Ministério da Saúde
Fundação Oswaldo Cruz
Instituto Oswaldo Cruz
Programa de Pós-Graduação em Biologia Parasitária

O papel da resistência a inseticidas e da densidade de *Aedes aegypti* na disseminação da *Wolbachia* em populações nativas do Rio de Janeiro, Brasil

Gabriela de Azambuja Garcia

Orientadores:

Dr. Rafael Maciel de Freitas (IOC/Fiocruz)

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Rio de Janeiro, agosto de 2017

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Tese apresentada como requisito à obtenção do título de Doutor em Biologia Parasitária, com área de concentração em Ecologia e Epidemiologia

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Garcia, Gabriela de Azambuja.

O papel da resistência a inseticidas e da densidade de Aedes aegypti na disseminação da Wolbachia em populações nativas do Rio de Janeiro, Brasil / Gabriela de Azambuja Garcia. - Rio de janeiro, 2017.
ix, 303f f.

Tese (Doutorado) – Instituto Oswaldo Cruz, Pós-Graduação em Biologia Parasitária, 2017.

Orientador: Rafael Maciel de Freitas.
Co-orientador: Daniel Antunes Maciel Villela.

Bibliografia: f. 282-303

1. Aedes aegypti. 2. Controle de vetores. 3. arboviroses. 4. resistência a inseticidas. 5. Wolbachia. I. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da Biblioteca de Manguinhos/ICICT com os dados fornecidos pelo(a) autor(a).

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Tese submetida ao Programa de Pós-Graduação em Biologia Parasitária do Instituto Oswaldo Cruz como parte dos requisitos para obtenção de grau de Doutor em Biologia Parasitária, área de concentração: Ecologia e Epidemiologia.

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Este trabalho foi realizado no Laboratório de Mosquitos Transmissores de Hematozoários (Lathema) e no Laboratório de Fisiologia e Controle de Artrópodes Vetores (Laficave), ambos pertencentes ao Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro. Foram utilizados recursos do Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), da Fundação de Amparo à Pesquisa do Rio de Janeiro (Faperj), do Ministério da Saúde (DECIT/SVS), da Universidade de Monash (Austrália) e da Fundação Bill e Melinda Gates.

“Toda grande obra, em arte e em ciência, é o resultado de uma grande paixão colocada em serviço de uma grande ideia.” *Ramón y Cajal*

Ao meu pai, Eloi, e minha mãe, Patrícia, meus maiores incentivadores no mundo da ciência.

AGRADECIMENTOS

Aos meus orientadores Dr. Rafael e Dr. Daniel, por acreditarem em mim. Rafael, uma pessoa que admiro e respeito muito, foi um grande mestre que me estimulou e me fez ganhar a confiança necessária para desenvolver meus estudos. Sempre me dando a liberdade para criar e pensar sobre ciência. Muito obrigada por vibrar comigo a cada conquista, pelos conhecimentos a mim passados e pela amizade ao longo da minha trajetória. Ao Daniel, por me guiar pelo mundo da matemática. Obrigada pelos tantos ensinamentos, orientando sempre com muita calma e paciência. Uma parceria que certamente carregarei para o futuro.

Ao Programa de Pós-Graduação em Biologia Parasitária do Instituto Oswaldo Cruz/Fiocruz, por me proporcionar uma formação de excelência. Local onde tive a oportunidade de aprender, e me espelhar, em grandes cientistas.

A todos do Laboratório de Mosquitos Transmissores de Hematozoários, grupo que sinto muito orgulho em fazer parte. Aos pesquisadores, especialmente ao Dr. Ricardo Lourenço, por tantas discussões produtivas em nosso ambiente de trabalho. A Martha, Lilha, Roberto, Cátia e Bia pessoas que sempre estiveram do meu lado me ajudando nos momentos mais difíceis da minha jornada. Agradeço a todos os técnicos e alunos por toda a ajuda na realização desta tese.

Aos amigos do Laboratório de Fisiologia e Controle de Artrópodes Vetores, grupo que me sinto em casa e sigo trabalhando desde 2010. Um agradecimento em especial a Dra. Denise e ao Dr. Ademir, que estão sempre presentes me orientando em meus estudos. Ao Dr. Bento, pela sua ótima receptividade no laboratório. Aos técnicos e alunos, pelas tantas discussões produtivas.

Ao Professor Michael Turelli e a todos de seu laboratório na Universidade da Califórnia, Davis, que me receberam com todo o carinho. Obrigada pelas longas discussões científicas e pelos bons momentos vividos na minha visita.

A todos da equipe do Programa “Eliminar a Dengue: Desafio Brasil”, por toda a ajuda fornecida neste trabalho.

À minha família, primeiramente a meus pais por desde pequena introduzir a ciência em minha vida. E pelo apoio incondicional em todos os momentos. Obrigada à minha irmã e minha avó, pessoas que sempre estiveram me apoiando.

Ao Leonardo, meu grande parceiro, por todo amor, carinho e apoio incondicional. Estar com você torna tudo mais leve. À Brisa, minha cachorrinha que me acompanhou durante a graduação e mestrado, e que se foi no período final desta tese. Foi a minha grande companheira durante meus estudos.

A todos os amigos e mestres da Medicina Veterinária da Universidade Federal Rural do Rio de Janeiro (UFRRJ), que mesmo distantes, continuam torcendo por mim.

Ao apoio financeiro fornecido pelo Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Rio de Janeiro (Faperj), Programa de Pós-Graduação em Biologia Parasitária (IOC/Fiocruz), Ministério da Saúde (DECIT/SVS), Universidade de Monash (Austrália) e Fundação Bill e Melinda Gates.

Gostaria de agradecer à Dra. Flavia Bandeira do setor de Hemoterapia da UERJ pela concessão de bolsas de sangue para repasto sanguíneo dos mosquitos infectados com *Wolbachia*. Essa ajuda foi fundamental para o desenvolvimento de várias etapas deste projeto.

E, por fim, a todos que direta ou indiretamente fizeram parte da minha formação, serei eternamente grata.

