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LUCIANA OLIVEIRA OLIVA

ANÁLISE DA QUIESCÊNCIA EM *Aedes aegypti* (DIPTERA: CULICIDAE) E INFLUÊNCIA SOBRE PARÂMETROS BIOLÓGICOS

Recife
2018

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Tese apresentada ao Programa de Pós-Graduação em Biologia Animal da Universidade Federal de Pernambuco, como requisito parcial para a obtenção do título de doutora em Biologia Animal.

Área de Concentração: Zoologia Aplicada

Orientadora: Profa. Dra. Cleide Maria Ribeiro de Albuquerque

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A Deus, pois Dele, por Ele e para Ele são todas as coisas;

E à minha família,

Com imenso amor e gratidão, dedico.

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"Ainda que eu tenha o dom de profecia e saiba todos os mistérios e todo o conhecimento,
e tenha uma fé capaz de mover montanhas, mas não tiver amor, nada serei".
(BÍBLIA SAGRADA, 1 Coríntios 13:2)

RESUMO

Em resposta a condições ambientais desfavoráveis, ovos de *Aedes aegypti* (L.) podem entrar no estado de quiescência, característica que favorece a dispersão do mosquito e reinfestação de áreas tratadas, dificultando as medidas de controle. Intrinsecamente associada a esse processo está a formação da cutícula serosa (CS), camada que envolve e protege o embrião, particularmente contra a dessecação. No entanto, o impacto da quiescência sobre parâmetros biológicos associados à dinâmica populacional e capacidade vetorial, bem como o papel da CS na proteção do embrião contra a ação de larvicidas usados pelos programas de controle, ainda são pouco conhecidos. Nesse trabalho, comparou-se a viabilidade, tempo inicial de eclosão, desenvolvimento pós embrionário, taxa de emergência, proporção sexual, tamanho do adulto, fecundidade e fertilidade a partir de ovos quiescentes (10, 40, 70, 100, 130 e 160 dias) de duas populações de *Ae. aegypti* (Recife e Aracaju). Investigou-se também se o contato de ovos quiescentes (10, 40 e 70 dias) com o Sumilarv® 0,5 G (0,01 mg de pyriproxyfen / L), antes (2 h) e após a formação da CS (24 h), poderia afetar a sua viabilidade. Finalmente, foi determinado o perfil da expressão do gene *quitina sintase* (*AaCHS1a*) durante o desenvolvimento da CS, em ovos com idades entre 6-9, 11-13 e 15-18h após a oviposição, usando PCR quantitativa em tempo real. Resultados obtidos mostraram que a quiescência promoveu um maior custo energético para população de Aracaju (viabilidade máxima de 100 dias) do que em Recife (130 dias). A razão sexual foi alterada com maior produção de machos nas duas populações. Proporcionalmente, na população de Recife, a taxa de emergência foi mais elevada em ovos mais velhos comparada a Aracaju. Tempo inicial de eclosão, tamanho do adulto, fecundidade e fertilidade não foram afetados pela quiescência nas duas populações de mosquitos. Foram registradas diferenças interpopulacionais quanto à sensibilidade à ação do pyriproxyfen, com maiores reduções nas taxas de eclosão em ovos quiescentes na população de Aracaju. Nenhuma diferença significativa na viabilidade dos ovos foi registrada em função da ausência ou presença da CS. Perfis da expressão para *AaCHS1a* foram similares para Recife e Aracaju, com quantidade elevada de transcritos entre 15-18h. Esses dados indicam um custo diferencial da quiescência e do pyriproxyfen em populações distintas de *Ae. aegypti* e que esse efeito se mostrou independente da presença da CS.

Palavras-chave: Cutícula serosa. Dessecação. *Fitness.* IGR. Pyriproxyfen.

ABSTRACT

In response to unfavorable environmental conditions, *Aedes aegypti* (L.) eggs can enter the state of quiescence, a feature that favors mosquito dispersal and reinfestation of treated areas, making control measures difficult. Intrinsically associated with this process is the serosal cuticle formation (SC), a layer that surrounds and protects the embryo, particularly against desiccation. However, the impact of quiescence on biological parameters associated with population dynamics and vectorial capacity, as well as the role of SC in protecting the embryo against the action of larvicides used by control programs, are still little known. In this work, we compared the viability, initial hatching time, post-embryonic development, emergence rate, sexual proportion, adult size, fecundity and fertility from quiescent eggs (10, 40, 70, 100, 130 and 160 days) of two populations of *Ae. aegypti* (Recife and Aracaju). It was also investigated whether the contact of quiescent eggs (10, 40 and 70 days) with Sumilarv® 0.5 G (0.01 mg of pyriproxyfen / L), before (2 h) and after (24 h) SC formation, could affect their viability. Finally, the expression profile of the chitin synthase gene (*AaCHS1a*) was determined during the development of SC in eggs aged 6-9, 11-13 and 15-18h after oviposition, using quantitative real-time PCR. Results obtained showed that the quiescence promoted a higher energy cost for Aracaju population (maximum viability of 100 days) than in Recife (130 days). The sex proportion was altered with higher males production in both populations. Proportionally, in the population of Recife, the emergency rate was higher in older eggs compared to Aracaju. Initial hatching time, adult size, fecundity and fertility were not affected by quiescence in the two mosquito populations. Interpopulation differences were recorded regarding the sensitivity to the action of pyriproxyfen, with greater reductions in hatching rates in quiescent eggs in the Aracaju population. No significant difference in egg viability was recorded due to absence or presence of SC. Expression profiles for *AaCHS1a* were similar for Recife and Aracaju, with high transcripts between 15-18h. These data indicate a differential cost of quiescence and pyriproxyfen in different populations of *Ae. aegypti* and that this effect was independent of SC presence.

Keywords: Serosal cuticle. Desiccation. Fitness. IGR. Pyriproxyfen.

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1 INTRODUÇÃO

1.1 O PROCESSO DE QUIESCÊNCIA EM MOSQUITOS

O termo quiescência tem sido aplicado a diversas formas de dormência em animais e plantas, sendo considerado um processo comum na natureza (CONSIDINE; CONSIDINE, 2016; DINIZ et al., 2017; RUMMAN; DHAWAN; KASSEM, 2015). Ocorrendo sob várias formas, cada uma delas com um complexo mecanismo associado, esse processo pode ser estimulado em diferentes momentos na vida de um mesmo indivíduo a fim de alcançar condições favoráveis para a sua sobrevivência e desempenho biológico (*fitness*) (O'FARRELL, 2011).

Em mosquitos, o processo de quiescência tem sido registrado, principalmente, na fase de ovo (CHRISTOPHERS, 1960; CLEMENTS, 1992; REZENDE et al., 2008; SILVA; SILVA, 1999; VINOGRADOVA, 2007), podendo também ser denominado "*egg desiccation resistance* (EDR)", tomando-se como base o fato de que a capacidade de resistir à dessecação é uma propriedade do ovo e não especificamente do embrião (FARNESI et al., 2015). Independente da denominação usada, o processo de quiescência é induzido por condições ambientais desfavoráveis, tais como baixa umidade e altas temperaturas (CHRISTOPHERS, 1960; CLEMENTS, 1992; JULIANO et al., 2002; VINOGRADOVA, 2007). Ao receber os estímulos negativos do ambiente, o embrião já formado no ovo (também chamado de larva farata) reduz drasticamente o metabolismo, bloqueando a eclosão larval (MARTINS; LEWINSOHN; BARBEIROS, 2000; PEREZ; NORIEGA, 2013). Neste caso, a inibição da eclosão se dá de forma temporária, sendo imediatamente reversível após o retorno de condições favoráveis à sobrevivência da larva (GORDON; HEADRICK, 2001; MARTINS; LEWINSOHN; BARBEIROS, 2000; POELCHAU et al., 2013; VINOGRADOVA, 2007).

O efeito do tempo de quiescência sobre a viabilidade do embrião tem se mostrado variável entre espécies e populações da mesma espécie (DINIZ et al., 2015; FARNESI et al., 2015; FAULL; WILLIAMS, 2015; OLIVA et al., 2018; REZENDE et al., 2008; SILVA; SILVA, 1999; SOTA; MOGI, 1992), bem como entre ovos produzidos por uma mesma fêmea (MELO, 2016). Entre os fatores que contribuem para variação no tempo da viabilidade do ovo fora da água estão as diferenças nas camadas estruturais e no tamanho do ovo (FAULL; WILLIAMS, 2015; SOTA; MOGI, 1992; SUMAN et al., 2011), assim como a intensidade da melanização (FARNESI et al., 2017). Além desses, o período de dormência também pode ser

afetado por aspectos fisiológicos, principalmente pelas reservas nutricionais de origem materna acumuladas no vitelo (CONSOLI; OLIVEIRA, 1994; PEREZ; NORIEGA, 2013).

Ae. aegypti e *Ae. albopictus* (Skuse, 1894) têm sido as espécies de mosquitos mais estudadas quanto à resistência dos ovos a dessecação, sendo essa fase do desenvolvimento a mais susceptível a desidratação, particularmente nas primeiras horas pós-oviposição (FARNESI et al., 2015; REZENDE et al., 2008; VARGAS et al., 2014). Fêmeas dessas espécies tem por hábito depositar os ovos diretamente na água ou próximo à lâmina d'água (CHADEE; CORBET; TALBOT, 1995; FAY; PERRY, 1965) e, embora *Ae. albopictus* apresente maior plasticidade quanto à exploração de criadouros (SWAN; LOUNIBOS; NISHIMURA, 2018), ambas as espécies são consideradas antropofílicas, preferindo ovipositar, em recipientes com água limpa, tais como pratos de plantas, pneus e depósitos para armazenamento de água (ABÍLIO et al., 2018; GETACHEW et al., 2015; PINHEIRO; TADEI, 2002). Considerando a rápida redução nos níveis de água nesses tipos de criadouros e entre outros naturais (ocos de árvores e rochas, bromélias, etc.) devido à evaporação, ovos quiescentes podem se tornar um evento bastante comum no ambiente.

O processo de quiescência tem várias implicações negativas no combate ao vetor, uma vez que permite a dispersão passiva dos mosquitos e dificulta as medidas de controle. A introdução de *Ae. aegypti* no Brasil, por exemplo, ocorreu provavelmente pelo transporte de ovos quiescentes nos navios negreiros advindos da África (CONSOLI; OLIVEIRA, 1994; SILVA; SILVA, 1999). De modo similar, o estabelecimento de *Ae. albopictus* na Europa tem sido atribuído ao transporte devido ao comércio de pneus recondicionados e plantas (MARQUES, 2018). Originária da Ásia, *Ae. albopictus* atualmente pode ser encontrada nas Américas, parte da África, Austrália e em vários países europeus, aumentando o risco de transmissão de patógenos de importância médica (GOSSNER; DUCHEYNE; SCHAFFNER, 2018). Um outro ponto é que a reinfestação de áreas previamente controladas, devido a eclosão de larvas provenientes de ovos quiescentes após o restabelecimento das condições favoráveis nos criadouros, também tem dificultado as medidas de controle (DINIZ et al., 2017).

Epidemiologicamente, ovos quiescentes também podem permitir a sobrevivência de embriões infectados, favorecendo a transmissão viral dos diferentes arbovírus veiculados pelos vetores (CONSOLI; OLIVEIRA, 1994; ROSEN, 1987; YANG, 2014). Por exemplo, na ilha de Key West na Flórida, a detecção de 8,33% de ovos do mosquito *Ae. aegypti* infectados com o vírus DENV-1 levou Buckner et al. (2013) a sugerir que os surtos de dengue entre os anos de 2009 e 2010 nesta região teria sido facilitada por transmissão vertical. A transmissão

transovariana do vírus da dengue também foi detectada em 1,09% de imaturos coletados na Índia (ANGEL et al., 2016). Em tal situação, os ovos dormentes podem ter contribuído para sustentar as epidemias de dengue.

Outras formas de quiescência, menos frequentemente usadas como estratégias de sobrevivência em mosquitos, são a dormência no ovário de fêmeas e em espermatozoides nos machos. O estado de dormência em fêmeas de *Culex quinquefasciatus* (Say, 1823) tem sido registrada durante a estação fria na Califórnia (REISEN et al., 2010; REISEN; MEYER; MILBY, 1986). Um indicativo da ocorrência desse processo tem sido o registro de dilatações degenerativas contendo material granular nos ovários, estimulado pelas baixas temperaturas (NELMS et al., 2013; REISEN; MEYER; MILBY, 1986). Nessa condição, a quiescência contribui com o aumento da população após o inverno. Enquanto isso, a inatividade dos espermatozoides no trato reprodutivo dos machos da maioria dos animais, antes de ser ativado por substâncias produzidas pelas glândulas acessórias, tem sido considerada um tipo de quiescência. Em *Cx. quinquefasciatus,* os espermatozoides maduros saem da quiescência se tornando móveis em resposta a sinais químicos específicos mediados por uma protease semelhante à tripsina que requer influxo de íons de Ca^{+} (THALER et al., 2013).

Desse modo, podemos dizer que as várias formas de quiescência usadas pelos mosquitos podem ser mecanismos para a manutenção da sobrevivência das espécies, principalmente em condições desfavoráveis.

1.2 CUTÍCULA SEROSA EM MOSQUITOS: FORMAÇÃO E FUNÇÃO

A cutícula serosa (CS) é uma matriz extracelular presente nos ovos de várias espécies de insetos (PANFILIO, 2008; ROTH, 2004), sendo produzida pela membrana extraembrionária denominada serosa (CLEMENTS, 1992). Essa estrutura está localizada imediatamente abaixo das duas camadas que compõem a casca do ovo e que são produzidas ainda no ovário: o exócorio (mais externa e rígida) e o endocório (interna e flexível) (Figura 1) (CLEMENTS, 1992; FARNESI et al., 2015).

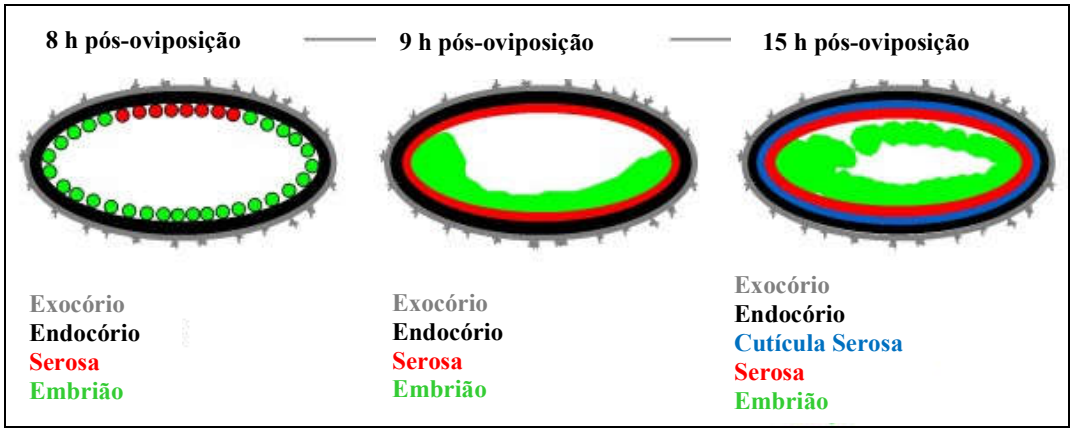

Figura 1. Organização dos envoltórios e do embrião no ovo de *Aedes aegypti*. No início da embriogêneseb (8 h após a oviposição), a casca do ovo é formada por duas camadas – o exocório (externa) e o endocório (interna) -, enquanto as células que irão compor a serosa e o embrião permanecem em uma posição periférica. A partir de 9 h de desenvolvimento, a serosa diferenciada circunda todo o embrião, dando início à formação da CS. Entre 13 e 15 h após a oviposição, a formação da CS está completa.
Fonte: Adaptado de Farnesi et al. (2015).

Em mosquitos, os ovócitos são fecundados pouco antes da oviposição, sendo a embriogênese iniciada a partir do momento da postura (CHAPMAN, 2013; CLEMENTbS, 1992). Seus ovos são centrolécitos (grande quantidade de vitelo no centro da célula) e sofrem clivagem do tipo meroblástica (separação parcial da célula) (CLEMENTS, 1992; GILBERT, 2000). Durante a segmentação, várias divisões mitóticas acontecem sem que haja a citocinese, acontecendo a migração desse conjunto células para a periferia celular, formando uma camada denomiada blastoderma sincicial. Nesse ponto, a separação entre as células finalmente ocorre, dando origem a uma blastordema celular (GILBERT; RAUNIO, 1997). Finalizado esse processo, é iniciada a definição das células que irão compor a banda germinal responsável pela formação do embrião propriamete dito, bem como àquela da membrana serosa que irá produzir a CS (CHRISTOPHERS, 1960; CLEMENTS, 1992; GOLTSEV et al., 2009). Durante a gastrulação, que ocorre paralelamente ao processo anterior, o embrião se dobrará dentro do vitelo e a serosa o envolverá completamente (CHRISTOPHERS, 1960; CLEMENTS, 1992; GOLTSEV et al., 2009), dando início à secreção da CS rica em quitina (GOLTSEV et al., 2009; REZENDE et al., 2008).

A CS tem se mostrado associada à capacidade do ovo em resistir à dessecação (GOLTSEV et al., 2009; REZENDE et al., 2008; VARGAS et al., 2014). Isso ocorre porque a CS forma uma barreira de proteção entre o embrião e o ambiente externo (CHRISTOPHERS, 1960), impedindo a perda de água (REZENDE et al., 2008). O período de formação dessa cutícula não é o mesmo entre as diferentes espécies o que resulta em uma capacidade

diferencial para suportar condições de ressecamento durante a embriogênese, sendo as espécies do gênero *Aedes* as mais resistentes (CARVALHO et al., 2017; SOTA; MOGI, 1992). Por exemplo, em *Ae. aegypti* a CS está formada entre 13 e 15 horas após a postura do ovo (REZENDE et al., 2008), enquanto em *Anopheles gambie* (Giles, 1902) esse processo ocorre cerca de oito horas após a fertilização (GOLTSEV et al., 2009), tornando seus ovos mais resistentes ao ressecamento.

Evidências moleculares e bioquímicas têm mostrado que a CS é composta de proteínas, lipídios e moléculas relacionadas à esclerotização (FURNEAUX; MCFARLANE, 1965; HARWOOD; HORSFALL, 1959; REZENDE et al., 2008). A inibição da síntese de quitina presente na CS de *Cx. quinquefasciatus* resultou em aumento na taxa de mortalidade larval durante a eclosão, possivelmente por interferir na formação da linha de deiscência onde ocorre o início da eclosão, principalmente entre os embriões mais jovens (MIURA et al., 1976). Embora a composição da CS possa ser similar entre mosquitos, a estrutura pode diferir entre as espécies, embora pouco se conheça sobre esse assunto. Por exemplo, experimentos de manipulação da CS de *Cx. quinquefasciatus* mostraram uma consistência gelatinosa e frágil, diferindo daquela de outros mosquitos como *Anopheles aquasalis* (Curry, 1932) e *Ae. aegypti* que é mais resistente (VARGAS et al., 2014).

Até o momento, não são conhecidas as rotas metabólicas ou hormônios que sejam associados à quiescência e, apenas o gene *quitina sintase* (*CHS*) está descrito na literatura como relacionado a esse processo em mosquitos. Esse gene promove a síntese de quitina que irá ser usada na formação da CS e, consequentemente, com a aquisição da resistência à dessecação (GOLTSEV et al., 2009; MERZENDORFER, 2006; REZENDE et al., 2008). Apesar de ter sido primeiramente estudado em *Anopheles gambiae* (*AgCHS*), esse gene possui elevada identidade com outras duas espécies de culicídeos – *Anopheles quadrimaculatus* (Say, 1824) e *Ae. aegypti*. O gene possui dois variantes editados, sendo apenas o alelo *AgCHS1* envolvido na embriogênese (GOLTSEV et al., 2009). Em *Ae. aegypti*, essas duas variações do gene foram também analisadas. Enquanto a isoforma *AaCHS1a* foi associado à formação da CS, a isoforma *AaCHS1b* foi relacionado com a formação da cutícula larval nessa espécie (REZENDE et al., 2008). Além disso, o surgimento da CS coincidiu com a aquisição da resistência à dessecação, entre 9 e 12h estando a estrutura totalmente formada entre 13 e 15 h após a oviposição (GOLTSEV et al., 2009; REZENDE et al., 2008). Apesar disso, de modo geral, parece que os genes envolvidos na formação da CS estão também associados à formação cutícula larval (GOLTSEV et al., 2009).

1.3 IMPORTÂNCIA DOS REGULADORES DE CRESCIMENTO NO CONTROLE DE INSETOS

Apesar dos significativos avanços nos métodos utilizados para o controle de *Ae. aegypti*, esta espécie persiste como a principal vetora de várias arboviroses, entre elas a dengue, chikungunya e Zika (KRAEMER et al., 2015; REZZA, 2014; SHRAGAI et al., 2017; WEAVER; REISEN, 2010). Atualmente, o combate ao mosquito tem sido prioritariamente realizado através do uso de larvicidas químicos que, ao longo dos anos, tem resultado em elevado nível de resistência nas diversas populações do mosquito, sendo um ponto crítico no estabelecimento de medidas eficazes de controle dessa espécie (DOLABELLA et al., 2016; LA CORTE et al., 2018; VIANA-MEDEIROS et al., 2017).

Recentemente, compostos com distintos mecanismos de ação e sítios alvos, como os reguladores de crescimento de insetos (IGR, do inglês *Insect Growth Regulator*), têm sido usados nos programas de controle de mosquitos. Como agentes inseticida, os IGRs atuam no sistema endócrino desses insetos, podendo atuar, principalmente, como inibidores da síntese de quitina (CSI, do inglês *Chitin Synthesis Inhibitors*), tais como o diflubenzuron e o novaluron, ou como análogos do hormônio juvenil (JHA, *Juvenile Hormone Analogue*s) a exemplo do pyriproxyfen e metopreno (GRAF, 1993). De modo geral, os IGRs podem afetar o desenvolvimento embrionário e dos imaturos (larvas e pupas), causando mortalidade, deformações ou inibindo a emergência do adulto (DEVILLERS, 2013). De acordo com a literatura, os primeiros IGRs surgiram na década de 70, o JHA metopreno e um CSI denominado benzoilfeniluréia (GILBERT, 2010) e

Produtos HJA são também conhecidos como “terceira geração” de inseticidas ou juvenóides, sendo moléculas sintetizadas artificialmente a fim de imitar os hormônios naturais produzidos pelos insetos da classe dos sesquiterpenóides (CONSOLI; OLIVEIRA, 1994; DEVILLERS, 2013). Esses hormônios, quando produzidos por insetos holometábolos como *Ae. aegypti*, atuam na metamorfose onde eventos morfológicos, fisiológicos e comportamentais acontecem de modo complexo e sincronizado, permitindo o desenvolvimento da ecdise da fase de larva até a formação do adulto. Produtos HJA atuam de modo similar sobre o indivíduo, e quando aplicados durante a fase pré-imaginal do inseto, inibem a emergência do adulto (MIAN; MULLA, 1982). Enquanto concentrações elevadas do HJ na hemolinfa mantém o animal em estágio juvenil, sua diminuição na circulação sinaliza o momento da ecdise (GILBERT; GRANGER; ROE, 2000; HARTFELDER, 2000; PALLI, 2009). Em *Ae. aegypti*, o par de glândulas endócrinas denominados corpos alados (*corpora*

allata) sintetizam e secretam JH III, cuja atuação se dará não apenas na regulação do desenvolvimento como também na reprodução das fêmeas (CHRISTOPHERS, 1960; LI et al., 2003; SHAPIRO et al., 1986). Estudos têm mostrado que essa classe de IGR atua com bastante especificidade sobre os mosquitos, com poucos relatos de impacto sobre outros insetos (MIURA; TAKAHASHI, 1974). Além disso, esses produtos são considerados mais seguros para manipulação pelos profissionais de saúde e de evolução lenta da resistência quando em comparação aos inseticidas convencionais (FEINSOD; SPIELMAN, 1980; KLOWDEN; BLACKMER, 1987; OKAZAWA et al., 1991; RESENDE; GAMA, 2006). Atualmente, a Organização Mundial da Saúde recomenda, e o Brasil adota, o larvicida HJA pyriproxyfen no combate a *Ae. aegypti* (CHAVASSE; HH, 1997).

Embora menos estudada, a ação de IGRs sobre os ovos também tem sido registrada, com fortes indícios da supressão da embriogênese, refletida na inibição da eclosão larval (MIURA; TAKAHASHI, 1974; SUMAN et al., 2013; VASUKI, 1990). Bloquear o ciclo de vida do mosquito na fase de ovo seria desejável, pois impediria a continuação do ciclo de vida do vetor e, consequentemente, a transmissão de doenças (FARNESI et al., 2017). Apesar disso, esse não tem sido um objetivo fácil de se alcançar. A ação de IGRs sobre ovos tem se mostrado dependente de inúmeros fatores, tais como o produto usado, a concentração aplicada, a idade do ovo e a espécie analisada. Reduções na viabilidade de ovos jovens (até 48h) expostos ao triflumuron (CSI) e pyriproxyfen foram observadas em concentrações entre 0,1 e 1,0 ppm, sendo as concentrações maiores aquelas com maior eficácia (SUMAN et al., 2013; VASUKI, 1990). De modo contrário, Suman et al. (2013) registrou ação ovicida do diflubenzuron (CSI) com concentrações bem menores do que as mencionadas anteriormente (entre 0,1 e 1,0 ppm). Variações interespecíficas na susceptibilidade de ovos expostos a IGRs também foram observadas por Suman et al. (2013). Esses autores mostraram que, de modo geral, ovos não embrionados apresentavam menor eclosão larval e sugeriram que o diflubenzuron afeta a quitinização da cutícula embriônica, tornando a larva farata inviável.

