.

MATERIALS AND METHODS

Mosquito Rearing- The *A. aegypti* specimens eggs used for this study, were originate from eggs of Rockefeller strain gently ceded by Dr José Bento Pereira Lima - from colonies of the Laboratory of Physiology and Control of Arthropod Vectors (LAFICAVE, -IOC/-FIOCRUZ); Dr José Bento Pereira Lima). Insects were reared until adult stage in the Laboratory of Insect Biochemistry and Physiology (LABFISI, IOC/FIOCRUZ) under standard conditions (temperature 26 ±1◦C, relative humidity 80 ± 5% and photoperiod 12:12h [L: D]). Newly hatched larvae derived from the same egg batch within 2 h of eclosion were fed Tetramin e® sprinkled on the distilled water surface until the nutritional trials being performed.

Screening of Microorganisms - The nutritional physiology experiments were performed based on the follow microorganisms: *Serratia marcescens* (SM365), *Escherichia coli* (D31) and *Staphyloccoccus aureus* isolated and cryopreserved in the LABFISI, *Saccharomyces cerevisiae* (S14) kindly donated by Dr. Pedro Soares de Araújo (Chemistry Institute, University of São Paulo), *Asaia* sp. (A1), *Ochrobactrum intermedium* (Om17), *Bacillus* sp. and *Pseudozyma* sp (Pa1) by Dr. Rod J. Dillon (Faculty of Health and Medicine, Lancaster University, UK), *Arthrospira platensis* (*Spirulina*) and *Chlorella* sp. by Dr. José Bonomi Barufi (Laboratory of Ficology, Federal University of Santa Catarina, Brazil).

Preparation of Microorganisms Diets– Aliquots of *S. marcescens*, *E. coli*, *Bacillus* sp, *O.* intermedium and *S. aureus* were inoculated in Luria-Bertani agar plates (LB) and incubated overnight for 24 hours at 30°C. *S. cerevisiae* and *Pseudozyma* were inoculated in YEPD agar plates (1% yeast extract, 2% peptone, 2% glucose/dextrose, 2% agar). Growth conditions: *S.cerevisiae* overnight for 24 hours at 30°C and *Pseudozyma* sp 48 hours at 30°C. *Asaia* sp. were inoculated on GCA agar plates (2% glucose, 0.8% yeast extract, 0.7% de CaCO3, 2% agar) and incubated overnight for 72 hours at 26°C (Sant'Anna et al., 2014). Bacteria single colonies were transferred to LB medium, yeast-like fungus to YPD medium and *Asaia* sp to GLY medium (glycerol 25 g/l, yeast extract 10 g/l, pH 5.0) in 50 mL polypropylene tubes. All strains grown according to the incubation temperatures of each strain in a shaking incubator (150 rpm). The microbial suspensions were centrifuged (20 minutes, 21.000 g, 4°C) and the supernatant was

discarded to fresh mass (FM) measurements. Cells harvested by brief spin were washed with sterile PBS three times and finally the bacterial and yeast pellet was resuspended in sterile water and adjusted in a concentration of 800 mg / 80 mL (w/v) per strain. *Chlorella* sp. were inoculated in Bold's Basal Medium (BBM) and *A. platensis* were inoculated in Spirulina Medium Modified (Anderson 2005). *Chlorella* sp and *A. platensis* were incubated at 21°C with photoperiod of 12:12 [L: D] h in a shaking incubator (100 rpm). They were centrifuged gently (5 minutes, 5,000 g), before the measure of their biomass. Cells harvested by brief centrifugation were resuspended in their respective medium and were adjust in a concentration of 150 mg / 15 mL (w/v).

Microorganisms Viability Trials- To evaluate the capacity of microorganisms used in this study remains alive in the aquatic environment, we observed the viability of these strains on water. Bacteria and yeast were inoculated in 50 mL polypropylene tubes in specific liquid media and growth conditions were described previously (see details in Preparation of Microorganisms Diets). Cells harvested by centrifugation (20 minutes, 21.000 g, 4°C) were resuspended in liquid media or sterile water. Twenty microliter aliquots of each suspension were placed in agar plates and the the number of colony forming unit, CFU were recorded after 0, 24, 48 and 120 hours. Five experiments were perfomed.

Experimental Nutrition Protocol - Ten diets were compared. Groups of 150 first instar larvae (L1) were manually counted and transferred to each of three sterile borosilicate glass recipients (22.5 cm x 12.8 cm x 3.59 cm). Under sterile conditions each container was filled with QSP 250 ml of sterile water or distilled water (density = 0.22 larvae/cm^2 of surface área; depth of 39 mm). The dietary supply was administered only at the L1, 80 mL (corresponding to 800 mg [w/v] and 16 mg / larva) of yeasts and bacterial and 15mL (corresponding to 150 mg [w/v] and 3 mg / larva) of microalges was added to glass recipients. A slurry by mixing the components of Tetramin® in distilled water was prepared to fed standard group. We used 800 mg of Tetramin® resuspended in distilled water QSP to final volume of 250 mL. Evaporated water was replaced as needed to maintain the initial volume. Three replicates were performed for each dietary experiment.

Effects of Diets on Development and Survivorship of *A. aegypti* – For comparison of diet effects, developmental rate and survivorship from eclosion to adult emergence were

measured. Larvae were observed daily until pupation and dead larvae and exuviae were removed. Pupae were collected daily, counted and transferred individually into 15 mL polypropylene tubes covered with mosquito netting and filled with 4 mL of breeding water until adult emergence. The number and the sex of adults emerged were determined. Adults received only cotton wool moistened with distilled water *ad libitum*. The median time in days for pupation, emergence of adults (males and females) and adult survivorship were calculated as the number of individuals that reached pupae or the adult stage deducted by the initial number of larvae. Development was estimated to pupation (proportion of larvae that survived from L1 to the pupal stage), time to metamorphosis (development duration in days, between pupa and adult stage), time to emergence (development duration in days, between L1 and adult stage), adult survival (the proportion of adults that survived from emergence to adult dead) and survivorship full span (the proportion of larvae that survived from L1 to dead adult stage). Sex ratio was estimated as the number of males relative to total emerged adults.

Wing length measurements- To evaluate possible morphological variation in body size of adults reared with microorganism diets, we measured size and shape of males and females wings separately. In this study, we used the wings of adults emerged from larvae fed with standard diet Tetramin®, *S. cerevisiae, Pseudozyma* sp., *E. coli* and *Asaia* sp. (Table S1). The bilateral wings were removed from the thorax of individuals, mounted on Canadian balsam microscope glass slides and processed as reported by Lorenz *et al*. (2012). Images of the slides were digitized by means of a digital camera Leica DFC320 coupled to a Leica S6 (40x) stereoscope. To each image were registered coordinates x and y of eighteen landmarks by means of TpsDig software V.2.05 (Rohlf 2006). Procrustes superimposition, discriminant analysis, and cross-validated reclassification test were used to assess the wing shape variations. Differences in wing shape were determined by canonical variate and performed with MorphoJ software (Klingenberg 2011). To estimate the metric distance, reclassification tests were realized using the Mahalanobis distances (MD) (cross-validated classification). The wing size variations were assessed using measurements of the centroid size (CS) (Bookstein 1991). Allometry is basically, the variation/escalation of some characteristic of organisms according to the body size (Thompson 1917, Huxley 1932, Gould 1966). To report the shape variations, the allometry influence was removed in all cases, and the analysis were performed with

and without it. To allometry extraction, a regression analysis was realized between the coordinate landmarks and CS for each comparison performed.

Microorganism Staining and Larval Feeding Behaviour- We decided to monitor the ingestion of live microorganisms labelled with fluorescein isothiocyanate (FITC) by *A. aegypti* larvae. The protocol of microorganism staining was performed according to Moraes et al (2012). FITC-labelled microorganisms were ressuspended in 3 mL of sterile water and added to 50 mL polypropylene tubes containing 7 mL of sterile water and 50 fourth instar larvae raised on Tetramin®. After 2 hours incubation at 26 ºC, 10 larvae were dissected and single guts were placed in microtubes with 100 µL of sterile NaCl 0.9% (w/v). Samples were homogenized by shaking the tube for 30 s at 25 Hz (MiniBeadBeater; Biospec products, Bartlesville, Oklahoma, US). The gut fluorescence detection was performed in a FlexStation 3 Multi-Mode Microplate Reader (Molecular Devices, San Jose, California, US) on λEx = 495 nm and λEm = 520nm. Aliquots (10 µL) of microorganisms FITC-labelled were mounted on microscope glass slides for fluorescence observation in a Nikon Elipse E200 (40x), fitted with a B-2A filter (Excitation Filter Wavelengths: 450–490; Dichromatic Mirror Cut-on Wavelength: 500; Barrier Filter Wavelengths: 515). Images were taken with a regular digital camera. Five experiments were performed.

Protein and Carbohydrate Contents of Microorganism-based Diets - Culture samples of 20 mL were centrifuged (7.500 x g, 20 min, 4°C). Supernatant was discarded and cells were ressuspended in 1 mL of water. Aliquots of 10 µL and 40 µL were withdrawn for protein and sugar measurements respectively. We assessed total protein content using the bicinchoninic acid method procedure (Smith, 1985) and total carbohydrates were measured with the phenol-sulfuric method (Dubois, 1956). Eight experiments were performed for each diet.

Measurement of the Energy Reserves in Single Individuals - Fourth instar newly molted larvae were individually weighed and immediately frozen for analysis of nutritional reserves. We quantified protein, total carbohydrate, glycogen and lipids in single individual fourth instar entire larvae, gut and rest of the body for comparison. Larvae were reared as reported in item ¨**Experimental Nutritional Protocol**¨ and dissected as described in ¨**Microorganism Staining and Larval Feeding Behaviour**¨.

The biochemical analysis were performed accordingly the Van Handel's method (1985) adapted by Foray et al 2012. Protein content was measured as Bradford (1976) method using ovalbumin as a standard. Carbohydrates and glycogen were detected by an anthrone procedure using glucose as a standard (Van Handel, 1985a). Total lipid was determined in chloroform–metanol solvent solution by a vanillin–phosphoric acid reaction (Van Handel 1985b, 1988) using Glyceryl trioleate as standard (Van Handel and Lum 1961). The assays were performed in 96-well microplates. Ten experiments were performed for each diet.

Statistical analysis- For all experiments, measurements were described using mean ± SEM. Developmental parameters (larval development, pupation, emergence and survivorship) were analyzed using the GraphPad InStat v.3.01 (San Diego, CA) and Excel®. The correlation among non-parametric variables was performed using the Log-rank (Mantel cox), Wilcoxon and Fisher tests. Tests for normality of the sample distribution were assessed by D'Agostino-Pearson omnibus test. Microorganism's viability significance and measurement of protein and carbohydrate content on diets were examined with a T-test. Analysis of variance (ANOVA 1) was used in ingestion of live microorganisms labelled with FITC and measurement of the energy reserves in whole larva. The wings morphometric statistical analyses were managed with the softwares MorphoJ, and GraphPad InStat v.3.01 (San Diego, CA). Using MorphoJ software, permutation test with 10,000 randomizations was applied to assess the significance of the allometry. Using Past3 software, the normality and homoscedasticity of the sample distribution were assessed by Shapiro-Wilks. In populations that had a Gaussian distribution, the parametric T-test was used based on means. In populations that did not, the non-parametric Mann-Whitney test was used based on medians.

RESULTS

To verify if all the strains that will be used in our experiments are viable on aquatic conditions we tested the viability of the microorganism cells in water. CFU counts revealed that resuspension in water does not affect the viability and number of cells ($p > 0.05$, paired T-test, n = 15, Figure 1). *S. cerevisiae*, *S. marcescens*, *Bacillus* sp. and *O. intermedium* remained viable after being incubated in water for 48 hours and *Pseudozyma* sp, *E. coli*, *Asaia* sp and *S. aureus* were viable until 24 hours (Figure 1). These data demonstrate that is possible to expose larvae to live cells and this microbial cells might be used as a nutritional source.

Estimates of development and survivorship for *A. aegypti* reared using exclusive microorganism-based diets

To assess the development and survivorship until adult stage of *A. aegypti* reared exclusively with microorganism-based nourishment at immature stages, four strains of gram negative bacteria were used: *S. marcescens* (SM365), *E. coli* (D31), *Asaia* sp. (A1) and *O. intermedium*. Two strains of gram negative bacteria: *Bacillus* sp. and *S. aureus*. We also used the yeasts: *S. cerevisiae* (S14) and *Pseudozyma* sp. (Pa1). A genus of microalga *Chlorella* sp. and a specie of cyanobacteria (blue-green algae), *A. platensis* (*Spirulina*), were also tested. The biological life attributes measured for the *A. aegypti* Rockefeller strain under controlled laboratory conditions were presented from Supplementary Tables 1 to 6, Tables 1 to 5, and summarized as follows.

The developmental time from L1 to pupae differed significantly ($p < 0.0001$) between the diets. Larvae of the standard group fed with Tetramin® developed in 5.3 ± 0.04 days. *Pseudozyma* sp and *S. cerevisiae* (mean 6.5 ± 0.12; 8.1 ± 0.05 days) developed faster than the larvae on other microorganism-based diets. Larvae fed with the *Chlorella* sp take longer to develop until pupation with mean time of 61.5 ± 2.09 days until pupa (Supplementary Table 1). *S. marcescens* (42.0 ± 3.0 days) and *Bacillus* sp (34.0 ± 0.0 days) showed the longest time to achieve pupal stage compared with the others bacterial diets.

The next biological parameter evaluated was the duration of the metamorphosis period of larvae in adult mosquitoes. No significant differences ($p > 0,005$) were detected in diets that used *Asaia* sp. (2.0 ± 0.04 days; p= 0.6804), *O. intermedium* (2.0 ± 0.00 days; p= 0.3652), *Chlorella* sp. (1.9 ± 0.07 days; p= 0.0978) and *A. platensis* (1.9 ± 0.09; p= 0.1042) compared to the standard group fed with Tetramin® (2.09 ± 0.02 days) (Supplementary Table 2). Time from L1 to adult emergence differed significantly ($p < 0.0001$) among each diet. Time until adult stage was higher to *Chlorella* sp (Supplementary Table 3).

The survival of adults maintained only with water was evaluated once a day until confirmation of the death of all insects. The diets containing *E. coli* (6.2 ± 0.3 days) and *Asaia* sp. (5.5 ± 7.52 days) showed the closest survival rates compared to the standard diet (8.9 ± 0.1 day). *Bacillus* sp. (2.0 ± 0.00), *S. aureus* (2.3 ± 6.1 days) and *O. intermedium* (2.5 ± 2.1 days) revealed the lowest survival rates (Supplementary Table 4). The full lifespan from L1 to adult death is significantly ($p < 0.0001$) different between diets (Supplementary Table 5).

Biological parameters were analyzed separately by gender to disclose possible sex-specific effects in development rates and survivorship. The development time of L1 to pupae differed significantly in females and males ($p < 0.001$; Table 1) from different diets. Larvae fed with yeast diet developed faster in both genders (mean 7.2 ± 0.22; 8.4 ± 0.06 days for females; 6.1 ± 0.13; 7.9 ± 0.07 days for males) than larvae on bacteria and microalgae diets. Male development time until pupa is shortest than female larvae in all diets used (Table 1). No significant differences ($p=0.7867$) were detected for female adult metamorphosis on diets containing *Asaia* sp. However, a significant ($p < 0.0001$) effect was observed among all the others diets compared to the standard group fed with Tetramin® (Table 2).

Male development time until adult metamorphosis differed significantly ($p < 0.0001$) solely on yeasts diets, and no significant differences were observed among the others diets (Table 3). Female average development time until adult emergence was longest than males. In both genders a significant difference ($p < 0.0001$) was detected when compared microorganism-based diets with the group fed with the standard diet Tetramin (Table 3). The survival of adults (males or females) differ significantly ($p < 0.0001$) between diets. The average survival time of each female adult varied from 2.6 ± 0.93 to 5.1 ± 0.73 days across the different dietary supply. Females fed with *A. plantensis* (5.1 ± 0.73 days), *Asaia*

sp. (4.8 ± 0.39 days) and *E. coli* (4.6 ± 0.40 days) exhibited an elongated average survival (Table 4).

The adult survival pattern observed in males differed partially from females. The average observed in the survival span for males varied from 2.1 ± 0.39 to 7.0 ± 0.44 days. Males fed with *E. coli* (7.0 ± 0.44) and *Asaia* sp. (6.1 ± 0.24 days) displayed the same longest survival observed for females. Additionally, males fed with *Pseudozyma* sp (5.6 ± 0.19 days) and *S. cerevisiae* (5.5 ± 0.28 days) also presented a long survival (Table 4). The full lifespan of males and females from L1 larvae to pupation differ significantly among diets ($p < 0.0001$). The average life span varied from 13.0 ± 0.23 to 73.5 ± 4.74 days for females and 14.0 ± 0.17 to 65.0 ± 2.32 days for males (Table 5). The overall adult sex ratio was more male biased (Supplementary Table 6). The development time of L1 to pupae, adult emergence, survivorship and lifespan of all dietary studied have been depicted graphically in Figures 2-5.

Yeast diets revealed a similar development time to standard diet Tetramin®. The diets of bacteria and microalgae, in opposite, presented a lethargic larval development (Figures 2 and 3). Regarding adult mortality, *E. coli* and *Asaia* sp presented the highest survival mean in days, surpassing even the yeast diets (Figure 4). The full lifespan was extended in immature stages fed with bacteria and microalgae diets. These results suggest a possible badly nourishment which could breed smaller larvae with difficulty to attain the critical mass that is necessary for metamorphosis (Figure 5; Telang et al 2007). All the compiled biological development data were detailed in the supplementary spreadsheet S1.

The geometric morphometry of mosquito wings was assessed on size and shape, separately. Canonical variate analysis revealed differences in wing shape between all comparisons, more evident in females and in the *Asaia* sp. x *E. coli* x Tetramin ® comparison (Figure 6). In general, all of the treatments showed shape differences when compared with the Tetramin control.

Mahalanobis distances were significant in all of the comparisons without allometry. Based on this distances, it was possible to observe that larvae feed with *E. coli* showed major dissimilarity from the control (Tetramin®) than *Asaia* sp. group (in both genders and wing sides). In females, *Pseudozyma* sp. showed in both wing sides more dissimilarity than *S. cerevisiae* when compared to Tetramin®. This same comparison in

male wings, showed a subtle difference: the right wings remained more dissimilar in *Pseudozyma* sp. than in *S. cerevisiae*, but the left wings of mosquitoes fed with *S. cerevisiae* showed more dissimilar than *Pseudozyma* sp. when compared to Tetramin®. (Supplementary Table 7).

Cross-validated reclassification, in other words, the capacity of the individuals of a determinate group be classified corrected in their own groups, varied from 47 to 95.23% with allometry and 41.17 to 100% without allometry in *Asaia* sp. x *E. coli* x Tetramin ® comparison; and from 63.33% to 100% with allometry and 69.56 to 100% without allometry in *Pseudozyma* sp. x *S. cerevisiae* x Tetramin ® comparison. Even though were a low allometry (from 1.26% to 8.13%, <10%), there was an expressive difference between the comparisons with and without allometry, the majority of groups were better reclassified (cross-validated reclassification) without allometry (Supplementary Table 7). When females and males were compared as for their wing geometric morphometry, females showed major allometry. And when the wing sides were compared, the allometry was bigger in the comparisons of right wings in all analysis, except for females of *Pseudozyma* sp. x *S. cerevisiae* x Tetramin® comparison.

Related to size analysis, the majority of samples showed normal distribution ($p>0.05$, Supplementary Tables 8 and 9). In general terms, the comparisons between microorganism-based diets and the standard group (with normal and non-normal distribution, respectively), showed significant difference of sizes in both genders and wing sides ($p<0.05$). The isometric size of males and females reared in Tetramin® showed larger adults than all the other diets (Figure 7). On average, among the experimental diets (excluding the standard group), the largest females were reared in *S. cerevisiae*, followed by *Pseudozyma* sp., *Asaia* sp. and *E. coli* diet. The males were largest in *S. cerevisiae*, *Asaia* sp., *Pseudozyma* sp. and *E. coli* diet respectively (Figure 7).

Nutritional evaluation of diets, energetic reserve accumulation measurement and feeding behaviour of *A. aegypti* larvae reared exclusively with microorganisms

Nutritional quality influences directly the physiology of *A. aegypti* immature stages. For evaluate the impact of quality of two important macronutrients on larval breeding, we

quantified the protein and carbohydrate contents present in the dietary supplies used. Differences in the nutritional composition of the microorganism diets were detected and may have influenced the immature stages development time (Table 6). *S. cerevisiae* and *Pseudozyma* sp diets have higher amounts of protein (116 ± 6.1 mg; 75 ± 6.9 mg) and carbohydrate (180 ± 9.2 mg; 98 ± 3,8 mg) than the bacterial diets (7.0 ± 0.6 mg to 15.4 ± 1.4 mg and 0.3 ± 0.1 mg to 1.6 ± 0.02 mg). Yeast diets, on the other hand, have less protein than *A. platensis* (341 ± 34.0 mg) and *Chlorella* sp (300 ± 17.5 mg) diets. Conversely, the amount of carbohydrates was higher on diets with yeasts (180 ± 9.2 mg; 98 ± 3.8 mg) compared to the diets based in *A. plantensis* (35 ± 4.0 mg) and *Chlorella* sp (26 ± 1.5 mg).

Four major energetic components (protein, carbohydrate, glycogen and lipids) and the body weight were measured in individual fourth instar entire larvae and compared with the contents recovered from their guts and rest of body tissues. The results are summarized in Table 7. The absolute values of the nutritional reserves differed significantly ($p < 0.0001$) among individuals compared with the standard group Tetramin®. Larvae fed with *S. cerevisiae, Pseudozyma* sp, *E. coli* and *Asaia* sp accumulate the highest amounts of protein (194 ± 18 mg to 89 ± 3 mg), carbohydrates (103 ± 1.3 mg to 16 ± 0.3 mg), glycogen (12 ± 0.8 to 3 ± 0.4 mg) and lipids (71 ± 4.9 to 27 ± 0.8 mg). The nutritional reserve accumulation in *A. platensis*, *Chlorella* sp, *S. aureus*, *S. marcescens*, *Bacillus* sp and *O. intermedium* showed the lowest amounts of energetic components (protein, carbohydrate, glycogen and lipid) and these results are in agreement with the longer developmental rates observed in the experiments above (Supplementary Tables 1 to 5, and Tables 1 to 5). Mean body weight differed significantly with larval diet (Table 7). Only *S. cerevisiae* and *Pseudozyma* sp did not differ significantly compared with the standard diet.

To further investigate the rates of ingestion of the different microorganisms by larvae, the consumption rate of each diet was measured by fluorescence in the gut of individual larvae after 2 hours of exposition to FITC-labelled cells. Larvae fed with *S. cerevisiae*, *Pseudozyma* sp and *E.coli* consumed more food than larvae fed with the other diets after 2 hours (Figure 8). Our results showed that *A. aegypti* larvae actively consumed all the microorganisms used, but with some preference for the microorganisms above.

Table 1. Average pupation time for females and males in days. Differences were analyzed with Log-rank and Wilcoxon.