Índice

Lista de siglas e abreviaturas

AChE – acetilcolinesterase

AJH – análogo de hormônio juvenil

Bti- *Bacillus thuringiensis israelensis*

CA – inseticidas carbamatos

CHIKV – vírus chikungunya

CL – concentração letal

CV – capacidade vetorial

DDT – dicloro-difenil-tricloroetano

DENV – vírus dengue

DNA - ácido desoxirribonucleico

ECSA – *East-Central South African*

EST – esterases

GST – glutationa S-transferases

IGR – regulador do desenvolvimento de insetos (do inglês "*Insect Growth Regulator*")

IIT – *Incompatible insect technique*

ISQ – inibidores de síntese de quitina

kdr – resistência tipo "*knock-down*"

MFO – oxidases de Função Múltipla (ou Mista)

MS – Ministério da Saúde

MSR – Experimentos de marcação, soltura e recaptura

mtDNA – DNA mitocondrial

Na_V – canal de sódio regulado por voltagem

OC – inseticidas organoclorados

OMS – Organização Mundial da Saúde

OP – inseticidas organofosforados

PCR – reação em cadeia da polimerase (do inglês "*Polymerase chain reaction*")

PDS – probabilidade de sobrevivência diária

PI – inseticidas piretroides

PIE – período de incubação extrínseco

RNA – ácido ribonucleico

Rock – Rockefeller, linhagem de referência de susceptibilidade a inseticidas

RR – razão de resistência

SIT – *sterile insect technique*

SNC – Sistema Nervoso Central

UBV – ultra baixo volume

*w*MelBr – linhagem de *Aedes aegypti* com *Wolbachia* (perfil de susceptibilidade a piretroides)

*w*MelRio – linhagem de *Aedes aegypti* com *Wolbachia* (perfil de resistência a piretroides)

*w*MelTet – linhagem de *Aedes aegypti* curada da *Wolbachia* pelo tratamento com tetraciclina

WHOPES – *WHO Pesticide Evaluation Scheme*

YFV – vírus da febre amarela (*yellow fever virus*)

ZIKV – vírus Zika

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Resumo

No Brasil encontra-se em andamento liberações de *Aedes aegypti* infectados com *Wolbachia*, uma bactéria que reduz, experimentalmente, a transmissão de arbovírus como os vírus dengue, chikungunya e Zika. Testes em áreas da Austrália, Indonésia e Vietnã confirmaram uma invasão rápida e estável de *Ae. aegypti* contendo a cepa *w*Mel sobre a população selvagem. No Rio de Janeiro, liberações semanais de *Ae. aegypti* com *Wolbachia* (linhagem *w*MelBr) foram iniciadas em setembro de 2014. Entretanto, diferentemente do observado naqueles países, a primeira liberação de *Ae. aegypti* infectados com *Wolbachia* na América Latina não logrou êxito. Durante 20 semanas consecutivas, a linhagem *w*MelBr foi liberada no Rio, mas um platô inesperado (devido à introdução semanal de milhares de mosquitos e ao efeito da incompatibilidade citoplasmática) foi observado nas semanas 7 a 19. Primeiramente, realizamos experimentos de marcação-soltura-recaptura durante as liberações para verificar o número de mosquitos liberados e a densidade populacional dos mosquitos selvagens. Após esses resultados, dobramos o número de indivíduos liberados. Comparamos também a aptidão física (*fitness*) dos mosquitos *w*MelBr com a dos nativos. Os mosquitos *w*MelBr apresentaram tamanho de asas maior, menor mortalidade e uma razão sexual de 1,2: 1 para as fêmeas e, a análise de mtDNA, não mostrou falha na transmissão materna da bactéria. Todas estas características favorecem a invasão da *w*MelBr. Entretanto, apesar da frequência de *w*MelBr atingir 65% em campo na semana 20, ela caiu dramaticamente nas quatro semanas seguintes ao fim das liberações. Animado pelo aumento de venda de inseticidas spray, o dono da mercearia local manifestou desejo de que as liberações fossem incessantes, chamando-nos atenção para o possível *status* da resistência a inseticidas na linhagem liberada. A *w*MelBr permanecera em endocruzamento por 17 gerações em laboratório e os genótipos de resistência a piretroides (PI) diminuíram de 68% para 3,5%. Ou seja, havíamos liberado mosquitos altamente suscetíveis a PI em uma área cuja população selvagem que era altamente resistente. Após isso, uma segunda linhagem (*w*MelRio), com alelos de resistência a PI, foi gerada em laboratório e liberada simultaneamente em dois locais no Rio de Janeiro. Os alelos de resistência foram mantidos em *w*MelRio, em laboratório, por introdução de 50% de machos selvagens a cada duas gerações. Após 18 meses sem liberações adicionais, a frequência da *w*MelRio em campo era > 90%, em ambos os locais, evidenciando o sucesso em campo. A linhagem *w*MelRio é resistente a PI e ao larvicida temephos, e suscetível ao malathion e ao diflubenzuron, os compostos químicos atualmente utilizados no controle vetorial. Modelos matemáticos foram aplicados para testar como diferentes estratégias de liberação (variações na suscetibilidade a inseticidas das linhagens, custo de *fitness* da resistência a inseticidas) aumentam a chance de invasão da *Wolbachia*. Nossos resultados indicam uma invasão bem sucedida em duas situações: 1) liberando-se mosquitos suscetíveis em um ambiente sem uso de inseticidas (pode causar uma reversão nos níveis de resistência aos inseticidas); e 2) liberando-se mosquitos resistentes (*w*MelRio) em uma população resistente (como no Rio de Janeiro), mesmo com um elevado uso de inseticidas. Portanto, a implementação da tecnologia de liberação de *Ae. aegypti* infectado com *Wolbachia* como potencial estratégia para redução da transmissão de arbovírus deve considerar o perfil de resistência aos inseticidas da população selvagem, e da linhagem liberada, para alcançar uma invasão bem sucedida em campo.