Um outro ponto na aplicação de IGRs sobre os ovos e que tem sido estudado é o transporte passivo dessas substâncias de um criadouro para outro, usando os próprios mosquitos como transportadores. Trabalhos têm mostrado, por exemplo, que o pyriproxyfen pode ser dispersado para diferentes criadouros através das patas contaminadas pelas próprias fêmeas dos mosquitos, ampliando seu o poder de ação para estes ambientes (DEVINE et al., 2009; GAUGLER; SUMAN; WANG, 2012; ABAD-FRANCH, 2015).

Apesar dos IGRs serem considerados uma alternativa viável e segura em relação aos atuais químicos utilizados no combate de vetores, o uso de qualquer agente de controle deve

ser feito de forma racional e integrada com outros métodos no âmbito de programas de controle de mosquitos.

1.4 OBJETIVOS

1.4.1 Geral

Caracterizar e avaliar o impacto da quiescência sobre aspectos biológicos de *Aedes aegypti* em diferentes populações do mosquito.

1.4.2 Específicos

- Determinar o período de quiescência dos ovos de *Ae. aegypti* provenientes dos municípios de Recife (PE) e Aracaju (SE);

- Estimar o tempo inicial de eclosão a partir de ovos quiescentes das duas populações de mosquitos estudadas;

- Avaliar o impacto da quiescência sobre a duração do desenvolvimento pós-embrionário, taxa de emergência e proporção sexual dos adultos em amostras de ovos de *Ae. aegypti* de Recife e Aracaju;

- Averiguar o tamanho do adulto, fecundidade e fertilidade nas populações estudadas;

- Analisar a influência do produto pyriproxyfen, um IGR, análogo do hormônio juvenil, na duração da quiescência das populações acima;

- Comparar a viabilidade dos ovos expostos ao pyriproxyfen, antes e após a formação da cutícula serosa;

- Avaliar o perfil de expressão do gene *AaCHS1a*, durante o período de formação da cutícula serosa nos ovos de *Ae. aegypti*, nas populações de Recife e Aracaju.

2 MANUSCRITO 1 – QUIESCENCE IN *Aedes aegypti*: INTERPOPULATION DIFFERENCES CONTRIBUTE TO POPULATION DYNAMICS AND VECTORIAL CAPACITY.

Este manuscrito foi publicado no formato de artigo na revista *Insects* (Apêndice A).

Quiescence in *Aedes aegypti*: Inter-population differences contribute to population dynamics and vectorial capacity.

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Abstract: The strategy of *Aedes aegypti* to prolong embryonic viability by quiescence has severe implications for geographic expansion and maintenance of mosquito populations in areas under control measures. We evaluated the effects of quiescence on biological parameters directly or indirectly associated with population dynamics and vectorial capacity in populations of this mosquito species from two Brazilian municipalities characterized as dengue, chikungunya, and Zika transmission areas. Egg viability, initial hatching time, post-embryonic development time, adult emergence rate, sexual proportion, adult size, fecundity, and fertility were analyzed using eggs stored for 10, 40, 70, 100, 130, and 160 d. Quiescence time reduced overall egg viability and post-embryonic development time in both municipalities but was more costly in Aracaju (100 d; 8 d) than in Recife (130 d, 7.5 d). Emergence rates increased in Recife when the eggs were older, but not in Aracaju. Significant

deviations in sexual proportion, with male predominance, were observed in both populations. Initial hatch, fecundity, fertility, and adult size did not significantly influence egg quiescence time. These results indicate intrinsic and differential characteristics for each *A. aegypti* population, suggesting a differential cost of quiescence for population dynamics parameters which can indirectly affect vectorial capacity and control measures.

Keywords: Biological cycle; Culicidae; desiccation resistance; development; egg dormancy; fitness; mosquito; plasticity; reproduction

Introduction

Arboviruses transmitted by mosquito vectors have been a major cause of global health problems, particularly in tropical and subtropical countries [1–5]. Viruses causing dengue fever, urban yellow fever, chikungunya fever, and Zika virus disease are some arboviruses whose main transmission vector in these areas is *Aedes aegypti* (L.) [6–10].

Over the course of its evolutionary history, *A. aegypti* has developed strategies favoring the explosive growth of natural populations, invasion, and dissemination worldwide [11–16]. One of these strategies is the capacity of eggs to resist desiccation, mainly due to low humidity and high temperatures, until conditions become favorable for hatching (non-seasonal quiescence) [17–21]. Resistant eggs can allow the pharate first instar larvae to survive inside the egg in unfavorable environments for up to over a year [12,22–24]. Thus, quiescent eggs constitute an important problem for vector control because these eggs can directly contribute to the maintenance of mosquito populations in treated areas, and may facilitate the transportation of eggs and the establishment of new populations [22,25]. In subtropical regions, low annual average temperatures are a limiting factor for the survival of *A. aegypti*. However, in a current global warming scenario, the elevation of temperatures in these areas can favor the introduction, dispersion, and expansion of *A. aegypti* [26,27]. Furthermore, increases in temperature may reduce the incubation period of some pathogens, including dengue virus, shortening the dengue transmission cycle [28,29]. Considering the existence of trans-ovarian transmission in this vector [30,31], quiescent eggs may play a crucial role in this situation since eggs can remain viable in the environment for long periods of time.

Although scarcely studied, there are indications that the quiescence process has a negative cost on the fitness of *A. aegypti* individuals. For instance, the extension of egg quiescence periods has been shown to negatively affect larval physiology and development by

reducing lipid reserves [32] and reducing female body masses and reproductive fitness [33]. Such observations raise a key question as to whether quiescence would shape parameters directly or indirectly associated to population dynamics, vector capacity, and competence (i.e., mosquito density, time of postembryonic development, sexual proportion, fecundity, and fertility), and how different such effects would be on different mosquito populations. Moreover, previous studies have shown that local and regional genetic differences between mosquito populations can affect different characteristics of the biology of vectors [34,35]. Thus, a role for inter-population variations in egg quiescence in population dynamics and vector capacity is a reasonable assumption, but this remains uninvestigated.

Using an approach that combines biological parameters capable of directly or indirectly affecting population dynamics and vector capacity we investigated: (1) the differences in the eclosion rate from quiescent eggs in distinct *A. aegypti* populations from transmission areas for dengue fever, chikungunya fever, and Zika; (2) whether the initial hatching time, post-embryonic development time, adult emergence rate and sexual proportion were differentially affected by the quiescence period in these populations; (3) whether fecundity, fertility, and adult size are altered as result of increased quiescence time. We tested the hypothesis that the duration of the quiescent period and its consequences on individual fitness are intrinsic characteristics of each population, differing between *A. aegypti* populations due to variations in genetic features and selective environmental pressures. Therefore, we predict that quiescence will negatively affect the initial hatching time by increasing the time required for larvae eclosion due to a greater necessity for rehydration. Similarly, post-embryonic development time will be longer, and emergence rate will be reduced as the quiescent period progresses due to smaller lipids reserves in the embryos. Meanwhile, since genetic mechanisms modulate the sexual proportions of mosquitoes, there will be no change in the proportion of males and females related to quiescence time. Finally, fecundity will be decreased as quiescence time increases, since low energy reserves result in smaller female body sizes, reducing their ability to ingest a blood meal and, consequently, egg production. In contrast, the fertility of females originating from quiescent eggs would not be affected because the eggs of these females were not exposed to the quiescence process.

Materials and methods

Mosquito strains

A. aegypti populations from two Brazilian municipalities 501 km apart from each other (Recife - 08°03' 03" S - 34°56' 54" W and Aracaju 10º 54' 40" S - 37º 04' 18" W) were studied in this work. Both cities are dengue fever, chikungunya fever, and Zika virus transmission areas, and are characterized as Am Tropical monsoon climates based on the Köppen-Geiger climatic classification system, where the temperature of the coldest month is > 18 °C and the precipitation on driest month is > 100 mm [36].

Recife (8 m above sea level - masl) is one of the oldest cities in Brazil and is considered one of the ten most-populous municipalities in the country. The city presents an average temperature of 25.9 ºC and average rainfall of 1800 mm throughout the year [37]. Its estimated population in 2017 was 1,633,697 inhabitants [38]. In contrast, Aracaju (4 masl) is one of the least populated capital cities (650,106 people) [38] in Brazil. On average, annual temperatures of 26 ºC and rainfall of 1,590 mm are registered [39].

Parental generation (PG)

The *A. aegypti* parental generation (PG) was obtained from eggs collected in urban areas, using oviposition traps (ovitraps) made of 500 mL black plastic pots, 15 x 12 cm. The oviposition trap contained water and a 15 x 5 cm strip of a wooden, Eucatex pallet as an oviposition substrate. In the laboratory, paddles containing eggs were placed in plastic trays (40 x 27 x 7 cm) covered with a mesh and left for drying for three days to complete embryonic development, at 26±1°C, 80±5% RH and photoperiod of 12:12 [light: dark] h. After this period, eggs were transferred to a new plastic tray, filled with filtered water to allow larvae to hatch. Larvae were fed with fish food (Tetra® Marine XL Granules, Melle, Germany) *ad libitum* until pupation. Pupae were transferred to plastic cups and placed inside breeding cages for adult emergence. Due to the presence of other *Aedes* species in both cities, a screening method based on the taxonomic key of Consoli and Lourenço-de-Oliveira [40] was used to identify adults of *A. aegypti* during the formation of the PG. After selection, mosquitoes were fed 10% sucrose solution *ad libitum* as a carbohydrate source. After sugar deprivation for 24-48 h, females were fed defibrinated and sterile sheep blood (EBE-FARMA Biológica e Agropecuária LTDA, Rio de Janeiro, Brazil) until full engorgement to stimulate ovogenesis. Three days after the blood meal, 50 mL plastic cups containing sterilized cone filter paper filled with 10 mL of water were placed inside the cages for oviposition for three days and exchanged every 24 hours.

Quiescent eggs

Eggs from F1-F3 generations were divided into groups and stored in plastic containers covered with a mesh for 10, 40, 70, 100, 130 and 160 days before use. The storage periods were chosen based on previous observations made in our laboratory [41]. From this, egg groups were formed for viability analyzes with a 30-day interval between groups. Eggs of 10 days old were used as a baseline (control) for the experiments. Although these eggs were in the beginning of the quiescent period, in our previous tests, hatching rate did not significantly differ from three days old eggs (freshly embryonated eggs) (mean of 49.2 ± 12.11; Kruskal-Wallis with Student-Newman-Keuls test *a posteriori*, $p \leq 0.05$). Ten replicates for each group were used totalizing approximately 1,000 eggs for each population and stored period.

Effect of quiescence on different biological parameters of A. aegypti

Egg viability

For each quiescence condition (10, 40, 70, 100, 130 and 160 days), the total number of larvae hatched following immersion of eggs in water was recorded after seven days (maximum period of larval hatching observed in previous tests). Egg viability was measured as the percentage mean of total egg hatching in each group.

Initial hatching time

The presence of the first larvae was recorded in hourly observations for each quiescence time to evaluate whether older eggs would require a longer water immersion time for larval hatching. Observations performed before an hour resulted in no larval hatching.

Post-embryonic developmental time

A maximum sample of 10% of the total number of larvae obtained in each replicate in the above experiments was used to investigate whether the time for larvae to reach adulthood would increase according to a longer quiescence period. This value was determined to keep the same proportion for each replicate, thus avoiding bias due to sampling errors. Larvae (up to 8 h after hatching) were individualized in plastic cups (50 mL capacity) with 40 mL of

water and fed with fish food, in proportion to 2 mg/larvae on alternate days. Upon reaching the pupa stage, the cups containing individuals were transferred to cages (transparent container with 15 cm diameter and 9 cm in height, and 1000 mL volume) separately for adult emergence. The period required to complete the life cycle (L1 to adult), in days, was registered.

Emergence rate and sexual proportion

After their emergence, males and females were quantified, and the male/female proportion was determined. Adults were left for mating in adult cages (40 x 40 cm) and used for fecundity and fertility trials described below. After females received a blood meal (as previously described), mosquitoes were fed on sugar solution ad libitum.

Fecundity and fertility

Immediately after a blood meal, engorged females were individualized into transparent plastic receptacles (1000 mL) fitted with a breeding site (50 mL plastic cup containing filter paper soaked in water). A cotton swab moistened with a 10% sucrose solution was offered *ad libitum*. After a week, breeding sites were removed, dried at room temperature, and eggs counted under a stereomicroscope using 10x magnification (LEICA Microsystems, model S8 AP0, Wetzlar, Germany). The total number of eggs laid by a female can be altered throughout the gonotrophic cycle (GC) [15]. Thus, for this study, the number of eggs deposited at the end of the first GC was used as an estimate of fecundity. Fertility assays were performed using eggs from the fecundity trials. Three days after the removal of the oviposition sites, larvae were hatched by submerging eggs in water, and the eclosion rate evaluated after seven days.

Adult size

To estimate adult size, left wings were detached and the distance from the axillary incision to the apical margin, excluding the fringe of scales, was measured using a stereomicroscope with a coupled camera [42,43] using a magnification of 25x (LEICA Microsystems, model S8 AP0, Wetzlar, Germany).

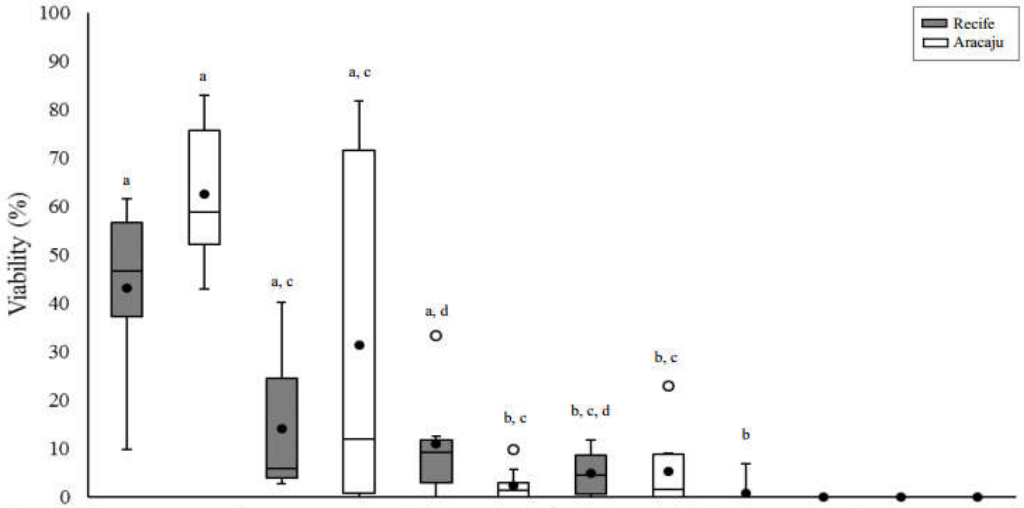
Statistical analyses

Lilliefors and Bartlett tests were used to verify the normality of distribution and homoscedasticity of all data. All analyses were performed using BioEstat software, version 5.3 [44], with values of $p \leq 0.05$ indicating statistical significance. To analyze whether the quiescence period affects viability, initial hatching time, post-embryonic development time, adult emergence rate, size, fecundity, and fertility we used the Kruskal-Wallis test followed by the Student-Newman-Keuls (SNK) multiple comparison tests. Differences in sexual proportion were determined using the Qui-Square Adhesion test for samples with the same expected proportions of 50:50% (males: females) (with a correction of Yates). Lastly, the Mann-Whitney U-test for independent samples and unequal sizes was used to calculate *p*-values and determine the significance between populations of mosquitoes. Results were expressed as average followed by confidence interval 95% (CI95%) and range. The graphical representation of the data was made using box plots and bar graphics were produced in the Microsoft Office Excel software, version 2016.

Results

Egg viability

Overall, egg viability significantly decreased in both populations as time progressed (Recife - H = 35.0000; df = 5; $p = 0.0000$ and Aracaju - H = 32.7493; df = 5; $p = 0.0000$), although a differential response in the reduction pattern of the eclosion rate had been observed. Maximum egg viability lasted a month longer in eggs from Recife (130 days; 0.8%) compared to the Aracaju population (100 days; 5.2%) (Figure 1). A significant reduction in egg viability was recorded after 40 and 70 days of quiescence in the Aracaju ($p = 0.0021$) and Recife ($p = 0.0077$) populations, respectively. A wide variation in hatching rate was observed, being more expressive at ten days in Recife (9.8 – 61.5 days) and 40 days in Aracaju (0 – 81.8 days). Significant differences between populations were observed in egg storage for 10 (U = 20; $p = 0.0412$) and 70 days (U = 23.5; $p = 0.0452$). In comparison with the Recife population, eggs from Aracaju showed a 70% higher eclosion rate at ten days and a 20% reduction after 70 days of storage.

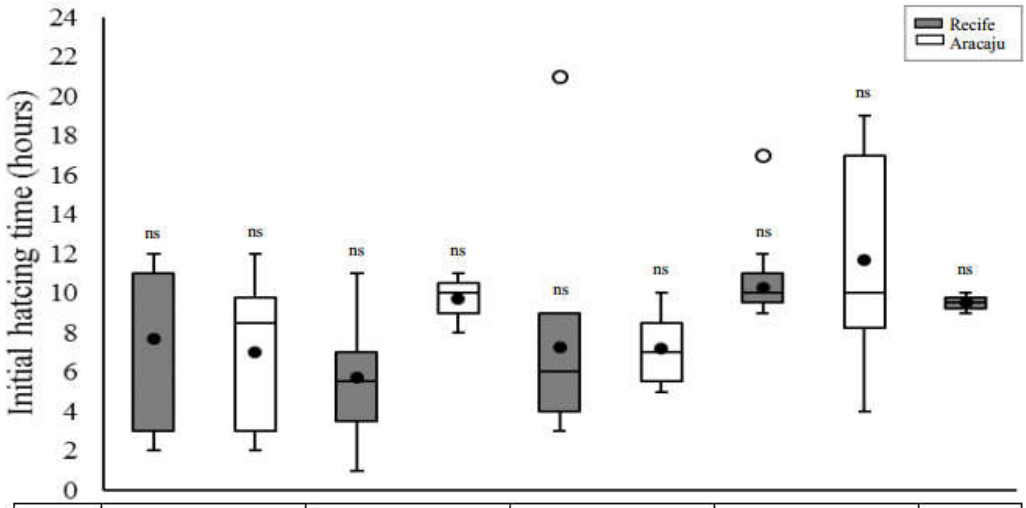


Days	10 (Control)*		40		70*		100		130		160	
Total of eggs	934	1162	1126	1059	1227	1083	1249	1113	1110	1048	1222	1070
Replicate	9	10	10	10	10	10	10	10	10	10	10	10
Mean	43.1	62.6	14.0	31.4	10.9	2.4	4.9	5.2	0.8	0.0	0.0	0.0

Figure 1. Eclosion rate (%) as a function of the quiescent period in two populations of *Aedes aegypti* – Recife (gray) and Aracaju (white). Replicates consist of approximately 100 eggs. The size of the boxes indicates the distance between the first (lower) and third (upper) quartiles, the central mark among them shows the median and the closed circle the mean; the bars indicates variability outside quartiles and outliers are plotted as empty circles. The letters indicate comparisons between the different quiescent periods within each population (Kruskal-Wallis with Student-Newman-Keuls test *a posteriori*). Different letters indicate significant differences at $p \leq 0.05$. Asterisks indicate significative difference inter-population in the same period of quiescence (Mann-Whitney test, $p \leq 0.05$).

Initial hatching time

The mean time (hour) required for the first larvae to hatch after all quiescent periods is presented in Figure 2. Neither quiescent eggs from Recife nor from Aracaju required extended immersion times for the first hatching due to their quiescence period (Recife, H = 6.1401; df =4; $p = 0.1889$ and Aracaju, H = 5.0897; df =3; $p = 0.1653$). However, we noted a differential hatching pattern between the different populations. Under the quiescent conditions used in this work, most larvae in both populations hatched after seven h (> 55%). Even so, eggs from Recife showed a greater range of hatching time (1 – 21 h) compared with eggs from Aracaju (2 – 19 h). The greatest difference between the two populations occurred in eggs stored for 40 days, with a significantly shorter mean hatching time in Recife population (5.7 ± 2.13 h) compared to Aracaju (9.7 ± 1.16 h) (U = 8; $p = 0.0084$).



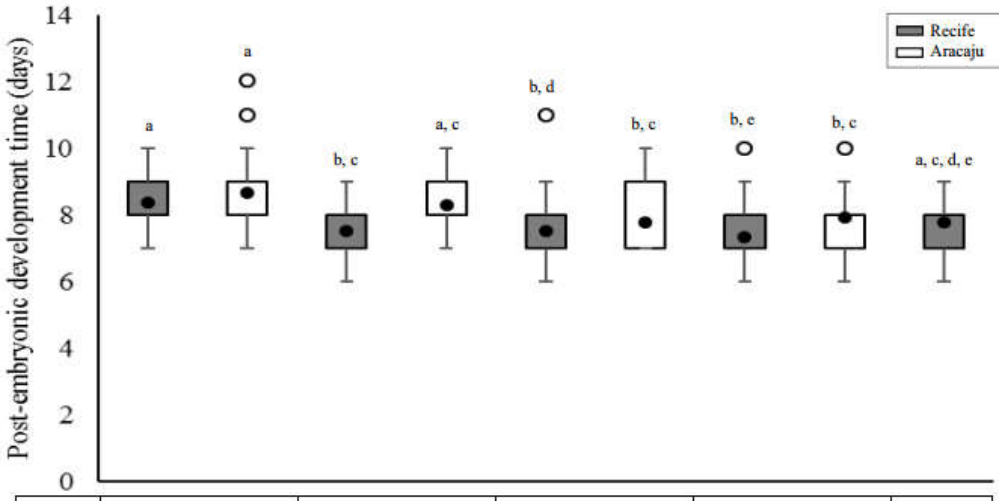
Days	10 (Control)		40*		70		100		130†
Total of eggs	934	1162	1126	740	1121	664	845	622	217
Replicate	9	10	10	7	9	6	7	6	2
Mean	7.7	7.0	5.7	9.7	7.2	7.2	10.3	11.7	9.5

Figure 2. Initial hatching time (hours) of *Aedes aegypti* larvae in different quiescent periods, in two mosquitoes populations – Recife (gray) and Aracaju (white). The number of replicates was obtained according to the viability test, each them consisting of approximately 100 eggs. Values are represented as average hatching time (closed circle); the size of the boxes indicates the distance between the first (lower) and third (upper) quartiles, the central mark among them shows the median; the bars indicates variability outside quartiles and outliers are plotted with empty circles. No significant difference (ns) was found between the quiescent period analyzed within each mosquito population separately (Kruskal-Wallis with Student-Newman-Keuls test *a posteriori,* $p \leq 0.05$). Asterisks indicate significative difference inter-population in the same period of quiescence (Mann-Whitney test, $p \leq 0.05$). † No hatching was observed for the Aracaju mosquito population from 130 days, and no hatching was observed with 160 days of quiescence for both populations (see viability tests, figure 1).

Post-embryonic development time

In both populations, larvae from older eggs reached adulthood in a shorter time compared to controls (Recife, H = 47.2926; df =4; $p = 0.0000$ and Aracaju, H = 16.1884; df =3; $p = 0.001$). Overall, the cost of quiescence to immature development was lower in Recife (shorter development time) compared to the Aracaju population (longer development time) (Figure 3) with a significant reduction at 40 d (U = 368; $p = 0.0001$) and 100 d (U = 406; $p = 0.0182$). In Recife, larvae from the control group averaged 8.4 ± 0.18 d to reach adulthood, a period significantly higher compared to intermediate quiescent periods (40 days, $p < 0.0001$; 70 days, $p < 0.0001$ and 100 days, $p < 0.0001$), even including outliers at 70 and 100 days. No difference was observed between day 10 and 130 ($p = 0.1046$). However, the difference in sample sizes may represent a bias in this result. Meanwhile, shorter larval development times

were observed for individuals from the Aracaju population eggs after 40 days of quiescence (70 days, $p < 0.0013$ and 100 days, $p < 0.0057$) when compared with control eggs.