	Females ♀				Males ♂			
Diet	**Pupae *n***	**Mean ± SEM**	**Median**	**P value**	**Pupae *n***	**Mean ± SEM**	**Median**	**P value**
Tetramin®	88	5.4 ± 0.05	5	-	61	5.2 ± 0.05	5	-
Pseudozyma sp	46	7.2 ± 0.22	7	p < 0.0001	83	6.1 ± 0.13	6	p < 0.0001
S. cerevisiae	62	8.4 ± 0.06	8	p < 0.0001	87	7.9 ± 0.07	8	p < 0.0001
E. coli	29	16.0 ± 0.80	15	p < 0.0001	58	15.6 ± 0.58	15	p < 0.0001
S. marcescens	0	NA	NA	NA	2	NA	NA	NA
Bacillus sp	0	NA	NA	NA	2	NA	NA	NA
Asaia sp	16	11.4 ± 0.64	10	p < 0.0001	25	9.9 ± 0.28	9	p < 0.0001
S. aureus	5	23 ± 3.09	22	p <0.0001	11	22 ± 2.96	17	p < 0.0001
O. intermedium	1	NA	NA	NA	7	28 ± 3.79	28	p < 0.0001
Chlorella sp	6	68 ± 4.57	69	p < 0.0001	21	59 ± 2.30	56	p < 0.0001
A. platensis	9	38 ± 1.37	39	p <0.0001	19	34 ± 1.26	34	p < 0.0001

NA: Not avaiable.

Table 2. Average metamorphosis time for females and males in days. Differences were analyzed with Log-rank and Wilcoxon.

	Females ♀				Males ♂			
Diet	**Adults *n***	**Mean ± SEM**	**Median**	**P value**	**Adult *n***	**Mean ± SEM**	**Median**	**P value**
Tetramin®	88	2.1 ± 0.03	2	-	61	2.1 ± 0.04	2	-
Pseudozyma sp	46	3.0 ± 0.09	3	p < 0.0001	83	2.4 ± 0.05	2	p < 0.0001
S. cerevisiae	62	2.5 ± 0.06	2.5	p < 0.0001	87	2.5 ± 0.05	3	p < 0.0001
E. coli	29	2.4 ± 0.09	2	p 0.0002	58	2.2 ± 0.05	2	p 0.0865
S. marcescens	0	NA	NA	NA	2	NA	NA	NA
Bacillus sp	0	NA	NA	NA	2	NA	NA	NA
Asaia sp	16	2.1 ± 0.09	2	p 0.7867	25	2.0 ± 0.04	2	p 0.4904
S. aureus	5	2.6 ± 0.24	3	p 0.0013	11	2.4 ± 0.2	2	p 0.0098
O. intermedium	1	NA	NA	NA	7	2.0 ± 0.00	2	p 0.4347
Chlorella sp	6	2.0 ± 0.00	2	p <0.0001	21	2.0 ± 0.00	2	p 0.1784
A. platensis	9	2.0 ± 0.00	2	p <0.0001	19	2.0 ± 0.00	2	p 0.2003

NA: Not avaiable.

Table 3. Average emergence time for females and males in days. Differences were analyzed with Log-rank and Wilcoxon.

	Females ♀				Males ♂			
Diet	**Adults *n***	**Mean ± SEM**	**Median**	**P value**	**Adults *n***	**Mean ± SEM**	**Median**	**P value**
Tetramin®	88	7.5 ± 0.07	7	-	61	7.3 ± 0.08	7	-
Pseudozyma sp	46	9.7 ± 0.22	9	p < 0.0001	83	8.5 ± 0.12	8	p < 0.0001
S. cerevisiae	62	10.9 ± 0.06	11	p < 0.0001	87	10.4 ± 0.07	10	p < 0.0001
E. coli	29	18.4 ± 0.80	18	p < 0.0001	58	17.8 ± 0.57	17	p < 0.0001
S. marcescens	0	NA	NA	NA	2	NA	NA	NA
Bacillus sp	0	NA	NA	NA	2	NA	NA	NA
Asaia sp	16	13.6 ± 0.46	13	p < 0.0001	25	12.0 ± 0.27	11	p < 0.0001
S. aureus	5	25.8 ± 2.92	24	p < 0.0001	11	23.9 ± 2.93	20	p < 0.0001
O. intermedium	1	NA	NA	NA	7	30.0 ± 3.79	30	p < 0.0001
Chlorella sp	6	69.8 ± 4.57	70	p < 0.0001	21	61.3 ± 2.30	58	p < 0.0001
A. platensis	9	40.2 ± 1.37	41	p < 0.0001	19	35.6 ± 1.26	36	p < 0.0001

NA: Not avaiable.

Table 4. Adult average survival time in females and males. Differences were analyzed with Log-rank and Wilcoxon.

	Females ♀				Males ♂			
Diet	**Adults *n***	**Mean ± SEM**	**Median**	**P value**	**Adults *n***	**Mean ± SEM**	**Median**	**P value**
Tetramin®	88	8.4 ± 0.18	8	-	61	9.5 ± 0.19	10	-
Pseudozyma sp	46	3.3 ± 0.14	3	p < 0.0001	83	5.6 ± 0.19	6	p < 0.0001
S. cerevisiae	62	3.6 ± 0.12	4	p < 0.0001	87	5.5 ± 0.28	4	p < 0.0001
E. coli	29	4.6 ± 0.40	4	p < 0.0001	58	7.0 ± 0.44	7	p < 0.0001
S. marcescens	0	NA	NA	NA	2	NA	NA	NA
Bacillus sp	0	NA	NA	NA	2	NA	NA	NA
Asaia sp	16	4.8 ± 0.39	4	p < 0.0001	25	6.1 ± 0.24	7	p < 0.0001
S. aureus	5	2.6 ± 0.93	2	p < 0.0001	11	2.1 ± 0.39	2	p < 0.0001
O. intermedium	1	NA	NA	NA	7	2.7 ± 0.61	2	p < 0.0001
Chlorella sp	6	3.7 ± 0.27	4	p <0.0001	21	3.5 ± 0.15	3	p < 0.0001
A. platensis	9	5.1 ± 0.73	4	p <0.0001	19	3.8 ± 0.28	4	p < 0.0001

NA: Not avaiable.

Table 5. Full lifespan of females and males in days. Differences were analyzed with Log-rank and Wilcoxon.

	Females ♀				Males ♂			
Diet	**Adults *n***	**Mean ± SEM**	**Median**	**P value**	**Adults *n***	**Mean ± SEM**	**Median**	**P value**
Tetramin®	88	15.9 ± 0.19	16	-	61	16.8 ± 0.21	17	-
Pseudozyma sp	46	13.0 ± 0.23	13	p < 0.0001	83	14.0 ± 0.17	14	p < 0.0001
S. cerevisiae	62	14.0 ± 0.19	14	p < 0.0001	87	15.9 ± 0.28	15	p < 0.0001
E. coli	29	23.0 ± 0.83	21	p < 0.0001	58	24.8 ± 0.61	25	p < 0.0001
S. marcescens	0	NA	NA	NA	2	NA	NA	NA
Bacillus sp	0	NA	NA	NA	2	NA	NA	NA
Asaia sp	16	18.4 ± 0.55	18	p < 0.0001	25	18.1 ± 0.08	18	p < 0.0001
S. aureus	5	28.4 ± 2.94	26	p < 0.0001	11	26.0 ± 2.84	26	p < 0.0001
O. intermedium	1	24.0 ± 0.00	24	NA	7	33.0 ± 3.76	33	p < 0.0001
Chlorella sp	6	73.5 ± 4.74	75	p < 0.0001	21	65.0 ± 2.32	62	p < 0.0001
A. platensis	9	43.3 ± 1.77	45	p < 0.0001	19	39.0 ± 1.29	40	p < 0.0001

NA: Not avaiable.

Table 6. Protein and carbohydrate contents in microorganism-based diets.

Diets	Proteins (mg)	Carbohydrates (mg)
Tetramin®	212 ± 3,1	207 ± 2,3
S. cerevisiae	116 ± 6,1****	180 ± 9,2****
Pseudozyma sp	75 ± 6,9****	98 ± 3,8****
E. coli	15,4 ± 1,4****	1,6 ± 0,02****
S. aureus	7,4 ± 0,4****	1,0 ± 0,1****
S. marcescens	7,0 ± 0,6****	0,5 ± 0,05****
Bacillus sp	6,7 ± 0,8****	0,3 ± 0,1****
Asaia sp	10,3 ± 1,6****	1,4 ± 0,04****
O. intermedium	7,7 ± 0,6****	1,0 ± 0,04****
Chlorella sp	300 ± 17,5****	26 ± 1,5****
A. platensis	341 ± 34,0****	35 ± 4,0****

(ANOVA 1; **** $p < 0.001$)

Table 7. Nutritional reserve amounts of proteins, soluble carbohydrates, glycogen and total lipids in larvae and their tissues.

Protein (mg)	**Tetramin®**	***S.cerevisiae***	***Pseudozyma sp***	***A.platensis***	***Chlorella sp***	***E.coli***	***S. aureus***	***S.marcescens***	***Bacillus sp***	***Asaia sp***	***O.intermedium***
Larvae	253 ± 31	194 ± 18*	171 ± 6**	60 ± 7.9****	54 ± 19.1***	117 ± 6 ****	64 ± 0.7****	48 ± 1.3****	52 ± 0.8****	89 ± 3****	55 ± 0.7****
Gut	77 ± 1.6	44 ± 2.4***	48 ± 1.9***	4 ± 0.4****	6 ± 0.4****	14 ± 2.1****	9 ± 0.3****	6 ± 0.3****	3 ± 0.5****	10 ± 1.2****	4 ± 0.2****
Carcass	169 ± 2.1	133 ± 5.6	116 ± 9.1**	51 ± 10.6****	43 ± 4.7*	89 ± 5.8****	50 ± 2.3****	39 ± 1.7****	46 ± 1.9****	76 ± 3.1****	51 ± 2.3****
Carbohydrate (mg)	**Tetramin®**	***S.cerevisiae***	***Pseudozyma sp***	***A.platensis***	***Chlorella sp***	***E.coli***	***S. aureus***	***S.marcescens***	***Bacillus sp***	***Asaia sp***	***O.intermedium***
Larvae	125 ± 3.1	103 ± 1.3	82 ± 0.8	10 ± 0.3****	9.2 ± 0.4****	21 ± 3.2****	13 ± 1.3****	5 ± 0.3****	5 ± 0.4****	16 ± 0.3****	11 ± 0.1****
Gut	23 ± 2.6	17 ± 0.8***	16 ± 0.6****	2 ± 0.1****	2 ± 0.1****	1.4 ± 0.1****	3 ± 0.1****	0.7 ± 0.1****	0.8 ± 0.1****	1 ± 0.1****	2 ± 0.1****
Carcass	82 ± 3.4	77 ± 1.0**	61 ± 0.4***	6.9 ± 0.3****	6.7 ± 0.3****	13 ± 0.6****	7 ± 0.4****	4 ± 0.2****	4 ± 0.2****	14 ± 0.4****	8 ± 0.1****
Glycogen (mg)	**Tetramin®**	***S.cerevisiae***	***Pseudozyma sp***	***A.platensis***	***Chlorella sp***	***E.coli***	***S. aureus***	***S.marcescens***	***Bacillus sp***	***Asaia sp***	***O.intermedium***
Larvae	20 ± 0.5	12 ± 0.8***	10 ± 0.3***	2 ± 0.1****	3 ± 0.2****	4 ± 0.9****	3 ± 0.4****	1 ± 0.5****	1 ± 0.3****	3 ± 0.4****	2 ± 0.1****
Gut	5 ± 0.2	3 ± 0.5	3 ± 0.5	0.8 ± 0.05*	0,9 ± 0.11*	0.3 ± 0.04*	0.3 ± 0.1*	0.2 ± 0.1*	0.1 ± 0.04**	0.2 ± 0.02*	0.3 ± 0.03**
Carcass	11 ± 0.5	6 ± 0.3**	6 ± 0.5**	1 ± 0.1**	2 ± 0.2**	3 ± 0.3***	3 ± 0.2***	1 ± 0.1***	1 ± 0.1***	2 ± 0.3***	1 ± 0.1***
Lipid (mg)	**Tetramin®**	***S.cerevisiae***	***Pseudozyma sp***	***A.platensis***	***Chlorella sp***	***E.coli***	***S. aureus***	***S.marcescens***	***Bacillus sp***	***Asaia sp***	***O.intermedium***
Larvae	111 ± 1.9	71 ± 4.9*	79 ± 5.2**	19 ± 1.8**	16 ± 0.7***	36 ± 4.7*	23 ± 4.4****	7 ± 0.4****	6 ± 0.6****	27 ± 0.8**	17 ± 0.4****
Gut	32 ± 3.5	17 ± 1.1**	22 ± 0.6**	3 ± 0.5****	3 ± 0.4****	1 ± 0.4****	1 ± 0.1****	0.2 ± 0.04****	0.3 ± 0.03****	2 ± 1.5****	1 ± 0.1****
Carcass	70 ± 4.3	53 ± 1.3**	51 ± 2.3**	14 ± 1.4***	12 ± 0.9***	28 ± 0.2***	17 ± 2.1***	6 ± 0.2****	5 ±0.2 ****	22 ± 0.7***	15 ± 0.8***
Fresh Weight (mg)	**Tetramin®**	***S.cerevisiae***	***Pseudozyma sp***	***A.platensis***	***Chlorella sp***	***E.coli***	***S. aureus***	***S.marcescens***	***Bacillus sp***	***Asaia sp***	***O.intermedium***
Larvae	7 ± 0.0004	6 ± 0.001	6 ± 0.0004	3 ± 0.0003*	2 ± 0.0003*	3 ± 0.0003*	3 ± 0.0003 *	3 ± 0.001 *	3 ± 0.001 **	3 ± 0.0003*	3 ± 0.0003**
Gut	3 ± 0.001	3 ± 0.0002	3 ± 0.0003	1 ± 0.0003 **	1 ± 0.0003 **	1 ±0.0002**	1 ±0.0002**	1 ± 0.0001 ***	1 ± 0.0002 ***	1 ± 0.0001 **	1 ± 0.0003 **
Carcass	5 ± 0.002	4 ± 0.0001	4 ± 0.0003	2 ± 0.001*	2 ± 0.0003*	2±0.0003***	2±0.0003***	2 ± 0.001 ***	3 ± 0.001 ***	2 ± 0.0003 *	2 ± 0.0001 **

DISCUSSION

The nutrition environment experienced by larvae strongly influences the phisiology and behavior of mosquitoes. The current work evaluated the impact of nourishment from live microbes in the development and survival of *A. aegypti*. The organisms selected for feeding were strains of gram negative bacteria *S. marcescens* (SM365), *E.coli* (D31), *Asaia* sp. (A1) and *O. intermedium* (Om17), gram positive bacteria *Bacillus* sp. and *S. aureus*, yeasts-like fungi *S. cerevisiae* (S14) and *Pseudozyma* sp. (Pa1), the cyanobacteria (blue-green algae) *A. platensis* (also known as *Spirulina*), and the marine microalga *Chlorella* sp. All microorganisms used in this paper showed stability in water until 120h. Therefore, we decided to perform the feeding experiments by dispersing the strains directly in rearing water under sterile conditions.

All diets showed important differences in developmental and survival rates when compared individually to the standard group Tetramin®. Larva fed with yeast take less time to achieve pupation than all the others microorganism-based diets tested (Figure 2). Parameters as adult emergence (Figure 3), survival (Figure 4) and full lifespan (Figure 5) showed a slight delay in developmental rates when compared to the standard group Tetramin®. Due to the satisfactory developmental rates in all life parameters tested, and the rapid growth speed of cultures in low-cost media, *S. cerevisiae* and *Pseudozyma* sp seem to be suitable candidates for diets in the mass rearing of mosquitoes or for the regular laboratory breeding (Imam et al 2014). In 1935[a, b], Trager's studies pioneered larval nutrition, demonstranting that *A. aegypti* larvae are able to reach adulthood being fed only with yeast powder. Confirming the results obtained by Trager, Golberg and Lavoipierre (1945) showed that autoclaved yeasts suspended in $CaCl_2$ 0.01% are sufficient for the full larval lifespan of *A. aegypti*. Souza et al (2016) conducted a study based on feeding larvae with a specific diet containing only live or dead *S. cerevisiae* cells. The data obtained in this paper, corroborated the works described previously, that highlighted the ability of *A. aegypti* larvae to feed and digest living yeast cells through the enzyme beta-1,3-glucanase. Yeast and fungi can be also suitability as a paratransgenic vehicles or integrated pest management (IPM) tools. Interestingly, the symbiont *Wickerhamomyces anomalus (Saccharomycetales)* can be found in the gut and reproductive organs of some mosquito vector species. This symbiont can be easily

cultured in cell free media and seem to be a good candidate for the expression of effector molecules in the gut of mosquito vectors (Ricci et al 2011). Murphy et al (2016) demostrated that genetically modiefed *S. cerevisiae* can be used as biopesticide through oral delivery of specie specific dsRNA. This application as biopesticide decreases larval survivorship, reduces locomotor activity and reproductive fitness in the insect pest *Drosophila suzukii*. This yeast biopesticide approach could be adapted to a large number of species once many interactions have been observed between sylvatic yeasts and insect species as Diptera, Coleoptera and Hymenoptera (Gonzalez et al 2014, Abrieux and Chiu 2016). The authors also postulate that biopesticide design may be favored in the managing of an insect pest that both consumes yeast as food and has systemic RNAi.

Studies of biological control agents based on entomopathogenic fungi have been reported in several vector mosquitoes. The potential of *Metarhizium anisopliae* fungus was tested in *Anopheles gambiae, A. aegypti, Aedes albopictus, Culex quinquefasciatus* (Alves et al 2002, Scholte et al 2007, de Paula et al 2008, Pereira et al 2009, Fang et al 2011). Genetic-engineered *M. anisopliae* inhibited *Plasmodium sp* development within the mosquito and prevented malaria infection in *Anopheles* (Fang et al 2011). In *A. aegypti*, *A. albopictus* and *C. quinquefasciatus*, the full lifespan of *M. anisopliae*-contaminated mosquitoes was significantly reduced and showed high mortality rates compared to uninfected mosquitoes (Alves et al 2002, Scholte et al 2007, de Paula et al 2008, Pereira et al 2009). Recently, two strains of *M. anisopliae* were tested against *A. aegypti* and besides the increase in mortality, the fungus also reduced egg laying (Jemberie et al 2018). The fungi: *Lagenidium giganteum* and *Leptolegnia chapmanii* were also tested as promising biological control agents for use against *A.* aeg*ypti* adults (McCray et al 1973, McInnis and Zattau 1982). The development of innovative strategies using yeast has therefore a potential as an eco-productive alternative for the management of mosquito borne diseases.

Bacteria are considered the most commonly microorganism present in the nourishment of mosquito larvae (Laird 1956 and 1988, Christophers 1960). Previous studies reported that bacteria could be used as a unique food requisite to mosquito growth (Hinman 1932, Rozeboom 1935). Larvae fed with *S. marcescens*, *Bacillus* sp, *S. aureus*, *O. intermedium*, *S. aureus*, *E. coli* and *Asaia* sp. showed a severe delay to achieve pupal stage when compared to the standard group Tetramin® (Supplementary Table 1). Other biological parameters as metamorphosis (Supplementary Table 2), emergence (Figure 3), survival (Figure 4) and full lifespan also also strongly affected (Figure 5). Studies obtained by

Dickson et al (2017) provide the concept that larval exposure to different bacterial communities during larval development can drive variation in *A. aegypti* adult traits. Therefore, the results observed here are similar to others studies reported. Interestingly, *E. coli* and *Asaia* sp take result in shorter developmental times into pupa than the others bacterial diets (Figure 2). The survival span for larvae fed with *E. coli* and *Asaia* sp. were superior than those observed in diets using *S. cerevisiae* and *Pseudozyma* sp. *E. coli* and *Asaia* sp. seem to be the best bacterial models for laboratory reared larvae until now.

Food stress was expected to reduce survival, however larva fed with *Asaia* sp. and *E. coli* had higher adult survival (Supplementary Table 4). There are different ways to interprete these results. Nutritional stress can increase the life-span by an hormetic model (Mattson 2008). Larvae fed with *E. coli* and *Asaia* sp. might be presenting a stress-induced response, hormesis, that can be an overcompensation to nutritional environmental stress (Calabrese 2001). Hormesis induces cellular protective mechanisms through an increased in gene expression, working as a key regulator of many cellular defenses that allow survival in response to stress (Lin et al 2000, Heilbronn et al 2005a, Motta et al 2004). Enhanced levels of heat shock proteins (HSPs) and antioxidants to cellular maintenance are also considered as part of the hormetic mechanism (Mattson 2008, Gems and Partridge 2008). The beneficial effects of hormesis on survival and longevity have been described for years and our results might thus exemplify a beneficial carry-over effect of the hormetic stress on larval development, reinforcing the *A. aegypti* phenotypic plasticity in limiting environments (Aznar et al 2018). Previous studies had already shown that larvae reared in restricted diets might be associated with prolonged life in *A. aegypti* (Joy et al 2010, Zeller and Koella 2016) and *Anopheles* sp (Vantaux et al 2016).

The positive correlations between the presence of microbiota and larval development might be another way to explain the results observed in larvae fed with *Asaia* sp. and *E. coli*. The acetic acid bacterium genus *Asaia* have been shown to be stably associated with larvae and adults of anophelines and *A. aegypti* (Favia et al 2007, Damiani et al 2010, Ricci et al 2012, Rossi et al 2015). Mitraka et al (2013), showed that a diet supplemented with *Asaia* in the *Anopheles gambiae* larval environment had a significant boost in developmental rate and Chouaia et al (2012) observed a delayed larval development in *Anopheles* mosquitoes deprived of *Asaia* bacterial symbionts. Besides the effects on development rates, a possible mutual exclusion or a competition between *Asaia* and *Wolbachia* may contribute to explain the inability of *Wolbachia* to colonize the female

reproductive organs of anophelines, inhibiting its vertical transmission and explaining the absence of *Wolbachia* infection in *A. aegypti* and in the majority of natural populations of *Anopheles* mosquitoes (Rossi et al 2015). These results drive us to belief that *Asaia* may play a significant role in mosquito larval development. Although the molecular nature of the developmental improvement caused by the *Asaia* symbiote needs to be identified, these bacteria can be considerate as candidate paratransgenic vehicle for the control of mosquito-borne diseases (Favia et al 2008, Ricci et al 2012, Mitraka et al 2013).

Coon et al (2016) showed that each mosquito species including *A. aegypti* contains a simple bacterial community and that the composition of bacterial gut communities can also be strongly influenced by diet. Their results also showed that axenic larvae can not develop, but several community members and *E. coli* are able to rescue the larval development. Using *E. coli* K-12 as a model for studies of molecular interactions that underlie bacteria-dependent growth of larvae into adults, Coon et al (2017) unveiled one of the molecular mechanisms involved in mosquito development. They showed that bacteria through the cytochrome b oxidase gene mediate a reduction of oxygen levels in the digestive tract of larvae, working as a signal for ecdysone-induced molting. Thereby, *E. coli* plays as essential role in mosquito development and may have important implications to be used in symbiont-based control techniques for disabling the growth of larvae into mosquito adults.