Abstract

In Brazil are underway releases of *Aedes aegypti* infected with *Wolbachia*, a bacteria that reduces, experimentally, the transmission of arboviruses such as dengue, chikungunya and Zika viruses. Previous data reported stable and rapid invasion into wild population in Australia, Indonesia and Vietnam using *Ae. aegypti* with *w*Mel strain. In Rio de Janeiro, we started weekly releases of *Ae. aegypti* with *Wolbachia* (*w*MelBr strain) in Sep/2014. However, unlike in other countries that obtained a rapid invasion of this bacteria, the first release of *Ae. aegypti* infected with *Wolbachia* in Latin America did not succeed. During 20 consecutive weeks, the *w*MelBr was released in Rio de Janeiro, but an unexpected plateau (due to the weekly incoming of mosquitoes and strong cytoplasmic incompatibility) from weeks 7-19 was observed. First of all, we carried out a mark-release-recapture experiment during *Wolbachia* releases to check the number of released mosquitoes and wild population density. After these results, we doubled the number of mosquitoes released. We also compared the fitness of *w*MelBr released mosquitoes with wild ones. Released *w*MelBr presented larger wing sizes, lower mortality and 1.2:1 biased sex ratio to females and mtDNA analysis showed no leakage on maternal transmission of the bacteria. All these characteristics favor *w*MelBr invasion. However, despite the frequency of *w*MelBr reached 65% in the field at week 20, it dropped dramatically in the following four weeks after releases stopped. Stunned by the increasing of insecticide sprays selling, the local grocery owner wished releases to be endless, shading light on the possible status of insecticide resistance of released material. *w*MelBr colony remained inbreed for 17 generations and resistant genotypes decreased from 68 to 3.5% in the colony lab. Thus, we released highly susceptible mosquitoes in an area whose wild population was highly resistant to pyrethroids. A second strain (*w*MelRio), with pyrethroid resistance alleles was produced in the laboratory and released simultaneously in two sites in Rio de Janeiro. Resistance alleles were maintained in lab colony by outbreeding 50% wild males every two generations. After 18 months without additional releases, *w*MelRio frequency was >90% in both sites, evidencing its success. *w*MelRio strain is resistant to pyrethroids and the larvicide temephos and susceptible to malathion and diflubenzuron, the chemical compounds currently used in vector control. Mathematical models were applied to test whether different releasing strategies (variations in susceptibility to insecticides of released strains, fitness cost of insecticide resistance) would enhance *Wolbachia* invasion. Our results indicate a successful invasion in two situations: 1) releasing susceptible mosquitos in an environment without insecticide use (may causing a reversal in insecticide resistance levels); and 2) releasing resistant mosquitoes (wMelRio) into a resistant population (such as Rio de Janeiro), even with a high insecticide use. Therefore, the implementation of *Ae. aegypti* infected with *Wolbachia* as a potential strategy to reduce arbovirus transmission should consider the insecticide resistance profile of the wild population and the released strain, to achieve a successful invasion in the field.

1. Introdução

1.1) O cenário das arboviroses no mundo

Arbovírus (termo cunhado a partir da expressão em inglês "**Ar**thropod-**bo**rne **virus**") são vírus mantidos na natureza por meio da transmissão biológica entre hospedeiros vertebrados suscetíveis e seus respectivos vetores, artrópodes hematófagos (Kuno e Chang 2005). Em termos de classificação viral, podem estar distribuídos em sete famílias, seis delas compostas por agentes que têm genoma composto exclusivamente por ácido ribonucleico (RNA): *Togaviridae*, *Flaviviridae*, *Bunyaviridae, Reoviridae, Rhabdoviridae* e *Orthomyxoviridae*. Faz exceção o vírus da febre suína africana, da família *Asfarviridae*, que possui um genoma constituído por ácido desoxirribonucleico (DNA) (Weaver e Raisen 2010).

Os arbovírus apresentam uma alta frequência de mutações, o que possibilita adaptações à infecções em diferentes espécies de hospedeiros vertebrados e invertebrados (Coffey et al. 2013). Consequentemente, podem ser encontrados em uma gama de animais vertebrados como mamíferos, aves e répteis, além de diversos artrópodes vetores, em especial aqueles das ordens Diptera, Anoplura, Hemiptera e carrapatos ixodídeos (Kuno e Chang 2005). Os artrópodes adquirem o arbovírus, preferencialmente, ao realizar repasto sanguíneo em um hospedeiro vertebrado em viremia suficientemente alta. No organismo do vetor susceptível, o vírus pode se replicar em diferentes tecidos e, após um período de incubação, já pode ser encontrado nas glândulas salivares. Assim, em uma próxima alimentação, ocorre a inoculação viral juntamente com a saliva no hospedeiro vertebrado, infectando-o (Liang et al. 2015; Wilson et al. 2017). Uma vez infectados, os vetores susceptíveis assim permanecem por toda sua vida, potencialmente inoculando o vírus em todos os repastos sanguíneos subsequentes. Além disso, alguns arbovírus

podem ser mantidos apenas entre vetores, sem passagem pelo hospedeiro vertebrado, via transmissão vertical (ou transovariana) ou venérea. Entretanto, a frequência deste tipo de transmissão vem se mostrando bem baixa, menor que 5%, para alguns vírus (Gubler e Kuno 1997; Grunnill e Boots 2016).

Os arbovírus, em sua grande maioria, circulam entre animais silvestres em matas e florestas, mantendo-se em ciclos enzoóticos entre vertebrados e vetores, com certa especificidade por hospedeiros. Mas podem ser classificadas como zoonoses caso infectem o homem, que geralmente é classificado como hospedeiro acidental. Das mais de 545 espécies de arbovírus conhecidas, cerca de 150 podem causar doenças em humanos (Liang et al. 2015). Todavia, alguns arbovírus estabeleceram-se com sucesso em ciclos urbanos, com sua manutenção exclusivamente entre humanos e vetores antropofílicos (Mayer et al. 2017). Destes, os arbovírus de maior importância médica pertencem aos gêneros *Flavivirus* (família *Flaviviridae*) e *Alphavirus* (família *Togaviridae*), sendo transmitidos por culicídeos, principalmente dos gêneros *Aedes* e *Culex* (Weaver e Reisen 2010; Mayer et al. 2017).

A ocorrência de surtos epidêmicos de arboviroses está geralmente relacionada à introdução ou reemergência de um dado arbovírus ou sorotipo em regiões com elevada presença de hospedeiros humanos suscetíveis (*naïve*), sem ocorrência do fenômeno de imunidade de rebanho (*herd immunity*) (Kuno e Chang 2005; Fine et al. 2011). Isso porque, uma vez recuperado de uma infecção, o indivíduo aparentemente adquire imunidade por toda sua vida ao arbovírus ou sorotipo com que foi infectado.