Days	10 (Control)		40*		70		100*		130
Replicate	54	61	62	25	61	17	47	27	9
Mean	8.4	8.7	7.5	8.3	7.5	7.8	7.3	7.9	7.8

Figure 3. Post-embryonic development time (days) for immature *Aedes aegypti* obtained from eggs with different quiescence periods in two mosquitoes populations – Recife (gray) and Aracaju (white). Replicates represents the number of larvae obtained in the viability tests (approximately 10% of total hatchling). Values are represented as average post-embryonic time (closed circle); the size of the boxes indicates the distance between the first (lower) and third (upper) quartiles, while the central mark among them shows the median; the bars indicates variability outside quartiles and outliers are plotted with empty circles. The letters indicate comparisons between the different quiescent periods within each population (Kruskal-Wallis with Student-Newman-Keuls test *a posteriori*). Different letters indicate significant differences at $p \leq 0.05$. Asterisks indicate significative difference inter-populational for comparisons of values of the same period of quiescence (Mann-Whitney test, $p \leq 0.05$).

Emergence rate and sexual proportion

Overall, the proportion of adults emerging from pupae was dependent on the studied population and the quiescent period. The effect of the quiescent period was clearly seen in the mosquitoes from Recife ($H = 9.9393$; $df = 4$; $p = 0.0415$), but not in mosquitoes from Aracaju ($H = 5.0264$; $df = 3$; $p = 0.1699$). Also, although the proportion of viable eggs had decreased during the quiescent periods in both populations (Figure 1), proportionately more adults were obtained from older eggs in the Recife population. For instance, from a total of 94 L1 larvae (10 days of quiescence) individualized in a viability trial (shown above), 54 emerged as adults (57.5%). This percentage increased to 86.8% (n = 75) after 40 days of quiescence, reaching 100% (n= 9) at 130 days (Table 1). Conversely, the effect of the quiescence period on adult

emergence showed no clear pattern for the Aracaju population. When the samples from each quiescent period were compared between the studied populations, significant differences in the emergence rate was observed in eggs after 40 days of quiescence (U = 6; p = 0.0047). In this situation, the total number of adults was 2.5 times greater in the Recife population (86.8%) than Aracaju (34.2%) (Table 1).

Table 1. Emergence rate means ± CI 95% (range of values) from *Aedes aegypti* eggs of the Recife and Aracaju populations according to quiescence periods.

Period of quiescence	Population		Mann-Whitney test	p-value
	Recife	Aracaju		
10 days	57.5 ± 22.76 (10.0 - 100) [a] n = 94	52.8 ± 23.13 (0 - 91.7) [a] n = 117	41.5	0.7751
40 days	86.8 ± 9.74 (61.5 – 100) [b,c] n = 75	34.2 ± 30.48 (0 - 90) [a] n = 66	6.0	0.0047
70 days	74.5 ± 15.40 (40.0 - 100) [a,c] n = 88	61.7 ± 35.37 (0 - 100) [a] n = 26	18.5	0.3165
100 days	84.5 ± 12.29 (66.7 - 100) [a,c] n = 58	73.6 ± 30.14 (22.2 - 100) [a] n = 42	17.0	0.5700
130 days	100 ± 0.00 (100 - 100) [b,c] n = 9	No emergence	NA	NA
160 days	No emergence	No emergence	NA	NA

n = Total number of L1 larvae that were individualized; Small letters indicate comparisons of values (Kruskal-Wallis with Student-Newman-Keuls test *a posteriori;* $p \leq 0.05$) in the same column. Values identified by the same letter types are not significantly different; The Mann-Whitney test ($p \leq 0.05$) was used to assess inter-populational differences between the emergence rate means; NA = Not applicable.

In general, quiescence did not affect the ratio of females and males within the timelines of both *A. aegypti* populations studied. However, when the expected sexual proportion of 50:50% was evaluated, significant differences were observed, particularly, at 70 and 100 days (Figure 4). No difference between populations was recorded.

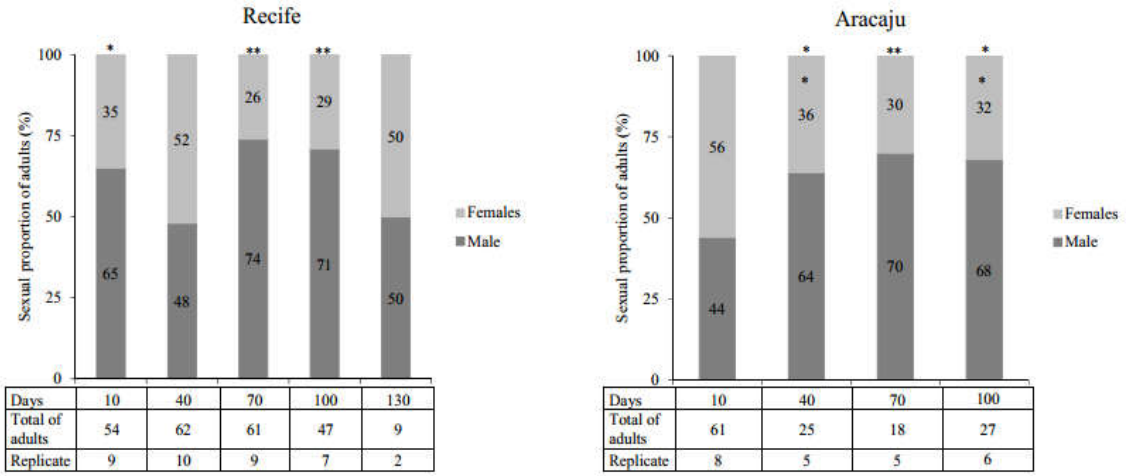


Figure 4. The proportion of *Aedes aegypti* adults (%) observed from eggs with different periods of quiescence. Replicates indicates the number of samples that presented emergence of adults from the viability tests. Asterisks identify within each population the periods of quiescence with significantly different deviations from the expected 50:50% (males: females) sexual proportion (χ 2 test; *$p < 0.005$, **$p < 0.0001$).

Fecundity and fertility

Fecundity was not affected by the quiescence period in either *A. aegypti* population (Recife, H = 4.5183; df =3; p = 0.2107 and Aracaju, H = 1.2361; df =3; p = 0.7444). Independent of the quiescence period, females from the Recife population (n = 77 females) laid an average of 46.3 ± 6.60 eggs / female (range 1 – 132,), while those of Aracaju (n = 53) presented a mean rate of 44.2 ± 8.05 (range of 3 – 123,). In both populations, maximum and minimum fecundity rates were observed at 70 (Recife – 56.2 ± 10.00 and Aracaju – 54.8 ± 32.88) and 100 days (Recife – 40.3 ± 24.98 and Aracaju – 29.2 ± 22.19), respectively. No significant difference was found when comparing each period of quiescence between populations.

Fertility was also similar among groups and populations (Recife, H = 6.8691; df =3; p = 0.0762 and Aracaju, H = 0.3967; df =3; p = 0.9409). Approximately, 28% ± 4.44 (n = 2159 eggs) and 27.9% ± 5.81 (n = 1478) of all eggs hatched from Recife and Aracaju, respectively. The first population presented maximum fertility rates when females were emerged from eggs after 70 days (34.7 ± 6.20), while the for the second population the optimal time was ten days (28.6 ± 8.41). Conversely, the minimum larval hatching rates were recorded for females from young eggs (10 days) in Recife and old eggs (100 days) in Aracaju. Significant differences between *A. aegypti* populations were otherwise not identified.

Size

Male and female body sizes were not affected by quiescence. However, females from the Recife population were larger than those from Aracaju, particularly those that emerged after 70 (U = 34.5; p = 0.0127) or 100 days (U = 53; p = 0.0136) quiescent eggs. Intrapopulation and interpopulation differences in male and female sizes are summarized in Table 2. Overall, females originating from quiescent eggs in the Recife population averaged 2.7 ± 0.03 cm (range 2.3 - 2.9 cm) and males 2.1 ± 0.06 cm (range 1.2 - 2.8 cm). The average wing size of females from eggs from the Aracaju population was 2.6 ± 0.05 cm (range 2.0 - 2.9 cm) and 2.1 ± 0.05 cm for males (1.8 - 2.7 cm).

Table 2. Average size ± CI 95% (range of values) of adult males and females from *Aedes aegypti* of the Recife and Aracaju populations according to quiescence periods.

Period of quiescence	Population				Mann-Whitney test		*p*-value	
	Recife		Aracaju					
	Males	Females	Males	Females	Males	Females	Males	Females
10 days	2.1 ± 0.13 (1.6 - 2.7) [a,A] n = 15	2.7 ± 0.09 (2.3 - 2.9) [a,B] n = 15	2.1 ± 0.06 (2.0 - 2.3) [a,A] n = 15	2.7 ± 0.09 (2.3 - 2.9) [a,B] n = 15	99.0	94.5	0.5755	0.4553
40 days	2.2 ± 0.11 (2.0 - 2.5) [a,A] n = 15	2.6± 0.09 (2.4 - 2.9) [a,B] n = 15	2.2 ± 0.10 (1.9 - 2.5) [a,A] n = 15	2.6± 0.17 (2.0 - 2.9) [a,B] n = 10	100.5	69.5	0.6187	0.7603
70 days	2.1 ± 0.10 (1.9 - 2.6) [a,A] n = 15	2.7 ± 0.05 (2.5 - 2.9) [a,A] n = 15	2.2 ± 0.33 (1.9 - 2.7) [a,A] n = 06	2.5 ± 0.12 (2.2 - 2.8) [a,B] n = 11	37.0	34.5	0.5334	0.0127
100 days	2.2 ± 0.15 (1.9 - 2.8) [a,A] n = 15	2.8 ± 0.04 (2.7 - 2.9) [a,B] n = 15	2.1 ± 0.11 (1.8 - 2.7) [a,A] n = 15	2.6 ± 0.08 (2.3 - 2.8) [a,B] n = 15	51.0	53.0	0.9158	0.0136
130 days	2.0 ± 0.36 (1.2 - 2.4) [a,A] n = 07	2.7 ± 0.52 (2.7 - 2.8) [a,B] n = 02	NA	NA	NA	NA	NA	NA

n = Total number of adult mosquitoes; Small letters indicate comparisons of values (Kruskal-Wallis with Student-Newman-Keuls test *a posteriori;* $p \leq 0.05$) in the same column, whereas capital letters indicate comparisons of values (Kruskal-Wallis with Student-Newman-Keuls test *a posteriori;* $p \leq 0.05$) in the same row. Values identified by the same letter types are not significantly different ($p > 0.05$); The Mann-Whitney test ($p \leq 0.05$) was used to assess inter-populational differences between males and females; NA = Not applicable.

Discussion

Studying different populations of *A. aegypti* we found a negative quiescent cost to egg viability, and sexual rate, which was dependent on the population studied and egg age (quiescence period). However, important parameters for population dynamics and vectorial capacities such as fecundity, fertility, and mosquito size were not affected by quiescence. In agreement with previous studies on egg quiescence in *A. aegypti* indicating differential egg viability [12,17,21,22,45–47], in our study, the *A. aegypti* population from Recife proved to be more resistant to desiccation (130 days, with high egg viability until three months of quiescence), than mosquitoes from the Aracaju population (100 days, with a high hatching rate up to 40 days of quiescence). Even so, the overall quiescence was shorter compared with other similar studies in which maximum larvae hatching was recorded from 150 days up to more than a year [12,22,46–48].

The reasons for the observed differences in mosquito egg resistance to desiccation are complex and involve a range of different intrinsic and extrinsic egg characteristics [17,19,21,22,49,50]. Variations in abiotic factors, particularly, those related to climate events (temperature and humidity), associated with egg water loss [51], and geographic characteristics (altitude), or causing variations in oxygen availability [52,53] have been associated with egg resistance to desiccation. Variations in rainfall (Recife 1804 mm; Aracaju 1409 mm), but not in temperature (Recife 25.8 °C and Aracaju 25.6 °C) observed between the two cities indicate that humidity is a more important factor in egg desiccation than the temperature for these populations. Also, additionally, Recife, at 7 m above sea level, has a slightly higher altitude compared with Recife, at 4 m above sea level. Egg viability in both populations was shorter compared with mosquito egg viability from Manaus (north Brazil), which has a higher average environmental humidity due to rainfall throughout the year and an altitude of 92 m [38]. Elevation of altitude decreases the oxygen available for absorption by individuals [52,53], and lower quantities of dissolved oxygen in breeding sites may affect egg hatching [54–56]. Although our data originates from laboratory observations, the use of eggs from the F1-F3 generations implies that many characteristics intrinsic to each population remain in the analyzed samples.

In addition to the variation of the maximum egg quiescence period, our results also demonstrate the cost of quiescence on the rate of emergence and sexual proportion in individuals of both populations, but a gain in the duration of post-embryonic development. All these biological parameters were modified across the time of quiescence for both

mosquito populations - Recife and Aracaju. Relatively few studies have specifically addressed the effects of quiescence on the time of larval development after hatching, and divergent results have been reported. According to Silva and Silva [12], egg quiescence has no cost for development, ranging from 7.7 to 10.3 days. Conversely, Perez and Noriega [32] showed a significant increase in the duration of larval development in individuals from older eggs (7.33 days) compared with younger eggs (5.95 days), attributing such differences to a nutritional and physiological cost of quiescence time on larval development related to decreased lipids. Contrasting with these studies, we found that larvae from both populations developed 16-24h faster in older compared with younger eggs whose averages were 8.4 ± 0.18 (range of 7 – 10) and 8.7 ± 0.26 (7 – 12) days to reach adulthood in Recife and Aracaju, respectively. These results may indicate an adaptative response for recolonization after starvation. These changes could be an adaptation in response to selection that favors the rapid production of mosquitoes. Intrinsic differences of larval homeostatic control of metabolite levels and greater efficiency in decreasing the rates of energy depletion may improve survival of larvae from older eggs, as observed for *Drosophila* [57,58].

Also, such efforts seem to have no negative effects on adult formation since the time of egg quiescence increased the number of adults in the Recife population but had no influence on the Aracaju population. Considering that adult formation is an important aspect in population density, the differential impact of quiescence in adult formation between the two populations implicate a distinct contribution to population dynamics and a lesser extent, to vectorial capacity. Thus, in the wild, mosquitoes from the Recife population could achieve a 100% increase of emergent adults after 130 days, while those from the Aracaju population would achieve a smaller proportionate increase (73.6%) but in a shorter period (100 days).

Overall, in our study, the proportion of males in both populations was greater than females in most of the quiescent periods. Despite this, no significant variations in gender proportions were observed over time. There was a sexual disproportion, particularly after 70 and 100 days quiescence, resulting in an excess number of males. These results are consistent with previous reports [22,59]. Recent research has confirmed that sex determination in *A. aegypti* does not involve an XY chromosome but an endogenous meiotic driver system that can cause sexual proportion distortion with a predominance of males over females [60,61].

Contrary to our hypothesis that older eggs would require more time for rehydration and consequently a longer time to larval hatching compared with the younger eggs, the quiescence period did not affect the initial hatching time, which remained between 5.7 h and 11.7 h in both populations. In general, younger eggs tend to hatch between 5 and 20 minutes after

submersion, whereas older eggs, conditioned for months may require more than more 24 hours for the appearance of larvae [22]. Although considered long, the times observed in our study were also mentioned by Christopher [22] who noticed an irregular time for hatching that varied from 1 to 5 days or more in floating eggs before submersion in water.

The ability of *A. aegypti* eggs to resist desiccation has been assumed to contribute to the invasion and spread of this mosquito worldwide and, consequently, the arbovirus transmitted by this species [17–19,21,24]. The differential capacity of eggs to resist desiccation among populations implies a differential contribution to these events, also affecting the success of control measures. Over the last few years, huge strides have been made in reducing *A. aegypti* populations, with the creation of maps of distribution and maximum risk of their establishment [2,62]. However, lack of up-to-date information regarding the factors affecting the distribution of *Aedes* species hampers surveillance and control. The results presented here revealed that different populations of *A. aegypti* have distinct phenotypes in response to quiescence, by modifying parameters of viability, post-embryonic development time, adult emergence rate, and sexual proportion. As a consequence, quiescence may impact measures of control, population dynamics, vector capacity, and competence by re-infestation of previously-treated areas and an increase in the population density associated with a short life cycle. Additionally, our results provide information on the behavior of these populations for improvement of surveillance and control purposes, additional knowledge about egg resistance to desiccation, and provide support for new research aimed at fitness, geographic expansion, and transmission of arboviruses by *A. aegypti*.

Conclusions

In summary, our data show that out of eight parameters evaluated, four were affected by the quiescence (viability, post-embryonic developmental time, emergence rate and sexual proportion). These findings reinforce the idea that quiescent eggs are a public health problem, directly or indirectly affecting control programs, population dynamics, vector capacity, and competence of mosquitoes. Additionally, it is important for future research aimed at answering questions about the biology of *A. aegypti*, take into account the quiescence period of eggs since this factor can significantly influence these characteristics.

Author Contributions: L.O.O. designed the study, collected eggs, reared and maintained the mosquitoes colonies, performed the experiments, analyzed the data and draft the manuscript.

R.L.C. provided support for execution of experiments, assisted in the progress of the trials, analyzed the results and wrote and reviewed the manuscript. M.O.S. collected eggs, helped in the rearing and maintenance of colonies and the execution of trials, analyzed data and commented upon and improved the quality of the manuscript. C.M.R.A. conceived and designed the idea and trials, guided and followed the execution of the experiments, analyzed the data, and wrote and reviewed the final manuscript version. All authors read and approved the final manuscript.

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Conflicts of Interest: The authors declare that they have no conflict of interests.

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3 MANUSCRITO 2 – VIABILIDADE DE OVOS QUIESCENTES DE *Aedes aegypti* (DIPTERA: CULICIDAE) EXPOSTOS AO PYRIPROXYFEN NO INÍCIO DO DESENVOLVIMENTO EMBRIONÁRIO.

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Viabilidade de ovos quiescentes de *Aedes aegypti* (Diptera: Culicidae) expostos ao pyriproxyfen no início do desenvolvimento embrionário

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Resumo

Contexto: Em mosquitos, o pyriproxyfen é conhecido por afetar a metamorfose, a reprodução e inibir a emergência de adultos, além de relatos de atividade ovicida. Considerando a importância do fenômeno de quiescência de ovos de *Aedes aegypti* (Linnaeus, 1762) para a

ecologia, epidemiologia e controle dessa espécie e que, esse processo é influenciado pela formação da cutícula serosa (CS) no ovo, avaliamos se o contato de ovos com o IGR Sumilarv® 0,5 G (0,01 mg de ingrediente ativo / L) antes (2 h) e após a presença da CS (24 h), aumentaria a redução natural da viabilidade de ovos em quiescência por 10, 40 e 70 dias. As análises foram feitas com ovos de duas populações brasileiras do mosquito, oriundas de regiões de transmissão das arboviroses dengue, chikungunya e Zika (Recife e Aracaju). Em todas as situações, os ovos foram expostos por 30 min a uma solução de 0,01 mg IA / L do pyriproxyfen, antes do armazenamento. **Resultados:** Os resultados mostraram uma forte associação negativa entre período de quiescência e viabilidade, com ovos mais velhos e não tratados apresentado menores taxas de eclosão em ambas as populações. A susceptibilidade ao pyriproxyfen foi dependente da população, tendo influenciado negativamente a taxa de eclosão apenas em ovos provenientes de Aracaju com 10 e 40 dias, independente da CS. Ao contrário, exceto em um grupo (70 dias Pré-CS), baixa redução de viabilidade foi registrada nos ovos tratados de Recife comparado ao controle. Os resultados sugerem diferença interpopulacional na susceptibilidade dos ovos quiescentes de *Ae. aegypti* ao Sumilarv® 0,5 G, na concentração recomendada para seu uso em criadouros, e que a CS não é fundamental na proteção do embrião contra a ação do pyriproxyfen utilizado neste estudo.

Palavras-chave: Análogo do hormônio juvenil, controle de vetores, IGR, mosquito.

Introdução

Mosquitos do gênero *Aedes* (Diptera: Culicidae), particularmente *Aedes aegypti* (Linnaeus, 1762), estão amplamente distribuídos em regiões tropicais e subtropicais do mundo (Bhatt et al. 2013, Brown et al. 2014). A capacidade dos ovos em resistir à dessecação por longos períodos de tempo (quiescência) tem sido um fator decisivo na disseminação passiva dessa espécie, permitindo seu transporte à longas distâncias (Gubler 1998, Forattini 2002, Lounibos 2002, Diniz et al. 2017). Predições baseadas em modelos matemáticos sugerem que ovos quiescentes podem contribuir para o restabelecimento das populações de *Ae. aegypti* em áreas tratadas, além de aumentar o *fitness* da população de mosquitos, dificultando as medidas de controle (Yang 2014). Epidemiologicamente, o processo de quiescência pode ter implicações na transmissão de arboviroses como dengue, chikungunya e Zika, que têm *Ae. aegypti* como principal vetor. Além disso, a sobrevivência de embriões infectados nos ovos resistentes à dessecação pode favorecer a manutenção dos vírus na

população de vetores (Rosen 1987, Consoli and Oliveira 1994, Yang 2014). Bloquear o ciclo de vida do mosquito na fase de ovo seria, portanto, um método eficiente no controle populacional e na interrupção da transmissão de doenças (Farnesi et al. 2017), contudo, esse tem sido um desafio de pouco sucesso.

Atualmente, a maioria das ações de controle de *Ae. aegypti* são focadas nas fases de larva e adulto. Além disso, a eliminação dos ovos do ambiente é de grande dificuldade para os programas de controle, principalmente devido ao comportamento que essa espécie tem de distribuir a sua postura em vários sítios de oviposição (REF). Apesar disso. No entanto, o uso intensivo e prolongado de inseticidas, principalmente organofosforados e piretróides, diminuiu significativamente sua eficácia, culminando na seleção de resistência em várias populações desse mosquito (Diniz et al. 2015, Dolabella et al. 2016, Viana-Medeiros et al. 2017, La Corte et al. 2018). Como alternativa, inseticidas reguladores de crescimento de insetos (IGR, do inglês "*Insect Growth Regulator*") têm sido utilizados com a vantagem de serem mais específicos e, relativamente, pouco tóxicos ao ambiente (Pener; Dhadialla, 2012) quando comparado àqueles usados anteriormente, tais como o temefós. Dentre esses, o pyriproxyfen (análogo do hormônio juvenil) é recomendado pela OMS (2001) para uso no controle de mosquito, com a vantagem de poder ser dispersado para diferentes criadouros através das patas contaminadas das próprias fêmeas dos mosquitos (Devine et al. 2009, Gaugler et al. 2012), podendo assim atingir locais não identificados pelo homem. Desse modo, por apresentar potencial ação negativa sobre o ovo (Vasuki 1990, Suman et al. 2013) o pyriproxyfen se constitui em importante ferramenta de estudo para o controle de ovos nos criadouros, incluindo ovos quiescentes.

Em *Ae. aegypti*, tanto a resistência à dessecação (Rezende et al. 2008, Vargas et al. 2014, Farnesi et al. 2015) quanto a ação ovicida de produtos inseticidas (Vasuki 1990, Suman et al. 2013) tem se mostrado fortemente associada com a formação da cutícula serosa (CS) durante a embriogênese entre 13-15h após a postura (Rezende et al. 2008) quando o embrião atinge cerca de 18-21% de sua formação (Vargas et al. 2014). A CS protege o embrião prevenindo sua desidratação, minimizando a perda de água para o ambiente externo, fazendo com que sua viabilidade seja mantida por longos períodos de tempo (Harwood and Horsfall 1959, Kliewer 1961). Por ser protetora do embrião, e sendo ela uma barreira física e química do ovo (Rezende et al. 2008, Suman et al. 2013, Farnesi et al. 2015), é possível que a CS influencie negativamente na eficácia ovicida de muitos produtos, reduzindo o seu contato com o embrião (Farnesi et al. 2017).

Nesse trabalho avaliamos se a viabilidade de ovos quiescentes de *Ae. aegypti* seria alterada pelo contato com o pyriproxyfen pré (até 2h) e pós formação da CS (24h). Para facilitar o entendimento, a partir desse ponto, esses grupos serão referidos com Pré-CS (2h) e Pós-CS (24h). Considerando que variações genéticas e pressões ambientais seletivas são intrínsecas a cada população, avaliamos também se a resposta ao pyriproxyfen era dependente da população avaliada. Testamos as hipóteses de que (1) o contato dos ovos com o pyriproxyfen afeta negativamente a viabilidade dos embriões, sendo seu efeito maior naqueles que foram expostos antes da formação da CS; (2) Acreditamos ainda que esse efeito é permanente e que atue sinergicamente com o decréscimo natural da viabilidade de ovos envelhecidos, reduzindo significativamente a eclosão de ovos quiescentes expostos antes da formação da CS e que (3) características intrínsecas de cada população decorrentes de variações de pressão de seleção ambiental levarão a uma resposta diferencial à exposição aos inseticidas.

Material e métodos

Mosquitos

Amostras de ovos das gerações F2 das populações de *Ae. aegypti* provenientes das cidades de Recife - PE (08 ° 03 ' 03 ” S - 34 ° 56 ' 54 ” W) e Aracaju - SE (10º 54' 40” S - 37º 04 '18 ” W) foram usadas nesse trabalho. Ovos da geração parental foram coletados entre abril e junho de 2018. A formação e manutenção das colônias dos mosquitos seguem o descrito em Oliva et al. (2018). As larvas foram criadas em bandejas com água destilada e alimentadas com ração para peixes (Tetra® marine XL Granules, Melle, Alemanha). Pupas foram separadas em copos descartáveis com água e colocadas em gaiolas de criação. Os adultos obtidos foram mantidos com solução de sacarose 10% (fonte energética). Fêmeas foram alimentadas com sangue de carneiro, para o processo de ovogênese, e permitidas ovipositar em papel filtro umedecido com água (substrato de postura).