S. marcescens, *Bacillus* sp., *O. intermedium* and *S. aureus* had a lower adult rate survival contrary to results described in diets with *Asaia* sp. and *E. coli* (Supplementary Table 4). The effect of a restricted diet in mosquito larva is a rather speculative issue. Previous studies reported that dietary restrictions can lead to longer development rates (Olivo et al 1979, Tun-Lin et al 2000, Arrivillaga and Barrera 2004, Tal Levi 2014, Couret et al 2014, Aznar et al 2018), with larvae extending time to achieve the pupal stage (Chambers and Klowden 1990, Telang et al 2007, Foster et al 2012, Banerjee et al 2015). Some individuals with slower growth rates try to counterbalance this deficit through compensatory growth (Wilson and Osbourn 1960). This ecological factor allows that once the same nutritionally deprived individuals have the opportunity to acquire more food, grow rapidly or slow down the growth rates to employ the food resources in maintenance of important biologicals traits as reproduction and survival (Dmitriew 2011, Dmitriew and Rowe 2011, Zeller and Koella 2016).

Bacillus sp. and *S. marcescens* presented only two adults (Supplementary Table 1). *S. marcescens* is recognized by its entomopathogenic properties in some conditions (Flyg et al 1980 and 1983). However, it does not appear to being associated with any type of infection in *A. aegypti* larvae. In the presente study, larvae fed with bacterial diets, including *S. marcescens*, remained in the third instar for several weeks. Other studies have been shown that withstand to starvation could be measured by the time spent in the third larval instar without adequate nourishment (Wigglesworth 1942). The duration of development from the L1 larval stage to adult mosquito is faster when food is abundant (Tu-lin et al 2000), thus, the prolongation in third instar larvae observed in our results may indicate a state of malnutrition. We did not observe the presence of melanized-killed larvae or any specific phenotype more related to pathogenicity. However, previous studies have already reported that food restriction alters immunological traits in *A. aegypti* (Grimstad and Walker 1991, Alto et al 2005 and 2008[a], Muturi et al 2011, Telang 2012). More studies are necessary to evaluate the influence that the diets used in this study exert on the immune system of *A. aegypti* larvae.

Lowering of growth rates may be a response to dietary stress and an adaptive behavior in calorie-depleted environments (Arendt 1997, Badyaev 2005). It is possible that *A. aegypti* larvae raised under bacterial diets suffer nutritional depletion, resulting in developmental delays. In natural environments, mosquitoes are commonly found with reduced sizes and low energy reserves. This remarkable capacity of withstand starvation situations and recovery later in more favorable conditions can justify the great success for the establishment of these insects in the environment (Barrera 1996, Barrera and Medialdea 1996, Zeller and Koella 2016). Therefore, our data shows that *A. aegypti* larva is able to develop in all bacterial diets tested, even at a higher ecological cost.

In natural conditions, the biomass of algae is the major content of mosquito larvae guts (Hinman 1930, Garros et al 2008). Thus, algae seem to play an important role in development and survival of larvae. *A. aegypti* larvae were not able to develop in microalgae diets with the standard concentration established for the other diets (16 mg / larva), so we adjusted the values offered to lower concentrations (3 mg / larvae). The development failure observed in the standard concentration (data not shown) might have been caused by several factors among them the junction between a controlled photoperiod environment as well a low larval density. Together these factors might have accelerated the exponential microalgae growth triggering a toxicological response by larvae which

impaired their development. Larvae fed with the microalgae *Chlorella* sp or with *A. platensis* (3 mg / larvae) showed a delay in development similar to the observed in bacterial diets, but with shorter adult survivals (Supplementary Table 4). Kivuyo et al (2014) evaluated larval survival and the development rate of the aquatic stage using green filamentous algae and dry powdered filamentous algae as diet. The survivorship and pupation rates in algae food diet had the worst performance between all foods assessed. Some species of algae seem to be resistant to digestion and are discarded entirely after passage through the larval gut (Laird 1988). However, there is no evidence that *Chlorella* sp. and *A. platensis* belong to this specific group. Actually, a genus of *Chlorella* was used as a larvicide against *A. aegypti* by Borovsky et al (2016). *Chlorella desiccata* was engineered to express an insect peptide hormone, the trypsin modulating oostatic factor (TMOF), that is recognized by *A. aegypti* ovaries and controls the translation of the gut's trypsin mRNA. Feeding mosquito larvae with transformed *C. desiccata* cells kill by starvation 60% of *A. aegypti* larvae in 4 days (Borovsky et al 2016). *A. platensis* (*Spirulina*) have never been used in nourishment experiments with mosquito larvae before. Our results showed that *Chlorella* and *A. platensis* can be used as a food source by mosquito larvae, but with slower developmental rates. Their rapid grow under heterotrophic culture, with a relatively low cost, make these microalgae interesting candidates for future methods of vector control through genetic engineering (Dawson et al 1997, Madkour et al 2012).

The biological parameters evaluated in *A. aegypti* were analyzed separately for males and females in all diets (Tables 1 to 5 and Supplementary Table 6). The results showed that males take less time to reach adulthood and survive longer. When mosquitoes are fed only water as adults, the only nutritional resources available lie in the reserve acquired during immature stages. The transference of reserves from the larval nutrition to the mosquito adult stage using only microbial cells as nourishment has never been investigated before. Thus, it is possible that males show more withstand to starvation during the larval stage and a lower nutritional threshold for pupation than females. These traits might have influenced the better developmental rates observed in males and increased their survival. The measured lifespan of adult males and females fed with nectar is approximately 9 weeks, and around 12 to 17 weeks for females that were also blood fed (Putnam and Shannon 1934). We were not able to find available data in the literature to compare the mortality rates of males and females under laboratory conditions without any nutritional

stimulus. Aznar et al (2018) reported that treatments with scarcity or excess of food might preferentially influence the proportions and survival of females over males. They have shown that females exhibit a larger extension of development time in response to food deprivation than males, and relate this result to a male fitness advantage, ability of an individual to survive, reproduce and spread genes, this advantage being obtained when males emerge early and can copulate with non-mated females. The longer development time on females are similar to the observed under a competitive environment (Bedhomme et al 2003). Under variated conditions, it is expected that females take more time to develop and spend more time to enhance their fitness abilities, achieving larger body sizes and thus boost their fecundity (Bedhomme et al 2003, Wormington and Juliano 2014). Previous field studies already reported a faster development in male over female mosquito larvae, that showed a slower development and a higher mortality rate (Yates 1979).

Imbalances in sex ratio (males: females) were observed in all diets tested (Supplementary Table 6). This male-biased sex ratio may result from underfeeding. Some studies support a sex-related difference in larval nutrient metabolism, possibly due to the earlier ecdysteroid peak in *A. aegypti* male during pupal-adult development (Brust et al 1967, Whisenston et al 1989, Chambers and Klowden 1990, Puggioli et al 2013, Balestrino et al 2014b). In field studies, distortion in sex ratio are frequent and associated to the slow development of female larvae and differential response of the sexes to egg hatching stimuli (Yates 1979, Shroyer and Craiger 1981, Sims and Munstermann 1983, Frank et al 1985, Lounibos and Escher 2008). Other studies observed a density-dependent alteration and a sex-specific response to a critical day period time, trough feedback mechanisms that are dependent on density or mortality by selective sexual predation (Frank et al 1985, Chambers 1985, Alto et al 2012). The highest proportion of males observed in this study seem favorable to future studies using the Sterile Insect Technique (SIT). A greater male pupae production is important to SIT mass rearing and determine the quantity of males that can be selected for release in the natural environment (Puggioli et al 2016).

In this study it was also possible to realize that microbial diets (yeast and gram-negative bacteria) influenced both wing shape and size of *A. aegypti* adults in different degrees and in both genders. The existence of differences in wing sizes and shapes that are inherent to each gender (wing sexual dimorphism) has been already described, including some studies that showed the species-specific phenotypic expression of wing shape and size (Devicari et al. 2011, Virginio et al. 2015; Christe et al 2016). In addition, there is some

discussion about the relationship between body size and shape (allometry) and the sexual dimorphism (Virginio et al 2015). However, we aimed to study how different diets might impact in wing size and shape, and how this is reflected in different genders, and not necessarily to assess the sexual dimorphism directly. The correlation of wing size with food concentration is interesting, mainly because the "centroid size" (Bookstein 1991) is considered a more informative estimate of body size than the traditional size measurements (Jirakanjanakit and Dujardin 2005; Jirakanjanakit et al 2007). When Tetramin® diet was compared to both gram-negative bacteria (*Asaia* sp. and *E. coli*) or to yeasts (*Pseudozyma* sp. and *S. cerevisiae*), based on Canonical Variates 1 and 2 (the first and the second most relevant CVs), there are almost no overlap. This suggests that the diets can influence differently the wing shapes and corroborate the other findings of this study. Considering just the CV1, it is possible to observe that *Asaia* sp. is more similar to Tetramin® than *E. coli* in both genders. In Tetramin® and yeast comparisons, males of *Pseudozyma* sp. are more similar to the control diet than *S. cerevisiae*; in females the opposite was observed. As previously mentioned, we quantified the wing centroid size as a read-out of the adult size. In general, the wings of the mosquitoes fed with Tetramin® in their immature stages, were larger than those that were fed with microorganism-based diets. In addition, in all comparisons females showed larger wings than males. The sexual dimorphism present in wing shape and size of *A. aegypti* has been studied (Sánchez and Lira, 2017, Virginio et al 2015). However, independently of the inherent wing sexual dimorphism, and some asymmetry, we recorded some patterns of differentiation. In males, the group fed with *E. coli* showed slightly lower sizes than the *Asaia* sp. group, which in turn was more similar to Tetramin®. In females, *Asaia* and *E. coli* were lower than Tetramin®, although *Asaia* sp. has been closer to the Tetramin® diet. On the yeast diets, males of *Pseudozyma* sp. and *S. cerevisiae* showed similar scores, and females of the *Pseudozyma* sp. were lower than *S. cerevisiae*, both being lower than the Tetramin® score. Jong *et al* (2017), showed significant shorter wings in *A. albopictus* under sub-optimal food availability. Jirakanjanakit et al (2007) and Aznar et al (2018) reported that low food concentration in *A. aegypti* immature stages could alter mosquito size. These results support this study, suggesting a strong influence of microbial food composition and wing length.

The differences reported in the developmental time and adult size with the microbial diets are consistent with other studies on environment stress (Badyaev 2005). Response to

stress conditions such as nutritional limitation (Bubliy et al 2000), extreme temperatures (Sisodia and Singh 2009) and larval crowding (Imasheva and Bubliy 2003) in *Drosophila* have been studied and related to genetic modifications. Schneider et al (2011) demonstrated a genotype variation in *A. aegypti* adult size in response to larval food depletion, reporting a wide phenotypic plasticity and an adaptive behaviour to changed environments. Aznar et al (2018), suggested that the variety in food stress conditions in the natural habitat can increase genetic modifications in *A. aegypti*. This genetic variety, as mutation and recombination rates, are usually hidden under regular food conditions, and facilitates the development of novel adaptations to adverse environments (Badyaev 2005).

The next step was to analyze the nutritional quality through the measurement of main energetic components of diets. Our goal was to observe if these developmental and size variations are a result of malnourishing. The nutritional requirements of mosquitoes are divided into two majority classes: macronutrients (energetic nutrients) and micronutrients (non-energetic nutrients) (Singh and Brown 1957, Foster 1995, Canavoso et al 2011, Arrese and Soulages et al 2010, Rivera-Pérez et al 2017). Aquatic environments with abundant nutritional richness supply all the energy necessary to mosquito larvae metamorphosis. Carbohydrates and proteins are among the main nutritional requirements of *A. aegypti* (Singh and Brown 1957).

The nutritional values of the diets were evaluated through protein and total sugar quantification (Table 6). *Chlorella* sp and *A. platensis* showed higher protein amounts than the other diets, including the standard diet Tetramin®. These results were already expected once both species are rich in proteins and can be employed even for human consumption (Mühling 2000, Guccione et al (2014), Bleakley and Hayes et al 2017). Yeast diets also presented robust protein values. *S. cerevisiae* has more protein than *Pseudozyma* sp. Souza et al (2016) showed that *S. cerevisiae* contains higher protein amounts than the standard diet Cat food, however Tetramin® seems to be more nutritive and present a superior protein amount. *Bacillus* sp, *S. marcescens*, *S. aureus* and *O. intermedium* showed lower protein values than *E. coli* and *Asaia* sp. These results coincide with the developmental pattern observed (Supplementary Tables 1 to 5). As previously mentioned, protein and amino acids consumption are directly related to growth and development in mosquitoes (Golberg and De Meillon 1948b, Merrit et al 1992). Scarce consumption of proteins during the immature stages might interfere in life cycle

duration, adult emergence, body size and fecundity (Dadd 1977, Singh and Brown 1957). Thus, our results are in line with other studies, showing that badly nourishment leads to delayed larval development and to adults with low energetic reserves (Timmermann and Briegel 1999, Arrivillaga and Barrera 2003, Telang et al 2007, Zeller and Koella 2015, Banerjee et al 2015).

The sugar amounts in *S. cerevisiae* and *Pseudozyma* sp. were higher than in the others diets. *S. cerevisiae* did not show significant differences ($p < 0,005$) when compared to the standard diet (Table 6). All the bacterial diets had very low sugar contents and *E. coli* and *Asaia* sp. presented the best values, reinforcing our hypothesis that both bacteria are better sources for larval feeding. *Chlorella* sp. and *A. platensis* presented high sugar values. However, differently to the protein amounts, their sugar content is lower than the yeast or the standard diet. Carbohydrate is directly associated with pupation (Chambers and Klowden 1990, Telang et al 2007). Carbohydrate storage is associated to pupal commitment and larval growth (Sneller and Dadd 1977, Van Hendel 1988, Telang et al 2007), and the low levels observed in our results suggest that larvae have not sufficient sugar to achieve the pupal stage in these dietas, resulting in developmental delay. Although the microalgae species had a significant amount of protein and sugar, the development time in these diets were prolonged, leading us to believe that *A. aegypti* larvae may not assimilate nutrients adequately from these organisms.

Besides the nutritional quality measurements, we analyzed the nutrient reserves of *A. aegypti* larvae. We have undertaken a comparative study in the larval development rates and their storage of protein, free carbohydrates, glycogen and lipids in relation to different dietary conditions. There is a positive correlation between body mass and caloric reserves stored during the larval stage, triggering endocrine responses that lead to insect molting (Chambers and Klowden 1990). Insects must achieve a minimum weight in the immature stage in order for continue their development (Nijhout and Williams 1974a, Nijhout 1975, Lounibos 1979, Safranek and Williams 1984, Chambers et al 1990, Davidowitz et al 2003, Lan et al 2004, Mirth et al 2005). In *A. aegypti* larvae, the metamorphic capacity depends on nutritional reserves and needs a minimum critical mass that is estimated between 2.7 to 3.2 mg (Telang et al 2007). Critical mass is defined as the mass that result in 50% of starved larvae achieving the pupal stage. That occurs usually 24 h after the transformation into the final fourth instar, in optimal conditions (Chambers and Klowden 1990, Davidowitz et al 2003, Lan and Grier 2004, Telang et al 2007). This is the minimum

period of time these larvae require to acquire food so that at least 50% of them pupate and emerge as adults, and at this age the ecdysteroid production begins to rise (Telang et al 2007). Nutrient intake and energetic accumulation are important factors to metamorphosis. Larvae that are starved after reaching their critical weight generally will molt, but if larvae are starved before they have achieved the critical weight, metamorphosis is delayed or they eventually die without initiating this process (Chamber and Klowden 1990, Davidowitz et al 2003, Telang and Wells 2004).

Insects fed exclusively with bacteria and microalgae seem unable to accumulate sufficient nutrients during the larval stage to reach the minimum critical mass required for pupation in regular time (Table 7). Restrictive food amount in *A. aegypti* larvae delay pupation, but larvae remains able to pupate even in underfeeding conditions if sufficient energetic accumulation have been done in previous larval stages (Telang et al 2007). Thus, the delay observed in our results in the metamorphosis rates might afford to larvae an additional period to feed, grow and meet the critical mass to achieve the pupal stage. In all diets tested, the larval energetic contents were lower than in larvae fed with the standard diet Tetramin®. These results were expected, once the insect physiological parameters were strongly affected (Supplementary Tables 1 to 5). Additional evidence for developmental delays comes from studies in *Manduca sexta*. The underfeeding in *M. sexta* during the last larval stage, resulted in death or a delay in metamorphosis (Nijhout and Williams 1974b).

The protein storage in larvae fed with bacteria or algae showed the lowest proteins levels and *Asaia* sp. and *E. coli* showed better levels than the others including *Chlorella* sp. (54 ± 19.1 mg) and *A. platensis*. Although microalgae have high protein content, *A. aegypti* seems not able to store this protein satisfactorily. The energetic reserves in gut and rest of body of all diets tested were proportional to values obtained in whole larvae, and the rest of body showed higher contents than the gut (Table 7). Yeast-fed larvae showed the highest levels of protein, suggesting that *A. aegypti* larvae can process and store this protein efficiently. In immature stages proteins play an important role in metabolic processes as well for adults of several insect species (Hagen et al 1984, Zucoloto 1988). In immature stages ingestion of proteinaceous food is essential for growth, survival, nutritional reserve for the pupation and for utilization in adult stage, principally for egg production (Chan et al 1990). Our results are in line with the decline in body size observed

in *A. aegypti*, *Culex pipiens*, *Anopheles albimanus* and *Anopheles gambiae* caused by different feeding regimes (Timmermann and Briegel 1999).

Carbohydrates content in bacterial and microalgae diets showed significant storage differences ($p < 0.0001$). Larvae fed with *S. cerevisae* and *Pseudozyma* presented carbohydrates reserves similar to the standard diet, showing no significant diferences ($p > 0,005$). As expected, *E. coli* and *Asaia* sp. had the better carbohydrates storage amounts than other bacteria and microalgae, reinforcing the potential of these diets. Carbohydrate is necessary for optimal growth and developmental rates in larvae, and nutritional environment with low levels of sugar leads to a substantially delayed growth (Sneller and Dadd 1977). Previous studies with *M. sexta* suggested the dependence of sugar ingestion to reaching the pupa stage and the requirement to both dietary sugar and protein to growth until the adults (MacWhinnie et al 2005). Carbohydrate role in *A. aegypti* larvae development is associated with pupal commitment and an inverse relation between hemolymph trehalose levels and juvenile hormone titers has been described (Jones et al 1981, Chambers and Klowden 1990, Telang et al 2007). Therefore, our data confirm that sugar and other digestible carbohydrates are required for adult stage maintenance and for larval development.

The larvae glycogen storage showed significant differences ($p = 0.0001$) in all diets tested. Bacterial diets resulted in low glycogen contents, and *Asaia* sp. and *E. coli* showed the higher rates than other bacterias diets. Larvae fed with *S. cerevisiae* and *Pseudozyma* sp. showed better glycogen levels, confirming the excellent storage capacity of *A. aegypti* in yeast diets. Past studies reported a critical threshold of larval glycogen as stimulant to metamorphosis through a drastic drop in juvenile hormone titer and concomitant increase in ecdysone that trigger molting (Chambers and Klowden 1990, Timmermann and Briegel 1998). Telang et al (2007) reported that the timing of ecdysteroid release is not critical to start the larval–pupal molt for *A. aegypti* larvae, however both the ecdysteroid titer and the nutritional condition of fourth instars are determinant factors in initiating the metamorphic molt. Earlier studies indicated that larval nutrient reserves (protein, lipid and glycogen) are important for egg production and the endocrine regulation of egg development in *A. aegypti* and *Ochlerotatus atropalpus* (Telang et al 2006). High levels of glycogen and protein overtake a threshold set in the insect nervous system that activates ovarian ecdysteroid production and inhibits juvenile hormone biosynthesis by the *corpora allata*, which together enable vitellogenesis and egg production. Without sufficient

threshold levels, the *corpora allata* increase the juvenile hormone levels secretion, decreasing ovarian ecdysteroid production and as consequence egg maturation is delayed (Telang et al 2006).

The low glycogen reserve in *A.aegypti* larvae fed with bacteria and microalgae might have affected the insect neuroendocrine system, resulting in the delaying of pupation time, due to a lack of the minimum glycogen threshold required for a successful molt (Nijhout and Wheeler 1982, Chambers and Klowden 1990, Noriega 2004, Lan and Grier 2004, Telang and Wells 2004, Telang et al 2006, 2007, Margam et al 2006). Glycogen storage seems to be a larval strategy valid to withstand starvation when the larva is waiting for additional nutrition during the last larval instar, under badly nourishment conditions (Timmermann and Briegel 1998). This strategy might be used by larvae fed with *E. coli* and *Asaia* sp, which showed higher glycogen levels and prolonged their survival.

Levels of lipids were lowest in larvae fed with *S. marcescens* and *Bacillus* sp. These diets showed the worst developmental rates (Supplementary Table 1) with a severe delay in all biological parameters evaluated (Supplementary Tables 1 to 5). Although, the other experimentals diets have also shown significant and substantial diferences ($p > 0,005$) in the lipid contents. Lipid reserve influence pupal commitment, and the endocrine regulation of egg development in autogenous and anautogenous female mosquitoes (Briegel 1990, Briegel et al 2002, Foster 1995, Zeigler and Ibrahim 2001, Zhou et al 2004, Telang et al 2006). Under favorable nutritional conditions, lipids start accumulating after glycogen has reached a plateau (Van Handel 1984). Environments with nutritional stress lead to drastic reductions in whole body lipid, protein and carbohydrate contents (Briegel 1990). Due to the diets poor in nutrients (Table 6), *A. aegypti* larvae storage lesser amounts of energetic components (Table 7), affecting directly the developmental rates. Clearly, the metamorphic capacity depends on all four nutrient reserves in different ways.

Nutrients accumulated by larvae are correlated with adult emergence and body size (Telang et al 2007, Zeller and Koella 2016). Nutrition, temperature and larval density also influence growth, development, energy reserves, egg production, longevity of adult females, immunity, vector capacity and insecticide-resistance (Stearns and Koella 1986, Briegel 1990a, Briegel 1990b, Lyimo et al 1992, Kitthawee et al 1992, Juliano and

Stoffregen 1994, Ameneshewa and Service 1996, Murray et al 2005, Telang et al 2006, 2007, Murrell and Juliano 2008, Reiskind and Lounibos 2009, Arrese and Soulages 2010, Muturi et al 2011, Kulma et al 2013, Price et al 2015). Larvae fed with *S. cerevisiae* and *Pseudozyma* sp. were not affected in body weight, what is expected once these diets showed good developmental rates, high nutritional quality and efficient energetic accumulation. The fresh mass of larva fed with bacteria and microalgae were significant lower ($p<0,005$) when compared to controls. In natural environments it is common to find mosquitoes with reduced sizes and low energy reserves (Zeller and Koella 2016). Telang et al (2007) showed that nutritional stress leads to smaller adults with reduced hemocyte numbers. Muturi et al (2011) reported that nutritional stress during larval development cause changes in phenotype and immunity of mosquitoes and increased susceptibility of these adults to pathogens. Therefore, the nutritional quality of larvae in diets with microorganisms can not only affect the entire life history of *A.aegypti*, but also their size, shape, immunity and vector competence.

Our results showed that *A. aegypti* larvae consumed all microorganisms used in different rates (Figure 8). The guts of larvae fed with *S. cerevisiae*, *Pseudozyma* sp and *E. coli* presented high mean fluorescent intensity after 2 h of feeding. The lower consumption rates in other diets might suggest a possible food preference. Despite that, more studies must be performed, increasing the feeding exposure time. We have not found studies involving food preference in *A.aegypti* larvae, hence exploring this subject is necessary for a better understanding of larval phisiology.