Nas últimas décadas, o número de epidemias de arboviroses vem aumentando significativamente pelo mundo (Mayer et al. 2017). Fatores como altos índices de mobilidade entre

países, crescimento urbano desordenado, desmatamentos e mudanças climáticas em escala global têm favorecido a disseminação tanto de vetores quanto de arbovírus, promovendo aumento da incidência destes agravos (Liang et al. 2015; Mayer et al. 2017; Wilder-Smith et al. 2017). Atualmente, entre estes arbovírus com distribuição pandêmica e, portanto, com potencial de causar epidemias urbanas, destacam-se os vírus dengue (DENV), Zika (ZIKV) e chikungunya (CHIKV) (Figura 1) (Patterson et al. 2016; Mayer et al. 2017). Evidências de campo e de laboratório têm confirmado que estes arbovírus sejam transmitidos por mosquitos do gênero *Aedes*, sendo o *Aedes aegypti* o principal vetor, e o *Aedes albopictus* um vetor potencialmente secundário, quiçá com importância epidemiológica em algumas regiões do mundo (Lourenço-de-Oliveira et al. 2004; Vega-Rúa et al. 2015; Ferreira-de-Brito et al. 2016).

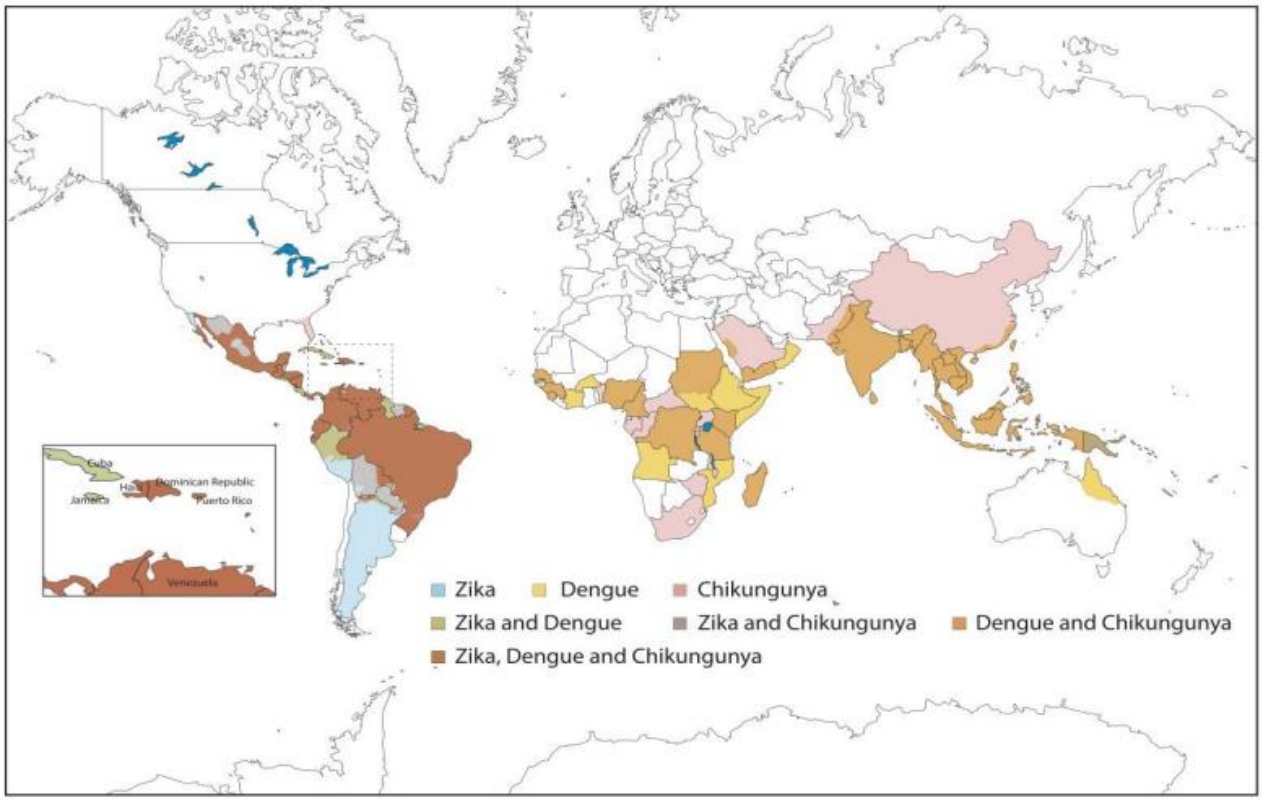

Figura 1. Distribuição global estimada de dengue, Zika e chikungunya (Figura adaptada de Patterson et al. 2016).

1.1.1. Dengue e Zika

Os vírus DENV e ZIKV são vírus de RNA, fita-simples positiva, do gênero *Flavivirus* (família *Flaviviridae*). Infecções por esses arbovírus em humanos podem ser assintomáticas (Bhatt et al. 2013; Grange et al. 2014). Quando sintomáticas, os sintomas são similares aos de outras arboviroses, e inclui febre, dores no corpo (principalmente cabeça e articulações), exantema com pápulas, náuseas, vômitos e conjuntivite (Simpson 1964; Hayes 2009).

A dengue é considerada uma das principais doenças que acometem o ser humano, sendo endêmica em mais de 100 países de regiões tropicais e subtropicais do planeta (OMS 2017a). É a arbovirose com maior prevalência global, pois estima-se que ocorram aproximadamente 390 milhões de infecções por ano, das quais 96 milhões se manifestam clinicamente (Bhatt et al. 2013). O DENV possui quatro sorotipos principais (DENV-1, DENV-2, DENV-3 e DENV-4). Importante ressaltar que qualquer um dos sorotipos pode causar a doença, desde seu espectro mais brando, com sintomas inespecíficos, como também espectros mais severos, com evolução para a dengue grave, com hemorragias, choques e até levar a óbito (Teixeira et al. 2013; MS 2016a). Após 50 anos sem detecções de novos sorotipos, foi descrito o DENV-5 em um surto pontual em humanos na Malásia, em 2007. Entretanto, este sorotipo foi considerado de circulação silvestre em primatas e sua importância epidemiológica ainda carece de informações mais precisas (Mustafa et al. 2015). Importantes surtos de dengue já foram relatados nas Américas, Sudeste da Ásia e África, com um a taxa de letalidade chegando a até 15% (Gubler 2002). No entanto, diagnósticos rápidos e precisos da doença, em conjunto com tratamento médico adequado, podem diminuir esta taxa para menos de 1% (OMS 2017a).