Ambas as cidades são áreas de transmissão autóctone das arboviroses dengue, chikungunya e Zika. De acordo com o sistema de classificação de Köppen-Geiger o clima é do tipo tropical de monções (am) cuja temperatura do mês mais frio é > 18 °C e a precipitação no mês mais seco é > 100 mm (Peel et al. 2007). De modo geral, a cidade de Recife apresenta como médias anuais uma temperatura de 25,9 ºC e pluviometria de 1.800 mm (Prefeitura da Cidade de Recife), com população estimada em 2017 de 1.633,697 habitantes (IBGE 2018). O

município de Aracaju registra ao longo do ano temperatura média de 26,0 ºC e precipitação média de 1.590 mm (Prefeitura de Aracaju), sendo sua população estimada em 650.106 pessoas em 2017 (IBGE 2018). De acordo com o PNCD (Plano Nacional Controle da Dengue), enquanto os criadouros de *Ae. aegypti* da população de Recife são tratados apenas com o biolarvicida Bti desde 2002, nos de Aracaju é aplicado o IGR pyriproxyfen desde 2014.

Inseticida

Neste estudo foi usado um produto a base de pyriproxyfen (Sumilarv® 0,5 G, Sumitomo Chemical Co., Tóquio, Japão), registrado sob o número 3.2586.0009.001-1 na ANVISA / Ministério da Saúde do Brasil (MS), doado pela Secretaria Municipal da Saúde de Aracaju. O produto foi usado na dosagem recomendada pelo MS para controle de *Ae. aegypti* em condições de campo (0,01 mg de ingrediente ativo / L).

Efeito do pyriproxyfen na viabilidade de ovos quiescentes e o papel da CS neste processo

O efeito do pyriproxyfen foi avaliado em ovos quiescentes (10, 40 e 70 dias de armazenamento) tratados com Sumilarv® 0,5 G, no início da embriogênese: até 2 h após a oviposição (Pré-CS) e 24h (Pós-CS). Papel de filtro contendo posturas para cada condição dos grupos citados foram expostos a 20 mL de solução larvicida em placas de Petri por 30 min, sendo em seguida lavados três vezes consecutivas com 40 mL de água de osmose reversa. Como tratamento controle, para cada grupo, foi usado um lote de ovos expostos a água, sem qualquer outro tratamento. Após esse procedimento, sob microscópio estereomicroscópio, três grupos de 30 ovos foram transferidos, com o auxílio de um pincel, para um novo papel de filtro (1 x 1 cm) umedecido com água de osmose reversa. Esses ovos foram armazenados, separadamente, em recipientes plásticos cobertos com tecido tipo *voil*, secando naturalmente, por 10, 40 e 70 dias para os estudos de quiescência. Para cada tempo de armazenamento, em cada uma das populações (Recife e Aracaju), réplicas foram executadas em triplicatas (n = 90) e repetidas em três dias diferentes (n = 270), totalizando 810 ovos/ população.

As condições ambientais foram mantidas em 26 ± 1 ° C, 80 ± 5% UR e fotoperíodo de 12:12 [claro: escuro] h. Após cada um dos períodos de quiescência, os ovos foram imersos em 180 mL de água de osmose reversa para a eclosão das larvas. A viabilidade foi medida como a porcentagem de eclosão ao final de sete dias de observação, após contato dos ovos com a

água. Reduções na viabilidade dos grupos tratados em comparação ao controle foram expressas com base na seguinte fórmula (WHO 2009, Ducheyne et al. 2018).

$$R = \left(1 - \frac{T}{C}\right) * 100$$

Onde: R é a redução da viabilidade dos grupos tratados em relação ao controle, T e C referem-se ao percentual de eclosão dos grupos tratado e controle, respectivamente.

Para calcular as reduções na viabilidade entre os grupos tratados, dentro de cada período, a diferença entre os valores de R foram usadas.

Análises estatísticas

A normalidade de distribuição e homogeneidade de variância dos dados foram verificadas pelos testes de Shapiro-Wilk e Bartlett, respectivamente. As médias apresentadas são seguidas pelo intervalo de confiança de 95% e dos valores mínimo e máximo. O teste não-paramétrico de Kruskal-Wallis, com o Student-Newman-Keuls (SNK) *a posteriori* foi usado para avaliar a influência do inseticida sobre a taxa de eclosão dos ovos (viabilidade). Regressão linear foi usada para descrever a associação entre idade e taxa de eclosão ao longo do período de quiescência. Todas as análises foram feitas no programa BioEstat (Ayres et al. 2014), versão 5.3 para Windows, com nível de significância de $p \leq 0,05$.

Resultados

Eclosão em ovos quiescentes das populações de Recife e Aracaju, pré e pós CS, sem exposição ao pyriproxyfen

Ovos das duas populações reduziram a viabilidade com o envelhecimento progressivo, apresentando uma significante associação linear negativa entre idade dos ovos e porcentagem de eclosão. Isso ocorreu tanto nas amostras de ovos com 2 h (Recife, Pré-CS: $F = 54.1483$; $p < 0,0001$; Aracaju, Pré-CS: $F = 152.8788$; $p < 0,0001$) quanto nos grupos com 24 h (Recife, Pós-CS: $F = 159.4366$; $p < 0,0001$; Aracaju, Pós-CS: $F = 155,6308$; $p < 0,0001$) (Figura 1). O percentual de explicação das variações nas taxas de eclosão associado ao período de

estocagem (R^2) foi de 69% (2 h) e 86% (24 h) para Recife, e de 85% para Aracaju com 2 ou 24h.

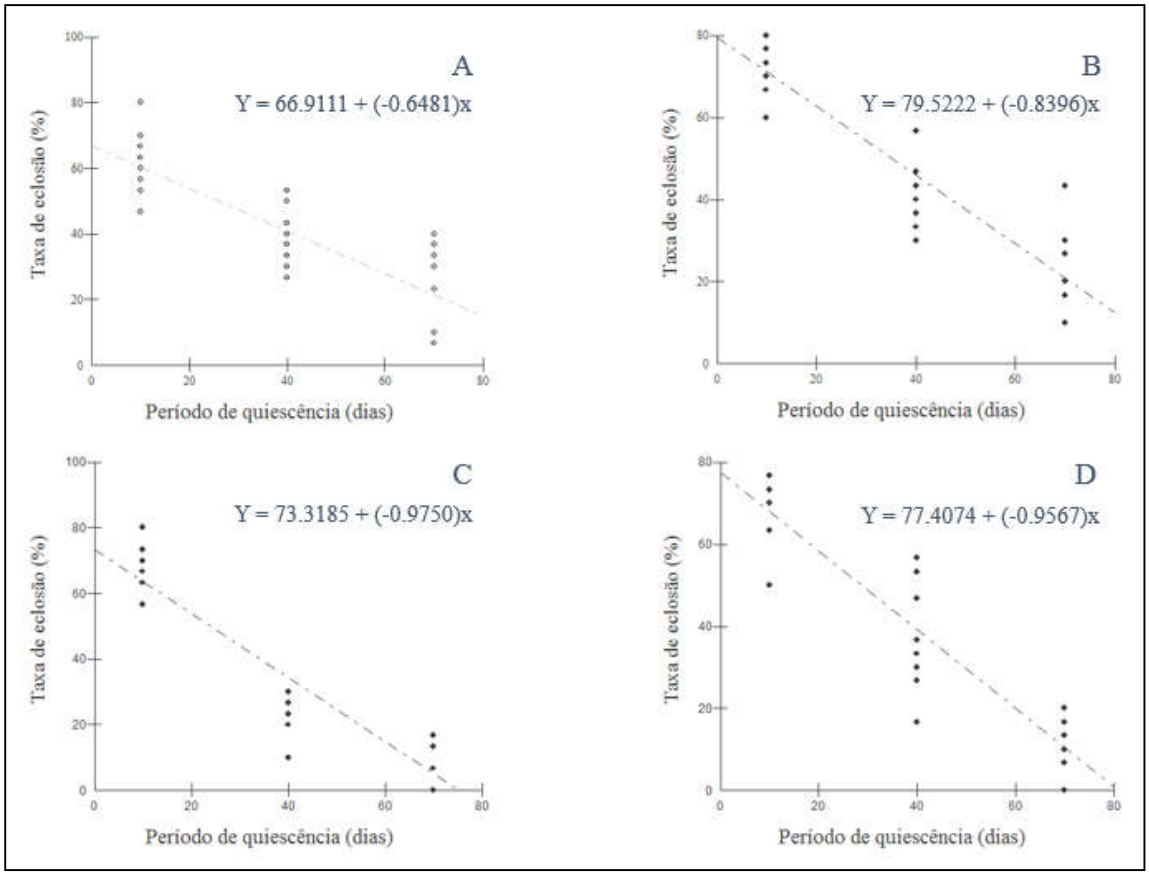

Figura 1. *Scatterplot* das taxas de eclosão (%) em ovos quiescentes de *Aedes aegypti* nas populações de Recife (A e B) e Aracaju (C e D). Reduções em ovos com 2 h (esquerda) e com 24 h (direita). Cada ponto corresponde a três replicatas.

Em ambas as populações, a taxa média de eclosão registrou percentuais similares nas comparações pareadas de cada período de quiescência quando os ovos foram armazenados com 2h (Pré-CS) ou 24h (Pós-CS) após a postura (Tabela 1).

Tabela 1. Viabilidade (%) de ovos quiescentes (média ± intervalo de confiança 95% seguidos de variação) de *Aedes aegypti* nas populações de Recife e Aracaju, expostos ao pyriproxyfen, antes e após a formação da cutícula serosa (CS).

Período de quiescência (dias)	População							
	Recife				Aracaju			
	Controle		Pyriproxyfen		Controle		Pyriproxyfen	
	Pré-CS	Pós-CS	Pré-CS	Pós-CS	Pré-CS	Pós-CS	Pré-CS	Pós-CS
10	$61,1 \pm 7,8^{a,A}$ (46,7 - 80,0)	$73,0 \pm 4,9^{a,A}$ (60,0 - 80,0)	$57,4 \pm 15,6^{a,A}$ (23,3 - 83,3)	$61,9 \pm 12,2^{a,A}$ (33,3 - 83,3)	$68,9 \pm 5,4^{a,A}$ (56,7 - 80,0)	$69,3 \pm 6,4^{a,A}$ (10,3 - 50,0)	$17,4 \pm 5,3^{b,A}$ (6,7 - 30,0)	$35,2 \pm 12,0^{b,A}$ (10,0 - 56,7)
40	$39,6 \pm 6,8^{a,B}$ (26,7 - 53,3)	$42,2 \pm 6,3^{a,B}$ (30,0 - 56,7)	$29,6 \pm 8,9^{a,A}$ (13,3 - 50,0)	$35,9 \pm 15,5^{a,B}$ 10,0 - 63,3)	$23,7 \pm 5,0^{a,c,B}$ (10,0 - 30,0)	$36,3 \pm 10,3^{a,B}$ (16,7 - 56,7)	$14,8 \pm 5,8^{b,A}$ (0,0 - 23,3)	$22,2 \pm 6,4^{b,c,A}$ (6,7 - 36,7)
70	$22,2 \pm 10,2^{a,B}$ (6,7 - 40,0)	$22,6 \pm 7,5^{a,C}$ (10,0 - 43,3)	$7,0 \pm 3,9^{b,B}$ (0,0 - 16,7)	$12,6 \pm 4,0^{a,b,C}$ (6,7 - 23,3)	$10,4 \pm 4,3^{a,C}$ (0,0 - 16,7)	$11,9 \pm 4,6^{a,C}$ (0,0 - 20,0)	$4,4 \pm 2,6^{b,B}$ (0,0 - 10,0)	$7,0 \pm 3,7^{a,b,B}$ (0,0 - 13,3)

O número total de réplicas (n = 09), bem como a quantidade de ovos analisada em cada réplica (n = 30) foram os mesmos em todos os grupos e períodos. Letras minúsculas, após as médias, indicam comparações de valores (teste de Kruskal-Wallis, com o teste de Student-Newman-Keuls *a posteriori*, $p \leq 0,05$) nas linhas horizontais para cada uma das populações separadamente, enquanto que, letras maiúsculas se referem a comparações de valores (teste de Kruskal-Wallis, com o teste de Student-Newman-Keuls *a posteriori*, $p \leq 0,05$) ao longo do período de quiescência, em cada uma das linhas verticais. Valores identificados pela mesma letra não são significativamente diferentes. Pré-CS = exposição dos ovos antes da formação da cutícula serosa (duas horas pós-oviposição); Pós-CS = exposição dos ovos após a formação da cutícula serosa (24 horas pós-oviposição).

Eclosão em ovos quiescentes das populações de Recife e Aracaju, pré e pós CS, com exposição ao pyriproxyfen

Assim como aconteceu nos ovos sem tratamento, reduções significativas na média de eclosão foram observadas ao longo do tempo de quiescência para todos os grupos, em cada uma das populações (Recife, Pré-CS: H = 19,9334; gl = 2; p = 0,0000; Recife, Pós-CS: H = 16,4257; gl = 2; p = 0,0003; Aracaju, Pré-CS: H = 13,2419; gl = 2; p = 0,0013; Aracaju, Pós-CS: H = 16,0017; gl = 2; p = 0,0003) (Tabela 1).

Na população de Aracaju, reduções significantes na eclosão em função do contato com o pyriproxyfen foram registradas aos 10 dias (H = 27,3068; gl = 3; p = 0,0000) e 40 dias (H=15,2624; gl=3; p=0,0016), tanto na ausência quanto na presença da CS (10 dias- Pré-CS, p < 0,0001; Pós-CS, p = 0,0027) e 40 dias (Pré-CS, p < 0,0365; Pós-CS, p = 0,0260). Ao contrário, na população de Recife, a taxa média de eclosão em cada período de quiescência após tratamento foi similar aos respectivos controles, independente da CS (Tabela 1), exceto com 70 dias (H = 14,6880; gl = 3; p = 0,0002), com reduções significantes no grupo onde a CS estava ausente (p = 0,0038).

Comparando-se as duas populações, ovos dos mosquitos provenientes de Aracaju foram mais sensíveis à exposição ao pyriproxyfen (Tabela 1), apresentando menores médias de eclosão nos grupos tratados, particularmente em ovos com 10 (Pré-CS, U = 2,5; p = 0,0008; Pós-CS, U = 8,5; p = 0,0047) e 40 dias (Pré-CS, U = 2,5; p = 0,0008; Pós-CS, U = 8,5; p = 0,0047). Essa população atingiu índices de redução da eclosão de 69% (sem CS) e 34% (com CS). Na população de Recife, a redução de eclosão em função da quiescência variou de 6% a 68% nos ovos tratados antes da CS e 15% a 44% nos expostos ao pyriproxyfen após a CS.

Discussão

A resistência dos ovos de *Ae. aegypti* a longos períodos de dessecação representa um desafio para os programas de controle por facilitar a dispersão dessa espécie. Produtos reguladores do crescimento de insetos (IGRs) como o pyriproxyfen, têm sido preconizados pela Organização Mundial da Saúde (OMS) como métodos de controle adicionais aos inseticidas químicos convencionais, devido aos altos níveis de resistência ao temefós e organofosforados desenvolvidos por essa espécie de mosquito (Viana-Medeiros et al. 2017, La Corte et al. 2018). Avaliando o efeito do pyriproxyfen (Sumilarv® 0,5 G) na viabilidade

de ovos quiescentes (10, 40 e 70 dias) e a importância da CS nesse processo em duas populações brasileiras de *Ae. aegypti* (Recife e Aracaju), nós encontramos uma sensibilidade diferencial ao produto nas populações analisadas e ausência de proteção da CS.

De um modo geral registramos viabilidade de ovos quiescentes em todos os períodos analisados com uma associação negativa entre o período de estocagem e a taxa de eclosão. Isso ocorreu em ambos, ovos que tiveram contato ou não com o pyriproxyfen com reduções no número de larvas ao longo do tempo. Contudo, nossa premissa de que o dano causado pelo pyriproxyfen no início da embriogênese (Pré CS) maximizaria a redução natural da viabilidade dos ovos com a quiescência só foi corroborada para ovos da população de Aracaju, , particularmente em ovos com 10 e 40 dias. A redução natural na viabilidade de ovos quiescentes de *Ae. aegypti* tem sido um resultado descrito por vários autores (Christophers 1960, Clements 1992, Silva and Silva 1999, Vinogradova 2007, Rezende et al. 2008, Brown et al. 2017, Oliva et al. 2018). No entanto, não encontramos relatos na literatura sobre o efeito de inseticidas na viabilidade de ovos quiescentes. Comumente, a resistência a dessecação tem sido fortemente relacionada com a formação da CS no início da embriogênese (13-15h), reduzindo a perda de água pelo embrião permitindo a sobrevivência em ambientes em condições de dessecação por longos períodos (Rezende et al. 2008, Vargas et al. 2014, Farnesi et al. 2015). No entanto, a alta taxa de eclosão obtida em nosso trabalho em ovos armazenados em ambientes secos por 10, 40 e 70d após 0-2 h de oviposição e, portanto, quando a CS ainda não estava presente nos ovos, sugere que outros fatores estejam envolvidos na proteção do embrião contra dessecação. Isso corrobora estudos que mostram a importância de vários outros fatores no sucesso da eclosão de larvas provenientes dos ovos de *Ae. aegypti* como alta umidade do ambiente (Kliewer 1961, Costa et al. 2010) e a melanização do ovo (Farnesi et al. 2017). Além disso, como estratégia de sobrevivência a longos períodos de dessecação, ovos de *Ae. aegypti* são capazes de modular seu metabolismo para resultar em elevadas taxas de sobrevivência (da Silva et al. 2018).

A relação entre CS e ação de IGRs ainda é pouco conhecida. No entanto, a exposição de ovos em diferentes momentos da embriogênese (correspondentes a pré e pós formação da CS), a produtos como triflumuron, diflubenzuron e pyriproxyfen tem apresentado reduções diferenciais nas taxas de eclosão, afetando principalmente ovos mais jovens (Vasuki 1990, Suman et al. 2013). Expondo ovos de *Ae. aegypti* (0-1h e 12-18h) a concentrações de 0.1 ppb do pyriproxyfen (OMS 3019- ethoxypyridine) registrou inibição de eclosão mais acentuada em ovos mais jovens. Resultado similar foi encontrado na população de *Ae. aegypti* de Aracaju em nosso trabalho com o uso de Sumilarv®, na mesma concentração. Contudo, ao

contrário do observado nessa população, a viabilidade da grande maioria dos ovos quiescentes provenientes de mosquitos da cidade de Recife não foi afetada pelo contato com o pyriproxyfen, evidenciando uma resposta populacional diferenciada. Esse resultado difere, não apenas dos achados de Vasuki (1990), mas também dos obtidos por Suman et al. (2013), em ovos de diferentes espécies de *Aedes*, expostos a 1,0 ppm de pyriproxyfen durante a postura e após 48h da oviposição. Esses autores mostraram que, de modo geral, ovos recém-embrionados apresentavam menor eclosão larval e sugeriram que o pyriproxyfen altera o equilíbrio hormonal afetando o desenvolvimento do embrião se constituindo na principal causa da redução na eclosão larval. Esses autores também encontraram relação entre dose do IGR e eclosão. A taxa similar de eclosão dos ovos de *Ae. aegypti* na presença e ausência da CS em nossos resultados sugere que essa matriz não é fundamental para proteção do embrião contra o pyriproxyfen, embora efeitos deletérios em sua sobrevivência tenham sido observados, nas condições analisadas, Estudos mais detalhados são necessários para saber se dosagens mais elevadas teriam papel ovicida como sugerido por Vasuki (1990) que registrou um efeito dose dependente do pyriproxyfen sobre ovos de *Ae. aegypti*, ou se corrobora os resultados de Sihuincha et al. (2005) que não registrou efeito inibitório desse IGR na eclosão das larvas dessa espécie no Peru, mesmo em concentração > 30,000 ppb.

A diferença interpopulacional na sensibilidade dos ovos de *Ae. aegypti* ao pyriproxyfen encontradas nesse trabalho entre as populações de Recife e Aracaju, amplia o número de variáveis biológicas que podem ter um papel nas diferenças encontradas entre elas. Em estudo anterior, com as mesmas populações de mosquitos, Oliva et al. (2018) demostraram diferenças nos efeitos da quiescência sobre vários parâmetros biológicos importantes para a capacidade vetorial e dinâmica populacional desse mosquito. Nesse estudo, o custo da quiescência foi maior para a população de Aracaju do que Recife que apresentou menor período de quiescência, menor taxa de emergência de adultos e maior tempo de desenvolvimento pós-embrionário. Esses dados sugerem que populações distintas possuem especificidades importantes que podem influenciar e / ou modular determinadas estratégias de controle.

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Conflito de Interesses

Autores declaram que não há conflito de interesses.

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4 MANUSCRITO 3 – PERFIL DA EXPRESSÃO DO GENE Q*UITINA SINTASE* (*AACHS1A*) DURANTE O PROCESSO DE FORMAÇÃO DA CUTÍCULA SEROSA EM OVOS DE *Aedes aegypti* (DIPTERA: CULICIDAE).

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Perfil da expressão do gene *quitina sintase* (*AaCHS1a*) durante o processo de formação da cutícula serosa em ovos de *Aedes aegypti* (Diptera: Culicidae)

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Resumo

Contexto: A aquisição da resistência à dessecação em ovos de *Aedes aegypti* (L.) tem relação direta com a formação da cutícula serosa (CS), mas até o momento, a base genética envolvida na formação dessa estrutura em mosquitos é pouco conhecida. O presente estudo teve como objetivo avaliar o perfil da expressão do gene *quitina sintase* (*AaCHS1a*) envolvido na síntese de quitina na CS, durante o período de formação dessa estrutura em ovos de *Ae. aegypti* e a ocorrência de variação interpopulacional. Para tanto, ovos de *Ae. aegypti* provenientes das cidades brasileiras de Aracaju e Recife foram coletados em diferentes intervalos de tempo após a postura: precoce (6 – 9 h), médio (11 – 13 h) e tardio (15 – 18 h). As análises de expressão gênica foram realizadas por PCR em tempo real (qPCR), utilizando como controles endógenos os genes constitutivos *Rp49* e *ACT* **Resultados:** a expressão do gene *AaCHS1a* foi detectada em ambas as populações de mosquitos, embora tardiamente no primeiro tempo de desenvolvimento (6 – 9 h). A maior quantidade de transcritos foi observada com 15 a 18 h após a oviposição, sendo os valores similares para Aracaju e Recife. **Conclusões:** O gene *AaCHS1a* é expresso durante a formação da CS e o padrão de expressão é similar entre as amostras das populações de Aracaju e Recife, sugerindo que diferenças interpopulacionais podem não ser determinantes nesse processo.

Palavras-chave: Dessecação, desenvolvimento, embriogênese, mosquito, quiescência.

Introdução

Aedes aegypti é o principal transmissor de inúmeras arboviroses de importância médica em áreas tropicais e subtropicais (Kraemer et al. 2015), entre elas a dengue, chikungunya e Zika (Rückert et al. 2017). Seus ovos possuem a capacidade de resistir à dessecação (quiescência), sendo um mecanismo de sobrevivência em condições adversas (Christophers 1960, Clements 1992). Os ovos quiescentes podem permanecer viáveis por longos períodos de tempo (Silva and Silva 1999, Faull and Williams 2015), podendo as larvas eclodirem quando condições ambientais forem favoráveis, reinfestando áreas anteriormente tratadas, dificultando as medidas de controle do mosquito (Yang 2014).

Estudos mostram que a aquisição da resistência à dessecação em ovos de *Ae. aegypti* tem relação direta com a formação da cutícula serosa (CS), uma matriz extracelular sintetizada entre 13 e 15 h após a oviposição (Rezende et al. 2008, Farnesi et al. 2017). Pouco

se sabe sobre a base genética envolvida na formação da CS em mosquitos, e como variações intraespecíficas podem atuar nesse processo. Para *Ae. aegypti*, o gene *quitina sintase* (*AaCHS1a*) foi implicado na formação da CS (Rezende et al. 2008), sendo responsável pela síntese de quitina nessa estrutura (Moussian et al. 2005).

Dessa forma, o presente estudo teve como objetivo avaliar o perfil de expressão do gene *AaCHS1a* durante o período de formação da CS de *Ae. aegypti*, em diferentes populações do mosquito. Assumimos a hipótese de que o perfil de expressão do gene *AaCHS1a* será diferente entre as populações analisadas, uma vez que o comportamento biológico entre elas se mostrou contrário em vários aspectos (Oliva et al., 2018).