CONCLUSION

In conclusion, our results provide new knowledge into the effect of microorganism-based diets in different larval biological parameters as developmental rates, pupation time, emergence, survivorship, lifespan, wing and shape size. Larvae fed with bacteria and microalgae shown lethargic development and low survival, due a badly nourishment and a low energetic reserve accumulation. *Asaia* sp. and *E.coli* seem the best bacterial models for future studies aiming develop symbiont-based control. Larvae fed with yeasts showed developmental rates that are similar to the standard diet Tetramin®, being nutritional rich and providing high energetic storage. *S. cerevisiae* and *Pseudozyma* sp. seem suitable candidates to improve mosquito laboratorial breeding and a low-cost diet to mosquito mass rearing. *A. aegypti* larvae ingested all microorganisms after 2h. Therefore, *A. aegypti* larvae showed a very high plasticity in relation to feeding, being able to develop under different microbial diets.

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E-H: Comparison between control diet (Tetramin (R)) and Yeasts (*Pseudozyma* sp. and *S. cerevisiae*).

Figure 8. A. aegypti larvae consume different microorganisms offered in diets. **(A)** Mean fluorescent intensity in the intestine of larvae 2 h after placement of FITC-labeled microorganisms into rearing water. Columns present mean values with 95% confidence intervals for each diet (ANOVA 1; * $p<0.01$). Figures are means ± SEM of 5 experiments with 10 larvae each. **(B)** Epifluorescent images of microscope slide glass with FITC-labeled E.coli (D31) and individual larva 2 h after placing FITC-labeled E.coli (D31) into rearind water.

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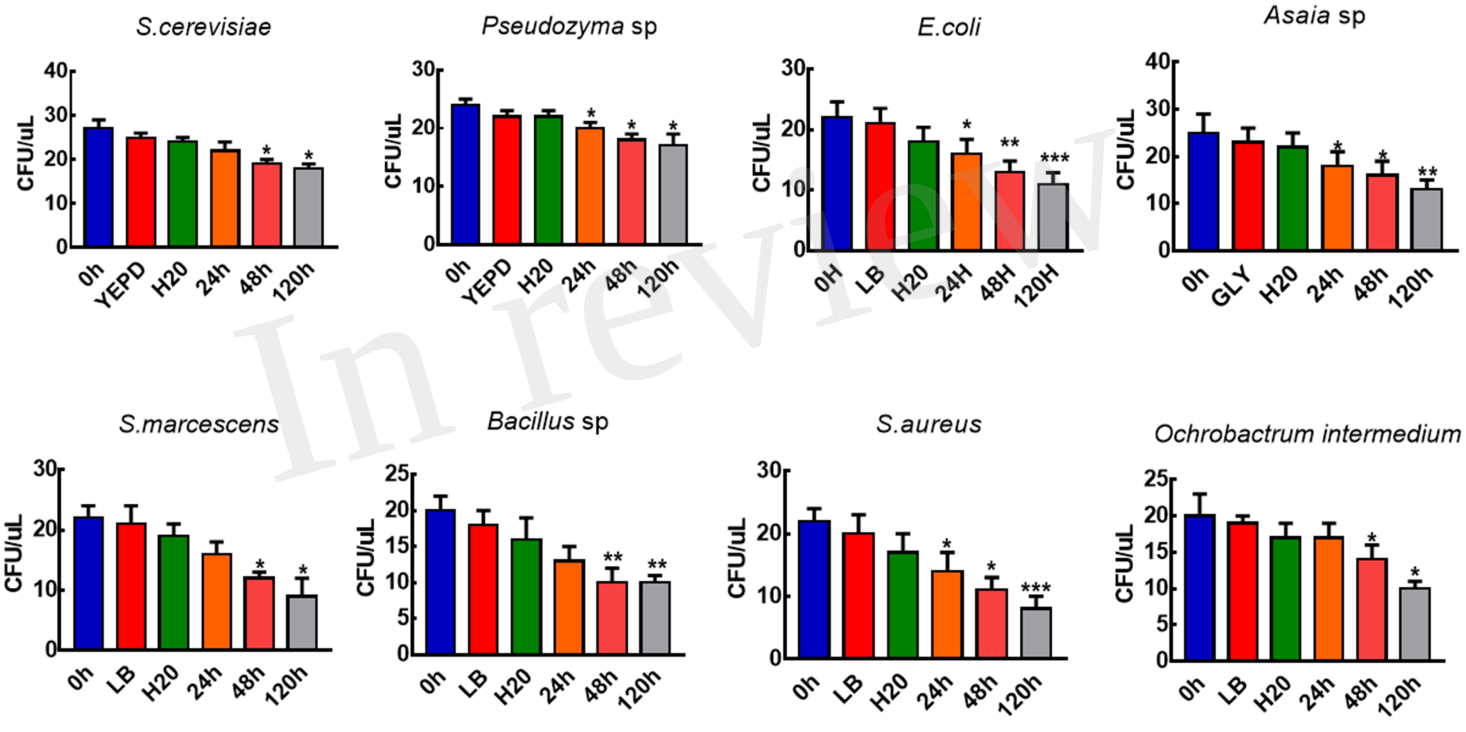
Figure 1.TIF



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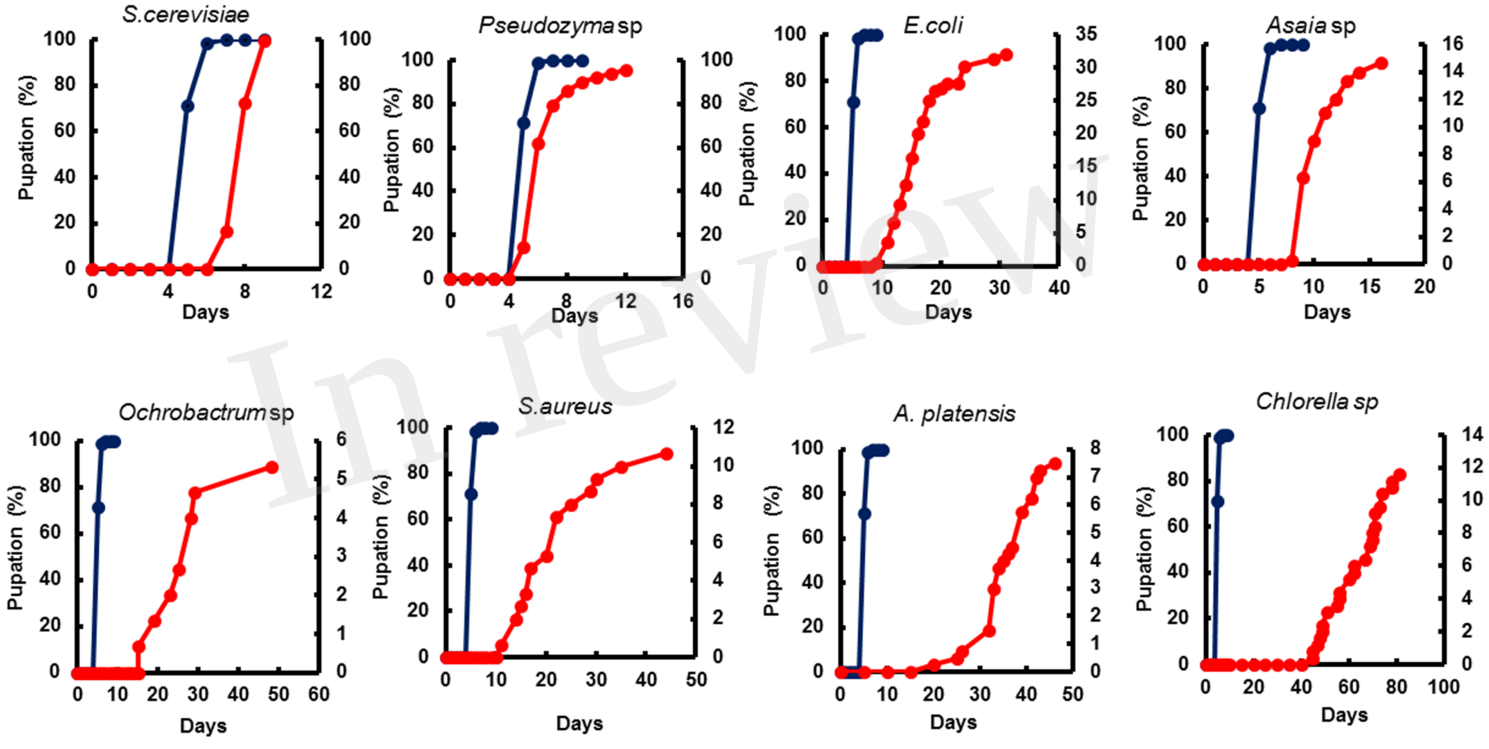

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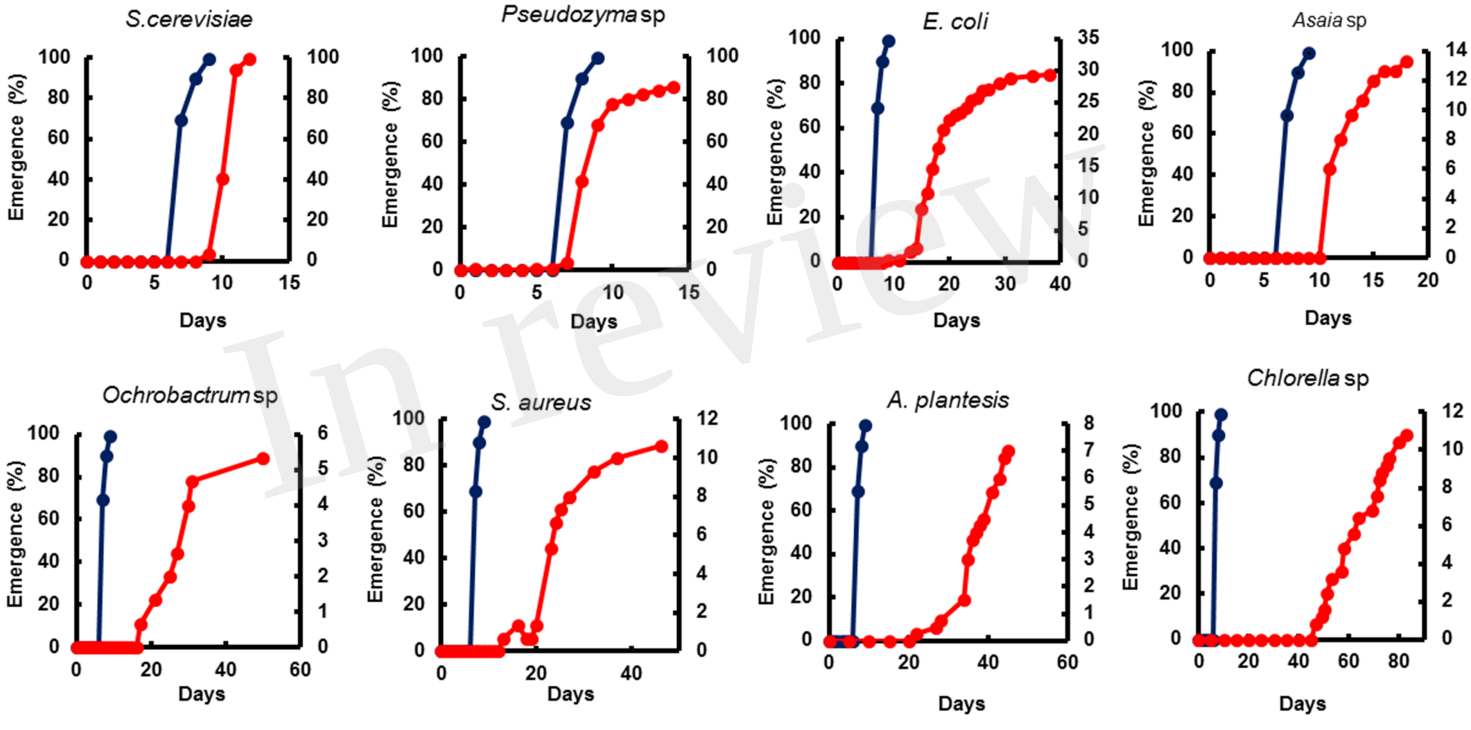

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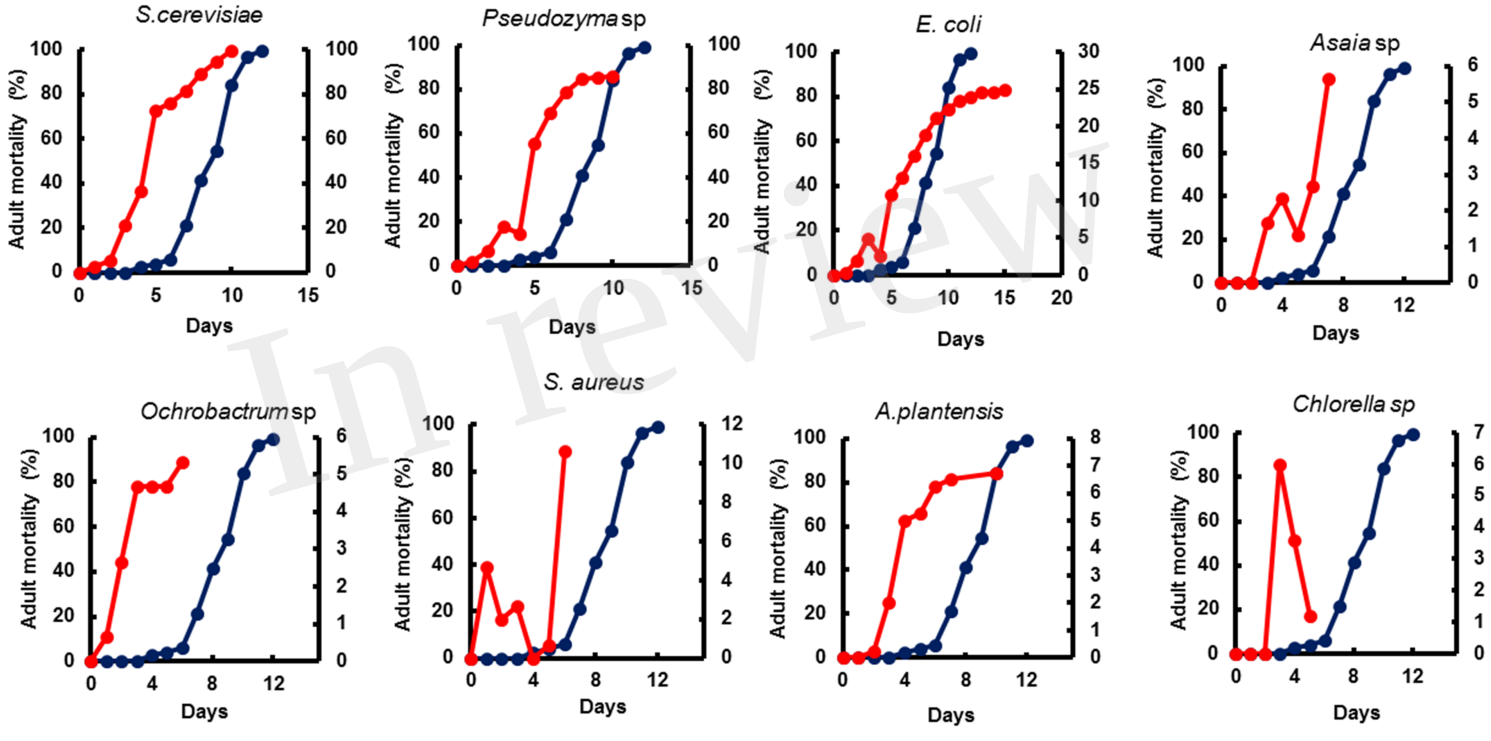

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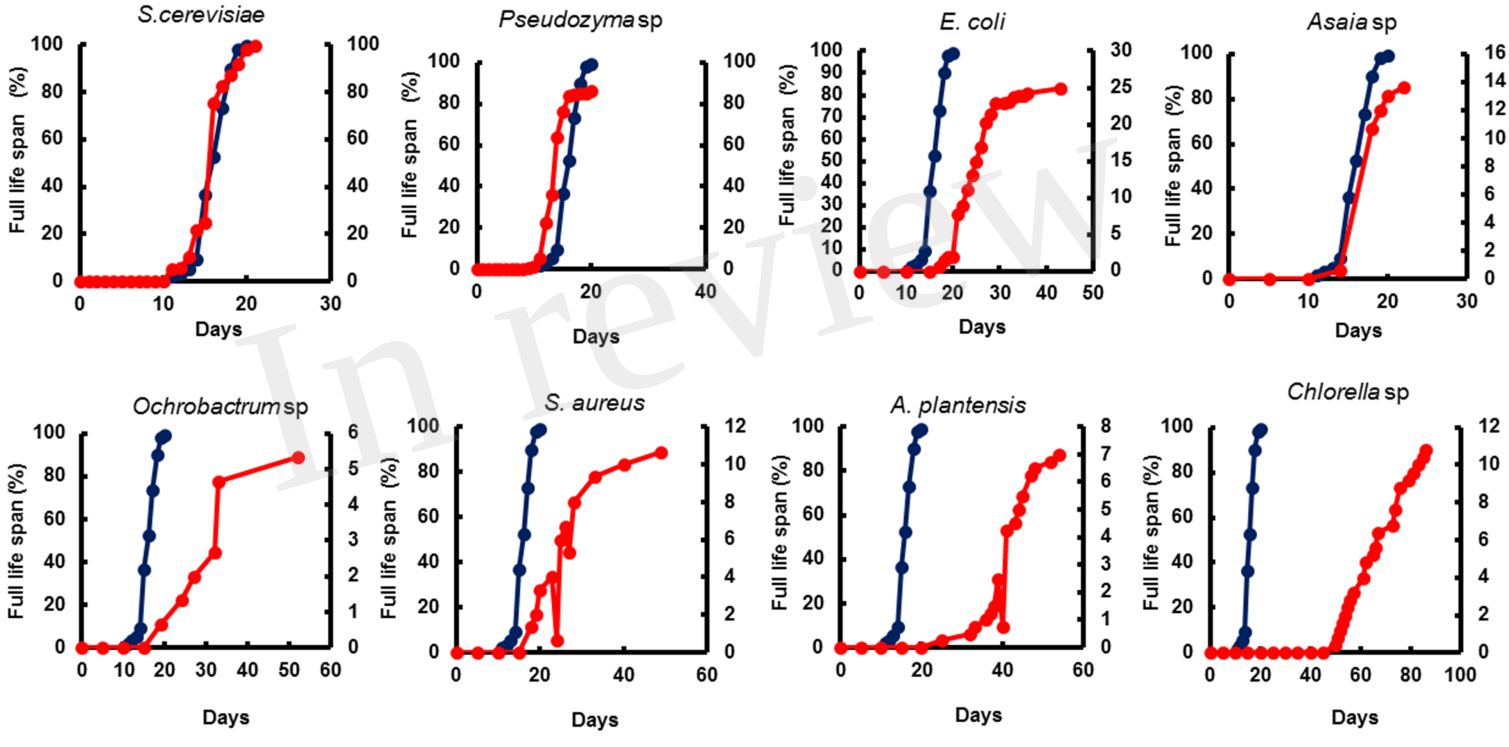

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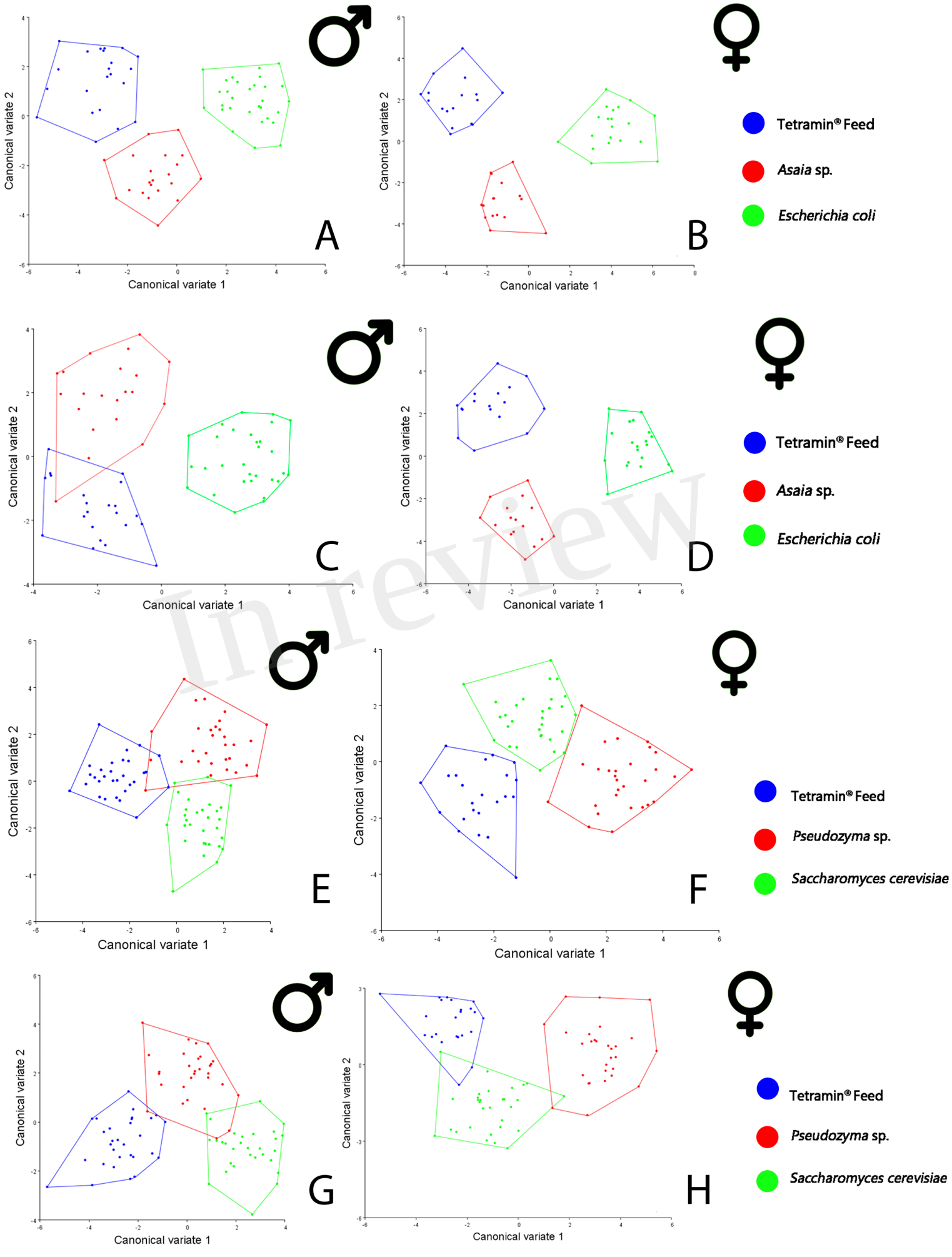