No Brasil, existem indícios de possíveis surtos de dengue desde o século XIX até o início do XX. Posteriormente a este período, a ausência de casos entre 1920 e 1980 provavelmente ocorreu em consequência da campanha bem sucedida de erradicação do *Ae. aegypti* que visava, sobretudo, à eliminação da transmissão da febre amarela urbana (Figueiredo et al. 2000). Após a reinfestação do vetor no país, provavelmente em idos dos anos 70, em 1981-82 ocorreu a primeira evidência de epidemia de dengue no Brasil, quando foram isolados os sorotipos DENV-1 e DENV-4, em Boa Vista (RO) (Osanai et al. 1983). Após um silêncio epidemiológico, os sorotipos DENV-1, -2 e -3 foram introduzidos pelo Estado do Rio de Janeiro, nos anos de 1986, 1990 e 2001, respectivamente, acarretando em sucessivas ondas epidêmicas no Brasil (Schatzmayr et al. 1986; Nogueira et al. 1999, 2001, 2005; Teixeira et al. 1999). Por fim, como já comentado, após breve circulação no país em um surto local no Estado de Roraima, em 1982 (Osanai et al. 1983), o DENV-4 ressurgiu em 2010 no mesmo Estado, o que resultou na sua disseminação para diversas regiões do país (Temporão et al. 2011; Souza et al. 2011). Neste contexto, o Brasil se tornou o país das Américas mais afetado pela dengue, com a cocirculação de seus quatro sorotipos (Fares et al. 2015).

O ZIKV apresenta três linhagens principais: Leste da África, Oeste da África e Asiática (Faye et al. 2014). Foi originalmente foi isolado em 1947, de uma fêmea de macaco *Rhesus* na Floresta Zika (por isso o nome), em Uganda (Dick et al. 1952; Karabatsos 1985). Até recentemente, apenas casos humanos esporádicos foram registrados no continente africano e asiático. O primeiro surto relevante de ZIKV ocorreu na Micronésia em 2007, desde então, sua área de transmissão se expandiu para as ilhas do Oceano Pacífico, com destaque para uma grande epidemia na Polinésia Francesa em 2013 (Duffy et al. 2009; Roth et al. 2014). Possivelmente, sua circulação no Brasil iniciou-se em 2015, nos estados do Rio Grande do Norte (Zanluca et al. 2015),

Bahia (Campos et al. 2015; Zammarchi et al. 2015) e Rio de Janeiro (Ferreira-de-Brito et al. 2016; Metsky et al. 2017). A introdução deste vírus no país gerou uma enorme preocupação para a Saúde Pública por sua rápida expansão, sua importante associação a casos graves de microcefalia e outros comprometimentos neurológicos em recém-nascidos, além da síndrome de Guillain-Barre, independente de idade e gênero (MS 2016b; Ali et al. 2017).

1.1.2. Chikungunya

O CHIKV foi descrito pela primeira vez em 1950, durante um surto atribuído inicialmente ao vírus dengue na Tanzânia (Ross 1956). O nome "chikungunya" é oriundo de um dialeto regional e seu significado faz alusão a "aquele que se dobra", pela postura curvada que o infectado adota (Robinson 1955; Enserink 2007). Este vírus pertence ao gênero *Alphavírus* (família *Togaviridae*) e apresenta três genótipos distintos: Oeste da África, Leste-Centro-Sul da África (ECSA, sigla em inglês) e Asiático. A partir de 2005, o CHIKV rapidamente se espalhou pelas ilhas do sudoeste do Oceano Índico (Weaver e Forrester 2015). Muitos casos importados foram observados em países ocidentais não tropicais, como na Itália, onde ocorreu em 2007 um surto por CHIKV (Angelini et al. 2008). Os casos continuaram a acontecer, e em 2013 o CHIKV foi introduzido na região do Caribe, expandindo-se para as áreas continentais das Américas (Weaver e Forrester 2015).

Estima-se que este arbovírus tenha sido introduzido no Brasil em 2014, com a circulação de dois genótipos, o Asiático via Caribe e Oiapoque/AP, e o ECSA via Feira de Santana-BA (Nunes et al. 2015). Este último aparentemente com uma maior disseminação no país, sendo associado ao surto que ocorreu no Rio de janeiro recentemente, em 2016 (Souza et al. 2017; Costa-da-Silva et al. 2017). Assim sendo, devido à inexistente imunidade de rebanho a esse arbovírus

por conta de sua recente entrada no país, os casos de infecção por CHIKV vem se expandindo rapidamente (Azevedo et al. 2015; MS 2017). Sua infecção em humanos pode causar sintomas similares à dengue, como febre, dores de cabeça e exantema, entretanto, a artralgia pode ser bem mais intensa. Embora a taxa de mortalidade decorrente a este arbovírus seja baixa, em torno de 1%, é uma doença que pode gerar um grande impacto econômico devido ao seu alto potencial incapacitante, ocasionalmente gerando sequelas que podem durar por anos (OMS 2017b).

Neste contexto, o cenário epidemiológico do Brasil se agrava em função das arboviroses apresentarem sintomas clínicos muito similares, que dificultam um diagnóstico preciso, gerando problemas de identificação e controle destas doenças. Portanto, atualmente o país se encontra em situação muito preocupante, visto que o vetor *Ae. aegypti* está disseminado por todos os Estados do país, promovendo surtos com cocirculação de dengue, chikungunya e Zika em grandes centros urbanos, além de haver risco da reintrodução da febre amarela (YFV, gênero *Flavivirus*) em seu ciclo urbano, em virtude da circulação de hospedeiros infectados no ciclos silvestre nas cidades infestadas por populações competentes desse vetor (Couto-Lima et al. 2017).

1.2) O mosquito *Aedes aegypti*

1.2.1. Distribuição geográfica e introdução no Brasil

O *Ae. (Stegomyia) aegypti* (Linnaeus 1762) é um mosquito pertencente à ordem Diptera, família Culicidae e subfamília Culicinae, oriunda do Velho Mundo, sendo originalmente descrita no Egito, na África. A distribuição geográfica desta espécie está interligada com a migração do homem para diversas regiões do mundo, acompanhada pelo estabelecimento do vetor em locais

onde as alterações antrópicas propiciaram a sua proliferação (Consoli e Lourenço-de-Oliveira 1994). Hoje, é um mosquito considerado cosmopolita, tendo sua ocorrência principal em regiões tropicais e subtropicais (Figura 2) (Kraemer et al. 2015).