Material e métodos

Ovos de *Ae. aegypti* (geração F1-F3), provenientes das colônias de Aracaju (10°54'40" S; 37°04'18" O) e Recife (08°03'03" S; 34°56'54" O) mantidas no Laboratório de Entomologia e Parasitologia Tropical da Universidade Federal de Sergipe, foram usados nesse estudo. As cidades são áreas de transmissão autóctone das arboviroses dengue, chikungunya e Zika, sendo o clima de ambas classificado pelo sistema de Köppen-Geiger como tropical de monções (Peel et al. 2007). Para a obtenção dos ovos, foi adotada a metodologia descrita em Oliva et al. (2018). Brevemente, cartelas (papéis de filtro) contendo ovos foram submersas em água destilada para eclosão das larvas, que foram alimentadas com ração para peixe (Tetra® Marine XL Granules, Melle, NI, Germany), os adultos (machos e fêmeas) foram mantidos com solução de sacarose a 10%, e as fêmeas receberam também alimentação sanguínea (sangue de carneiro). Substratos de oviposição constituídos por papel filtro umedecido em água, e colocados em copos plásticos descartáveis (50 mL), foram disponibilizados às fêmeas para postura. Os experimentos foram realizados em condições controladas de 26 ± 1 ºC, 80 ± 5% UR e fotofase de 12 horas. Os ovos foram coletados em intervalos de tempo denominados precoce (6 – 9 h), médio (11 – 13 h) e tardio (15 – 18 h) em relação ao tempo de formação da CS (Rezende et al., 2008). *Pools* de ovos (200 a 500) foram transferidos, com o auxílio de um pincel, do substrato de postura para microtubos (1,5 mL) onde, posteriormente, foram feitas as extrações do RNA.

Os genes usados neste estudo foram selecionados com base na literatura e no banco de dados GenBank® (http://www.ncbi.nlm.nih.gov). O gene alvo *quitina sintase* (*AaCHS1*) codifica a enzima quitina sintase responsável pela síntese de polímeros de quitina na CS de

Ae. aegypti (Rezende et al. 2008). Os genes constitutivos *Rp49* (Gentile et al. 2005) e *ACT* (Dzaki et al. 2017) atuam na constituição estrutural do ribossomo e na formação dos filamentos de actina no músculo, respectivamente, e foram usados como controles endógenos para as análises de PCR em tempo real (qPCR). Todos os genes, números de acesso, sequências de *primers* e tamanho dos *amplicons* foram listados na Tabela 1.

Tabela 1. Genes e respectivos *primers* utilizados para análise dos seus níveis da expressão em ovos de *Aedes aegypti* por qPCR.

Gene	ID GenBank	Sequência dos *primers*	Tamanho do amplicon (pb)
AaCHS1a (*Quitina sintase*)	XM_021849205.1	F 5′ CAAGGACAATATCCACGTCAAG 3’ R 5′ AGGCCAAGATATGCGACAG 3’ (Rezende et al. 2008)	209
Rp49 (*Gene da proteína ribossômica 49*)	XM_001656684.2	F 5′ GCTATGACAAGCTTGCCCCCA 3’ R 5′ TCATCAGCACCTCCAGCTC 3’ (Gentile et al. 2005)	189
ACT (*Actina-1*)	AAEL011197	F 5′ CGTTCGTGACATCAAGGAAA 3’ R 5′ GAACGATGGCTGGAAGAGAG 3’ (Dzaki et al. 2017)	175

ID = Identificador do gene no banco de dados GenBank.

O RNA total dos ovos (200-500 ovos / amostra) de *Ae. aegypti* foi extraído com TRIzol@ (Invitrogen™, Ambion™, Life Technologies, Carlsbad, CA, USA), de acordo com o fabricante, sendo o seu volume ressuspendido em 80 µL de água DEPC livre de RNAse. Após a extração, o RNA foi quantificado em espectrofotômetro (BioTec™ Epoch, Winooski, VT, USA) e a qualidade avaliada pelas razões de absorbância 260:280 e 260:230. A integridade do RNA foi verificada em gel de agarose 1% em tampão TAE (1x), corado com brometo de etídio. Para a síntese do cDNA, foi usado o kit *High-Capacity cDNA Reverse Transcription* (Applied Biosystems™, Foster, CA, USA), com ~ 1 µg de RNA (normalização feita usando uma concentração de 100 ng / µL) em 20 µL da reação de transcriptase reversa (RT-PCR), seguindo o protocolo do fabricante. *Primers* para as reações de qPCR foram otimizados e validados pelo método da curva de dissociação, usando diluições seriadas do cDNA a ~ 50 ng, com fator 2 (1:1, 1:2, 1:4, 1:8, 1:16), em duas concentrações de *primers* (0,4 e 0,5 µM). O *mix* das reações foi feito utilizando 2x *Power SYBR Green PCR Master Mix* (Applied Biosystems™), *primers* conforme concentração descrita acima (0,4 µM) e cDNA (1:4), em um volume final de 10 µL. Em cada uma das reações foram acrescentados três *mixes* com água que serviram como controle negativo.

As reações foram feitas em quadruplicatas utilizando o equipamento de PCR em Tempo Real *StepOnePlus*™ (Applied Biosystems™), submetidas a um ciclo de 95 °C por 10 minutos, seguidos por 40 ciclos de 95 °C a 15 segundos e 59 °C por 1 minuto. A curva de Melting foi feita para confirmação da especificidade dos *primers*, usando a programação de 95 °C por 15 segundos, 59 °C por 1 minuto e 95 °C a 15 segundos. Resultados da qPCR foram expressos em valor de C_T e apresentados em tabela como média seguida de desvio padrão. O método comparativo $2^{-\Delta\Delta CT}$ (Livak and Schmittgen 2001) foi usado para determinar as alterações relativas nos níveis de expressão gênica de *AaCHS1a* nas populações de *Ae. aegypti* (Recife e Aracaju). Para a normalização desses níveis em cada população, os genes de referência *Rp49* e *ACT* foram usados como controles internos.

Resultados

O gene *AaCHS1a* apresentou expressão nas duas populações de *Ae. aegypti*, tendo iniciado tardiamente sua expressão (valores de Ct acima de 32) no primeiro tempo de desenvolvimento (6 – 9 h) (Tabela 2). Maior quantidade de transcritos foi observado associado ao tempo 3 (15 – 18 h), sendo os valores de Ct aproximadamente iguais para Aracaju e Recife, o que pode indicar que a completa formação da CS nessas populações ocorre pelo menos 18 h após a postura dos ovos (Tabela 2). Os genes endógenos *Rp49 e ACT* apresentaram pouca variação nos tempos de desenvolvimento na população de Recife, enquanto que na de Aracaju a variação foi maior (Tabela 2).

Tabela 2. Valores de C_T médio e desvio padrão (DP) referentes à expressão dos genes alvo (*AaCHS1a*) e de referência (*Rp49* e *ACT*) determinados por qPCR, a partir dos ovos de *Aedes aegypti* das populações de Aracaju e Recife, em três diferentes tempos do desenvolvimento embrionário.

Gene	Tempo de desenvolvimento embrionário	População			
		Recife		Aracaju	
		C_T Médio	DP	C_T Médio	DP
AaCHS1a (*Quitina Sintase*)	T1	32,75	0,39	32,86	0,31
	T2	28,02	0,11	0,00*	0,00
	T3	25,86	0,04	26,52	0,30
Rp49 (*Proteína ribossomal 49*)	T1	16,95	0,03	17,89	0,17
	T2	16,34	0,29	20,76	0,18
	T3	16,71	0,19	18,14	0,12
ACT (*Actina1*)	T1	16,85	0,18	18,31	0,09
	T2	16,73	0,10	21,01	0,10
	T3	16,94	0,16	19,93	0,14

T1 = 6 a 9 h pós-oviposição; T2 = 11 a 13 h pós-oviposição; T3 = 15 a 18 h pós-oviposição.
* Possível falha metodológica, havendo necessidade de repetição.

O perfil de expressão para o gene *AaCHS1a* em nosso estudo, seguiu o mesmo comportamento, independente da população de origem dos ovos, aumentando a expressão do gene alvo ao longo do tempo de desenvolvimento embrionário, evidenciado pela diminuição do valor médio de C_T (Tabela 2). Não foi observada, porém, expressão deste gene no T2 na população de Aracaju. Ovos de Recife, de modo contrário, exibiram a presença de transcritos, não seguindo o mesmo padrão de Aracaju para esse tempo (Tabela 2). Além disso, embora os genes de referência em Aracaju tenham se expressado no T2, não houve uma forte estabilidade como a observada em Recife, variando nos três tempos analisados (Tabela 2). De modo contrário, ovos de Recife apresentaram expressão do gene *AaCHS1a* no T2, aumentando gradativamente a expressão do gene alvo entre os tempos T1 a T3 (Tabela 2).

Em cada tempo de desenvolvimento, embora a expressão do gene alvo a partir dos ovos de Aracaju e Recife tenha ocorrido de forma similar nos períodos analisados, níveis de expressão maiores foram observados nos ovos da população de Aracaju (Tabela 2), registrando duas vezes mais transcritos em T1 e quase três vezes mais em T3 (Figura 1). Quando T3 foi relacionado com T1, o nível de expressão de *AaCHS1a* foi 26% maior nos ovos de Aracaju em comparação a Recife (Figura 2).

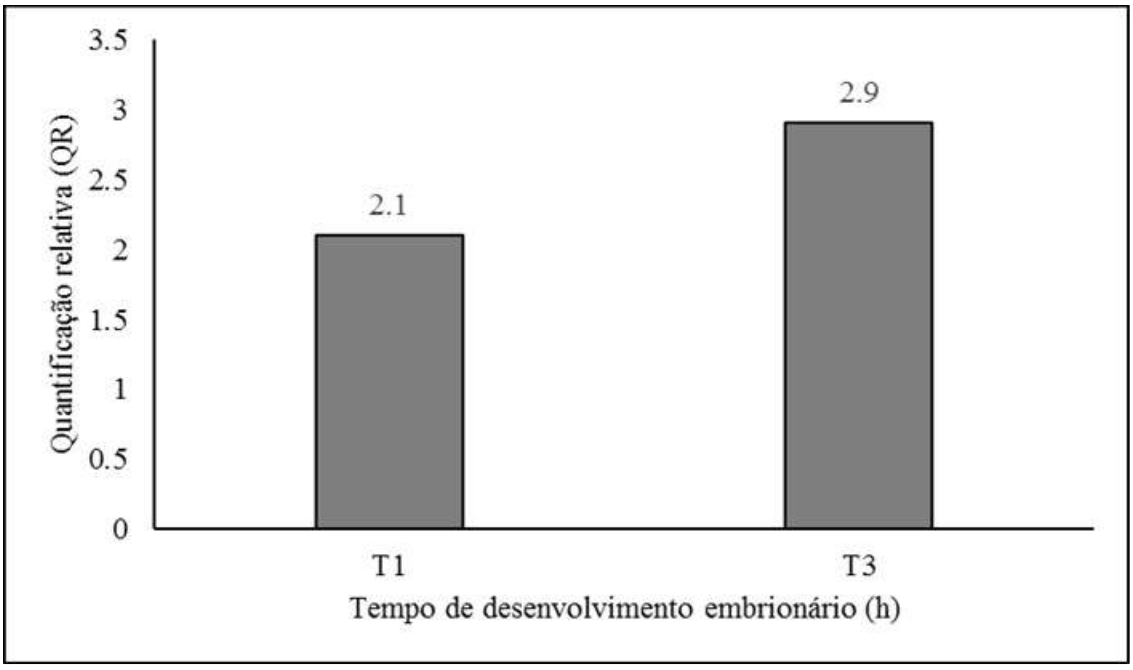

Figura 1. Quantificação relativa (QR) da expressão gênica de *quitina sintase* (*AaCHS1a)* em ovos de *Aedes aegypti* na população de Aracaju em relação a Recife, em diferentes tempos de desenvolvimento embrionário. Para cálculo da QR foi usado o método comparativo $2^{-\Delta\Delta CT}$ (Livak; Schmittgen, 2001), onde $\Delta\Delta C_T = \Delta C_{T\ \text{Tempo x Aracaju}} - \Delta C_{T\ \text{Tempo x Recife}}$. T1 = 6 a 9 h pós-oviposição; T3 = 15 a 18 h pós-oviposição.

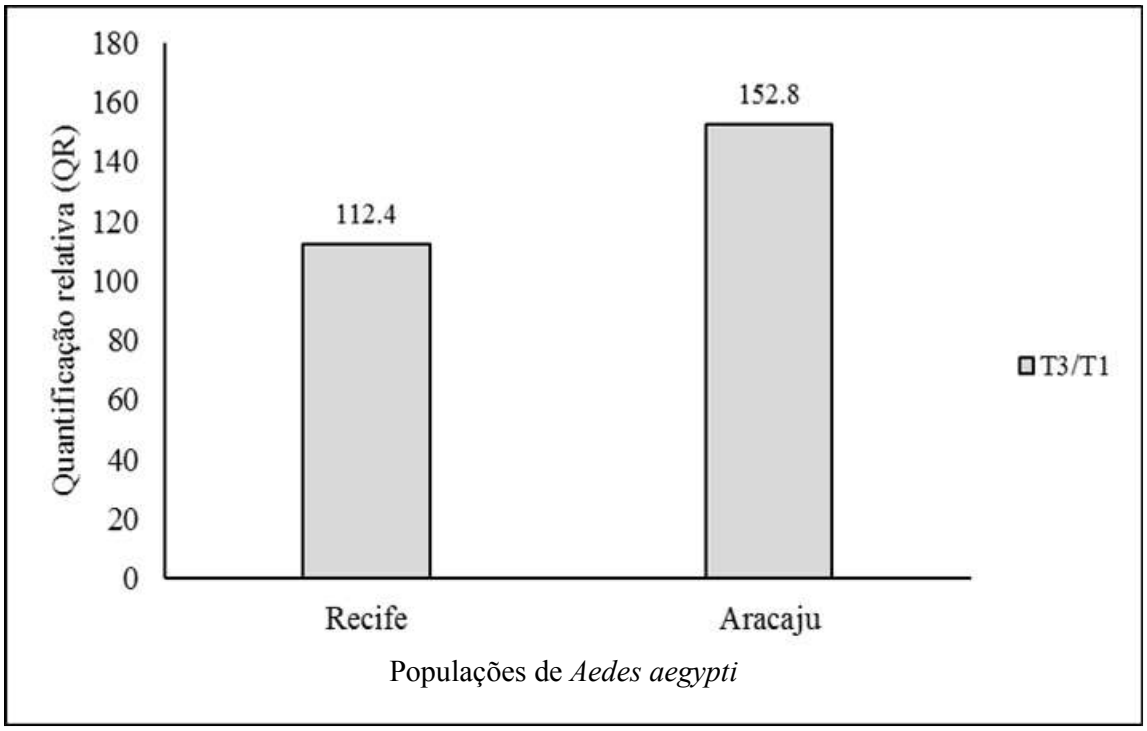

Figura 2. Quantificação relativa (QR) da expressão gênica de *quitina sintase* (*AaCHS1a)* em ovos de *Aedes aegypti*, em cada uma das populações (Aracaju e Recife), comparando o tempo de desenvolvimento T3 com o tempo T1. Para cálculo da QR foi usado o método comparativo $2^{-\Delta\Delta CT}$ (Livak; Schmittgen, 2001), onde $\Delta\Delta C_T = \Delta C_{T\ Tempo\ x} - \Delta C_{T\ Tempo\ 1.}$ T1 = 6 a 9 h pós-oviposição; T3 = 15 a 18 h pós-oviposição.

Discussão

Em nosso trabalho, encontramos que tanto as amostras de ovos de *Ae. aegypti* da população de Recife quanto àquelas de Aracaju apresentaram expressão do gene *AaCHS1a*. Esse gene é considerado ortólogo (Arakane et al. 2004, 2005) de outros genes expressos durante o desenvolvimento embrionário de outros insetos, tais como o mosquito *Anopheles gambiae* (Giles, 1902) (Goltsev et al. 2009) e o coleóptero *Tribolium castaneum* (Herbst, 1797) (Arakane et al. 2004, 2005). Além disso, analisando as duas populações deste estudo, observamos que ambas se comportaram de modo similar, exibindo um perfil da expressão de *AaCHS1a* crescente a partir do período inicial (6 – 9 h) até o último período analisado (15 – 18 h). Esse mesmo padrão da expressão foi observado por Rezende et al. (2008) utilizando mosquitos *Ae. aegypti* da linhagem Rockfeller, que registraram uma discreta expressão de *AaCHS1a* em ovos com 6 h após a postura, aumentando significativamente entre 9 e 12 h e alcançando um nível máximo com aproximadamente 25 h. Como não analisamos todo o processo de embriogênese, que nessa espécie pode variar de 48,4 a 489,3 h, dependendo da condição de temperatura utilizada (Farnesi et al. 2009), é possível que valores ainda maiores

da expressão, em estágios mais tardios do desenvolvimento, possam ocorrer nas populações de Aracaju e Recife, necessitando a inclusão de avaliações após o período de 18 h para a confirmação dessa hipótese. Além disso, como os perfis da expressão foram semelhantes independente da população analisada (Aracaju ou Recife), é possível que as condições usadas na manutenção das colônias de *Ae. aegypti*, bem como na obtenção dos ovos, tais como temperatura, umidade, fotoperíodo, alimentação, etc., sejam mais relevantes para a expressão e perfil da expressão do gene *AaCHS1a* do que as características genéticas intrínsecas de cada população.

Em *Ae. aegypti*, o primeiro estudo sobre a base genética envolvida na formação da CS foi de Rezende et al. (2008) os quais observaram que o gene *AaCHS1* apresentava dois variantes de *RNAm* editados, mas que apenas o *AaCHS1a* era transcrito durante a formação da CS. Os autores concluíram ainda que a completa formação dessa estrutura entre 13 e 15 h pós-oviposição, coincidia com níveis significativos da expressão de *AaCHS1a*, revelando o papel fundamental desse gene no processo. Período similar para a formação estrutural da CS (14 a 16 h) foi observado por Farnesi et al. (2017), sem, contudo, avaliar algum aspecto genético em seu estudo. Em nosso trabalho, embora não tenhamos registrado o período exato da completa formação da CS, os padrões da expressão do gene *AaCHS1a* foram muito similares entre as nossas populações e a de Rezende et al. (2008) sugerindo que a formação da CS nos ovos nas amostras de Aracaju e Recife pode ocorrer em intervalos de tempo próximos ao registrado por esses autores (13 a 15 h após a oviposição).

Dessa forma, é possível concluir que o perfil da expressão de *AaCHS1*a nos intervalos de tempo analisados e nas populações de *Ae. aegypti* de Aracaju e Recife é similar, apresentando uma discreta expressão no início do desenvolvimento, que aumenta ao longo do tempo (15 a 18 h). Essas informações indicam que diferenças interpopulacionais podem não ter influência no tempo de formação da CS em *Ae. aegypti*, podendo esse mecanismo estar bem estabelecido dentro dessa espécie.

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Conflito de Interesses

Autores declaram que não há conflito de interesses.

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5 CONCLUSÕES

- A quiescência dos ovos de *Ae. aegypti* afeta, negativamente, quatro (viabilidade, tempo de desenvolvimento pós-embrionário, taxa de emergência de adultos e proporção sexual) dos oito parâmetros biológicos avaliados neste estudo, sendo o custo energético dessa quiescência maior para mosquitos do município de Aracaju do que Recife. Tempo inicial de eclosão, fecundidade, fertilidade e tamanho do adulto não são afetados pela quiescência nas duas populações de mosquitos avaliadas;

- O pyriproxyfen afeta, negativamente, a viabilidade dos ovos quiescentes de *Ae aegypti*, com este efeito ocorrendo independente da formação da cutícula serosa. Neste estudo, o efeito varia de acordo com os diferentes tempos de quiescência e cidade de origem dos mosquitos estudados;

- O gene *quitina sintase* (*AaCHS1a*) inicia sua expressão no período precoce (entre 6 e 9 h após a postura) nas populações de *Ae. aegypti* de Recife e Aracaju, apresentando maior quantidade de transcritos no período tardio (entre 15 a 18 h após a postura).

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APÊNDICE A – ARTIGO PUBLICADO NA REVISTA *INSECTS*: QUIESCENCE IN *Aedes aegypti*: INTERPOPULATION DIFFERENCES CONTRIBUTE TO POPULATION DYNAMICS AND VECTORIAL CAPACITY

insects

Article

Quiescence in *Aedes aegypti*: Interpopulation Differences Contribute to Population Dynamics and Vectorial Capacity

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Abstract: The strategy of *Aedes aegypti* to prolong embryonic viability by quiescence has severe implications for geographic expansion and maintenance of mosquito populations in areas under control measures. We evaluated the effects of quiescence on biological parameters directly or indirectly associated with population dynamics and vectorial capacity in populations of this mosquito species from two Brazilian municipalities characterized as dengue, chikungunya, and Zika transmission areas. Egg viability, initial hatching time, post-embryonic development time, adult emergence rate, sexual proportion, adult size, fecundity, and fertility were analyzed using eggs stored for 10, 40, 70, 100, 130, and 160 d. Quiescence time reduced overall egg viability and post-embryonic development time in both municipalities but was more costly in Aracaju (100 d, 8 d) than in Recife (130 d, 7.5 d). Emergence rates increased in Recife when the eggs were older, but not in Aracaju. Significant deviations in sexual proportion, with male predominance, were observed in both populations. Initial hatch, fecundity, fertility, and adult size did not significantly influence egg quiescence time. These results indicate intrinsic and differential characteristics for each *A. aegypti* population, suggesting a differential cost of quiescence for population dynamics parameters that can indirectly affect vectorial capacity and control measures.

Keywords: biological cycle; Culicidae; desiccation resistance; development; egg dormancy; fitness; mosquito; plasticity; reproduction

1. Introduction

Arboviruses transmitted by mosquito vectors have been a major cause of global health problems, particularly in tropical and subtropical countries [1–5]. Viruses causing dengue fever, urban yellow fever, chikungunya fever, and Zika virus disease are some arboviruses whose main transmission vector in these areas is *Aedes aegypti* (L.) [6–10].

Over the course of its evolutionary history, *A. aegypti* has developed strategies favoring the explosive growth of natural populations, invasion, and dissemination worldwide [11–16]. One of these strategies is the capacity of eggs to resist desiccation, mainly due to low humidity and high temperatures, until conditions become favorable for hatching (non-seasonal quiescence) [17–21]. Resistant eggs can allow the pharate first instar larvae to survive inside the egg in unfavorable

environments for up to over a year [12,22–24]. Thus, quiescent eggs constitute a significant problem for vector control because these eggs can directly contribute to the maintenance of mosquito populations in treated areas, and may facilitate the transportation of eggs and the establishment of new populations [22,25]. In subtropical regions, low annual average temperatures are a limiting factor for the survival of *A. aegypti*. However, in a current global warming scenario, the elevation of temperatures in these areas can favor the introduction, dispersion, and expansion of *A. aegypti* [26,27]. Furthermore, increases in temperature may reduce the incubation period of some pathogens, including dengue virus, shortening the dengue transmission cycle [28,29]. Considering the existence of trans-ovarian transmission in this vector [30,31], quiescent eggs may play a crucial role in this situation since eggs can remain viable in the environment for long periods of time.

Although scarcely studied, there are indications that the quiescence process has a negative cost on the fitness of *A. aegypti* individuals. For instance, the extension of egg quiescence periods has been shown to negatively affect larval physiology and development by reducing lipid reserves [32] and reducing female body masses and reproductive fitness [33]. Such observations raise a key question as to whether quiescence would shape parameters directly or indirectly associated to population dynamics, vector capacity, and competence (i.e., mosquito density, time of post-embryonic development, sexual proportion, fecundity, and fertility), and how different such effects would be on different mosquito populations. Moreover, previous studies have shown that local and regional genetic differences between mosquito populations can affect different characteristics of the biology of vectors [34,35]. Thus, a role for interpopulation variations in egg quiescence in population dynamics and vector capacity is a reasonable assumption, but this remains uninvestigated.

Using an approach that combines biological parameters capable of directly or indirectly affecting population dynamics and vector capacity we investigated: (1) the differences in the eclosion rate from quiescent eggs in distinct *A. aegypti* populations from transmission areas for dengue fever, chikungunya fever, and Zika; (2) whether the initial hatching time, post-embryonic development time, adult emergence rate and sexual proportion were differentially affected by the quiescence period in these populations; (3) whether fecundity, fertility, and adult size are altered as result of increased quiescence time. We tested the hypothesis that the duration of the quiescent period and its consequences on individual fitness are intrinsic characteristics of each population, differing between *A. aegypti* populations due to variations in genetic features and selective environmental pressures. Therefore, we predict that quiescence will negatively affect the initial hatching time by increasing the time required for larvae eclosion due to a greater necessity for rehydration. Similarly, post-embryonic development time will be longer, and emergence rate will be reduced as the quiescent period progresses due to smaller lipids reserves in the embryos. Meanwhile, since genetic mechanisms modulate the sexual proportions of mosquitoes, there will be no change in the proportion of males and females related to quiescence time. Finally, fecundity will be decreased as quiescence time increases, since low energy reserves result in smaller female body sizes, reducing their ability to ingest a blood meal and, consequently, egg production. In contrast, the fertility of females originating from quiescent eggs would not be affected because the eggs of these females were not exposed to the quiescence process.