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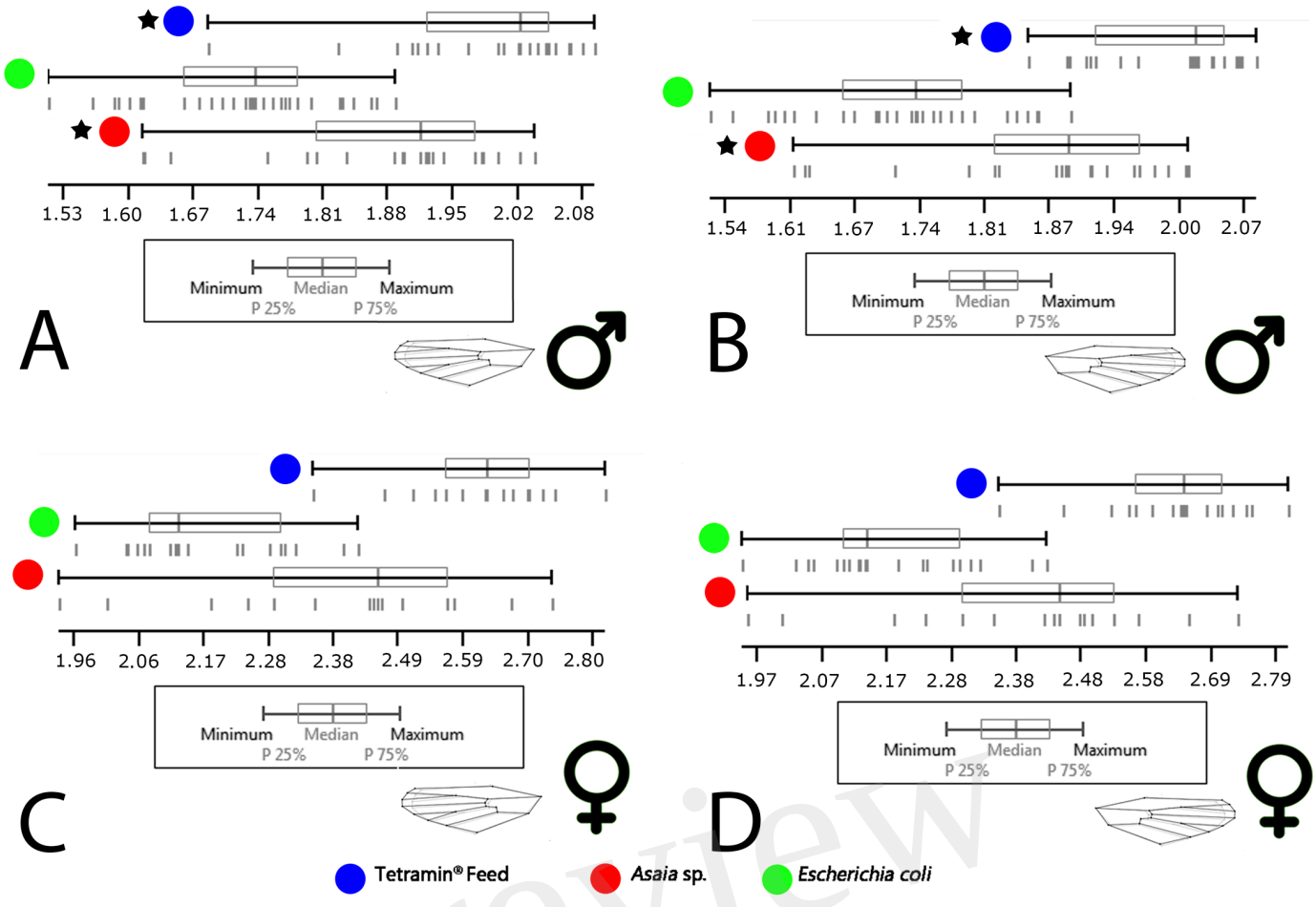

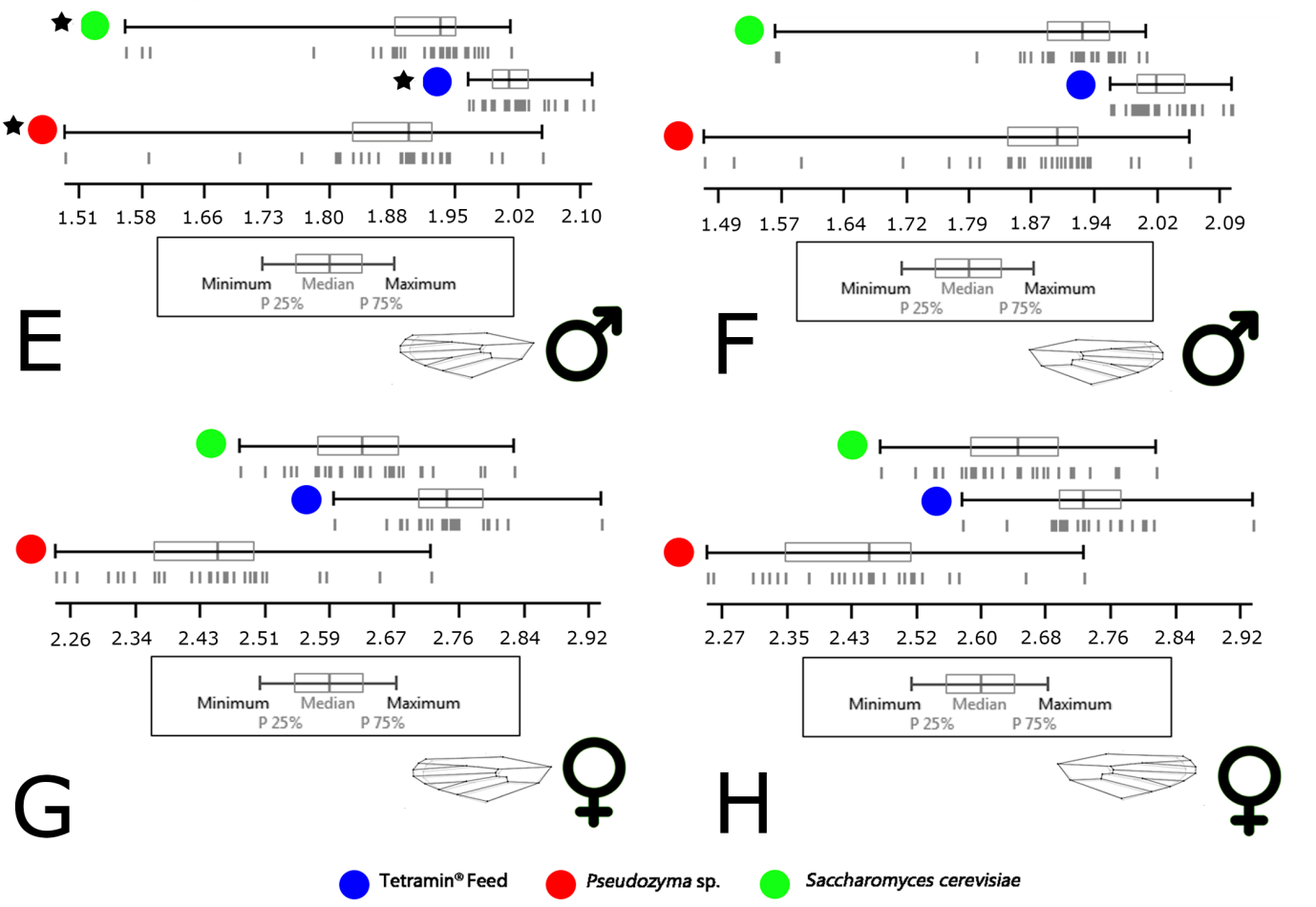

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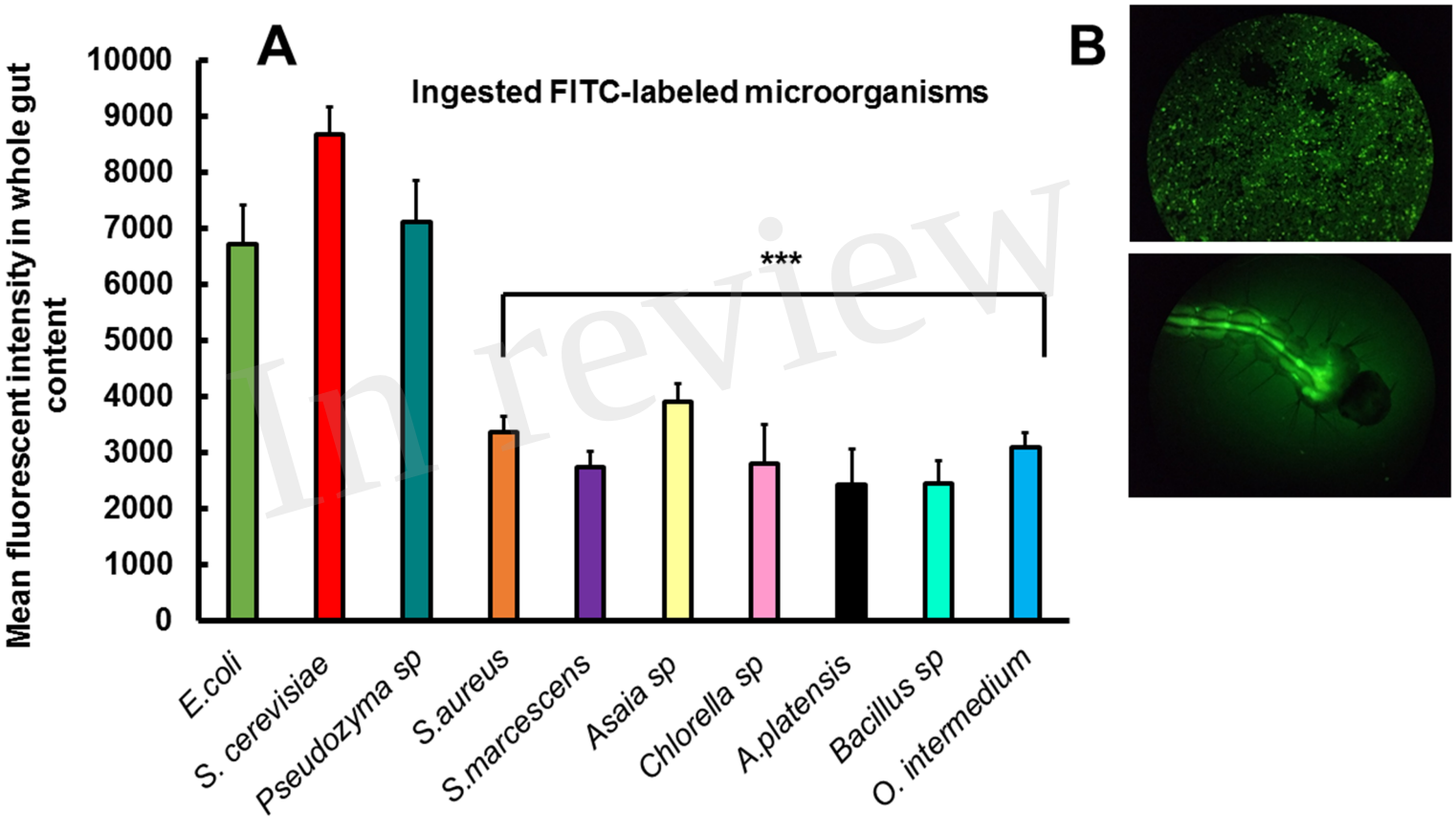

Artigo 4

Transcriptome Sequencing based analysis of compartmentalization in the midgut of Aedes aegypti larvae

Autores: Raquel Santos Souza, Caroline da Silva Moraes, Maiara do Valle Faria Gama, Fábio Faria da Mota, Christiane Cardoso, Clélia Ferreira, Walter Terra, Fernando Ariel Genta

Transcriptome Sequencing-based analysis of compartmentalization in the midgut of Aedes aegypti larvae

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Keyword:	Aedes aegypti, digestion, transcriptome, larvae, mosquito
Theme:	Molecular Biology, Medical Entomology, Dengue, Genomics, Biochemistry
Note: The following files were submitted by the author for peer review, but cannot be converted to PDF. You must view these files (e.g. movies) online.	
Supplementary Tables.rar	

Ministério da Saúde

FIOCRUZ
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Laboratório de Bioquímica e Fisiologia de Insetos

Rio de Janeiro, 16th September 2018.

To the Editor of Memórias do Instituto Oswaldo Cruz,

I would like to submit for your appreciation the manuscript “Transcriptome Sequencing based analysis of compartmentalization in the midgut of *Aedes aegypti* larvae” by Souza *et al*. In this work, we describe the high-throughput sequencing of cDNA libraries from different sections of the gut of *A. aegypti* larvae – Gastric Caeca, Anterior Midgut, Posterior Midgut and Hindgut. We were able to prospect differentially expressed genes for each of these compartments, suggesting that the physiological compartmentalization of mosquito digestion has an important transcriptional background. These findings, besides contributing to a deeper understanding of the digestive physiology of mosquito larvae, may build the basis for the development of future strategies for control of these insects, as it describes new targets for inhibition and interference in the development..

I am signing on behalf of all authors, which have revised the manuscript and are in agreement with all aspects of this work. Do not hesitate in contact me if any issue needs clarification. Sincerely Yours,

Fernando Ariel Genta

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Prof. Fernando Ariel Genta, Ph.D., M.Sc.

Running Title: Transcriptome compartmentalization in the intestine of *Aedes aegypti* larvae

Title: Transcriptome Sequencing based analysis of compartmentalization in the midgut of *Aedes aegypti* larvae

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Abstract

BACKGROUND. Transcriptomic studies are important for analysis of gene expression, and may result in a better understanding of the biology of insect vectors and to the development of new control methods. OBJECTIVES. Characterization of differentially expressed genes along the gut of *Aedes aegypti* larvae. METHODS. A global analysis of the transcriptome of the different intestinal compartments of *Aedes aegypti* larvae was performed by cDNA pyrosequencing in a Roche platform 454. Properties of differentially expressed genes in each compartment were analysed through several bioinformatic tools. Annotation was performed manually. FINDINGS. We sequenced cDNA libraries obtained from the anterior midgut (AM), posterior midgut (PM), hindgut (HG), and whole gut. We also sequenced a library corresponding to the whole gut without the gastric caeca (GC). We observed overlaps between the differentially expressed genes of the intestinal compartments GC/AM; AM/PM/HG, AM/HG and PM/HG. No overlaps were observed between GC and PM or HG. The data suggest a greater specialization of CG and lesser differentiation of the posterior portions of the intestine. A customized categorization of intestinal genes was created. MAIN CONCLUSIONS. We observed that each gut compartment presents a characteristic distribution of differentially expressed genes, which corroborates the assumption that the physiological compartmentalization of the intestine of *A. aegypti* larvae has a transcriptional basis.

Keywords: *Aedes aegypti*, transcriptome, digestion, larvae, mosquito

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Introduction

Arthropod-borne viruses have high prevalence in the tropics and subtropics due to environmental, ecological and socioeconomic factors. The World Health Organization believes that 17% of all human infectious diseases are transmitted by insect vectors (WHO 2017, Genta et al., 2016). *Aedes aegypti* is the major global vector of dengue virus in humans (Rodhain and Rosen 1997) and a key vector of viruses that cause urban yellow fever, chikungunya and zika fever (Jents et al., 2010; Pialoux et al., 2007; Leparc et al. al., 2014, Freitas et al., 2016). The global expansion of these arboviruses was preceded by a worldwide spread of *A. aegypti* (Bhatt *et al*., 2013). Blocking the life cycle of the mosquito is an effective way to hinder the transmission of these diseases (WHO 2013).

Dengue is currently considered the most important mosquito-borne viral disease, causing 100 million apparent annual infections worldwide, with almost half of the world's population at risk of infection (Brady et al., 2012; Bhatt et al., 2013). In the last decades, Brazil has faced a drastic emergence and reemergence of some arboviruses, particularly dengue, chikungunya and zika virus (Brathwhite et al., 2012, Leparc et al., 2004, Freitas et al., 2016). Besides that, these viral outbreaks intensified, increased in frequency and magnitude (WHO 2016). Due to the lack of effective medical treatments, the management of these diseases is mainly done by vector control through the reduction of larval breeding sites and the use of chemical insecticides. (Maciel-de-Freitas et al., 2012).

Given the medical importance of *A. aegypti*-transmitted diseases, control of larval breeding sites for mosquito population control (Kay 1999) is essential. Studies on larval digestive physiology may provide new targets for the development and/or improvement of control strategies (Oviedo et al., 2008, Coutinho-Abreu 2010). In insects, both digestion and absorption occur mainly in the intestine (Terra and Ferreira 2005). The intestine of the insect exhibits a high degree of compartmentalization and is considered one of the main contact surfaces with the environment (Terra and Ferreira 1994; Hakin et al., 2010; Bragatto 2010).

The anatomical structure of the intestine is shared between different classes of insects. However, some segments may present structural changes due to the multivariate eating habits of each species (Chapman 2013). *A. aegypti* larvae are considered detritivores, feeding on solid particles present in their natural habitat (Merrit et al 1990). Among the particles ingested

by mosquito larvae, several microorganisms, such as bacteria, algae, fungi and protozoa, were found in the intestine of the larvae (Walker et al., 1988; Merrit et al., 1990). The mechanisms used by the larvae for degradation of these nutritional sources are not clear. The knowledge of the digestion of mosquito larvae is still vague, but remains an essential issue when we consider the larval stages as targets for the control of disease vectors.

The anatomic structure of the intestine of mosquito larvae is divided into: foregut, midgut and hindgut (HG). The midgut is divided in Gastric Caeca (GC), anterior midgut (AM) and posterior midgut (PM) (Wigglesworth 1942; Christophers 1960; Clements 1992). Digestion in mosquito larvae is similar to that observed in other Diptera Nematocera (Terra et al., 1996). The midgut is the main area of nutrient digestion and absorption (Ferreira and Terra 1982), maintaining a highly alkaline luminal pH potentially related to the solubilization and extraction of complex proteins from ingested debris (Terra 1990). HG absorbs water, minerals, and other essential molecules prior to fecal elimination (Gullan 2010; Hakim et al., 2010).

The availability of *A. aegypti* genome sequencing (Nene *et al.*, 2007) allows the identification of relevant transcripts and processes associated with physiological compartmentalization. To date, studies of gene expression in *A. aegypti* larvae are rather scarce (Vogel *et al*., 2016). A detailed understanding of gene expression and tissue specialization in the compartmentalization of the digestive system in mosquito larvae is necessary to elucidate the molecular mechanisms involved in the maintenance of homeostasis (Oviedo et al., 2007).

Here we present the transcriptomic profile of gastric caeca (GC), anterior midgut (AM), posterior midgut (PM) and hindgut (HG) of *A. aegypti* larvae. For this purpose, we used a Roche 454 pyrosequencing platform and identified which transcripts are differentially expressed in each larval intestinal compartment, and the potential physiological roles associated with the corresponding gene products through manual annotations.

Material and Methods

Rearing and Dissection of Mosquito Larvae - *A. aegypti* eggs of the Rockfeller strain were obtained from the colony of the Laboratory of Physiology and Control of Arthropod Vectors

(LAFICAVE / IOC-FIOCRUZ, Dr. José Bento Pereira Lima). The mosquito larvae were kept in distilled water at 26 ° C, 12 h: 12 h light cycle: dark, and fed with 0.1 g of cat food (Whiskas, Purina, Brazil). For preparing each sample, 2500 initial fourth instar larvae were anesthetized on ice and dissected in sterile PBS. The intestines, full with food, were removed and anterior midgut (AM), posterior midgut (PM) and hindgut (HG) were separated (Figure 1). Each sample was transferred to a 1.5 ml RNase-free tube. Two corresponding groups of whole gut (WG), and whole gut without gastric caeca (WG-GC) were prepared for comparison between the samples.

Total RNA was extracted from the anterior midgut (AM), posterior midgut (PM), posterior gut (HG), whole gut (WG) and whole gut without gastric caeca (WG-GC) using TRIzol (Invitrogen) according to the manufacturer's protocol. Possible DNA remnants of the RNA samples were treated with DNAase using the Turbo-DNAfree kit (Life Technologies). Subsequently, RNA integrity was evaluated in a BioAnalyzer (Agilent) NanoVue ™ Plus (GE) using a RiboGreen® RNA (Invitrogen) Quant-iT ™ RNA kit. For purification and concentration of the total RNA samples, we used RNA Clean Up and Concentrator (Quiagen). Extraction of messenger RNA was performed using the Dynabeads ™ mRNA purification kit according to the manufacturer's protocol. Integrity of the mRNA was assessed using an Agilent RNA 6000 Pico kit (Agilent Technologies).

Library preparation and sequencing - The cDNA libraries were constructed using the Creator ™ SMART ™ kit (Clontech Laboratories Inc., Mountain View, CA, USA). Briefly, the mRNA was reverse-transcribed to the first cDNA stand coupled with tail initiator (dC) and CDS III sequences (both containing SfiI digestion site sequences) following a template switchover step and reverse transcription extension. The second strand cDNAs were then amplified using long-distance PCR with the Advantage 2 PCR enzyme system (Clontech Laboratories Inc.). The cDNA samples were quantified with RiboGreen® (Thermo Fisher Scientific). The quality of the cDNAs was then visually verified by running the cDNAs on a 1.1% (w/v) agarose gel. The cDNAs were then purified using the QIAquick PCR purification kit (Qiagen; Carlsbad, CA, USA) according to the manufacturer's instructions. The second

strand cDNAs were submitted to sequencing. Sequencing utilized the FLX System protocol in a 454 biological science sequencer pyrosquencing (Roche Applied Science).

Data analysis - After 454 pyrosequencing the readings were assigned to each sample and filtered using the TRIMMER tool (Joshi and Fass 2011). The readings were then mapped to the *A. aegypti* genome (Nene et al. 2007; Aedes-aegypti-Liverpool_PEPTIDES_AaegL3.4.fa) using HISAT2 (https://ccb.jhu.edu/software/hisat2/index.shtml). The readings and differential expression counts were determined using DESeq2 (Love et al 2014) with scores Log2fold <-1 for (WG-GCxWG) and > 1 for (AMxWG), (PMxWG) and (HGxWG). Venn diagram with the selected gene identifiers was drawn with InteractiVenn (Heberle et al., 2015).

The coding sequences of differentially expressed genes were analysed using a series of bioinformatic tools. Homologues were searched with the BLASTp tool (Boratyn et al., 2013) and Conserved Domains in each sequence were searched with the CDD tool (Marchler-Bauer A et al., 2017). The presence of putative signal peptide was verified with the SignalP tool (Nielsen, 2017), and putative GPI anchor sequences were searched with PredGPI (Pierleoni et al., 2008). Transmembrane regions were predicted with the TMHMM Server (Krogh et al., 2001), theoretical pI and molecular weight were calculated with the Expasy Server (Gasteiger et al., 2005), N-linked glycosylations were predicted with the NetNGlyc Server (Gupta et al., 2004), and O-linked glycosylations were predicted with the NetOGlyc Server (Steentoft et al., 2013).

Results and discussion

General sequencing results

Three cDNA libraries were constructed from specific intestinal compartments (AM, PM, and HG) and sequenced, resulting in a number of reads ranging from 163,000 to 185,000 (Table 1). In addition, 230,000 reads were obtained from the library of the digestive tract without the gastric caeca (GC), and 125,871 reads were obtained from the library corresponding to the complete digestive tract (WG). Between 87 and 117 thousand reads of each library were

mapped against known genes in the *A. aegypti* genome (available in the Vector Base database), resulting in the identification of transcripts corresponding to about 5,000 genes for each library (Table 1).

Differential Expression Analysis

Data on the libraries were loaded into the DESeq2 program to search for genes that were differentially expressed in each compartment. The following comparisons were made:

- AM x WG;
- PM x WG;
- HG x WG;
- WG-CG x WG;

In the first three cases, since the AM, PM and HG libraries were constructed with these tissues, we selected genes with an expression ratio above 2 (Log2FoldChange > 1). In the WG-GC vs. WG comparison, since the WG-GC library was constructed with the digestive tract without the gastric cecum, we looked for genes with an expression ratio below 50% (Log2FoldChange <-1). Thus, we were able to filter our data to find which genes would be particularly expressed in each compartment, obtaining 11, 23, 69 and 235 genes differentially expressed in the PM, HG, AM and GC, respectively (Table 1).