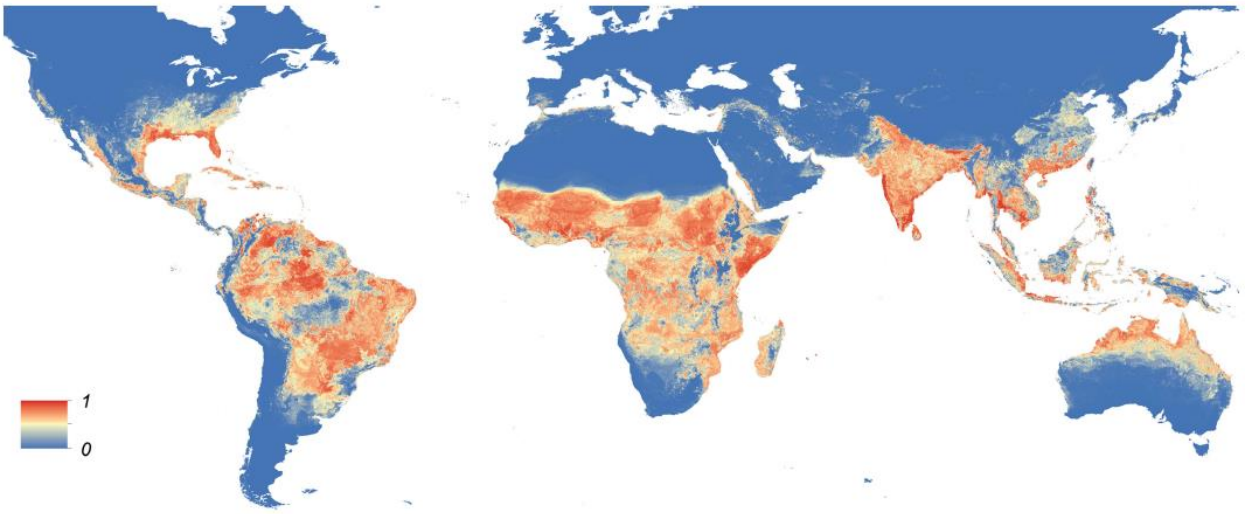

Figura 2. Distribuição geográfica global do *Aedes aegypti*. Probabilidade de ocorrência do vetor variando de 0 (azul) até 1 (vermelho) (Figura adaptada de Kraemer et al. 2015).

A introdução do *Ae. aegypti* no Brasil ocorreu, provavelmente, durante o período colonial, chegando por embarcações que aportaram no país com o tráfico de escravos africanos (Christophers 1960; Powell e Tabachnick 2013). A descoberta de que a febre amarela urbana era transmitida pelo *Ae. aegypti* foi feita em 1900 e, já em 1902-07, Oswaldo Cruz iniciou a primeira campanha pública contra esse vetor. Outras fortes campanhas ocorreram posteriormente, como a de meados de 1930-40. Presume-se que o controle da febre amarela no início do século XX também tenha impactado na transmissão de outras arboviroses, como a dengue, que não existiam no Brasil como um problema aparentemente relevante na época (Löwi 1990; Braga e Valle 2007a).

Como consequência das fortes campanhas contra o *Ae. aegypti*, esta espécie foi considerada erradicada por duas vezes no país, uma em 1955 (com reintrodução em 1967) e outra em 1973. Entretanto, muitos países vizinhos ao Brasil não realizaram tal erradicação, como as Guianas, Venezuela, Estados Unidos e Cuba. A partir de 1976, o *Ae. aegypti* voltou a ser encontrado no Brasil, colocando em suspeita se realmente sua erradicação havia sido eficiente em 1973 (Penna 2003; Braga e Valle 2007a). Após estes acontecimentos, o *Ae. aegypti* disseminou-se rapidamente pelo território nacional e hoje pode ser encontrado em todos os Estados (Kraemer et al. 2015).

1.2.2. Aspectos biológicos do vetor

O *Ae. aegypti* é um inseto holometabólico que possui quatro ínstares larvais, seguidos pela transformação em pupa e adulto (Figura 3). O tempo decorrente do seu ciclo de vida é rápido e ocorre em média de 7-10 dias, da fase larval até a adulta. Na fase imatura, apresenta ciclo aquático, desenvolvendo-se em reservatórios de água limpa e parada com a presença de pouca matéria orgânica (Consoli e Lourenço-de-Oliveira 1994). Embora a fêmea do *Ae. aegypti* tenha predileção por realizar postura de seus ovos em criadouros artificiais, localizados no intra e peridomicílio, como latas, vasos de plantas, caixas d'água e pneus, essas condições são sensíveis a mudanças por causa de variáveis ambientais, do perfil socioeconômico e do hábito cultural de cada região (Honório e Lourenço-de-Oliveira 2001; Maciel-de-Freitas et al. 2007a). Os ovos desta espécie possuem uma importante característica: após concluírem rapidamente o seu desenvolvimento embrionário podem permanecer viáveis por longos períodos em ambientes secos (até um ano), por serem resistentes à dessecação (Consoli e Lourenço-de-Oliveira 1994; Farnesi et al. 2015). A eclosão da larva acontece somente quando o ovo entra em contato direto com a água. Após a fase

larval, ocorre a transformação em pupa e, enfim, o mosquito adulto emerge iniciando o seu ciclo terrestre. Na fase adulta, os mosquitos podem se alimentar de seiva de plantas ou, no caso específico das fêmeas, procurar um hospedeiro para a realização do repasto sanguíneo, essencial para a maturação de seus ovos (Forattini 2002).