2. Materials and Methods

2.1. Mosquito Strains

A. aegypti populations from two Brazilian municipalities 501 km apart from each other (Recife, 08°03′03″ S and 34°56′54″ W; Aracaju, 10°54′40″ S and 37°04′18″ W) were studied in this work. Both cities are dengue fever, chikungunya fever, and Zika virus transmission areas, and are characterized as Am Tropical monsoon climates based on the Köppen–Geiger climatic classification system, where the temperature of the coldest month is >18 °C and the precipitation on driest month is >100 mm [36].

Recife (8 m above sea level masl) is one of the oldest cities in Brazil and is considered one of the ten most-populous municipalities in the country. The city presents an average temperature of 25.9 °C and average rainfall of 1800 mm throughout the year [37]. Its estimated population in 2017 was 1,633,697 inhabitants [38]. In contrast, Aracaju (4 masl) is one of the least populated capital cities (650,106 people) [38] in Brazil. On average, annual temperatures of 26 °C and rainfall of 1590 mm are registered [39].

2.2. Parental Generation (PG)

The *A. aegypti* parental generation (PG) was obtained from eggs collected in urban areas, using oviposition traps (ovitraps) made of 500 mL black plastic pots, 15 × 12 cm. The oviposition trap contained water and a 15 × 5 cm strip of a wooden pallet as an oviposition substrate. In the laboratory, paddles containing eggs were placed in plastic trays (40 × 27 × 7 cm) covered with a mesh and left for drying for three days to complete embryonic development, at 26 ± 1 °C, 80 ± 5% RH and photoperiod of 12:12 [light:dark] h. After this period, eggs were transferred to a new plastic tray, filled with filtered water to allow larvae to hatch. Larvae were fed with fish food (Tetra® Marine XL Granules, Melle, Germany) ad libitum until pupation. Pupae were transferred to plastic cups and placed inside breeding cages for adult emergence. Due to the presence of other *Aedes* species in both cities, a screening method based on the taxonomic key of Consoli and Lourenço-de-Oliveira [40] was used to identify adults of *A. aegypti* during the formation of the PG. After selection, mosquitoes were fed 10% sucrose solution ad libitum as a carbohydrate source. After sugar deprivation for 24–48 h, females were fed defibrinated and sterile sheep blood (EBE-FARMA Biológica e Agropecuária LTDA, Rio de Janeiro, Brazil) until full engorgement to stimulate ovogenesis. Three days after the blood meal, 50 mL plastic cups containing sterilized cone filter paper filled with 10 mL of water were placed inside the cages for oviposition for three days and exchanged every 24 h.

2.3. Quiescent Eggs

Eggs from F1–F3 generations were divided into groups and stored in plastic containers covered with a mesh for 10, 40, 70, 100, 130 and 160 days before use. The storage periods were chosen based on previous observations made in our laboratory [41]. From this, egg groups were formed for viability analyzes with a 30-day interval between groups. Eggs of 10 days old were used as a baseline (control) for the experiments. Although these eggs were in the beginning of the quiescent period, in our previous tests, hatching rate did not significantly differ from three days old eggs (freshly embryonated eggs) (mean of 49.2 ± 12.11; Kruskal–Wallis with Student–Newman–Keuls test a posteriori, $p \leq 0.05$). Ten replicates for each group were used totalizing approximately 1000 eggs for each population and stored period.

2.4. Effect of Quiescence on Different Biological Parameters of A. aegypti

2.4.1. Egg Viability

For each quiescence condition (10, 40, 70, 100, 130 and 160 days), the total number of larvae hatched following immersion of eggs in water was recorded after seven days (maximum period of larval hatching observed in previous tests). Egg viability was measured as the percentage mean of total egg hatching in each group.

2.4.2. Initial Hatching Time

The presence of the first larvae was recorded in hourly observations for each quiescence time to evaluate whether older eggs would require a longer water immersion time for larval hatching. Observations performed before an hour resulted in no larval hatching.

2.4.3. Post-Embryonic Development Time

A maximum sample of 10% of the total number of larvae obtained in each replicate in the above experiments was used to investigate whether the time for larvae to reach adulthood would increase according to a longer quiescence period. This value was determined to keep the same proportion for each replicate, thus avoiding bias due to sampling errors. Larvae (up to 8 h after hatching) were individualized in plastic cups (50 mL capacity) with 40 mL of water and fed with fish food, in proportion to 2 mg/larvae on alternate days. Upon reaching the pupa stage, the cups containing individuals were transferred to cages (transparent container with 15 cm diameter and 9 cm in height, and 1000 mL volume) separately for adult emergence. The period required to complete the life cycle (L1 to adult), in days, was registered.

2.4.4. Emergence Rate and Sexual Proportion

After their emergence, males and females were quantified, and the male/female proportion was determined. Adults were left for mating in adult cages (40 × 40 cm) and used for fecundity and fertility trials described below. After females received a blood meal (as previously described), mosquitoes were fed on sugar solution ad libitum.

2.4.5. Fecundity and Fertility

Immediately after a blood meal, engorged females were individualized into transparent plastic receptacles (1000 mL) fitted with a breeding site (50 mL plastic cup containing filter paper soaked in water). A cotton swab moistened with a 10% sucrose solution was offered ad libitum. After a week, breeding sites were removed, dried at room temperature, and eggs counted under a stereomicroscope using 10× magnification (LEICA Microsystems, model S8 AP0, Wetzlar, Germany). The total number of eggs laid by a female can be altered throughout the gonotrophic cycle (GC) [15]. Thus, for this study, the number of eggs deposited at the end of the first GC was used as an estimate of fecundity. Fertility assays were performed using eggs from the fecundity trials. Three days after the removal of the oviposition sites, larvae were hatched by submerging the eggs in water, and the eclosion rate was evaluated after seven days.

2.4.6. Adult Size

To estimate adult size, left wings were detached and the distance from the axillary incision to the apical margin, excluding the fringe of scales, was measured using a stereomicroscope with a coupled camera [42,43] using a magnification of 25× (LEICA Microsystems, model S8 AP0, Wetzlar, Germany).

2.5. Statistical Analyses

Lilliefors and Bartlett tests were used to verify the normality of distribution and homoscedasticity of all data. All analyses were performed using BioEstat software, version 5.3 [44], with values of $p \leq 0.05$ indicating statistical significance. To analyze whether the quiescence period affects viability, initial hatching time, post-embryonic development time, adult emergence rate, size, fecundity, and fertility we used the Kruskal–Wallis test followed by the Student–Newman–Keuls (SNK) multiple comparison tests. Differences in sexual proportion were determined using the Qui-Square Adhesion test for samples with the same expected proportions of 50:50% (males:females) (with a correction of Yates). Lastly, the Mann–Whitney U-test for independent samples and unequal sizes was used to calculate *p*-values and determine the significance between populations of mosquitoes. Results were expressed as average followed by confidence interval 95% (CI 95%) and range. The graphical representation of the data was made using box plots and bar graphics were produced in the Microsoft Office Excel software, version 2016.

3. Results

3.1. Egg Viability

Overall, egg viability significantly decreased in both populations as time progressed (Recife, H = 35.0000; df = 5; p = 0.0000 and Aracaju, H = 32.7493; df = 5; p = 0.0000), although a differential response in the reduction pattern of the eclosion rate had been observed. Maximum egg viability lasted a month longer in eggs from Recife (130 days; 0.8%) compared to the Aracaju population (100 days; 5.2%) (Figure 1). A significant reduction in egg viability was recorded after 40 and 70 days of quiescence in the Aracaju (p = 0.0021) and Recife (p = 0.0077) populations, respectively. A wide variation in hatching rate was observed, being more expressive at ten days in Recife (9.8–61.5 days) and 40 days in Aracaju (0–81.8 days). Significant differences between populations were observed in egg storage for 10 (U = 20; p = 0.0412) and 70 days (U = 23.5; p = 0.0452). In comparison with the Recife population, eggs from Aracaju showed a 70% higher eclosion rate at ten days and a 20% reduction after 70 days of storage.

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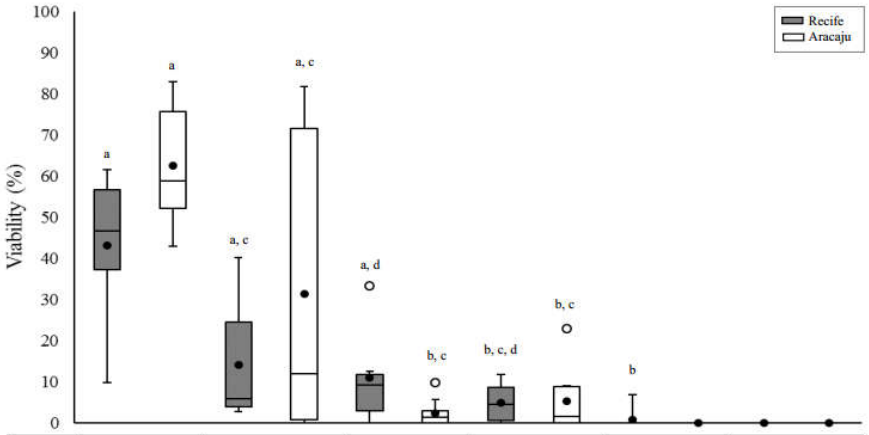


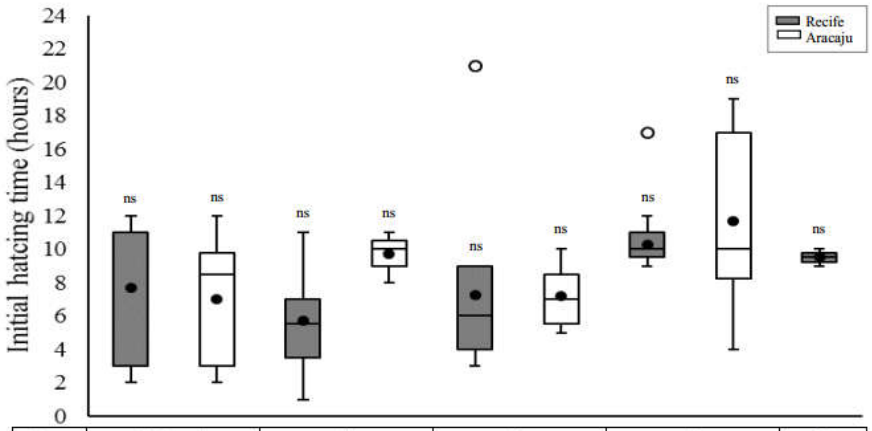
Days	10 (Control)*		40		70*		100		130		160	
Total of eggs	934	1162	1126	1059	1227	1083	1249	1113	1110	1048	1222	1070
Replicate	9	10	10	10	10	10	10	10	10	10	10	10
Mean	43.1	62.6	14.0	31.4	10.9	2.4	4.9	5.2	0.8	0.0	0.0	0.0

Figure 1. Eclosion rate (%) as a function of the quiescent period in two populations of *Aedes aegypti*—Recife (Gray) and Aracaju (White). Replicates consist of approximately 100 eggs. The size of the boxes indicates the distance between the first (lower) and third (upper) quartiles, the central mark among them shows the median and the closed circle the mean; the bars indicates variability outside quartiles and outliers are plotted as empty circles. The letters indicate comparisons between the different quiescent periods within each population (Kruskal–Wallis with Student–Newman–Keuls test a posteriori). Different letters indicate significant differences at $p \leq 0.05$. Asterisks indicate significative difference interpopulation in the same period of quiescence (Mann–Whitney U-test, $p \leq 0.05$).

3.2. Initial Hatching Time

The mean time (hour) required for the first larvae to hatch after all quiescent periods is presented in Figure 2. Neither quiescent eggs from Recife nor from Aracaju required extended immersion times for the first hatching due to their quiescence period (Recife, H = 6.1401; df = 4; p = 0.1889 and Aracaju, H = 5.0897; df = 3; p = 0.1653). However, we noted a differential hatching pattern between the different populations. Under the quiescent conditions used in this work, most larvae in both populations hatched after seven h (>55%). Even so, eggs from Recife showed a greater range of hatching time (1–21 h) compared with eggs from Aracaju (2–19 h). The greatest difference between the

two populations occurred in eggs stored for 40 days, with a significantly shorter mean hatching time in Recife population (5.7 ± 2.13 h) compared to Aracaju (9.7 ± 1.16 h) (U = 8; p = 0.0084).

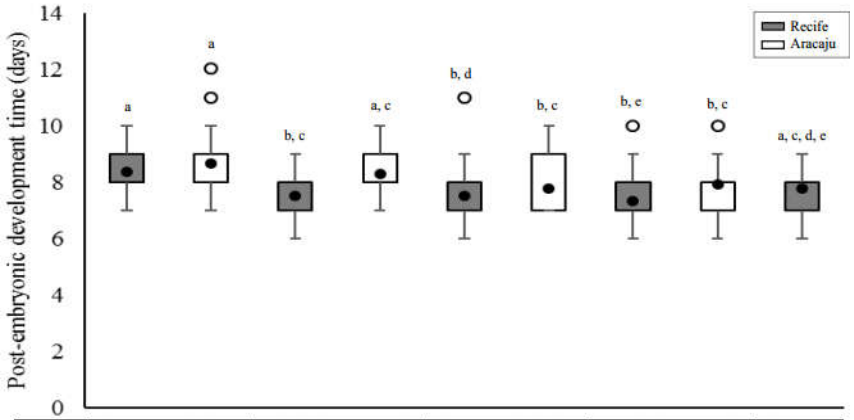


Days	10 (Control)		40*		70		100		130†
Total of eggs	934	1162	1126	740	1121	664	845	622	217
Replicate	9	10	10	7	9	6	7	6	2
Mean	7.7	7.0	5.7	9.7	7.2	7.2	10.3	11.7	9.5

Figure 2. Initial hatching time (hours) of *Aedes aegypti* larvae in different quiescent periods, in two mosquitoes populations—Recife (gray) and Aracaju (white). The number of replicates was obtained according to the viability test, each them consisting of approximately 100 eggs. Values are represented as average hatching time (closed circle); the size of the boxes indicates the distance between the first (lower) and third (upper) quartiles, the central mark among them shows the median; the bars indicates variability outside quartiles and outliers are plotted with empty circles. No significant difference (ns) was found between the quiescent period analyzed within each mosquito population separately (Kruskal–Wallis with Student–Newman–Keuls test a posteriori, $p \leq 0.05$). Asterisks indicate significative difference interpopulation in the same period of quiescence (Mann–Whitney U-test, $p \leq 0.05$). No hatching was observed for the Aracaju mosquito population from 130 days, and no hatching was observed with 160 days of quiescence for both populations (see viability tests, Figure 1).

3.3. Post-Embryonic Development Time

In both populations, larvae from older eggs reached adulthood in a shorter time compared to controls (Recife, H = 47.2926; df = 4; p = 0.0000 and Aracaju, H = 16.1884; df = 3; p = 0.001). Overall, the cost of quiescence to immature development was lower in Recife (shorter development time) compared to the Aracaju population (longer development time) (Figure 3) with a significant reduction at 40 d (U = 368; p = 0.0001) and 100 d (U = 406; p = 0.0182). In Recife, larvae from the control group averaged 8.4 ± 0.18 d to reach adulthood, a period significantly higher compared to intermediate quiescent periods (40 days, $p < 0.0001$; 70 days, $p < 0.0001$ and 100 days, $p < 0.0001$), even including outliers at 70 and 100 days. No difference was observed between day 10 and 130 (p = 0.1046). However, the difference in sample sizes may represent a bias in this result. Meanwhile, shorter larval development times were observed for individuals from the Aracaju population eggs after 40 days of quiescence (70 days, $p < 0.0013$ and 100 days, $p < 0.0057$) when compared with control eggs.



Days	10 (Control)		40*		70		100*		130
Replicate	54	61	62	25	61	17	47	27	9
Mean	8.4	8.7	7.5	8.3	7.5	7.8	7.3	7.9	7.8

Figure 3. Post-embryonic development time (days) for immature *Aedes aegypti* obtained from eggs with different quiescence periods in two mosquitoes populations—Recife (gray) and Aracaju (white). Replicates represent the number of larvae obtained in the viability tests (approximately 10% of total hatchling). Values are represented as average post-embryonic development time (closed circle); the size of the boxes indicates the distance between the first (lower) and third (upper) quartiles, while the central mark among them shows the median; the bars indicates variability outside quartiles and outliers are plotted with empty circles. The letters indicate comparisons between the different quiescent periods within each population (Kruskal–Wallis with Student–Newman–Keuls test a posteriori). Different letters indicate significant differences at $p \leq 0.05$. Asterisks indicate significative difference interpopulational for comparisons of values of the same period of quiescence (Mann–Whitney test, $p \leq 0.05$).

3.4. Emergence Rate and Sexual Proportion

Overall, the proportion of adults emerging from pupae was dependent on the studied population and the quiescent period. The effect of the quiescent period was clearly seen in the mosquitoes from Recife (H = 9.9393; df = 4; $p = 0.0415$), but not in mosquitoes from Aracaju (H = 5.0264; df = 3; $p = 0.1699$). Also, although the proportion of viable eggs had decreased during the quiescent periods in both populations (Figure 1), proportionately more adults were obtained from older eggs in the Recife population. For instance, from a total of 94 L1 larvae (10 days of quiescence) individualized in a viability trial (shown above), 54 emerged as adults (57.5%). This percentage increased to 86.8% ($n = 75$) after 40 days of quiescence, reaching 100% ($n = 9$) at 130 days (Table 1). Conversely, the effect of the quiescence period on adult emergence showed no clear pattern for the Aracaju population. When the samples from each quiescent period were compared between the studied populations, significant differences in the emergence rate was observed in eggs after 40 days of quiescence (U = 6; $p = 0.0047$). In this situation, the total number of adults was 2.5 times greater in the Recife population (86.8%) than Aracaju (34.2%) (Table 1).

In general, quiescence did not affect the ratio of females and males within the timelines of both *A. aegypti* populations studied. However, when the expected sexual proportion of 50:50% was evaluated, significant differences were observed, particularly, at 70 and 100 days (Figure 4). No difference between populations was recorded.

Table 1. Emergence rate means ± CI 95% (range of values) from *Aedes aegypti* eggs of the Recife and Aracaju populations according to quiescence periods.

Period of Quiescence (Day)	Population		Mann–Whitney U-Test	*p*-Value
	Recife	Aracaju		
10 days	57.5 ± 22.76 (10.0–100) [a] *n* = 94	52.8 ± 23.13 (0–91.7) [a] *n* = 117	41.5	0.7751
40 days	86.8 ± 9.74 (61.5–100) [b,c] *n* = 75	34.2 ± 30.48 (0–90) [a] *n* = 66	6.0	0.0047
70 days	74.5 ± 15.40 (40.0–100) [a,c] *n* = 88	61.7 ± 35.37 (0–100) [a] *n* = 26	18.5	0.3165
100 days	84.5 ± 12.29 (66.7–100) [a,c] *n* = 58	73.6 ± 30.14 (22.2–100) [a] *n* = 42	17.0	0.5700
130 days	100 ± 0.00 (100–100) [b,c] *n* = 9	No emergence	NA	NA
160 days	No emergence	No emergence	NA	NA

n = Total number of L1 larvae that were individualized; Small letters indicate comparisons of values (Kruskal–Wallis with Student–Newman–Keuls test a posteriori; $p \leq 0.05$) in the same column. Values identified by the same letter types are not significantly different; The Mann–Whitney test ($p \leq 0.05$) was used to assess interpopulational differences between the emergence rate means; NA = Not applicable.

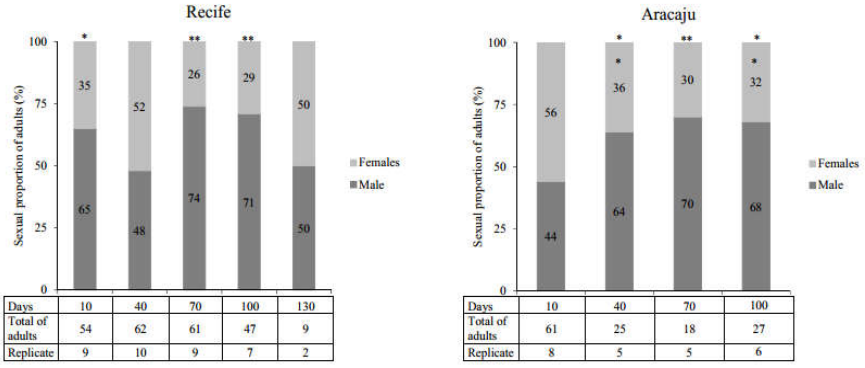


Figure 4. The proportion of *Aedes aegypti* adults (%) observed from eggs with different periods of quiescence. Replicates indicates the number of samples that presented emergence of adults from the viability tests. Asterisks identify within each population the periods of quiescence with significantly different deviations from the expected 50:50% (males: females) sexual proportion (Qui-Square test; * $p < 0.005$, ** $p < 0.0001$).

3.5. Fecundity and Fertility

Fecundity was not affected by the quiescence period in either *A. aegypti* population (Recife, H = 4.5183; df = 3; $p = 0.2107$ and Aracaju, H = 1.2361; df = 3; $p = 0.7444$). Independent of the quiescence period, females from the Recife population ($n = 77$ females) laid an average of 46.3 ± 6.60 eggs/female (range 1–132,), while those of Aracaju ($n = 53$) presented a mean rate of 44.2 ± 8.05 (range of 3–123). In both populations, maximum and minimum fecundity rates were observed at 70 (Recife, 56.2 ± 10.00 and Aracaju, 54.8 ± 32.88) and 100 days (Recife, 40.3 ± 24.98 and Aracaju, 29.2 ± 22.19), respectively. No significant difference was found when comparing each period of quiescence between populations.

Fertility was also similar among groups and populations (Recife, H = 6.8691; df = 3; $p = 0.0762$ and Aracaju, H = 0.3967; df = 3; $p = 0.9409$). Approximately, 28% ± 4.44 ($n = 2159$ eggs) and 27.9% ± 5.81 ($n = 1478$) of all eggs hatched from Recife and Aracaju, respectively. The first population presented maximum fertility rates when females were emerged from eggs after 70 days (34.7 ± 6.20), while for the second population the optimal time was ten days (28.6 ± 8.41). Conversely, the minimum larval hatching rates were recorded for females from young eggs (10 days) in Recife and old eggs (100 days) in Aracaju. Significant differences between *A. aegypti* populations were otherwise not identified.

3.6. Size

Male and female body sizes were not affected by quiescence. However, females from the Recife population were larger than those from Aracaju, particularly those that emerged after 70 (U = 34.5; $p = 0.0127$) or 100 days (U = 53; $p = 0.0136$) quiescent eggs. Intrapopulation and interpopulation differences in male and female sizes are summarized in Table 2. Overall, females originating from quiescent eggs in the Recife population averaged 2.7 ± 0.03 cm (range 2.3–2.9 cm) and males 2.1 ± 0.06 cm (range 1.2–2.8 cm). The average wing size of females from eggs from the Aracaju population was 2.6 ± 0.05 cm (range 2.0–2.9 cm) and 2.1 ± 0.05 cm for males (1.8–2.7 cm).

Table 2. Average size ± CI 95% (range of values) of adult males and females from *Aedes aegypti* of the Recife and Aracaju populations according to quiescence periods.

Period of Quiescence	Population				Mann–Whitney Test		*p*-Value	
	Recife		Aracaju					
	Males	Females	Males	Females	Males	Females	Males	Females
10 days	2.1 ± 0.13 (1.6–2.7) ^{a,A} $n = 15$	2.7 ± 0.09 (2.3–2.9) ^{a,B} $n = 15$	2.1 ± 0.06 (2.0–2.3) ^{a,A} $n = 15$	2.7 ± 0.09 (2.3–2.9) ^{a,B} $n = 15$	99.0	94.5	0.5755	0.4553
40 days	2.2 ± 0.11 (2.0–2.5) ^{a,A} $n = 15$	2.6 ± 0.09 (2.4–2.9) ^{a,B} $n = 15$	2.2 ± 0.10 (1.9–2.5) ^{a,A} $n = 15$	2.6 ± 0.17 (2.0–2.9) ^{a,B} $n = 10$	100.5	69.5	0.6187	0.7603
70 days	2.1 ± 0.10 (1.9–2.6) ^{a,A} $n = 15$	2.7 ± 0.05 (2.5–2.9) ^{a,A} $n = 15$	2.2 ± 0.33 (1.9–2.7) ^{a,A} $n = 06$	2.5 ± 0.12 (2.2–2.8) ^{a,B} $n = 11$	37.0	34.5	0.5334	0.0127
100 days	2.2 ± 0.15 (1.9–2.8) ^{a,A} $n = 15$	2.8 ± 0.04 (2.7–2.9) ^{a,B} $n = 15$	2.1 ± 0.11 (1.8–2.7) ^{a,A} $n = 15$	2.6 ± 0.08 (2.3–2.8) ^{a,B} $n = 15$	51.0	53.0	0.9158	0.0136
130 days	2.0 ± 0.36 (1.2–2.4) ^{a,A} $n = 07$	2.7 ± 0.52 (2.7–2.8) ^{a,B} $n = 02$	NA	NA	NA	NA	NA	NA

n = Total number of adult mosquitoes; Small letters indicate comparisons of values (Kruskal–Wallis with Student–Newman–Keuls test a posteriori; $p \leq 0.05$) in the same column, whereas capital letters indicate comparisons of values (Kruskal–Wallis with Student–Newman–Keuls test a posteriori; $p \leq 0.05$) in the same row. Values identified by the same letter types are not significantly different ($p > 0.05$); The Mann–Whitney test ($p \leq 0.05$) was used to assess interpopulational differences between males and females; NA = Not applicable.