After identifying the differentially expressed genes, these gene sets were analysed for their possible overlap. The intestinal compartments are physiologically related (Terra and Ferreira 2005), so it is possible that at the molecular level, the same gene is abundantly expressed in more than one compartment. This analysis was performed with the InteractiVenn software, resulting in Figure 1. We can observe that most of the genes found are expressed in only one compartment, with a discrete overlap between the sets. Of the total of 319 genes selected, 303 are differentially expressed in only one tissue, and 16 are more expressed in more than one compartment (Figure 2). It is interesting to note that overlaps in gene expression were found for GC + AM (3), AM + PM + HG (1), AM + HG (1), PM + HG (5), reflecting in some way the physiological structure of the gastrointestinal tract, since there were not observed overlaps between GC and PM or HG. The data suggest a greater specialization of

GC and a lesser differentiation of the posterior portions of the intestine. Another aspect observed was the reduced proportion of differentially expressed genes in each compartment, which varied from 0.2 to 4.9% of expressed genes (Table 1), which may be related to the approach used in this study or to the low functional differentiation between the compartments.

Bioinformatics analysis of differentially expressed transcripts

The sequences of differentially expressed transcripts in each intestinal compartment were analysed using several bioinformatic tools. Initially, we looked for the identification of homologues of each transcript using the BLASTp tool. All transcripts had homolog genes identified in the databases used. However, it was not possible to identify a known function for 9, 4, 5 and 25 AM, PM, HG and GC transcripts, respectively (Table S1).

Subsequently, conserved domains were identified in each of the differential transcripts through the CDD (Conserved Domain Database) tool. The results are shown in the Supplementary Table S2. In summary, 299, 30, 72 and 795 conserved domains were identified in the transcripts differentially expressed in AM, PM, HG and GC, respectively. The sequences obtained from AM have 0 to 9 conserved domains, whereas those from PM and HG show 0 to 6 CDDs. In GC, the DE transcripts had 0 to 14 conserved domains.

After that, the molecular properties of the proteins encoded by differentially expressed genes in each compartment were analysed. Most of the transcripts observed have an initial methionine residue (90-98%), and 18, 61, 10, and 28% have predicted peptide secretion signal in GC, AM, PM, and HG, respectively. A considerable fraction of the proteins presented prediction for anchoring via the GPI group, corresponding to 1-10% of the differentially expressed proteins. In addition, proteins with transmembrane domains were also observed, corresponding to 15-30% of the annotated protein pool. The targeting of most of the proteins differentially expressed in the AM is secretion to the extracellular medium, whereas for GC and PM most DE proteins are cytoplasmic with an important contribution of intracellular membrane proteins. In HG, the predominance of cytoplasmic proteins was observed, followed by proteins secreted to the extracellular medium.

Other characteristics observed for DE proteins were the theoretical size, molecular weigth, isoelectric point, and the presence of glycosylation sites. A large variation in the size and composition of these proteins was observed, with sequences ranging from 40 to 1866 amino acid residues, molecular masses ranging from 4.6 to 211 kDa, and isoelectric points ranging from 3.92 to 11.72. In addition, we observed that a significant fraction of the differentially expressed proteins had glycosylation sites (58-71% N-glycosylated and 64-80% O-glycosylated), in consonance with their probable targeting to the extracellular medium (Table 2).

After analysing the properties of each DE sequence, we decided to manually annotate all differentially expressed genes based on the information collected (Supplementary Tables S7 to S10). The annotation allowed us to perform the customized categorization of these genes, where specific functional classifications related to intestinal physiology in insects were created. This ontogeny was performed on two levels, with 30 categories and 6 macrocategories (Supplementary Tables S7 to S13). We observed that each compartment presents a characteristic distribution of differentially expressed genes, which corroborates the assumption that the physiological compartmentalization of the intestine of *A. aegypti* larvae has a transcriptional basis. The same phenomenon was described by Oviedo *et al*. (2007) using the microarray tool. However, in this previous work a much smaller number of differentially expressed genes was identified.

Our classification included an important part of the differentially expressed genes in all compartments in the category "others" (Figure 3). In addition, in the gastric caeca there is a predominance of the categories of transcription, energetic metabolism and protein digestion, while in the anterior midgut the categories of protein digestion, peritrophic matrix and carbohydrate digestion predominate. The number of genes differentially expressed in the posterior midgut is very small, and we observed a dispersion of these genes in all categories, so that no hegemony or specialization could be observed in any of them. In the hindgut, we observed the predominance of the categories of protein digestion, transcription and energetic metabolism, similar to that observed in gastric caeca. Despite this apparent similarity between gastric caeca and hindgut, it is important to highlight that these groups are completely different sets of genes, since there is no overlap between these sets (Figure 2).

In terms of macro-categories (Figure 4), while we observed a predominance of cellular processes (CEL) in the gastric caeca, in the anterior midgut we observed a predominance of the DIG macrocategory, mainly composed of digestive enzymes. In the posterior midgut, we found a predominance of the absorption categories (ABS) and cellular processes (CEL), and in the hindgut we observed the predominance of the MET category of cellular metabolism. This is compatible with the functional differentiation of each compartment, and suggests a specialization where the anterior compartments are responsible for the secretion of enzymes, with most of the absorption occurring in the posterior midgut. The posterior intestine, although not secreting enzymes or absorbing macronutrients, is shown to be metabolically active, which may be related to its role as excretory organ.

A study already carried out with a similar approach is the study by Oviedo et al. (2007), who analyzed the intestinal transcriptome of *Anopheles gambiae* larvae. However, in this paper, the authors compared the enrichment of transcripts from each region of the gut with a library obtained from whole larvae using the microarray technique. In this study 1211 more expressed genes were drawn in the Posterior Midgut and Malpighian Tubules, 707 in the gastric caeca, 603 in the Posterior Midgut and 208 in the Anterior Midgut. Of these genes, 833, 191, 120 and 53 are exclusively expressed in a single compartment. Although the number of genes observed by Oviedo et al. (2007) is higher than those we found, this is probably due to the differences in the experimental design, since we compared the libraries of each compartment with an entire intestine library. This makes the differences found to be much smaller, because theoretically our screening removed genes that are constitutively expressed along the intestine. Despite this difference, in some compartments we observed a number of unique genes very similar to the work of Oviedo et al. (2007), especially in the gastric caeca and the anterior midgut, where we found 232 and 57 DE and exclusive genes, respectively. This suggests that these two compartments are very differentiated in relation to the rest of the intestine, since the removal of the background of constitutive intestinal genes did not alter the number of DE genes. In the other compartments considered, the posterior midgut and the hindgut, we observed a significantly lower number of differentially expressed exclusive genes, respectively 4 and 10. In the case of the hindgut the comparison between both works is more difficult because Oviedo et al. (2007) combined the Malpighian tubules with the hindgut, which may be the reason for the observation of a larger number of DE

genes. On the other hand, the low number of DE genes found in these compartments can mean a smaller differentiation in the transcriptional level in relation to the rest of the intestine. In addition, it is important to consider that there may be differences in the intestinal physiology related to the biological species, between *A. gambiae* and *A. aegypti*.

The analyzes performed by us still require external validation regarding to expression levels. It will also be important in the future to have a more refined look at the nature of differentially expressed transcripts, since the categories we use differ from those described in Oviedo et al. (2007) and the GO base. It will be important to look at the absolute levels of gene expression in each functional category (for example, digestion of proteins, carbohydrates and lipids) for a more detailed analysis of the transcriptional patterns along the intestine.

Tetreau et al. (2012) analyzed the expression of genes in the intestine of Bti-challenged *A. aegypti* larvae, also using the DNA microarray technique. Harker et al. (2013) analyzed the gene expression in *A. aegypti* comparing different stages of insect development (larvae, pupae and adults) also with the microarray technique. Vogel et al. (2017) compared the gene expression of axenic larvae to gnotobiotic larvae (associated with *Escherichia coli*) and conventionally created larvae, using pyrosequencing on the Illumina platform. Venancio *et al.* (2009) analyzed sequencing data of whole larvae and observed the presence of specific larval genes. In order for more detailed comparisons between these surveys and our study to be carried out, it will be necessary to map the individual expression of each one of the genes observed.

In conclusion, it was possible to confirm and describe the compartmentalization of the digestive process in *A. aegypti* larvae with an alternative molecular technique. This analysis will allow functional studies of digestion in these insects with greater depth, aiming the development of specific strategies to control this important disease vector.

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Table 1. Number of total reads, mapped reads, mapped genes, and differentially expressed (DE) transcripts in each library. WG-GC – Whole Gut without Gastric Caeca; AM – Anterior Midgut; PM – Posterior Midgut; HG – Hindgut; WG – whole gut.

Library	Total Reads	Mapped Reads	Mapped Genes	DE Genes	% DE Genes
WG-GC	229830	117380	4826	235	4.9
AM	173302	87763	5282	69	1.3
PM	185195	107028	5502	11	0.2
HG	163337	92656	5246	23	0.4
WG	125871	75792	5140	ND	ND

Table 2. Summary of molecular properties of differentially expressed proteins in the intestinal compartments of *A. aegypti* larvae. Met - % of sequences starting with a Met residue at the N-terminus. Sig. Pep. - % of sequences with a putative signal peptide. GPI - % of sequences with a putative GPI anchor. TMHMM - % of sequences with putative transmembrane sequences. Extracellular secreted, Plasmatic membrane, Intracellular membrane and Cytoplasmic correspond to the % of proteins assigned to each cellular locatization. Residues (min, max) – minimal and maximum sizes of polypeptides coded by the transcripts in each group. MW (min, max) – minimal and maximum molecular weight of polypeptides coded by the transcripts in each group. pI (min, max) – minimal and maximum isoeletric point of polypeptided coded by the transcripts in each group. NGlyc - % of proteins with putative N-linked glycosylation sites. OGlyc - % of proteins with O-linked glycosylation sites.

Compartment	Met	Sig. Pep.	GPI	TMHMM	Extracellular secreted	Plasmatic membrane	Intracellular membrane	Cytoplasmic	Residues		MW		pI		NGlyc	Oglyc
									min.	max.	min.	max.	min.	max.		
Gastric Caeca	98	18	2	26	11	7	21	62	64	1237	6483	136339	3.65	11.72	58	65
Anterior Midgut	96	61	7	15	52	9	12	28	40	1866	4606	211247	3.92	11.22	71	80
Posterior Midgut	90	10	10	30	10	0	30	60	40	610	4606	67766	4.43	10.24	70	70
Hindgut	92	28	4	20	33	0	22	44	40	927	4606	105602	3.92	10.24	64	64

Legend of Figures

Figure 1. Gut compartments of A. aegypti larvae. GC – Gastric Caeca; AM – Anterior Midgut; PM – Posterior Midgut; HG – Hindgut.

Figure 2. Venn diagram of DE genes in each gut compartment. AM – Anterior Midgut; PM – Posterior Midgut; HG - Hindgut and GC – Gastric Caeca.

Figure 3. Percentage distribution by category of differentially expressed transcripts in the intestinal compartments of *A. aegypti* larvae. Categories: Categories: aa_transp - amino acids transporters; immune - immune response; aa_met - Amino acid metabolism; cytoskeleton - Cytoskeleton/Structure; carb_met - Carbohydrate metabolism; carb_dig - Carbohydrate digestion; redox - Redox metabolism; lip_lig – lipid ligands; transcription - Transcription; gen_reg - Gene regulation; PM - Peritrophic membrane; lip_dig – lipids digestion; ion_lig - ion ligands; ion_transp - Ion transporters; lip_met - Lipid metabolism; prot_dig - Protein digestion; carb_transp – carbohydrate transposrters; translation – protein translation; secretion – secretory pathways; energ_met - energetic metabolism; detox – detoxification pathways; Signaling – cellular signaling; others - others; cell cycle – cell cycle or apoptosis; vit_met - Vitamin metabolism; inhib_prot - Protease inhibitors; horm_met – hormone metabolism; protein turnover - Turn over of proteins; nuc_met - Nucleotide metabolism; nuc_dig - Nucleotide digestion.

Figure 4. Percentage distribution by macrocategories of differentially expressed transcripts in the intestine compartments of *A. aegypti* larvae. . Macrocategories: DIG – digestion; ABS – absortion; MET – metabolism; CEL – cellular processes; STR – structure; OTH – others.

Supplementary Material

Supplementary Table S1. Genes differentially expressed in the intestinal compartments of *A. aegypti* larvae and their homologues identified by the BLASTp tool (3 first hits), besides the identification parameters of this tool. AM - Anterior Midgut; PM - Posterior Midgut; HG - Hindgut; GC - Gastric Caeca.

Supplementary Table S2. Differentially expressed genes in the intestinal compartments of *A. aegypti* larvae and domains identified in their respective coding sequences according to the CDD (Conserved Domain Database) tool.

Supplementary Table S3. Differentially expressed Genes in the Anterior Midgut (AM) and molecular characteristics of proteins encoded by their sequences. ID - Identifier in the Vector Base Database; FASTA - amino acid sequence; Met - presence of initial methionine residue; Sig. Pep. - Presence of signal peptide (SignalP); GPI - GPI anchor presence (PredGPI); TMHMM - presence of transmembrane domains; Location - classification of protein addressing; Residues - number of amino acids; MW - molecular weight; pI - isoelectric point; NGlyc - presence of N-glycosylations (NetNglyc); Oglyc - presence of O-glycosylations (NetOGlyc).

Supplementary Table S4. Differentially expressed Genes in the Posterior Midgut (PM) and molecular characteristics of proteins encoded by their sequences. ID - Identifier in the Vector Base Database; FASTA - amino acid sequence; Met - presence of initial methionine residue; Sig. Pep. - Presence of signal peptide (SignalP); GPI - GPI anchor presence (PredGPI); TMHMM - presence of transmembrane domains; Location - classification of protein addressing; Residues - number of amino acids; MW - molecular weight; pI - isoelectric point; NGlyc - presence of N-glycosylations (NetNglyc); Oglyc - presence of O-glycosylations (NetOGlyc).

Supplementary Table S5. Differentially expressed Genes in the Hindgut (HG) and molecular characteristics of proteins encoded by their sequences. ID - Identifier in the Vector Base Database; FASTA - amino acid sequence; Met - presence of initial methionine residue; Sig. Pep. - Presence of signal peptide (SignalP); GPI - GPI anchor presence (PredGPI); TMHMM - presence of transmembrane domains; Location - classification of protein addressing; Residues - number of amino acids; MW - molecular weight; pI - isoelectric point; NGlyc - presence of N-glycosylations (NetNglyc); Oglyc - presence of O-glycosylations (NetOGlyc).

Supplementary Table S6. Differentially expressed Genes in the Gastric Caeca (GC) and molecular characteristics of proteins encoded by their sequences. ID - Identifier in the Vector Base Database; FASTA - amino acid sequence; Met - presence of initial methionine residue; Sig. Pep. - Presence of signal peptide (SignalP); GPI - GPI anchor presence

(PredGPI); TMHMM - presence of transmembrane domains; Location - classification of protein addressing; Residues - number of amino acids; MW - molecular weight; pI - isoelectric point; NGlyc - presence of N-glycosylations (NetNglyc); Oglyc - presence of O-glycosylations (NetOGlyc).

Supplementary Table S7 - Annotation and customized categorization (ontogeny) of differentially expressed genes in the Anterior Midgut of *A. aegypti* larvae. Categories: aa_transp - amino acids transporters; immune - immune response; aa_met - Amino acid metabolism; cytoskeleton - Cytoskeleton/Structure; carb_met - Carbohydrate metabolism; carb_dig - Carbohydrate digestion; redox - Redox metabolism; lip_lig – lipid ligands; transcription - Transcription; gen_reg - Gene regulation; PM - Peritrophic membrane; lip_dig – lipids digestion; ion_lig - ion ligands; ion_transp - Ion transporters; lip_met - Lipid metabolism; prot_dig - Protein digestion; carb_transp – carbohydrate transposrters; translation – protein translation; secretion – secretory pathways; energ_met - energetic metabolism; detox – detoxification pathways; Signaling – cellular signaling; others - others; cell cycle – cell cycle or apoptosis; vit_met - Vitamin metabolism; inhib_prot - Protease inhibitors; horm_met – hormone metabolism; protein turnover - Turn over of proteins; nuc_met - Nucleotide metabolism; nuc_dig - Nucleotide digestion.

Supplementary Table S8 - Annotation and customized categorization (ontogeny) of differentially expressed genes in the Posterior Midgut of *A. aegypti* larvae. Categories: aa_transp - amino acids transporters; immune - immune response; aa_met - Amino acid metabolism; cytoskeleton - Cytoskeleton/Structure; carb_met - Carbohydrate metabolism; carb_dig - Carbohydrate digestion; redox - Redox metabolism; lip_lig – lipid ligands; transcription - Transcription; gen_reg - Gene regulation; PM - Peritrophic membrane; lip_dig – lipids digestion; ion_lig - ion ligands; ion_transp - Ion transporters; lip_met - Lipid metabolism; prot_dig - Protein digestion; carb_transp – carbohydrate transposrters; translation – protein translation; secretion – secretory pathways; energ_met - energetic metabolism; detox – detoxification pathways; Signaling – cellular signaling; others - others; cell cycle – cell cycle or apoptosis; vit_met - Vitamin metabolism; inhib_prot - Protease inhibitors; horm_met – hormone metabolism; protein turnover - Turn over of proteins; nuc_met - Nucleotide metabolism; nuc_dig - Nucleotide digestion.

Supplementary Table S9 - Annotation and customized categorization (ontogeny) of differentially expressed genes in the Hindgut of *A. aegypti* larvae. Categories: aa_transp - amino acids transporters; immune - immune response; aa_met - Amino acid metabolism; cytoskeleton - Cytoskeleton/Structure; carb_met - Carbohydrate metabolism; carb_dig - Carbohydrate digestion; redox - Redox metabolism; lip_lig – lipid ligands; transcription - Transcription; gen_reg - Gene regulation; PM - Peritrophic membrane; lip_dig – lipids digestion; ion_lig - ion ligands; ion_transp - Ion transporters; lip_met - Lipid metabolism; prot_dig - Protein digestion; carb_transp – carbohydrate transposrters; translation – protein translation; secretion – secretory pathways; energ_met - energetic metabolism; detox – detoxification pathways; Signaling – cellular signaling; others - others; cell cycle – cell cycle or apoptosis; vit_met - Vitamin metabolism; inhib_prot - Protease inhibitors; horm_met – hormone metabolism; protein turnover - Turn over of proteins; nuc_met - Nucleotide metabolism; nuc_dig - Nucleotide digestion.

Supplementary Table S10 - Annotation and customized categorization (ontogeny) of differentially expressed genes in the Gastric Caeca of *A. aegypti* larvae. Categories: aa_transp - amino acids transporters; immune - immune response; aa_met - Amino acid metabolism; cytoskeleton - Cytoskeleton/Structure; carb_met - Carbohydrate metabolism; carb_dig - Carbohydrate digestion; redox - Redox metabolism; lip_lig – lipid ligands; transcription - Transcription; gen_reg - Gene regulation; PM - Peritrophic membrane; lip_dig – lipids digestion; ion_lig - ion ligands; ion_transp - Ion transporters; lip_met - Lipid metabolism; prot_dig - Protein digestion; carb_transp – carbohydrate transposrters; translation – protein translation; secretion – secretory pathways; energ_met - energetic metabolism; detox – detoxification pathways; Signaling – cellular signaling; others - others; cell cycle – cell cycle or apoptosis; vit_met - Vitamin metabolism; inhib_prot - Protease inhibitors; horm_met – hormone metabolism; protein turnover - Turn over of proteins; nuc_met - Nucleotide metabolism; nuc_dig - Nucleotide digestion.

Supplementary Table S11 - Summary of categorization of differentially expressed genes in intestine compartments of *A. aegypti* larvae. CG - Gastric Caeca; AM – Anterior Midgut; PM - Posterior Midgut; HG - Hindgut. Categories: aa_transp - amino acids transporters; immune - immune response; aa_met - Amino acid metabolism; cytoskeleton - Cytoskeleton/Structure; carb_met - Carbohydrate metabolism; carb_dig - Carbohydrate

digestion; redox - Redox metabolism; lip_lig – lipid ligands; transcription - Transcription; gen_reg - Gene regulation; PM - Peritrophic membrane; lip_dig – lipids digestion; ion_lig - ion ligands; ion_transp - Ion transporters; lip_met - Lipid metabolism; prot_dig - Protein digestion; carb_transp – carbohydrate transposrters; translation – protein translation; secretion – secretory pathways; energ_met - energetic metabolism; detox – detoxification pathways; Signaling – cellular signaling; others - others; cell cycle – cell cycle or apoptosis; vit_met - Vitamin metabolism; inhib_prot - Protease inhibitors; horm_met – hormone metabolism; protein turnover - Turn over of proteins; nuc_met - Nucleotide metabolism; nuc_dig - Nucleotide digestion.

Supplementary Table S12. List of categories that were collected in each of the Macrocategories used for the functional classification of differentially expressed transcripts. Categories: aa_transp - amino acids transporters; immune - immune response; aa_met - Amino acid metabolism; cytoskeleton - Cytoskeleton/Structure; carb_met - Carbohydrate metabolism; carb_dig - Carbohydrate digestion; redox - Redox metabolism; lip_lig – lipid ligands; transcription - Transcription; gen_reg - Gene regulation; PM - Peritrophic membrane; lip_dig – lipids digestion; ion_lig - ion ligands; ion_transp - Ion transporters; lip_met - Lipid metabolism; prot_dig - Protein digestion; carb_transp – carbohydrate transposrters; translation – protein translation; secretion – secretory pathways; energ_met - energetic metabolism; detox – detoxification pathways; Signaling – cellular signaling; others - others; cell cycle – cell cycle or apoptosis; vit_met - Vitamin metabolism; inhib_prot - Protease inhibitors; horm_met – hormone metabolism; protein turnover - Turn over of proteins; nuc_met - Nucleotide metabolism; nuc_dig - Nucleotide digestion.

Supplementary Table S13. Distribution in macrocategories of differentially expressed genes in the intestinal compartments of *A. aegypti* larvae. AM – Anterior Midgut; PM – Posterior Midgut; HG – Hindgut; GC – Gastric caeca. Macrocategories: DIG – digestion; ABS – absortion; MET – metabolism; CEL – cellular processes; STR – structure; OTH – others.

Figure 1. Gut compartments of A. aegypti larvae. GC – Gastric Caeca; AM – Anterior Midgut; PM – Posterior Midgut; HG – Hindgut.