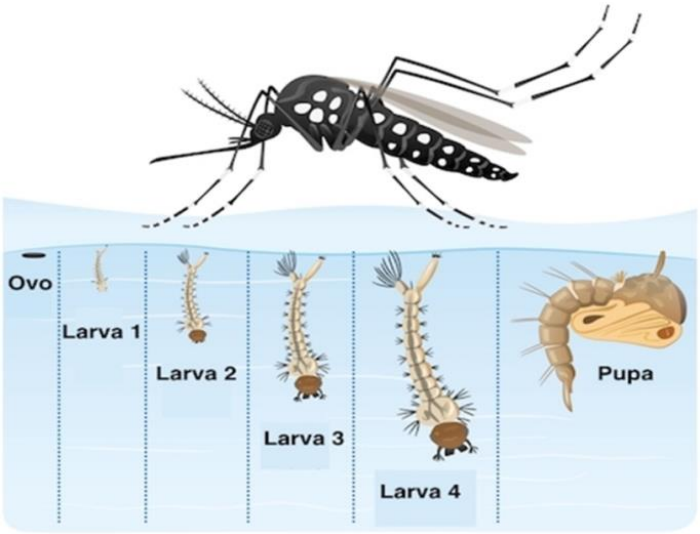

Figura 3. Ciclo de vida do mosquito *Aedes aegypti* (Figura adaptada de http://www.tuasaude.com/ciclo-de-vida-do-aedes-aegypti/).

São as fêmeas adultas do *Ae. aegypti* que desempenham papel chave na transmissão dos arbovírus. Estas possuem hábito hematofágico, destacadamente antropofílico, geralmente adquirindo o arbovírus quando se alimentam de sangue de seres humanos em viremia. Depois de um período de incubação extrínseco, que pode variar de 2 até 14 dias (Rudolph et al. 2014), dependendo do arbovírus e das condições abióticas em questão, as fêmeas tornam-se infectivas e são capazes de transmitir o vírus a indivíduos suscetíveis em todos os repastos subsequentes. Uma particularidade desta espécie é a habilidade da fêmea de se alimentar em diferentes indivíduos para completar seu ciclo gonadotrófico (tempo entre a ingestão de sangue e a postura de ovos),

aumentando assim a chance de transmissão de patógenos entre seres humanos (Consoli e Lourenço-de-Oliveira 1994).

1.3) Capacidade vetorial do *Aedes aegypti*

Um conceito importante em epidemiologia, proposto originalmente para malária e atualmente utilizado por pesquisadores para o modelo *Ae. aegypti*/arbovírus, é o de capacidade vetorial (CV). Este conceito é definido como sendo o número médio de contatos potencialmente infectantes feitos por uma população de mosquitos infectados a partir de hospedeiro infectado em função do tempo (Garret-Jones 1964). Em outras palavras, pode ser visto como a habilidade de um vetor em transmitir determinado patógeno a uma população humana susceptível (Klempner et al. 2007). A CV abrange diversos componentes diretamente envolvidos com a transmissão do agente infeccioso, como a densidade do vetor, susceptibilidade do vetor ao patógeno, taxa de picada, sobrevivência diária e período de incubação extrínseco do patógeno (Dye 1986, 1990). Assim sendo, a CV pode impactar diretamente no número básico de reprodução (R_0) de uma doença, que significa o número médio de casos secundários a partir de um caso primário numa população de hospedeiros susceptíveis (Service 1993).

A CV pode ser expressa pela fórmula matemática abaixo, seguida da definição e de breves considerações sobre suas variáveis:

$$CV = \frac{mbca^2 P^n}{-\ln(P)}$$

1.3.1) *m* = densidade do vetor: esta variável representa o número de fêmeas de mosquito por pessoa residente, em uma dada área. De um modo geral, a densidade de uma espécie como o *Ae. aegypti*, está sob forte influência sazonal, sendo maior nas estações quentes (pois aceleraram o ciclo de vida do vetor) e em períodos chuvosos (aumentam a oferta de criadouros). Outros fatores, tais como perfil socioeconômico das regiões, também podem influenciar na densidade e na distribuição espacial heterogênea do vetor, por aumentarem de maneira quantitativa os depósitos disponíveis (Consoli e Lourenço-de-Oliveira 1994). Maiores detalhes sobre esta variável podem ser encontrados no item 1.4.1.

1.3.2) *b*, *c* = susceptibilidade do vetor ao vírus (competência vetorial): *b* é a probabilidade que um mosquito infectado tem de transmitir o parasito ao picar um hospedeiro humano susceptível e *c* é a probabilidade que um mosquito tem de se infectar com o parasito ao picar um hospedeiro humano infectado. Variações na susceptibilidade do *Ae. aegypti* ao vírus dengue estão sob controle genético, o que resulta em populações com diferentes níveis de susceptibilidade (Bosio et al. 2000; Lourenço-de-Oliveira et al. 2004). Este mesmo padrão de variação de susceptibilidade entre populações dos vetores pode ser visto mais recentemente para outros arbovírus, como CHIKV e ZIKV (Vega-Rúa et al. 2015; Chouin-Carneiro et al. 2016).

1.3.3) *a* = taxa de picada: esse componente expressa a intensidade do contato entre vetor e hospedeiro humano: quanto mais intenso esse contato, maior a CV e a transmissão do vírus (Garret-Jones 1964; Maciel-de-Freitas et al. 2010; Sylvestre et al. 2013).

1.3.4) *P* = probabilidade de sobrevivência diária (PDS): determina a longevidade de fêmeas adultas e, juntamente com o período de incubação extrínseco (ver abaixo), é o parâmetro considerado mais importante em modelos de transmissão de arbovírus. Quanto mais longeva uma fêmea ou uma

população de mosquitos, maior chances têm de sobreviver o tempo necessário para que se tornem infectivas antes de morrerem. Vale ressaltar que pequenos aumentos de sobrevida podem influenciar exponencialmente a capacidade vetorial de mosquitos (Kuno 1995; Luz et al. 2003). Detalhes deste parâmetro podem ser encontrados no item 1.4.2.

1.3.5) *n* = período de incubação extrínseco (PIE): expressa o tempo, em dias, transcorrido desde a alimentação sanguínea da fêmea do mosquito em um hospedeiro virêmico até a chegada do vírus à sua glândula salivar. Acredita-se que o PIE esteja sob forte influência da temperatura, sendo este período mais curto em mosquitos são que mantidos em temperaturas mais elevadas (Watts et al. 1987; Turell e LundStrom 1990; Chan e Johansson 2012). Neste contexto, o termo P^n retrata o número de fêmeas que sobrevivem ao PIE.