4. Discussion

Studying different populations of *A. aegypti* we found a negative quiescent cost to egg viability, and sexual rate, which was dependent on the population studied and egg age (quiescence period). However, important parameters for population dynamics and vectorial capacities such as fecundity, fertility, and mosquito size were not affected by quiescence. In agreement with previous studies on egg quiescence in *A. aegypti* indicating differential egg viability [12,17,21,22,45–47], in our study, the *A. aegypti* population from Recife proved to be more resistant to desiccation (130 days, with high egg viability until three months of quiescence), than mosquitoes from the Aracaju population (100 days, with a high hatching rate up to 40 days of quiescence). Even so, the overall quiescence was shorter compared with other similar studies in which maximum larvae hatching was recorded from 150 days up to more than a year [12,22,46–48].

The reasons for the observed differences in mosquito egg resistance to desiccation are complex and involve a range of different intrinsic and extrinsic egg characteristics [17,19,21,22,49,50]. Variations in abiotic factors, particularly, those related to climate events (temperature and humidity), associated with egg water loss [51], and geographic characteristics (altitude), or causing variations in oxygen availability [52,53] have been associated with egg resistance to desiccation. Variations in rainfall (Recife 1804 mm; Aracaju 1409 mm), but not in temperature (Recife 25.8 °C and Aracaju 25.6 °C) observed between the two cities indicate that humidity is a more important factor in egg desiccation than the temperature for these populations. Also, additionally, Recife, at 7 m above sea level, has a slightly

higher altitude compared with Recife, at 4 m above sea level. Egg viability in both populations was shorter compared with mosquito egg viability from Manaus (north Brazil), which has a higher average environmental humidity due to rainfall throughout the year and an altitude of 92 m [38]. Elevation of altitude decreases the oxygen available for absorption by individuals [52,53], and lower quantities of dissolved oxygen in breeding sites may affect egg hatching [54–56]. Although our data originates from laboratory observations, the use of eggs from the F1–F3 generations implies that many characteristics intrinsic to each population remain in the analyzed samples.

In addition to the variation of the maximum egg quiescence period, our results also demonstrate the cost of quiescence on the rate of emergence and sexual proportion in individuals of both populations, but a gain in the duration of post-embryonic development. All these biological parameters were modified across the time of quiescence for both mosquito populations—Recife and Aracaju. Relatively few studies have specifically addressed the effects of quiescence on the time of larval development after hatching, and divergent results have been reported. According to Silva and Silva [12], egg quiescence has no cost for development, ranging from 7.7 to 10.3 days. Conversely, Perez and Noriega [32] showed a significant increase in the duration of larval development in individuals from older eggs (7.33 days) compared with younger eggs (5.95 days), attributing such differences to a nutritional and physiological cost of quiescence time on larval development related to decreased lipids. Contrasting with these studies, we found that larvae from both populations developed 16–24 h faster in older compared with younger eggs whose averages were 8.4 $\pm$ 0.18 (range of 7–10) and 8.7 $\pm$ 0.26 (7–12) days to reach adulthood in Recife and Aracaju, respectively. These results may indicate an adaptive response for recolonization after starvation. These changes could be an adaptation in response to selection that favors the rapid production of mosquitoes. Intrinsic differences of larval homeostatic control of metabolite levels and greater efficiency in decreasing the rates of energy depletion may improve survival of larvae from older eggs, as observed for *Drosophila* [57,58].

Also, such efforts seem to have no negative effects on adult formation since the time of egg quiescence increased the number of adults in the Recife population but had no influence on the Aracaju population. Considering that adult formation is an important aspect in population density, the differential impact of quiescence in adult formation between the two populations implicate a distinct contribution to population dynamics and a lesser extent, to vectorial capacity. Thus, in the wild, mosquitoes from the Recife population could achieve a 100% increase of emergent adults after 130 days, while those from the Aracaju population would achieve a smaller proportionate increase (73.6%) but in a shorter period (100 days).

Overall, in our study, the proportion of males in both populations was greater than females in most of the quiescent periods. Despite this, no significant variations in gender proportions were observed over time. There was a sexual disproportion, particularly after 70 and 100 days quiescence, resulting in an excess number of males. These results are consistent with previous reports [22,59]. Recent research has confirmed that sex determination in *A. aegypti* does not involve an XY chromosome but an endogenous meiotic driver system that can cause sexual proportion distortion with a predominance of males over females [60,61].

Contrary to our hypothesis that older eggs would require more time for rehydration and consequently a longer time to larval hatching compared with the younger eggs, the quiescence period did not affect the initial hatching time, which remained between 5.7 h and 11.7 h in both populations. In general, younger eggs tend to hatch between 5 and 20 min after submersion, whereas older eggs, conditioned for months may require more than more 24 h for the appearance of larvae [22]. Although considered long, the times observed in our study were also mentioned by Christopher [22] who noticed an irregular time for hatching that varied from 1 to 5 days or more in floating eggs before submersion in water.

The ability of *A. aegypti* eggs to resist desiccation has been assumed to contribute to the invasion and spread of this mosquito worldwide and, consequently, the arbovirus transmitted by this species [17–19,21,24]. The differential capacity of eggs to resist desiccation among populations implies

a differential contribution to these events, also affecting the success of control measures. Over the last few years, huge strides have been made in reducing *A. aegypti* populations, with the creation of maps of distribution and maximum risk of their establishment [2,62]. However, lack of up-to-date information regarding the factors affecting the distribution of *Aedes* species hampers surveillance and control. The results presented here revealed that different populations of *A. aegypti* have distinct phenotypes in response to quiescence, by modifying parameters of viability, post-embryonic development time, adult emergence rate, and sexual proportion. As a consequence, quiescence may impact measures of control, population dynamics, vector capacity, and competence by re-infestation of previously treated areas and an increase in the population density associated with a short life cycle. Additionally, our results provide information on the behavior of these populations for improvement of surveillance and control purposes, additional knowledge about egg resistance to desiccation, and provide support for new research aimed at fitness, geographic expansion, and transmission of arboviruses by *A. aegypti*.

5. Conclusions

In summary, our data show that out of eight parameters evaluated, four were affected by the quiescence (viability, post-embryonic development time, emergence rate and sexual proportion). These findings reinforce the idea that quiescent eggs are a public health problem, directly or indirectly affecting control programs, population dynamics, vector capacity, and competence of mosquitoes. Additionally, it is important for future research aimed at answering questions about the biology of *A. aegypti*, take into account the quiescence period of eggs since this factor can significantly influence these characteristics.

Author Contributions: L.O.O. designed the study, collected eggs, reared and maintained the mosquitoes colonies, performed the experiments, analyzed the data and draft the manuscript. R.L.C. provided support for execution of experiments, assisted in the progress of the trials, analyzed the results and wrote and reviewed the manuscript. M.O.S. collected eggs, helped in the rearing and maintenance of colonies and the execution of trials, analyzed data and commented upon and improved the quality of the manuscript. C.M.R.d.A. conceived and designed the idea and trials, guided and followed the execution of the experiments, analyzed the data, and wrote and reviewed the final manuscript version. All authors read and approved the final manuscript.

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Conflicts of Interest: The authors declare that they have no conflicts of interest.

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APÊNDICE B – ARTIGO DE REVISÃO PUBLICADO NA REVISTA *PARASITES & VECTORS*: DIAPAUSE AND QUIESCENCE: DORMANCY MECHANISMS THAT CONTRIBUTE TO THE GEOGRAPHICAL EXPANSION OF MOSQUITOES AND THEIR EVOLUTIONARY SUCCESS.

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REVIEW Open Access

Diapause and quiescence: dormancy mechanisms that contribute to the geographical expansion of mosquitoes and their evolutionary success

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Abstract

Mosquitoes are insects belonging to the order Diptera and family Culicidae. They are distributed worldwide and include approximately 3500 species, of which about 300 have medical and veterinary importance. The evolutionary success of mosquitoes, in both tropical and temperate regions, is due to the various survival strategies these insects have developed throughout their life histories. Of the many adaptive mechanisms, diapause and quiescence, two different types of dormancy, likely contribute to the establishment, maintenance and spread of natural mosquito populations. This review seeks to objectively and coherently describe the terms diapause and quiescence, which can be confused in the literature because the phenotypic effects of these mechanisms are often similar.

Keywords: Culicidae, Seasonality, Metabolism, Adaptation, Dispersion, Disease transmission

Background

Mosquitoes are arthropods that can cause considerable nuisance and affect human health worldwide [1, 2]. They are among the most prolific and invasive species, contributing to the spread of endemic diseases [3, 4]. These organisms are present in most places on the planet, from the Arctic to the most remote desert oases, except Antarctica due to its extremely low temperatures. Thus, mosquitoes are widely diverse and can easily be found in a wide variety of habitats, including forested, rural and urban environments [2, 5].

These insects have been intensely studied since the end of the nineteenth century due to their ability to act as hosts for many pathogens, including helminths, protozoans and arboviruses, that cause disease in humans and other vertebrates [2, 6]. However, only 10% of the approximately 3500 mosquito species are medically relevant [1, 7–11].

Mosquitoes, especially from the genera *Anopheles*, *Aedes* and *Culex*, include vectors for three major groups of human pathogens: parasites from the genus *Plasmodium*, which cause malaria; filarial worms from the genera *Wuchereria* and *Brugia*; and many arboviruses, including the agents of dengue, yellow fever, chikungunya, zika and others [12–14]. Estimates by the World Health Organization (WHO) indicate that diseases transmitted by mosquitoes are among the major causes of morbidity and mortality in developing countries [15], and high densities of mosquitoes severely challenge vector control programs [16]. The explosive growth of natural mosquito populations is strongly related to the survival and dispersion strategies that some species have acquired over the course of their evolutionary history [17].

Dormancy is a biological trait that may play an important role in the maintenance of natural populations and refers to a physiological phenomenon characterised by the interruption or reduction of metabolic activity in an organism. In mosquitoes, dormancy can occur at different stages of the life-cycle [18]. Diapause and quiescence represent different types of dormancy found in many species of mosquitoes. In this review, these terms are

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analysed for their conceptual principles and their respective delayed developmental effects; in addition, the mosquito species found to exhibit these phenomena will be noted.

Insect dormancy and its various types

Dormancy is a physiological phenomenon defined as a state of suspended development or suppressed metabolic activity in an organism [19]. Dormancy can occur in both plants and animals; in insects, it can manifest in the embryonic (pharate larvae), immature (larvae and pupae) and adult stages [18]. This phenomenon can be triggered by climactic signals, especially the photoperiod for temperate climate insects and relative humidity for tropical insects. This adaptation seeks to promote survival during and after unfavourable environmental conditions and is known in the literature as heterodynamic development [20]. In 1869, the term dormancy was first described as a period of inactivity caused by low temperatures by the French researcher Duclaux, who was studying silkworms (*Bombyx mori*) [20, 21]. According to a literature review by Danks [20] on the definitions and terminology of dormancy in insects, dormancy is divided into two major categories: diapause and quiescence. The terms diapause and quiescence have been reported to be synonymous in the literature [8, 22–27], but these survival strategies arise from distinct signalling pathways even though the strategies have the same goal: to ensure survival during and after environmental stress.

Mosquitoes belong to one of the most well-adapted taxa in the insect group; they are present across most of the planet, they occupy diverse niches and are potential disease vectors [2]. Diapause and quiescence are well characterised in several stages of the mosquito life-cycle. In the embryonic phase, for example, both strategies have the same effect: the inhibition of larval hatching. Conversely, only diapause drives dormancy in the larval and adult stages of mosquitoes [28].

Diapause in mosquitoes

Diapause is a well-studied seasonal survival strategy and is influenced by several factors, such as the species-specific ecological interactions, biogeography, life history and physiology of many insects [29]. The etymology of the word "diapause" comes from the Greek *diapausis* (pause), derived from the verb *diapauein*, which means to stop or to decrease activity at a time of constant action [30]. Biologically, Tauber et al. [31] defined the diapause phenomenon as a dynamic state of low metabolic activity that is genetically determined and mediated by neurohormones that phenotypically affect individuals by decreasing morphogenesis, blocking reproduction and metamorphosis, and increasing tolerance to extreme environmental conditions

The first studies on diapause in mosquitoes coincided with early studies of seasonality, diapause and photoperiod in other insects [17]. Early reviews on the topic were performed by Lees [32], Danilevskii [33], Tauber et al. [31] and Danks [20]. Studies at the time were motivated by the mosquito's hematophagous habit, which is linked to its ability to transmit the causative agents of several diseases such as malaria, filariasis, and many arbovirus infections (yellow fever, Western equine, St. Louis and Japanese encephalitis, and West Nile fever) [34].

Diapause is common in insects and other arthropods, especially in areas with harsh winters. Many aspects of diapause are critical for understanding the transmission cycle of vector-borne diseases, as this survival strategy contributes to the maintenance, establishment, growth and dispersion of natural vector populations after the end of an unfavourable season to their development [29]. The process of diapause seeks to reactivate development via external signals that control the genetic factors underlying the dormant phenotype. This can occur in several phases of the life-cycle, but most often only one developmental stage enters diapause [34].

What is the environmental signal that induces diapause in mosquitoes?

Species exhibiting the phenotypic plasticity to undergo diapause have the required information encoded in their genomes. The major stimuli inducing diapause in natural populations are changing photoperiod (short days and long nights) and gradual decreases in temperature [31, 35–40]. Mosquito species that use photoperiod to signal diapause include *Aedes albopictus*, *Aedes atropalpus*, *Aedes sollicitans*, *Aedes taeniorhynchus*, *Culex pipiens* and *Culex restuans* [38, 39, 41–44].

Preparation for diapause occurs in mosquitoes when pupae and/or adult females, which are thought to be the determining stages for this biological trait, are stimulated by exposure to the seasonal changes that typically occur during transitions between a favourable and unfavourable season [29, 39, 45–47]. For *Ae. albopictus*, for instance, induced females develop their offspring for diapause, which in turn present low metabolism in each life-cycle stage during the winter [48, 49]. However, for *Cx. pipiens,* the induced pupae females express diapause when they become adults [50]. Therefore, this ecological adaptation is indispensable for coordinating the growth, development and reproduction of mosquito species found in temperate zones [29].

Ecophysiological phases of diapause

The phenomenon of diapause consists of three ecophysiological phases [51]. The first is the diapause preparation or pre-diapause phase, which corresponds to the sensitive stage in which the insect is exposed to one or more

environmental signals (token-stimuli) that trigger and initiate the phenomenon in the offspring in the following season [19, 48]. In some species, this phase is favourable for the storage of energetic reserves that will be used for basal maintenance of the insect during dormancy and the reinitiation of development at the end of the process. In addition, morphophysiological, biochemical and behavioural changes can be observed in the individuals at this phase [19, 29, 51, 52]. This occurs because some mosquito species extend the developmental time of a specific life-cycle stage (delayed developmental effects) to increase their exposure to the stimulus, which is a favourable event for ensuring that the dormancy phenotype occurs in the offspring [29].

Culex pipiens females programmed for adult diapause have a longer larval phase, resulting in larger pupae and adults that have more lipids than their non-diapausing homologs [53]. The fat levels in females of this same species destined for diapause continue to increase significantly during the week following the emergence of the adults, reaching twice the level observed in non-diapausing females [54]. At the molecular level, this increase in energetic reserves is accompanied by an increased expression of genes associated with lipid reserve synthesis [55]. In *Ae. albopictus*, eggs in diapause are larger and contain more lipids than non-diapausing eggs, which is likely due to the increased expression of genes involved in lipid storage during pre-diapause [56].

Diapause programming (Fig. 1) involves the capture of photoperiod information by the central nervous system (CNS) of gravid females, followed by a cascade of biochemical events and culminating in the transfer of a molecular diapause regulator that promotes a dormancy state in embryos [29]. Thus, clock genes can reasonably be assumed to be involved in the regulation of circadian rhythms and, consequently, in the seasonal response based on the length of day and night [57, 58]. The main clock genes in mosquitoes that are involved in circadian rhythm regulation but are not necessarily related to diapause have been characterised in *Ae. aegypti*, *Ae. albopictus*, *Anopheles gambiae*, *Cx. quinquefasciatus* and *Wyeomyia smithii* [59–65].

Diapause specifically refers to the actual time when development is interrupted or significantly slowed, and the insect does not respond to environmental stimuli [29]. This is the second phase and can be divided into the following sub-phases: (i) the responsive phase-the beginning of the process when development is stopped at a specific life stage; (ii) the initiation stage-the phenomenon is maintained and controlled by endogenous and/or exogenous factors, and (iii) termination-the time when the individuals receive the signal to return to normal metabolic activity [19]. During diapause, various endogenous changes can be observed, but these depend on the species studied. In *Ae. albopictus* embryos, *Wy. smithii* larvae and *Cx. pipiens* adults, for example, lower lipid degradation

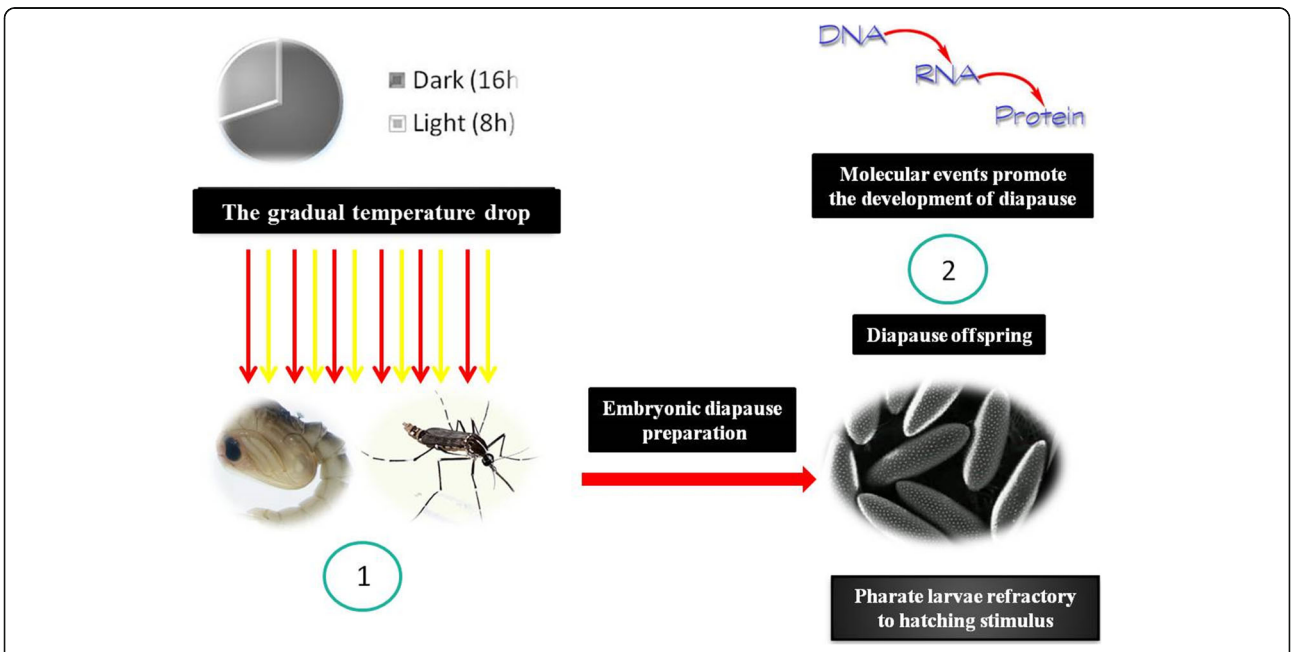

Fig. 1 Embryonic diapause induction in mosquitoes. 1 Exposure of pupae and/or adult females to short days, long nights and gradual temperature drop, and abiotic factors that promote the preparation of the embryonic diapause. 2 Expression of specific genes transferred from the female to the offspring allows diapause to be triggered and the embryos (pharate larvae) to become refractory to the hatching stimulus

and higher tolerance to desiccation and low temperatures are present [48, 66–70].

At the molecular level, a few genetic components that mediate these adaptive physiological traits have been reported in previous studies. In *Ae. albopictus*, resistance to desiccation, promoted by diapause, results from an increase in the external surface area of the egg with increased hydrocarbon levels, and this is caused by an overexpression of a transcript involved in lipid storage. However, the mechanisms responsible for cold tolerance in this species have not been determined [71]. In *Cx. pipiens*, increased tolerance to desiccation during diapause is primarily due to an increase in the hydrocarbon layer on the cuticular body surface of adults and an increase in trehalose production, which contribute to both desiccation and cold tolerance [66]. In contrast, the molecular events that promote the effects of diapause in *Wy. smithii* have yet to be discovered [29].

The last phase, termed post-diapause, is characterised by the complete reactivation of metabolism and development in the insect [51]. Although photoperiod is widely used as an environmental stimulus for entering diapause, it is less often used to signal the end of diapause; however, some exceptions exist, such as, for example, in *Wy. smithii*, where another change in photoperiod causes diapause to end [29, 72]. In *Ae. albopictus*, the termination of diapause in the eggs may be signalled by changes in photoperiod and by increasing temperature [73]. Another interesting characteristic, in addition to post-diapause, is a phenomenon known as post-diapause quiescence (Fig. 2), which is also present in *Ae. albopictus* [49, 73]. This process is considered to be a phenotypically indistinguishable phase from diapause. The insect remains in a state of dormancy, its metabolic rate continues to be low, and many of the same genes associated with diapause continue to be expressed. Thus, diapause and quiescence possibly have many molecular components in common, although the components for initial programming are exclusive to diapause [49]. Physiologically, the only difference is that during quiescence, the insect remains fully capable of responding to environmental stimuli [29, 74, 75].

Diapause in different mosquito species/life-cycle stages

Diapause can occur in different phases of the mosquito life-cycle, i.e. in the embryo (pharate larvae inside the

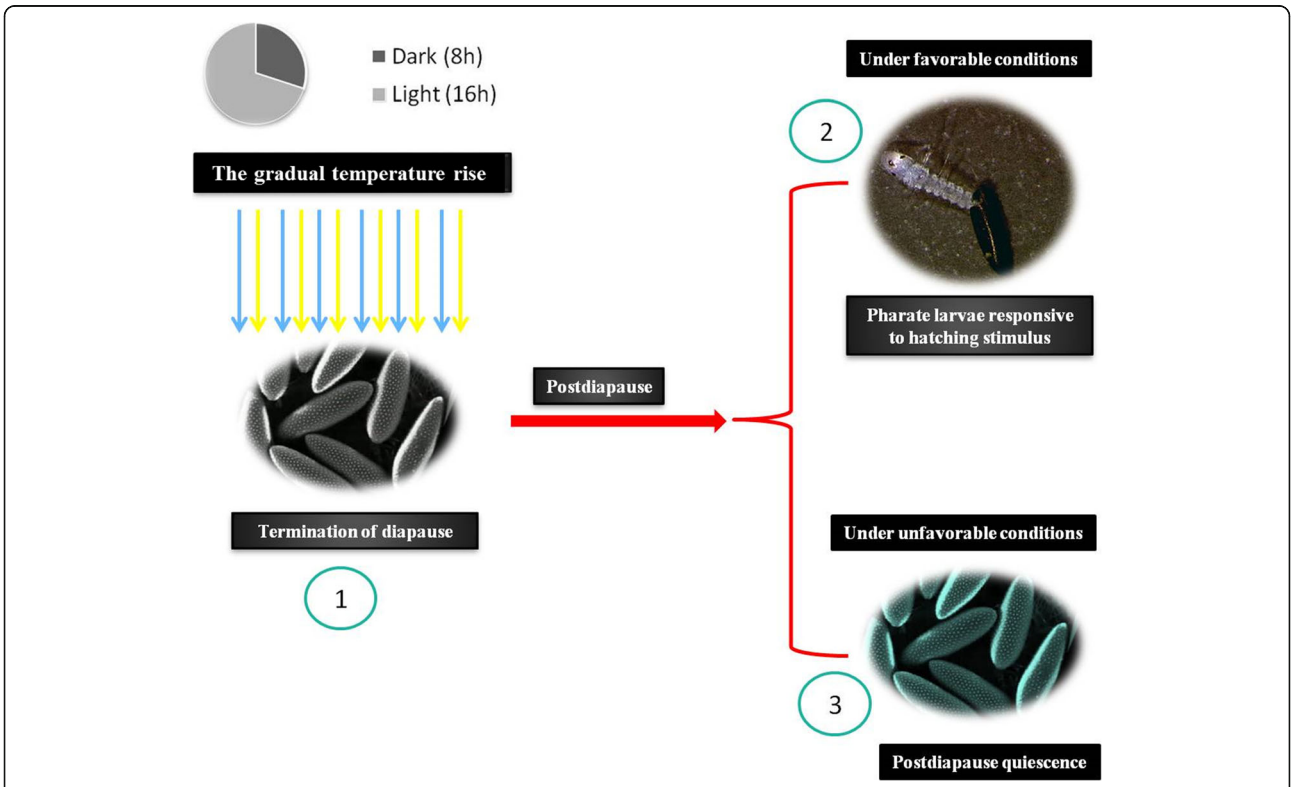

Fig. 2 Termination of the embryonic diapause in the mosquito (post-diapause). 1 Return to normal conditions (temperature and photoperiod) that signal the end of diapause. 2 Post-diapause embryo under favorable conditions responds to the stimuli of relative humidity increase and optimal temperature in the environment, resulting in larval hatching. 3 Post-diapause embryo under non-favorable abiotic conditions is sensitive to environmental stimuli but remains dormant in a quiescent state (post-diapause quiescence) until the temperature and relative humidity become ideal for larval hatching. The dynamics of quiescence are the same as post-diapause quiescence

egg), larval and adult stages. However, this type of dormancy tends to occur in a single stage of the life-cycle for a given species [19, 29, 34]. Furthermore, in some species, diapause can occur in more than one stage, more precisely, between the embryonic and larval stages [36, 62, 76–78].