235x152mm (300 x 300 DPI)

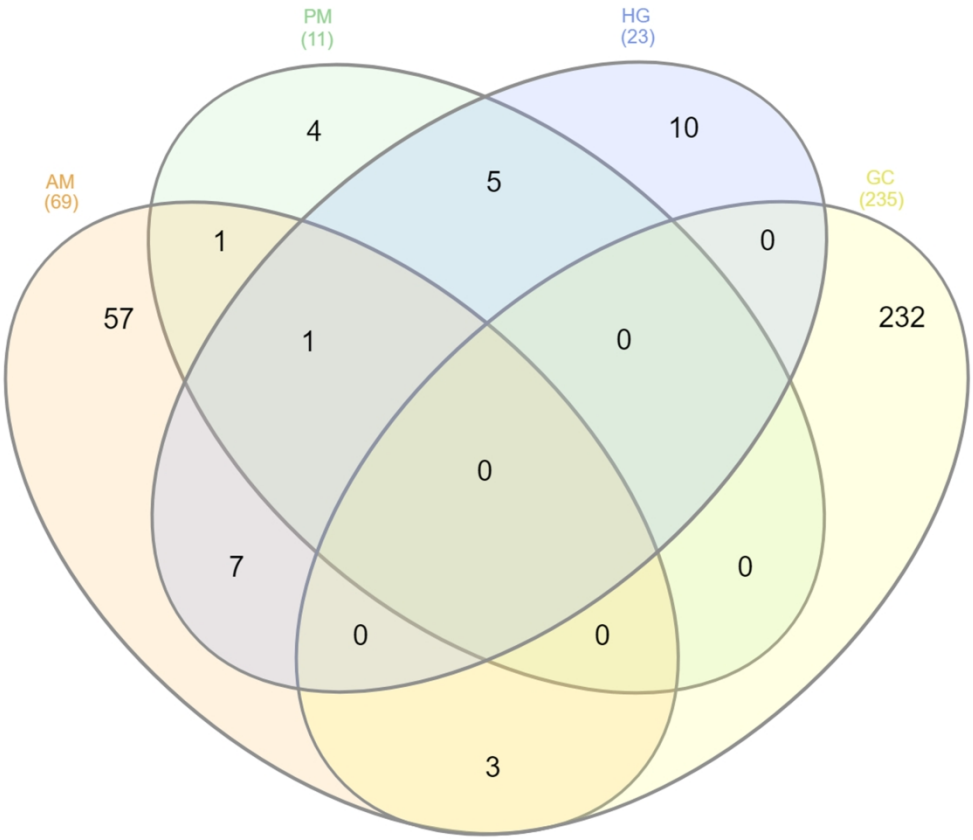

Figure 2. Venn diagram of DE genes in each gut compartment. AM – Anterior Midgut; PM – Posterior Midgut; HG - Hindgut and GC – Gastric Caeca.

423x407mm (300 x 300 DPI)

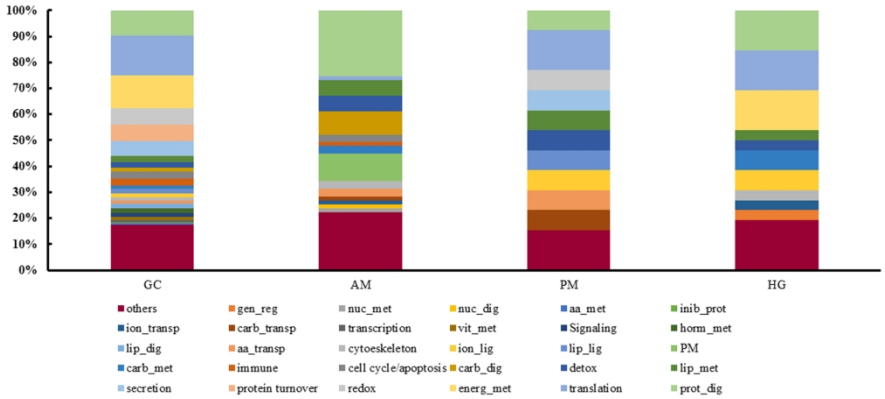

Figure 3. Percentage distribution by category of differentially expressed transcripts in the intestinal compartments of A. aegypti larvae. Categories: Categories: aa_transp - amino acids transporters; immune - immune response; aa_met - Amino acid metabolism; cytoskeleton - Cytoskeleton/Structure; carb_met - Carbohydrate metabolism; carb_dig - Carbohydrate digestion; redox - Redox metabolism; lip_lig – lipid ligands; transcription - Transcription; gen_reg - Gene regulation; PM - Peritrophic membrane; lip_dig – lipids digestion; ion_lig - ion ligands; ion_transp - Ion transporters; lip_met - Lipid metabolism; prot_dig - Protein digestion; carb_transp – carbohydrate transposrters; translation – protein translation; secretion – secretory pathways; energ_met - energetic metabolism; detox – detoxification pathways; Signaling – cellular signaling; others - others; cell cycle – cell cycle or apoptosis; vit_met - Vitamin metabolism; inhib_prot - Protease inhibitors; horm_met – hormone metabolism; protein turnover - Turn over of proteins; nuc_met - Nucleotide metabolism; nuc_dig - Nucleotide digestion.

338x190mm (300 x 300 DPI)

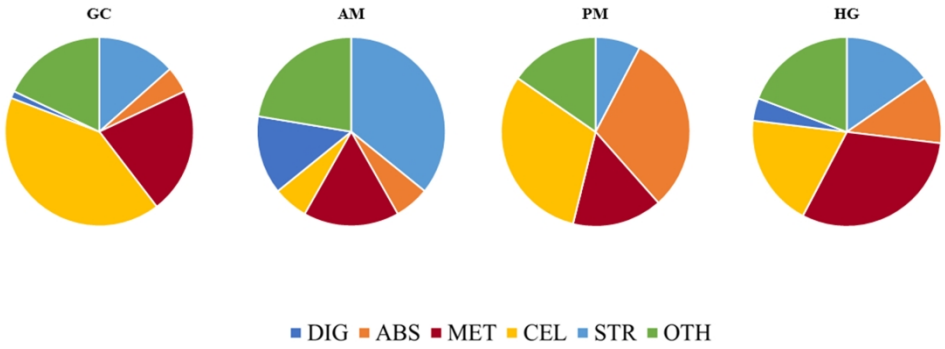

Figure 4. Percentage distribution by macrocategories of differentially expressed transcripts in the intestine compartments of A. aegypti larvae. . Macrocategories: DIG – digestion; ABS – absortion; MET – metabolism; CEL – cellular processes; STR – structure; OTH – others.

304x108mm (300 x 300 DPI)

4. Discussão Geral

No presente trabalho avaliamos aspectos fisiológicos e moleculares da digestão em larvas de *Ae.aegypti* criadas em laboratório. Em um primeiro momento, identificamos seqüências de codificação para GHF16 no genoma de *Ae.aegypti*, comparamos a expressão dos genes nos tecidos larvais e avaliamos o papel fisiológico de alguns desses genes através da ferramenta RNAi. Posteriormente, foram avaliados os efeitos de uma dieta exclusiva de *S. cerevisiae* no desenvolvimento de larvas de *Ae.aegypti* e o papel da beta-1,3-glucanase intestinal no processamento dessas células. Os resultados obtidos com a investigação de parâmetros fisiológicos frente à alimentação das larvas com leveduras conflagraram o terceito trabalho desenvolvido pelo grupo e estabeleceu novos detalhes sobre a biologia nutricional em larvas de mosquito. Nesse trabalho, os efeitos de dietas larvais baseadas apenas em microrganismos foram detalhados. Além disso, com esse trabalho de tese, analisamos o transcriptoma dos diferentes compartimentos intestinais de larvas *Ae.aegypti* corroborando com a suposição de que a compartimentalização observada no trato digestório dos insetos possui base transcriocional.

A diversificação e o sucesso evolutivo dos insetos se deve em grande parte a adaptação desses animais a diferentes recursos alimentares e em alguns casos a associações com microrganismos que fornecem nutrientes epecíficos. A nutrição diz respeito às substâncias químicas requeridas por um organismo para o crescimento, desevolvimento, reprodução e obtenção da energia necessária para a manutenção dessas funções. Muitas dessas substâncias são ingeridas com o alimento, mas outras são sintetizadas pelo próprio inseto. Atingir uma nutrição ideal envolve uma interação complexa entre o comportamento alimentar e o processamento pós-ingestão dos alimentos. Larvas de *Ae.aegypti* possuem hábitos alimentares pouco seletivos, ingerindo detritos diversos, principalmente matéria orgânica particulada e microrganismos como fungos, bactérias, microalgas e protozoários (Merrit et al 1992, Forattini 2002 Clements 2000). O processamento de dietas tão heterogêneas ocorre principalmente através da digestão com a transformação química de moléculas grandes e complexas de alimentos em moléculas menores que serão absorvidas na parede intestinal para a nutrição dos insetos (Terra e Ferreira 2005). Apesar de enzimas digestivas já terem sido descritas em *Ae.aegypti* (Frank *et al.* 1952, Geering e Freyvogel 1975, Marinotti e James 1990, Souza *et al*. 2016) pouco se conhece sobre como diferentes dietas podem influenciar o desenvolvimento larval em seu habitat natural e quais são as principais enzimas envolvidas no processamento desses nutrientes. Dada a importância médica do vetor *Ae.aegypti*, compreender a biologia nutricional e a fisiologia da digestão das larvas é imprenscíndivel.

Beta-1,3-glucanases são enzimas envolvidas na hidrólise de glucanas com ligações beta-1,3-, beta-1,3-1,6, ou beta 1,3-1,4. Esses polissacarídeos são frequentes na natureza e estão presentes na parede celular de fungos, algas e plantas superiores (Bartnicki-Garcia 1968, Gorin e Spencer 1968, Wessels e Sietsma 1981, Aspinall 1982, Bacic et al 1988, Krainer et al 1994, Schomburg e Salzmann 1997). Em insetos detrítivoros, beta-1,3-glucanases atuam na digestão inicial (Terra e Ferreira 1994) e já foram descritas em diversas ordens: Diptera (*Ae.aegypti*; *Lutzomyia longipalpis*; Souza et al 2016, Moraes et al 2014) Isoptera (Lucena et al 2011), Lepidoptera (*Spodoptera frugiperda*; Bragatto *et al.* 2010), Coleoptera (Tenebrio molitor; Genta et al 2009), Hymenoptera (*Acromyrmex subterraneus*; Erthal Jr. *et al.* 2007), Orthoptera (*Abracris flavolineata*; Genta et al 2007) e Blattodea (*Periplaneta americana*, Genta et al 2003). Além do papel digestivo observado em beta-1,3-glucanases, proteínas homólogas a essas enzimas foram descritas e relacionadas a resposta imune do inseto (Bulmer e Crozier 2006; Pauchet *et al*. 2009). Essas proteínas possuem receptores capazes de reconhecer moléculas presentes na superfície de patógenos como fungos (proteínas ligantes de beta-1,3-glucanas) e bactérias (proteínas ligantes de bactérias gram negativas) disparando a resposta imune inata (Ochiai 1988, 2000).

O hábito nutricional detritívoro encontrado nas larvas de *Ae.aegypti* foi a premissa investigativa para busca no genoma por genes envolvidos na digestão de carboidratos, especificamente genes que codifiquem proteínas pertencentes a GHF16, nas quais encontram-se associadas as beta-1-3-glucanases em insetos (Genta *et al*. 2009; Bragatto *et al*. 2010) (Artigo 1). O genoma de *Ae.aegypti* através do domínio PFAM-00722 (Bateman *et al*. 2004), evidenciou seis genes com sequências que possuem propriedades compartilhadas entre os integrantes da GHF16. Esses genes apresentaram de 2 a 6 éxons e cinco das seis sequências identificadas (AeGH16.1, AeGH16.2, AeGH16.3, AeGH16.5 e AeGH16.6) apresentaram resíduos conservados de glutamato na região consenso SGE (I / V) DL (M / L) ES (R/ K). Esses resultados estão de acordo com o que foi observado por Hahn *et al*. (1995), onde resíduos conservados nessa região específica atuam como doadores e aceptores de prótons no sítio ativo de beta-1,3-glucanases. A sequencia de AeGH16.4 não apresentou resíduos conservados ou presença de peptídeo sinal, o que pode indicar que essa proteína não está sendo secretada. Os resíduos de glutamato encontrados no sítio ativo de beta-1,3-glucanases são fundamentais para a atividade catalítica dessa enzima e conservados em todas as subfamílias de glucanases (Zhang *et al.* 2003), sendo assim, é bastante plausível que tenhamos identificado cinco sequencias de genes envolvidos em mecanismos de hidrólise das ligações glicosídicas e uma sequencia gênica associada às proteínas ligantes de beta-1,3-glucanas que não possuem essa característica. Mesmo sendo proteínas homólogas, ocorre uma grande dissimilaridade entre as funções observadas nas proteínas ligantes

e nas glucanases. Alguns autores sugerem que eventos de duplicação e posterior divergência gênica possam ter ocorrido a partir de um gene ancestral de beta-1,3-glucanase (Bragatto *et al*. 2010; Hughes 2012) fazendo com que as proteínas ligantes tenham perdido sua função catalítica em algum momento nesse período, porém mantido sua capacidade de reconhecer carboidratos através do acréscimo de uma região N terminal ou um domínio de reconhecimento e ligação a moléculas de polissacarídeos como as encontradas em beta-1,3-glucanas (Zhang *et al*. 2003; Pauchet *et al*. 2009).

Compreender as sequencias de aminoácidos propicia informações sobre a função, localização celular, evolução e estrutura tridimensional das proteínas. Através de análises filogenéticas comparando as sequencias obtidas nesse trabalho com sequencias homólogas de diferentes ordens de insetos, foi possível verificar a presença de dois grandes clados diferenciados: **(1)** um clado monofilético reunindo sequencias com resíduos catalíticos genuínos (atribuídos as beta-1-3-glucanases) e **(2)** um clado parafilético onde agruparam-se as sequencias de proteínas ligantes de beta-1,3-glucanas. A construção da árvore filogenética com o algoritmo Neighbor-Joining (Tamura *et al*. 2011) revelou muitas sequencias de dípteros nematoceros agrupados em sub-ramos monofiléticos de beta-1-3-glucanases, o que nos pareceu bastante interessante. Previamente, já se havia sido proposto que os insetos devem ter pelo menos duas cópias de genes pertencentes a GHF16 (Bragatto *et al*. 2010). Bulmer e Crozier (2006), observaram que várias espécies de cupins apresentam mais de uma cópia gênica e algumas dessas cópias apresentaram resíduos catalíticos verdadeiros, indicando uma possível relação positiva entre esses genes. Quando ocorre duplicação gênica, um par de genes inicialmente iguais dentro do genoma é criado e pode vir a divergir. Com mais de uma cópia, é possível que um desses genes sofra alguma mutação e especialize-se em outras funções dentro do mesmo organismo. Nossa hipótese é que duplicações gênicas tenham ocorrido nas sequencias de Anofelinos e Culícideos ocasionando uma expansão dos genes de beta-1,3-glucanases em Dipteros nematoceros, resultando em pelo menos cinco beta-1,3-glucanases parálogas nos genomas de *Aedes, Culex* e *Anopheles*.

Algumas análises bioquímicas voltadas para caracterização dessas enzimas foram realizadas. O pH ótimo observado nos diferentes tecidos das larvas coincidiu com o que foi relatado em outros insetos (Genta *et al.* 2003; Genta *et al*. 2009; Genta *et al*. 2007 e Moraes *et al*. 2012) e sugere uma disitinção entre as beta-1,3-glucanases nos tecidos. Os dados obtidos com os cálculos de massa molecular reforçam essa teoria, uma vez que os valores de cabeça e resto do corpo diferiram dos valores encontrados no insetino. O pH ótimo encontrado no intestino das larvas foi similar ao detectado em outras beta-1,3-glucanases de insetos (Genta *et al*. 2003, 2007,

2009, Bragatto *et al*. 2010). Além da descrição dos genes e de alguns experimentos de caracterização bioquímica, abordagens moleculares foram utilizadas para se compreender melhor o papel das beta-1,3-glucanases. Todos os genes apresentaram diferentes níveis de expressão relativa nos tecidos testados (cabeça, resto do corpo e intestino). Não existem muitos trabalhos de expressão dos genes da GHF16 em larvas de mosquito, mas com relação a aspectos interligados ao desenvolvimento do mosquito em geral, é possível sugestionar que os genes existentes são bastante específicos (Harker *et al*. 2013).

Como maneira de enterdermos se as beta-1,3-glucanases encontras em larvas possuem função intestinal realizamos experimentos com RNAi. Desde a sua descoberta inical em nematódeos (Fire *et al*. 1998), o RNAi vem sendo explorado como uma ferramenta molecular importante para compreensão da função de genes em insetos (Dietzl *et al*. 2007; Suzuki et al. 2008; Tian *et al.* 2009) e como agente potencial para controle de pragas (Huvenne e Smagghe 2010; Singh *et al.* 2013). Quatros dos seis genes identificados no genoma foram utilizados para o silenciamentos nas larvas (AeGH.1, AeGH.4, AeGH.5 e AeGH.6). Nossa decisão foi baseada nos resultados de expressão relativa, onde alguns genes apresentaram representações melhores.

O silenciamento do gene 5 foi o que resultou na menor taxa de pupação e na maior taxa de mortalidade dos insetos nos experimentos fisiológicos que foram performados. Esses parâmetros alterados foram atribuídos a um possível comprometimento das funções digestivas, uma vez que o desenvolvimento das larvas foi afetado. A investigação da função imune dos genes não foi explorada frente ao desafio com patógenos invasores nesse trabalho, pois esse não era o objetivo central. Ainda assim, mantivemos as larvas em condições de estresse nutricional e alta densidade durante cinco dias após o silenciamento e observamos alguns efeitos. Todas as larvas silenciadas para os genes AeGH.1 e AeGH.6 morreram, as larvas dos genes AeGH.5 também apresentaram um número elevado de mortes, mas em taxas semelhantes aos observados no controle com dsGFP. Nossa hipótese é que os genes AeGH1 e AeGH6 exerçam algum tipo de função imune nas larvas e situações de estresse e densidade populacional elevadas possam ter desafiado o sistema imune do inseto e ocaiosnado a sua morte. Telang *et al*. (2012) já havia demonstrado que o estresse nutricional em larvas de mosquito pode ocasionar adultos com imunodeficiência.

Apenas os genes AeGH.4, AeGH.5 e AeGH.6 apresentaram níveis de silenciamento efetivos, o gene AeGH.4 foi silenciado na larva inteira, AeGH.5 apresentou níveis de silenciamento significativos no intestino e AeGH.6 no resto do corpo. O gene AeGH.1 não apresentou níveis de silenciamento a nível de transcritos compatíveis, sugerindo que para esse gene não foi possível obter o silenciamento dentro das células, mesmo sem um silenciamento

significativo, conseguimos detectar importantes fenótipos nas larvas tratadas com dsRNA para AeGH1. Apesar de AeGH.6 aparentar ser relativamente mais expresso no intestino, o seu silenciamento foi verificado apenas no restante do corpo, uma explicação plausível para esse fenômeno seria que sua localização nas células esteja em tecidos limítrofes entre o intestino e o restante do corpo, como por exemplo, no corpo gorduroso, tecido que envolve os demais órgãos do inseto ou tenha se propagado para outros tecidos como já foi observado em outras investigações (Zangh *et al*. 2010; Singh *et al*. 2013).

A possível presença do gene AeGH.6 no corpo gorduroso é uma teoria interessante quando relacionamos o corpo gorduroso a sua importância como órgão ativamente imune em insetos, o que pode explicar o fenótipo observado sob condições de estresse nutricional. Estudos para verficar como ocorre a internalização do dsRNA em larvas de mosquito podem auxiliar na compreensão da dinâmica do silenciamento nesses insetos. Em outras espécies, sugere-se que mecanismos de internalização do dsRNA possam ocorrer de célula a célula através de proteínas transmembranar ou através de componentes envolvidos nas vias de receptores da endocitose (Winston *et al.* 2002; Feinberg e Hunter 2003; Ulvila *et al*. 2006; Saleh *et al.* 2006; Jose e Hunter 2007). Um outro fator que deve ser considerado nesse trabalho, foi a técnica de silenciamento utilizada. Na técnica de "*soaking*" as larvas são mergulhadas em uma solução com dsRNA por apenas duas horas, podendo não ter atingido todo potencial de silenciamento. Esse tipo de técnica é menos invasiva para o inseto e de fácil execução, no entanto é mais utilizada para experimentos com cultura de células.

Por fim, performamos ensaios enzimáticos dos genes silenciados AeGH.5 e AeGH.6, a fim de se observar se a função digestiva de beta-1,3-glucanases seria depletada em alguns dos tecidos verificados (intestino e resto do corpo). Larvas silenciadas com o gene AeGH.5 apresentaram atividades de beta-1,3-glucanses menores do que as observadas no intestino das larvas silenciadas para o gene AeGH.6 e os demais grupos controles (água e dsGFP). O mesmo padrão se repetiu nos experimentos de filtração em gel, onde as atividades referentes a atuação de beta-1,3-glucanses foi reduzida no intestino das larvas silenciadas para o gene AeGH.5. A atividade beta-1,3-glucanases não foi alterada no gene AeGH.6 em nenhum dos tecidos verificados e em nenhuma das técnicas utilizadas. Esses dados sugerem que larvas de *Ae.aegypti* possuem ao menos uma beta-1,3-glucase intestinal majoritária, com relação as demais funções imunes desses genes seriam necessários experimentos mais detalhados. De toda forma, os achados desse estudo contribuem para a compreensão da fisiologia digestiva e de aspectos evolutivos das larvas de *Culicidae*.

Corroborando com os dados moleculares e bioquímicos obtidos no Artigo 1, decidimos investigar os efeitos de uma dieta exclusiva de *S.cerevisiae* no desenvolvimento de larvas de *Ae.aegypti* e nas atividades de beta-1,3-glucanases. Nesse trabalho, experimentos bioquímicos e fisiológicos foram realizados e a compilação dos dados obtidos nesse estudo, revelaram que esta enzima é crucial para a atividade lítica desse microrganismo na larva (Artigo 2). Embora, larvas de culicídeos sejam bem generalistas no que se refere aos hábitos alimentares e precisem lidar com extremas variações na composição microbiona dos locais de criação, em determinadas ocasiões é difícil distinguir a ingestão passiva da seleção ativa dos alimentos. Leveduras já haviam sido utilizadas como fonte nutricional na dieta de larvas de *Ae.aegypti* (Trager 1935b; Golberg and Lavoipierre 1945) e outros trabalhos usando besouros e baratas como modelos experimentais, demonstraram que beta-1,3-glucanases são capazes de digerir células de leveduras (Genta *et al.* 2003; 2009). Primeiramente, avaliamos se larvas de *Ae.aegypti* são capazes de sobreviver em uma dieta exclusiva de leveduras vivas. Apesar de alguns trabalhos já terem utilizado levedura como suplemento alimentar na dieta de larvas, essa levedura estava liofilizada e suspensas em cloreto de cálcio (Trager 1935b). Até a publicação desse trabalho, não existiam evidências sobre a influência dessa dieta nos parâmetros de desenvolvimento de *Ae.aegypti*. Os resultados obtidos demonstraram que as larvas conseguem se desenvolver completamente até o surgimento dos adultos em dietas de leveduras vivas. Além disso, parâmetros nutricionais como os valores de proteína e açúcares totais indicaram que dietas com *S.cerevisiae* possuem dois dos macronutrientes indispensáveis para o pleno desenvolvimento do mosquito (Singh e Brown 1957). Além de testar leveduras vivas, realizamos ensaios fisiológicos comparativos com leveduras autoclavadas. O surgimento de pupas tanto em dietas com leveduras vivas quando em dietas com leveduras autoclavadas apresentou um atraso de dois dias em relação ao grupo controle (ração de gato), por outro lado, um percentual maior de adultos consegue emergir em dietas contendo leveduras vivas (80%) quando comparados as dietas contendo leveduras autoclavadas (58%). Como foi dito anteriormente, leveduras liofilizadas já haviam sido usadas como dieta em larvas, logo o desenvolvimento das larvas nessa dieta apenas corroborou com os dados encontrados na literatura. Outro estudo, dessa vez envolvendo larvas de *Culex pipens* também demonstrou que as larvas são capazes de se nutrir e desenvolver utilizando *S.cerevisiae* e várias outras espécies de leveduras (Steyn *et al*. 2016). Os dados obtidos para *Ae.aegypti* e *Cx.pipens* podem sugerir que a relação nutricional entre larvas de mosquitos e leveduras pode ser compartilhada entre os culicídeos. A contagem de leveduras após resusspensão na água revelou que as células permanecem viáveis mesmo após 24 horas de ressuspensão o que sugere que as larvas se alimentaram de células vivas. A marcação das células de levedura presentes no intestino

das larvas de quarto estádio com trypan blue indicaram que larvas de *Ae.aegypti* ingerem quantidades significativas de leveduras com duas horas de exposição a dieta, nas próximas 48 horas de exposição o número de leveduras contabilizadas no intestino das larvas descresce, indicando que essas células estão sendo processadas e absorvidas pelo inseto.

Trabalhos usando besouros e baratas como modelos experimentais, já haviam demonstrado que beta-1,3-glucanases são capazes de digerir células de leveduras (Genta *et al.* 2003; 2009). Uma vez que observamos que dietas com leveduras são favoráveis ao desenvolvimento das larvas do inseto, decidimos pesquisar se larvas de *Ae.aegypti* alimentadas com células vivas ou autoclavadas de *S.cerevisiae* seriam capazes de induzir uma atividade diferenciada em beta-1,3-glucanases. Atividades de beta-1,3-glucanases foram detectadas na cabeça, intestino e resto do corpo de larvas de *Ae.aegypti* e apresentaram poucas diferenciações quando comparamos dietas com células vivas x células autoclavadas e frações solúveis x frações insolúveis dos tecidos. A diferenciação entre os tecidos e tratamentos na performance do experimento se deu devido a discrepância dos papéis fisiológicos encontrados em beta-1,3-glucanases (Artigo 1). Essa dualidade parece mais evidente quando observamos a distribuição desigual das atividades enzimáticas entre os tecidos (Artigo 2). Beta-1,3-glucanases apresentaram atividades constitutivas no intestino das larvas em todos os tratamentos utilizados (células vivas x células autoclavadas e frações solúveis x frações insolúveis dos tecidos), o que sugere que fungos e plantas possam ser regularmente ingeridos por esses insetos, uma vez que essas enzimas são capazes de hidrolisar esses substratos. Valores de atividade enzimática basais no intestino coincidem com outros estudos de fisiologia intestinal em insetos (Terra e Ferreira 1994; 2005). As atividades de beta-1,3-glucanases no intestino das larvas podem estar sendo complementadas ou coordenadas por outras enzimas, como as quitinases intestinais que foram caracterizadas em mosquitos, essas enzimas estão supostamente envolvidas na digestão de fungos e outras partículas recobertas de quitina (Souza-Neto *et al*. 2003). As atividades detectadas na cabeça das larvas apresentaram atividades menores nas frações solúveis e insolúveis em larvas alimentadas com dietas contendo *S.cerevisiae* autoclavadas. Esse resultado foi atribuído a possíveis funções imunes desempemhadas pelas glândulas salivares que são encontradas na cabeça dos insetos. Alguns autores consideram que as glândulas salivares não somente atuam na digestão como exercem um importante papel na imunidade do inseto. Um padrão semelhante foi observado em beta-1,3-glucanases localizadas nas glândulas salivares do cupim *Naustitermes.corniger*. Nesse trabalho, os autores relacionaram essas atividades à defesa antimicrobiana nesses insetos (Bulmer *et al*. 2009). Além disso, genes de lisozima envolvidos na resposta antibacteriana tiveram sua expressão modulada nas glândulas salivares e no intestino médio de *Drosophila* sp. reforçando a hipótese

de que esses tecidos possam ser ativos imunologicamente quando desafiados com bactérias (Kylsten et al. 1992; Daffre *et al*. 1994). A inoculação de bactérias diretamente na hemocele do inseto reduz drasticamente a expressão desses transcritos de defesa (Kylsten *et al.* 1992). A queda da atividade das beta-1,3-glucanases na cabeça de larvas de *Ae.aegypti* coincidiram com outros trabalhos descritos na literatura sugerindo fortemente que essas enzimas sejam sensíveis a exposição de possíveis agentes infecciosos que possam estar associados ao alimento e assim, exerçam funções importantes tanto na digestão de leveduras, quanto no combate aos patógenos.

O resto do corpo apresentou as maiores atividades enzimáticas e não foram moduladas pelos diferentes tratamentos (células de leveduras vivas x células de leveduras autoclavadas e frações solúveis x frações insolúveis dos tecidos). As elevadas atividades encontradas no resto do corpo também foram atribuídas a funções imunes nas larvas, já que nenhum processo relacionado a digestão ou metabolismo de carboidratos ocorre nesse tecido. A caracterização da atividade de beta-1,3-glucanase de larvas de *Ae.aegypti* nos levou a investigar a relevância dessas enzimas na lise das leveduras ingeridas pelo inseto. Nós já havíamos observado que as células de levedura são estáveis sob as condições usadas no ensaio e que as mesmas eram processadas no intestino dos insetos, no entanto, nós não havíamos testado a atividade lítica dessas enzimas incubadas com leveduras e seu próprio substrato comercial (*laminarina digitata*). Os ensaios de competição resultaram em uma perda rápida de viabilidade quando os intestinos das larvas eram incubados com as células de leveduras vivas e em uma posterior prevenção desse efeito quando o intestino das larvas era incubado com células vivas acrescidas de *laminarina digitata*. Esses resultados demonstram que beta-1,3-glucanases lisam células de levedura e podem ser essenciais para digestão de uma dieta contendo fungos em larvas de mosquito. Com a fragmentação da parede celular fúngica as larvas podem acessar os macronutrientes intracelulares desse microrganismo e estocar energia suficiente para desenvolver-se normalmente. As conclusões obtidas nesse trabalho foram determinantes para o progresso dessa linha investigativa. Estudos envolvendo a biologia nutricional de larvas de mosquito são extremamente necessários para se compreender como ocorre a dinâmica da digestão e assim, revelar aspectos relacionados a manutenção da vida dessas espécies na natureza.

O efeito das variações da disponibilidade de alimentos na natureza na história de vida dos seres vivos é uma questão central da ecologia evolutiva. O tema central do terceiro artigo buscava investigar como aspectos nutricionais podem afetar o desenvolvimento de larvas de *Ae.aegypti* e assim, responder algumas questões sumárias, porém primordiais para um melhor entendimento da biologia do vetor. Decididos a buscar um conhecimento mais detalhado sobre como a biologia nutricional pode influenciar na fisiologia do desenvolvimento em larvas de *Ae.aegypti*, avaliamos

o status nutricional das larvas sob diferentes dietas microbianas. Estudos nutricionais voltados para a averiguação da ingestão de células microbianas vivas e sua dinâmica ao longo do tempo é algo inédito no âmbito da biologia descritiva em larvas de *Ae.aegypti*. Os diferentes gêneros testados incluíram espécies de leveduras (*S. cerevisiae* e *Pseudozyma* sp.), bactérias gram positivas (*S. aureus* e *Bacillus* sp.), bactérias gram negativas (*S. marscescens*, *E. coli*, *Ochrobactrum* sp. e *Asaia* sp.), algas filamentosas (*A. platensis*) e algas unicelulares *(Chlorella* sp.). Parâmetros de vida, como tempo de desenvolvimento, taxa de pupação, emergência, tamanho dos adultos, qualidade nutricional das dietas e reserva energética das larvas foram avaliados.

Análises mais detalhadas utilizando *S. cerevisiae* e *Pseudozyma* sp. como única fonte alimentar, resultaram em larvas com parâmetros de vida similares aos observados em larvas criadas com a dieta padrão Tetramin® e adultos um pouco menores. Bactérias são consideradas os mais importantes microrganismos presentes na dieta larval e o desevolvimento dos mosquitos também pode ocorrer apenas com esses organismos como fonte nutricional (Hinman 1932, Rozeboom 1935, Laird 1956;1988, Christophers 1960). Os resultados observados nesse trabalho corroboram com os dados encontrados na literatura e larvas de *Ae.aegypti* conseguem se desenvolver até a fase adulta, mas com taxas de desenvolvimento mais lentas, revelando atrasos severos e uma sobrevivência consideravelmente menor. No ambiente natural, a biomassa das algas parece ser o componente mais abundante e constituem a maior parte do conteúdo intestinal de larvas de *Ae.aegypti* (Hinman 1930, Garros et al 2008). Sendo assim, parece que as algas exercem um importante papel no desenvolvimento e na sobrevivência larval. Embora algumas espécies de algas pareçam ser resistentes à digestão e sejam descartadas integralmente do intestino das larvas (Laird 1988), não existem indícios de que *Chlorella* sp. e *A. platensis* pertençam a esse grupo específico. Larvas alimentadas com microalgas também apresentaram atrasos no desenvolvimento e adultos com baixa sobrevivência.

Durante o desenvolvimento larval as variações do conteúdo alimentar podem afetar o tamanho dos adultos, sua reserva energética, produção de ovos, longevidade das fêmeas adultas, imunidade e capacidade vetorial (Stearns e Koella 1986, Juliano e Stoffregen 1994, Barrera 1996, Juliano 1998, Daugherty et al 2000, Braks et al 2004, Alto et al 2005, 2008ª, Murrell e Juliano 2008, Reiskind e Lounibos 2009, Telang et al 2007, Muturi et al 2011). Os efeitos do estresse nutricional no desenvolvimento de patógenos em mosquitos já foi discutida em diversos trabalhos. Muturri *et al.* (2011) reafirmaram que o estresse nutricional durante o desenvolvimento larval pode causar alterações no fenótipo e na imunidade dos mosquitos e ainda acrescentaram traços preocupantes, como o aumento da susceptibilidade desses adultos a patógenos. Mosquitos bem nutridos durante a fase larval possuem reservas nutricionais maiores e isso pode induzir um

comportamento alimentar diferenciado durante a fase adulta (Naksathit *et al*. 1999). Adultos que emergem de larvas com baixa reserva nutricional são menores (Lehmann *et al*. 2006) e as fêmeas requerem mais repastos sanguíneos para produção de ovos (Briegel 1990). Larvas de mosquitos que sobrevivem a ambientes nutricionais restritos podem estender seu tempo de desenvolvimento na fase adulta e tornarem-se infecciosos por mais tempo (Vantaux *et al*. 2016), modulando a sua microbiota (Linenberg *et al*. 2016) e a permissividade aos parasitas (Linenberg *et al*. 2016; Takken *et al*. 2013), afetando assim, características imunulógicas (Suwanchaichinda e Paskewitz 1998; Telang *et al*. 2012). Shapiro *et al*. (2016) apresentaram uma vertente diferente das que foram observadas nos trabalhos citados acima. Nesse estudo eles observaram que o potencial de *Anopheles stephensi* na transmissão de *Plasmodium falciparum* foi melhorado em larvas bem alimentadas aumentando a taxa de infecção dessas fêmeas entre 260-330%. Investigações relacionadas à compreensão da ecologia do vetor abordando aspectos fisiológicos básicos para manutenção da vida do inseto, como alterações na disponibilidade ou natureza dos alimentos, podem proporcionar um melhor entendimento sobre como ocorre a dinâmica das doenças transmitidas por vetores. Larvas alimentadas com *E.coli* e *Asaia* sp. apresentaram taxas de desenvolvimento atrasadas, mas uma sobrevivência maior, mesmo sendo criadas em condições nutricionais aparentemente desfavoráveis. Esses resultados são extremamente importantes quando pensamos que larvas de mosquito provavelmente não possuem uma disponibilidade adequada de alimentos na natureza e ainda assim, conseguem se desenvolver e dispersar arboviroses. Sendo assim, a qualidade nutricional das larvas em dietas com microrganismos não apenas pode afetar todo o histórico fisiológico de vida dos insetos, como também alterar sua imunidade e capacidade vetorial. Além de apresentarem uma sobrevida maior dietas microbianas contendo, *E.coli* e *Asaia* sp. apresentaram adultos (machos e fêmeas) menores em comparação a dieta padrão. Os resultados obtidos podem indicar que as dietas estão interferindo diretamente no tamanho e na forma dos adultos. Resultados similares foram observados em outros estudos onde larvas criadas em dietas pouco nutritivas tornaram-se adultos menores e com baixa reserva energética (Zeller e Koella 2016).

Larvas de mosquito que dispõem de nutrientes suficientes durante o seu desenvolvimento aquático suprem todas as necessidades energéticas necessárias para a metamorfose. A capacidade metamórfica das larvas de *Ae.aegypti* depende da obtenção de uma massa crítica mínima, algo estimado entre 2,7 a 3,2 mg. Essa massa crítica pode ser alcançada 24 h após a transformação das larvas em quarto ínstar final (Telang *et al*. 2007). Em *Ae.aegypti* a massa crítica é definida como a massa com a qual 50% das larvas pupam em condições de restrição alimentar (Chambers e Klowden 1990). Carboidratos e proteínas estão entre os principais componentes nutricionais da

dieta larval de *Ae.aegypti* (Singh e Brown 1957). Cada elemento nutricional pode afetar traços específicos do inseto. Carboidratos, por exemplo, estão diretamente associados à pupação (Chambers e Klowden 1990, Telang *et al*. 2007). Já as proteínas são fundamentais para o surgimento de adultos (Golberg e De Meillon, 1948b). Dietas contendo microalgas e leveduras possuem quantidades maiores de proteínas e carboidratos, enquanto dietas contendo bactérias apresentaram valores bem menores de ambos os componentes energéticos. Insetos alimentados exclusivamente com leveduras são capazes de estocar proteínas, carboidratos, glicogênio e lipídeos suficientes para um desenvolvimento normal (levando em média de 7 a 14 dias para emergência de adultos), enquanto insetos alimentados com bactérias não apresentam valores energéticos cumulativos suficientes para se atingir a massa crítica mínima necessária para a pupação no período de tempo padrão. Apesar das microalgas apresentarem valores de proteínas e açúcares robustos, larvas de *Ae.aegypti* parecem ter dificuldades para processar esses alimentos de forma eficiente, apresentando taxas de desenvolvimento atrasadas e baixo acúmulo energético. Existe uma correlação positiva entre a massa corporal e as reservas calóricas adquiridas durante a fase larval, e um acúmulo energético adequado pode promover a pupação (Chambers e Klowden 1990). Alguns trabalhos já haviam demonstrado que larvas criadas em dietas restritas podem estar associadas a um tempo de vida prolongado em *Ae.aegypti* (Joy et al 2010, Zeller e Koella 2016). A capacidade de resistir a situações adversas pode justificar a grande ocorrência desses insetos na natureza. No ambiente é comum encontrar mosquitos com tamanhos reduzidos e baixas reservas energéticas que possivelmente conseguiram se estebelecer mesmo encontrando situações pouco favoráveis (Zeller e Koella 2016).

O aprofundamento dos estudos da fisiologia intestinal de larvas de *Ae.aegypti* pode ajudar a sugerir quais seriam efetivamente os alimentos desses insetos na natureza, como ocorre sua digestão e como a dieta pode influenciar fisiologicamente e molecularmente o desenvolvimento do inseto em condições laboratoriais. Além disso, estudos sobre o sistema digestivo de larvas são potencialmente estratégicos ao considerarmos que uma compreensão mais refinada da biologia do vetor pode auxiliar no surgimento de novas formas de inibição desse vetor.

Com a facilidade de acesso aos bancos de dados com informações genéticas sobre as diferentes espécies de microrganismos existentes, a identificação de genes e transcritos importantes para a fisiologia dos insetos passou a ser realizada em um fluxo cada vez maior. Um dos nossos objetivos nesse trabalho foi buscar informações mais detalhadas sobre como ocorre a compartimentalização do sistema digestivo de larvas de Ae.aegypti. A disponibilidade do sequenciamento do genoma de *Ae.aegypti* (Nene *et al*. 2007), juntamente com a acessibilidade a sequenciamentos de nova geração com custos menos onerosos, proporcionou que investigações

do padrão de expressão global dos diferentes compartimentos do intestino médio das larvas de *Ae.aegypti* fossem realizadas (Artigo 4).

O intestino dos insetos é uma estrutura subdividida em compartimentos e possui uma das maiores áreas de contato do organismo com o meio exterior, sendo o alvo mais suscetível a agentes de controle (Genta 2004; Hakin *et al*. 2010; Bragatto 2010). Nosso grupo de pesquisa vem investigando quais seriam as enzimas responsáveis pela digestão em larvas de *Ae.aegypti* ao longo dos últimos anos, com a identificação e caracterização dos transcritos envolvidos na digestão das larvas esses estudos seriam otimizados e inúmeros mecanismos e moléculas relacionadas à digestão seriam finalmente revelados.

Como em outras áreas do conhecimento, os estudos da expressão gênica em larvas de *A. aegypti* são bastante escassos (Vogel *et al*. 2016). No artigo 4 apresentamos o perfil transcriptômico dos cecos gástricos (GC), intestino médio anterior (AM), intestino médio posterior (PM) e intestino posterior (HG) de larvas de *Ae.aegypti*. Para este propósito, utilizamos uma plataforma 454 da Roche, composta de pirose e identificamos o padrão global dos transcritos diferencialmente expressos em cada compartimento intestinal larval, buscando associar os potenciais papéis fisiológicos desses compartimentos aos níveis de transcritos correspondentes através de anotações manuais. Três bibliotecas de cDNA foram sequenciadas para compartimentos intestinais específicos (AM, PM e HG), resultando em um número de reads variando entre 163 mil e 185 mil (Tabela 1). Além disso, 230 mil reads foram obtidos da biblioteca de tubo digestivo sem os cecos gástricos (GC), e 125871 mil reads foram obtidos da biblioteca correspondente ao tubo digestivo completo (TD). De acordo com as nossas comparações obtivemos 235, 69,11 e 23 genes DE para TD-CG, AM, PM e HG respectivamente. Dos 338 genes DE apenas 16 genes apresentaram uma sobreposição discreta entre os compartimentos e similar ao que já foi observado no trato intestinal de outros insetos não ocorreram sobreposições entre TD-CG e PM e HG. Esses resultados foram apresentados na forma de diagrama de Venn, facilitando a observação de sobreposição entre os genes. A reduzida proporção de genes diferencialmente expressos em cada compartimento pode ser atribuída ao tipo de técnica utilizada nesse estudo, à pouca diferenciação funcional entre os compartimentos. Mais investigações devem ser realizadas para testar essas hipóteses.

As análises das sequencias por bioinformática apresentaram particularidades sobre o conjunto de proteínas DE nas diferentes bibliotecas sequenciadas. A identificação de homólogos, domínios conservados e diversos parâmetros moleculares indicaram que essas proteínas possuem características próprias e diferentes endereçamentos celulares o que pode sugerir uma diferenciação individual em cada compartimento observado. Após a análise bioinformática das sequências, anotações dos genes DE foram performadas manualmente. As anotações permitiram

que os genes fossem categorizados e customizados de acordo com as características funcionais específicas relacionadas à fisiologia intestinal em insetos. Foram criadas 30 macrocategorias (com uma categorização mais pormenorizada) e 6 microcategorias (com uma categorização setorizada e condensada). Observamos que cada compartimento apresenta uma distribuição característica de genes DE, o que corrobora o pressuposto de que a compartimentalização fisiológica do intestino de larvas de *A. aegypti* têm base transcricional. O mesmo fenômeno foi descrito por Oviedo *et al*. (2007) utilizando a ferramenta de microarray. No trabalho de Oviedo *et al*. (2007) números bem menores de genes DE foram identificados, possivelmente devido ao tipo de técnica utilizada pelos autores. No geral, o padrão de predominância das possíveis funções dos genes DE estão de acordo com o que já foi descrito na literatura onde os compartimentos intestinais dos insetos estão fisiologicamente relacionados (Terra e Ferreira 2005). Observamos nos GC uma predominância de processos celulares (CEL), no AM observamos uma predominância da macrocategoria DIG, composta principalmente por enzimas digestivas. No PM, verificamos uma predominância conjunta das categorias de absorção (ABS) e processos celulares (CEL), e HG observamos a predominância da categoria MET, de metabolismo celular. Reunidos, esses dados são coerentes com a diferenciação funcional conhecida de cada compartimento, podendo sugerir uma especialização nos compartimentos anteriores indicando que os mesmos sejam responsáveis pela secreção de enzimas, com a maior parte da absorção ocorrendo no PM. apesar de não secretar enzimas ou absorver macronutrientes, o HG parece ser metabolicamente ativo, o que pode estar relacionado ao seu papel como órgão excretor.

Os dados obtidos nessse artigo sugerem que a compartimentalização intestinal em larvas de *Ae.aegypti* possuam base transcricional, no entanto, muitas outras análises precisam ser realizadas para se detalhar o nível de diferenciação entre os locais de digestão e absorção de nutrientes. Venancio *et al*. (2009) analisaram dados de sequenciamento de larvas inteiras e obseravaram a presença de genes larvais específicos, sugerindo que as diferenciações observadas entre as diferentes fases do inseto são finamente reguladas a nível molecular. Demais trabalhos como Tetreau *et al*. (2012) analisaram a expressão de genes no intestino de larvas de *Ae.aegypti* desafiadas com Bti, Harker *et al*. (2013) investigaram a expressão gênica em *Ae.aegypti* comparando diferentes estágios de desenvolvimento dos insetos (larvas, pupas e adultos) e mais recentemente, Vogel *et al* (2017) compararam a expressão gênica de larvas axênicas com larvas gnotobióticas (associadas a *E. coli*) com larvas criadas convencionalmente. Pesquisas envolvendo a expressão gênica de larvas de mosquito são fundamentais para melhorar o entendimento da dinâmica de importantes processos fisiológicos no vetor, sendo assim, para que comparações mais detalhadas entre as informações de expressão gênica em larvas de mosquito presentes na literatura

e os resultados obtidos nesse estudo sejam realizadas, torna-se necessário mapear a expressão individual de cada um dos genes DE selecionados. A perspectiva de mais análises proporciona novos horizontes para pesquisas futuras.

5. Conclusões

Larvas de *Ae.aegypti* possuem genes que codificam proteínas da GHF 16 e esses genes parecem ter sofrido duplicação em Dipteros nematoceros. Beta-1,3-glucanases são enzimas importantes para digestão do inseto e estão diretamente relacionadas a lise de polissacarídeos presentes nas paredes celulares de leveduras, pondendo estar envolvida também na defesa do inseto contra patógenos. Dietas larvais baseada em microrganismos como bactérias, microalgas e fungos afetam diversos parâmetros de desenvolvimento desses insetos o que pode sugerir como larvas de *Ae.aegypti* conseguem se desenvolver na natureza sob condições desfavoráveis. O sequenciamento intestinal das larvas propõe que a compartimentalização observada no trato intestinal dos insetos possui base transcrional.

6. Perspectivas Futuras

- Explorar mais detalhadamente a linha investigativa voltada a biologia nutricional das larvas, observando aspectos fisiológicos e moleculares do sistema imunológico desses insetos;

- Caracterizar a dinâmica da digestão dos microrganismos oferecidos e as enzimas digestivas envolvidas nesse processo;

- Identificar enzimas no transcriptoma intestinal das larvas que possam ser testadas como potenciais alvos de controle do vetor;

- Sequenciar o intestino das larvas alimentadas com microrganismos e observar se ocorrem modificações a nível transcricional no intestino das larvas ao longo da digestão.

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