Embryonic diapause

This is the most common type of dormancy and occurs in the mosquito embryo. Using *Ae. albopictus,* a model organism for diapause, as a reference, the embryo is completely formed inside the egg chorion, but a metabolic depression of post-embryonic development occurs due to genetic programming; thus, the larva is unable to respond to any abiotic signals, that is, the larva is refractory to hatching stimuli [29, 45, 46, 79]. Embryos in diapause are more tolerant to desiccation and tend to have a higher total lipid content than normal embryos [57, 68, 80]. The overexpression of ecdysteroid transcripts, found by transcriptomic analysis of mature oocytes, likely regulates embryonic diapause in *Ae. albopictus* and other mosquitoes [48, 49]. The genera of mosquitoes with embryonic diapause are *Aedes*, *Anopheles*, *Psorophora* and *Ochlerotatus* [29, 34], and the major species reported in the literature for each genus are listed in Table 1.

Diapause in larval stages

This physiological process is known in the literature as the syndrome of larval diapause, which is characterized in mosquitoes by the prolongation of the third- or fourth-instar. The induction of diapause in larvae is directly stimulated by a gradual decrease in environmental temperature, and the metabolic activity rapidly returns to normal in response to its normalisation in the wild, although changes in photoperiod also play a role in its induction [34]. The behaviour of the larvae is characterised by reduced locomotor and feeding activities, consequently promoting an increased accumulation of body reserves that, in turn, provide increased cold tolerance [34]. Under normal conditions, the progression of the development of the larval stages occurs biochemically through the periodic release of the steroid hormone ecdysone by the prothoracic gland, which culminates in the moults. When the larvae are in diapause, ecdysone release is lacking, and therefore, the larvae do not advance from one stage to the next [29]. Currently, no molecular studies have explained the hormonal basis for diapause in mosquitoes, but some studies have reported the absence of ecdysone as a major cause of larval diapause in other insects, which is likely similar to mosquitoes [81]. Mosquito species in which this type of dormancy has been observed are listed in Table 2.

Table 1 Embryonic diapause in different mosquito species

Species	References
Aedes albopictus	[46]
Aedes atropalpus	[41]
Aedes campestris	[150]
Aedes canadensis	[151]
Aedes caspius	[76]
Aedes dorsalis	[152]
Aedes fitchii	[153]
Aedes geniculatus	[77]
Aedes hendersoni	[78]
Aedes hexodontus	[154]
Aedes impiger	[129]
Aedes japonicus	[155]
Aedes mariae	[35]
Aedes nigripes	[156]
Aedes nigromaculis	[152]
Aedes sierrensis	[36]
Aedes sticticus	[157]
Aedes taeniorhynchus	[44]
Aedes triseriatus	[158]
Aedes vexans	[159]
Anopheles walkeri	[160]
Psorophora ferox	[151]
Ochlerotatus dorsalis	[152]
Ochlerotatus nigromaculis	[152]
Ochlerotatus hexodontus	[154]
Ochlerotatus flavescens	[34]
Ochlerotatus triseriatus	[75]
Ochlerotatus togoi	[75]

Diapause in adult females

Diapause in adult female mosquitoes involves a set of important characteristics, such as the interruption of gonadal development, reduced biting behaviour, negative phototaxis and changes in total metabolism, leading to the gradual accumulation of body fat. Mosquitoes can enter diapause in many habitats, such as caves, soil cavities, burrows, vegetable store-houses, empty sheds, basements, and catacombs [34]. In adult females, a type of dormancy occurs, known as reproductive diapause, where sexual immaturity is prolonged because the ovarian follicles do not differentiate completely and hence, delay the blood feeding activity [8, 34, 74].

The majority of studies on diapause in adult mosquitoes has been performed on the species *Cx. pipiens*, which is considered a model organism [29]. Under normal conditions, after the emergence of the winged form, juvenile hormone (JH) is synthesised and released,

Table 2 Larval diapause in different mosquito species

Species	References
Aedes caspius	[76]
Aedes geniculatus	[77]
Aedes hendersoni	[78]
Aedes sierrensis	[36]
Aedes togoi	[68]
Aedes triseriatus	[159]
Anopheles barberi	[161]
Anopheles plumbeus	[162]
Anopheles pulcherrimus	[85]
Armigeres subalbatus	[163]
Culiseta melanura	[164]
Orthopodomyia alba	[165]
Orthopodomyia puchripalpis	[166]
Orthopodomyia signifera	[167]
Toxorhynchites rutilus	[168]
Wyeomyia smithii	[169]

Table 3 Adult diapause in different mosquito species

Species	References
Anopheles atroparvus	[85]
Anopheles earlei	[86]
Anopheles freeborni	[87]
Anopheles superpictus	[85]
Anopheles gambiae	[88]
Anopheles hyracanus	[87]
Anopheles maculipennis	[85]
Anopheles messeae	[85]
Anopheles punctipennis	[89]
Anopheles sacharovi	[75]
Culex bitaeniorhynchus	[75]
Culex apicalis	[75]
Culex modestus	[75]
Culex pipiens	[39]
Culex restuans	[39]
Culex tarsalis	[90]
Culex tritaeniorhynchus	[91]
Culiseta alaskaensis	[92]
Culiseta impatiens	[93]
Culiseta inornata	[94]

promoting ovarian follicle growth within 3 days, and shortly after, the female is ready for its blood meal, which will contribute to oocyte maturation. In contrast, females programmed for diapause do not release JH immediately after emergence, and the follicles remain atrophied. The females also have a reduced aggressiveness [82]. Experiments on diapausing *Cx. pipiens* females treated with JH have exhibited ovarian growth stimulation, confirming the importance of inhibiting this hormone to initiate diapause in adult mosquitoes [54, 83]. It is important to highlight that in this species, males do not undergo diapause, thus, they inseminate females and then die, as they cannot overwinter [31, 33, 84]. The *Anopheles* and *Culex* species reported as exhibiting adult diapause are listed in Table 3 [38, 40, 75, 85–94].

The molecular biology of diapause in mosquitoes

Most studies on the genetic basis of diapause in mosquitoes have focused on two species, *Ae. albopictus* and *Cx. pipiens*, which are considered model organisms for this approach. Early studies were performed in the fly *Drosophila melanogaster*; however, although this species is a reference for basic genetic studies, it did not yield good results in the gene expression studies, as the insect showed highly variable responses and high variance between individuals [95–97].

Diapause in *Cx. pipiens*, according to breeding experiments, is polygenetically regulated and involves genes on all three chromosomes [98, 99]. A more detailed study on the species using suppressive subtractive hybridization to determine the expression profile of diapause genes revealed that a set of 40 genes were differentially expressed. Most of these genes were implicated in the expression of structural components and responses to the environmental stress [100]. One of the upregulated genes was a stress tolerance gene expressing a heat-shock protein (HSP70), which functions as a chaperone to inhibit abnormal protein folding in harsh environmental conditions, including desiccation and cold [101]. In addition, metabolic genes are overexpressed in *Cx. pipiens* during diapause, including the mitochondrial malate dehydrogenase (*mmd*), methylmalonate-semialdehyde dehydrogenase and cytochrome oxidase (*cox*) genes. These genes may be involved in the specific metabolic events associated with diapause and have been implicated in increased cold tolerance. The expression of certain cytoskeletal genes was also upregulated by preparation for diapause. The actin gene, for example, is overexpressed during the diapause preparation stage, likely due to increased flying activity before dormancy begins, and the expression levels of this gene decrease gradually during diapause and are low at diapause termination. Downregulated genes included the ribosomal genes S3A, rpS6 and rpS24, which are involved in gene regulation (translation initiation) and inhibit or reduce the expression of several other metabolic genes [102].

Most information on changes in gene expression associated with diapause in mosquitoes is based on recent high-throughput sequencing studies (such as RNA-seq) examining the transcriptome of *Ae. albopictus* at

different stages [29, 48, 49, 102]. Early studies on the differential expression of transcripts were performed in the ovary cells of this mosquito (oocytes), and later, the molecular mechanisms during embryogenesis were investigated.

A study by Poelchau et al. [48], who sequenced the oocyte transcriptome of diapausing *Ae. albopictus* females, and another more recent similar study from the same group, Poelchau et al. [49], who used diapausing embryos from *Ae. albopictus*, revealed the overexpression of genes involved in various biological processes. The following are included among these genes: the gene *ing1*, which encodes for the inhibitor of growth protein and is involved in the interruption of the cell division cycle [52, 103]; the gene *rack1*, a putative receptor for activated protein kinase C, which may bind to several signaling molecules, including transcription factors related to ecdysone (20-hydroxyecdysone), and is probably associated with the preparation for diapause [104, 105]; the gene *pepck* (phosphoenolpyruvate carboxykinase), whose product participates in the glycogen pathway to move from aerobic to anaerobic metabolism in diapausing mosquitoes [106, 107]; and the gene GPCR (G protein-coupled receptor), which is involved in increased resistance to environmental stress [108].

Quiescence in mosquitoes

Quiescence is a type of irregular dormancy (non-seasonal) characterised by slowed metabolism and directly resulting from unfavourable environmental conditions, including low humidity and high temperatures [22, 74, 109, 110]. This adaptive trait is often confused with diapause, especially when referring to embryonic dormancy, but quiescence is a less complex biological trait that does not depend on endogenous control for its initiation. Stimuli that trigger quiescence are referred to as acyclic environmental changes [19]. Quiescence also differs from diapause because it is neither a previously programmed event, nor is it hormonally controlled; once the stimulus that induces the process ceases, physiological activity is restored [29, 34, 73]. Because quiescence is controlled exogenously, it is possible that rapid gene activation and macromolecule synthesis or degradation are not required for entry into the quiescent state [109].

In mosquitoes, as in other organisms, the term quiescence is applied to various biological events. Most commonly studied in the egg, quiescence in mosquitoes can be stimulated in different stages or structures, enabling the insect to attain favourable conditions for survival. In the mosquito *Cx. quinquefasciatus*, for example, mature spermatozoids are maintained in quiescence in the male reproductive tract and are activated in response to specific chemical signals [111]. In this species, motility is stimulated by substances from the accessory glands in males and is possibly controlled by protein phosphorylation and Ca^{2+} levels [111]. In addition, in females, degenerative dilations may develop in the ovary, which contains granular material during winter, and the presence of these expansions in the ovaries is thought to be indicative of quiescence [112, 113].

In the family Culicidae, quiescence, unlike diapause, has been primarily observed in the egg, reflected in the resistance to desiccation that allows the embryo to survive in dry conditions. The process begins when the embryo (pharate larvae) receives an external stimulus, such as a rapid drop in humidity or change in temperature, which signals unfavourable environmental conditions and impedes larval hatching [19, 34, 74]. In this case, the developmental arrest is temporary and immediately reversible, as contact with water induces rapid hatching; that is, the quiescent embryo is not refractory to hatching stimuli as is found in diapausing embryos [18, 19, 34, 49, 114]. As shown in Table 4, the genera reported exhibiting quiescence are *Aedes*, *Anopheles* and *Culex* [23, 49, 68, 80, 115–124]. The species *Ae. aegypti* is prominent among mosquitoes due to its strategy of prolonged viability by embryonic quiescence, significantly contributing to the constant expansion of populations in

Table 4 Embryonic quiescence in different mosquito species

Species	References
Aedes aegypti	[68]
	[23]
	[116]
	[170]
	[118]
	[119]
	[120]
	[121]
	[122]
	[115]
	[123]
Aedes albopictus	[68]
	[117]
	[71]
	[48]
Aedes flavopictus	[68]
Aedes galloisi	[68]
Aedes riversi	[68]
Anopheles aquasalis	[121]
	[123]
Anopheles gambiae	[124]
Culex quinquefasciatus	[123]
	[123]

the wild [74, 75]. However, several studies have erroneously reported this trait as diapause [8, 22–24, 26].

Egg quiescence or embryonic desiccation resistance

Egg quiescence is commonly referred to as "embryonic desiccation resistance" (EDR) and depends on several factors that range from differences in eggshell composition and structure to physiological changes, resulting in reduced metabolism in the larvae contained within the egg [22, 116, 121, 125]. However, because the ability to resist desiccation is a property of the egg and not of the embryo and because desiccation can occur at other stages of development, the term "egg resistance to desiccation (ERD)" has been suggested as more appropriate for referring to this phenomenon [123].

The three layers that form the eggshell, the exochorion, endochorion and serosal cuticle, are particularly important for ERD [116, 123]. The first two layers are produced in the ovary, by females, and are, therefore, present at laying [74, 123, 126]. The serosal cuticle (the innermost layer), in turn, is an extracellular matrix produced by the extraembryonic serosa during early embryogenesis. In *Ae. aegypti*, secretion of the serosal cuticle occurs between 11 and 13 h after oviposition and approximately 8 h post-fertilization in *An. gambiae* [115, 124].

This cuticle likely secretes a chitin-containing material under the chorion, the external layer of the egg, making it impermeable and protecting the embryo from desiccation [116, 123]. Changes in the amounts of the eggshell components are associated with water loss regulation and are fundamental for determining the intensity of egg dehydration. *Aedes albopictus* females exposed to short day length in temperate regions produce eggs in photoperiodic diapause, unlike populations in tropical regions, which enter quiescence. One of the characteristics of the egg that permits this adaptation is the high quantity of fatty acyl-CoA elongase in the tissue of mature oocytes responsible for producing hydrocarbons in the eggshell [71]. These hydrocarbons regulate water loss in insect eggs, and the abundance of this enzyme varies in the eggs of *Ae. albopictus* exposed to long and short days in temperate populations but is maintained at relatively constant levels in tropical populations [80, 123]. In addition to several hydrocarbons in the eggshell, the amount of chitin is another factor involved in ERD in mosquitoes, such as *Cx. quinquefasciatus*, *An. aquasalis* and *Ae. aegypti*. Eggshells with higher amounts of chitin are more resistant to desiccation [123].

Quiescence patterns in container-inhabiting mosquitoes

ERD has been more commonly studied in container-inhabiting mosquitoes, including *Ae. aegypti* and *Ae. albopictus*. In urban areas, females often lay their eggs in containers with clean water, especially disposable containers, tires, plant pots and water storage containers [127, 128]. Because the eggs are laid near the water surface, this developmental phase is very susceptible to dehydration, particularly during the first few hours of development [129].

First-instar larvae that remain inside quiescent eggs have been referred to as pharate first-instar quiescence [34, 74]. Normal development finishes approximately 3 days after oviposition and larval survival depends on maternal reserves [119]. Throughout the quiescent period, the larval developmental period is significantly prolonged, and lipid reserves are reduced, incurring fitness costs for larval viability, compromising the reproductive performance of the adult [34, 74].

Minimally studied, quiescence in mosquito eggs does not appear to have a uniform pattern, exhibiting variability between species or even among populations of the same species [69, 123, 130]. Under similar low-moisture conditions, the pharate first instars of *Cx. quinquefasciatus*, *An. aquasalis* and *Ae. aegypti* can survive for a few hours, 1 day or several months, respectively [123]. These differences may be due to traits inherent to the eggs of each species, such as size, the structure of the exochorion and endochorion, differences in metabolite quantity and formation of the serosal cuticle [68, 121, 131, 132].

Brazilian colonies of *Ae. aegypti* maintained at a temperature of 28 ± 1 °C, a relative humidity of 80 ± 5% and a photoperiod of 12 h had a viability period of up to 492 days, with high hatching rates between three and 121 days [23]. A similar pattern with high larval hatching rates (80%) was reported by Diniz et al. [115] in quiescent *Ae. aegypti* eggs that had been stored for up to 150 days. The authors compared eggs from laboratory and wild populations with different susceptibilities to the insecticide temephos, which were then maintained for up to 180 days at 26 °C, with a photoperiod of 12 h and at 50–60% humidity. The high viability of quiescent eggs from temephos-resistant females suggests a high contribution to the maintenance of resistant individuals in the wild. Similarly, in Australia, quiescent *Ae. aegypti* eggs remained viable for more than a year with a hatching rate of approximately 2–15%, allowing its dispersion to new locations [133]. Species inhabiting forests have been shown to be less resistant to changes in humidity. *Aedes riversi*, *Ae. galloisi* and *Ae. flavopietus* eggs have different survivability rates in very humid conditions but were less resistant than *Ae. aegypti* and *Ae. albopictus* under low humidity. Intraspecific differences in ERD were also observed among these species, as *Ae. riversi* and *Ae. flavopietus* strains from subtropical regions had lower viability than strains from temperate regions [68].

The molecular biology of quiescence in mosquitoes

Although much is known about the metabolic mechanisms and molecular biology of diapause, very little is known about these aspects during quiescence in mosquitoes. A study by Poelchau et al. [49] that compared the transcriptomes of quiescent and diapausing *Ae. albopictus* eggs found that the genetic expression profile between these samples converged over time; that is, the transcription profile in eggs during late diapause (40 days) is similar to that in quiescent eggs [49]. An important aspect of this study is that expression levels of genes related to lipid metabolism were always higher in eggs in diapause, demonstrating the likely importance of this reserve for maintaining embryonic diapause and explaining why eggs in diapause have more lipid reserves than quiescent eggs [49].

Currently, the metabolic pathways or hormones associated with quiescence are unknown, and only the chitin synthase (CHS) gene has been described as being related to this phenomenon in mosquitoes. This gene promotes the synthesis of chitin, which is then secreted into the extracellular space of the egg, with direct implications for the formation of the serosal cuticle and consequently the resistance to desiccation [116, 124, 134]. Despite being primarily cited for *An. gambiae* (AgCHS), this gene is highly conserved in two other species of mosquitoes, *An. quadrimaculatus* and *Ae. aegypti*. The gene has two variants, but only the allele AgCHS1 is involved in embryogenesis. In *Ae. aegypti*, for example, the expression of the gene peaks between nine and 12 h after oviposition, coinciding with the acquisition of resistance to desiccation through the complete covering of the embryo by the chitinized serosal cuticle [116, 124].

Eco-epidemiological importance of quiescence

In Europe, a considerable increase in invasive mosquito propagation has been observed since the end of the 1990s, with the species *Ae. albopictus*, *Ae. aegypti*, *Ae. japonicus*, *Ae. atropalpus* and *Ae. koreicus* already established on the continent [131]. In addition to increased population densities, the distribution of *Ae. albopictus* has continued to increase, and several other species of *Aedes* are being reported in new countries each year [135]. For example, recently, a research group from Brock University reported the detection of *Ae. aegypti* for the first time in Canada [136]. In addition, Lima et al. [137] reported a permanent *Ae. aegypti* local population in the Capitol Hill neighbourhood in Washington DC that can overwinter. This is contrary to the previous hypothesis that different introductions of *Ae. aegypti* every year maintain that local population. All these species are well adapted to the urban environment, exploiting a variety of container habitats that proliferate near human settlements, and both quiescence and diapause may be contributing to the maintenance of these populations. In addition to the annoyance of their bites, these mosquitoes are potential vectors for agents that cause tropical diseases, including Zika, dengue, chikungunya and yellow fever [138]. Quiescence in *Ae. aegypti* may also allow the survival of infected embryos, favouring virus survival and its maintenance in nature [8, 122, 139]. For example, DENV-1 was detected and isolated in 8.33% of *Ae. aegypti* eggs in Florida, suggesting that maintenance of dengue outbreaks in 2009 and 2010 in Key West may have been facilitated by vertical transmission [140]. Transovarian transmission of DENV in the field was also detected in larvae and adults originating from larvae collected in domestic containers in Rajasthan, India. Approximately 1.09% of the reservoirs contained larvae with the virus, detected by the indirect fluorescence antibody test and reverse transcriptase polymerase chain reaction. In this case, dormant eggs may have contributed to prolonging dengue epidemics [141]. Furthermore, Zika virus, a flavivirus that has recently caused large outbreaks in several countries and has been linked to microcephaly cases and other neurological complications, has also been reported as being transferred via transovarian transmission by *Ae. aegypti* and *Ae. albopictus* [142–144, 145].

Although the implications of quiescence on the ecology of mosquito vectors and public health are well established, its effects on physiology, behaviour and life history are less understood. Maternal reserves accumulated in eggs directly influence the period of dormancy in the first-instar larvae contained within the eggs [8, 120]. Thus, quiescent eggs pose an important problem for vector control because these eggs can directly contribute to the maintenance of mosquito populations in treated areas. *Aedes aegypti* eggs from a single laying, at the same age, and maintained under the same environmental conditions had different hatching rates during the same period of quiescence. *Aedes aegypti* embryos employ a hedge betting mechanism not all eggs hatch at the first stimulus; some need a second wetting stimulus to hatch [146]. This will ensure that in the event of sudden unfavourable conditions, such as cold temperatures or a dry spell, following the oviposition of the egg batch, the entire batch is not lost [34]. Another explanation could be that not all larvae hatch simultaneously because of competition for space and resources as noted by Livdahl et al. [147] for *Ae. triseriatus*. Furthermore, Ebrahimi et al. [148] showed that the eggs of *An. gambiae* embryos are not stimulated to hatch when the water surface is agitated, demonstrating that environmental factors could indicate the best time for hatching. Sota & Mogi [68] suggested that intraspecific variation in the survival time of eggs is an inherited trait dependent on environmental pressures. Variations in the length of quiescence of eggs and variable hatching rates

may be mechanisms that *Ae. aegypti* employs to produce continuous, although fluctuating, populations of adults in the wild at various stages, depending on the existence of favourable or unfavourable environmental conditions [23].

Therefore, quiescence provides a high adaptive potential to *Ae. aegypti* and *Ae. albopictus* populations, increasing the viability of their eggs and the chances of surviving in nature [148, 149]. This trait has contributed to the geographical expansion of these two species at a global level, an issue that is closely related to the spread of diseases [122].

Conclusions

As presented in this review, dormancy, especially diapause and quiescence, has a significant impact on the life history of mosquitoes, as well as of many other arthropods. Dormancy is part of the life history of many mosquito species, providing a mechanism to overcome unfavorable seasons in tropical and temperate zones. This trait may have independently evolved several times in the family Culicidae, as the phenomenon occurs at various developmental stages in different species. These adaptive strategies provide, on an evolutionary scale, mechanisms for species survival, as offspring continue to be produced, even when exposed to the various types of stress found in a habitat, and this, in turn, contributes to the territorial expansion of natural populations, consequently increasing their invasive potential. Diapause and quiescence are not the same biological phenomenon but have been treated as synonymous in previous studies. In addition, these different types of dormancy likely aid the propagation of the transmission cycles of diseases caused by different types of arboviruses, as these etiological agents can be transferred via the transovarian route. Both of these biological phenomena could play important roles in the ecology and evolution of many insect species, such as, for example, the mosquito *Ae. albopictus*, which has both phenotypes. Thus, the phenotypic plasticity generated by these intrinsic characteristics results in the reproductive success and survival of mosquitoes in the face of adverse environmental conditions and the different control measures practised by humans. These mechanisms are also fundamental for adapting to more frequent changes in climate. These phenomena are possibly still developing and need to be more thoroughly studied, as the information generated from associated research may be applied to innovative control strategies.

Abbreviations

AgCHS: Chitin synthase of *Anopheles gambiae*; CHS: Chitin synthase; *cox*: Cytochrome oxidase; EDR: Embryonic desiccation resistance; GPCR: G protein-coupled receptor; *ing1*: Inhibitor of growth protein; JH: Juvenile hormone; MMD: Mitochondrial malate dehydrogenase; *pepck*: Phosphoenolpyruvate carboxykinase; *rack1*: Receptor for activated protein kinase C

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DFAD, MAVMS and CFJA designed this study; DFAD conducted the review and wrote the manuscript; CMRA and LOO participated in writing the quiescence topic; and CFJA and MAVMS reviewed the manuscript. All authors read and approved the final manuscript.

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