Alguns trabalhos demonstram como a CV de *Ae. aegypti* pode ser impactada por variações na estimativa de seus componentes. Por exemplo, para que ocorra transmissão, a longevidade de fêmeas precisa ser maior que a duração do PIE dos arbovírus (Maciel-de-Freitas et al. 2007b). Uma duração curta do PIE de poucos dias aumenta drasticamente a CV dos mosquitos. Em laboratório, já foram observadas durações variadas de PIE para DENV, ZIKV e CHIKV. Embora o DENV tenha o maior tempo de PIE, cerca de 10 a 14 dias (Watts et al. 1987; Gluber e Kuno 1997), o vírus já foi detectado a partir do quarto dia pós-infecção nas glândulas salivares de mosquitos (Salazar et al. 2007). Para ZIKV o PIE pode durar de 5 a 10 dias (Chouin-Carneiro et al. 2016) e para CHIKV de 2 a 9 dias (Rudolph et al. 2014; Vega-Rúa et al. 2015).

1.4) Experimentos de marcação, soltura e recaptura

Experimentos de marcação, soltura e recaptura (MSR) permitem estimar, indiretamente em campo, parâmetros da capacidade vetorial de *Ae. aegypti*, como sua densidade populacional e sobrevivência diária (Guerra et al. 2014). Além disso, outros aspectos ecológicos também podem ser estudados, como dispersão do vetor, duração do ciclo gonotrófico, etc (Service 1993; Maciel-de-Freitas et al. 2010). A metodologia de MSR tem uma vasta literatura de modelos matemáticos que são aplicados em estudos sobre ecologia de animais, desde mamíferos até insetos vetores (Le Cren 1965; Service 1993; Guerra et al. 2014). A partir da década de 1960, experimentos de MSR com mosquitos começaram a ter mais destaque e, desde então, diversos estudos foram realizados com os gêneros *Aedes*, principalmente para *Ae. aegypti* (Sheppard et al. 1969; Harrington et al. 2008; David et al. 2009; Maciel-de-Freitas et al. 2008, 2010) e *Ae. albopictus* (Marini et al. 2010; Cianci et al. 2013), *Culex* (Reisen et al. 1977; Tsuda et al. 2008) e *Anopheles* (Touré et al. 1998; Dos Santos et al. 2004; Midega et al. 2007). Para tal, os desenhos mais frequentes com mosquitos envolvem a soltura de uma ou mais coortes de fêmeas marcadas com pó fluorescente, de cores distintas, seguidas de capturas diárias por armadilhas, como BG-Sentinela, ou por aspiração manual (Service 1993; David et al. 2009; Maciel-de-Freitas et al. 2007b, 2008, 2010; Guerra et al. 2014).

1.4.1. Densidade populacional

Petersen foi o primeiro a estimar a densidade populacional de animais pelo método de MSR, dividindo o número de animais marcados e liberados, pela proporção de marcados no grupo

capturado (Lincoln 1930). Este primeiro modelo matemático mencionado, chamado de "Índice de Lincoln" ou "Lincoln-Petersen", é o modelo mais simples e considera as populações fechadas, ou seja, sem recrutamento (nascimento e imigração) e perdas (morte e emigração) de indivíduos. O modelo é definido pela fórmula matemática $\frac{M}{m} = \frac{N}{n}$, onde *M* é o número de fêmeas liberadas marcadas, *m* é o número de indivíduos marcados capturados, *n* é o total de mosquitos selvagens capturados e *N* é a densidade populacional em campo. Esta mesma ideia foi posteriormente modificada para outros modelos mais complexos, como Fisher-Ford, que adiciona a variável "mortalidade dos indivíduos" (Fisher 1947), e Jolly-Seber, que exige múltiplas capturas de um mesmo indivíduo (Service 1993).

Dando enfoque às arboviroses, a abundância de mosquitos apresenta uma intensa correlação com o curso temporal e espacial de uma epidemia. Por isso, uma estimativa precisa da densidade populacional de *Ae. aegypti* é de suma importância para um melhor entendimento sobre a transmissão de patógenos em campo, bem como para o planejamento e direcionamento de atividades de controle. Todavia, fazer a contagem de uma população de mosquitos não é uma tarefa trivial.

Atualmente, para quantificação de *Ae. aegypti* em campo, e consequentemente definição de risco de transmissão e direcionamento das atividades de controle, são coletadas amostras de imaturos (larvas e pupas) por meio de vistorias em busca de recipientes que possam albergá-los. Para isto, são amostradas cerca de 10% das casas de uma região, selecionadas aleatoriamente, onde são feitas inspeções de 4-6 vezes por ano (Coelho et al. 2008). A partir daí, são gerados índices de infestação, sendo o Índice de Infestação Predial (percentual de imóveis que tem criadouros positivos) e o Índice de Breteau (número de criadouros positivos por imóvel visitado) os dois mais

comumente empregados. Contudo, ambos apresentam baixa correlação com a população de mosquitos adultos, pois negligenciam a mortalidade das larvas e a produtividade dos criadouros (Morrison et al. 2008). Um índice baseado em pupas, por exemplo, teria maior representatividade da população de adultos, pois, neste estágio, os mosquitos apresentam mortalidade irrelevante. Dessa maneira, a associação entre a população de pupas com a de adultos costuma ser mais fidedigna (Tun-Lin et al. 1995; Focks e Chadee 1997).

Alternativamente, a quantificação de mosquitos na natureza pode ser realizada por experimentos de MSR com a utilização de estimadores simples, como o de Lincoln (Guerra et al. 2014). Uma das vantagens de se usar esta metodologia é que se estima diretamente a densidade populacional de adultos, a fase de vida do mosquito mais relevante epidemiologicamente por ser a responsável pela transmissão de patógenos. Estudos usando o método de MSR em *Ae. aegypti* ainda são importantes para otimizar estratégias de controle que aplicam a liberação de mosquitos em campo, como no controle biológico utilizando a bactéria *Wolbachia* (item 1.13.4). Nestes casos, conhecer a densidade populacional de mosquitos na área trabalhada é uma informação de grande valia. A partir da realização de MSR é permitido um cálculo mais preciso do número de mosquitos que devem ser soltos, baseados exclusivamente na quantidade estimada de mosquitos selvagens na área. Portanto, este cálculo é importante para se encontrar uma densidade ótima a ser solta, evitando-se que, ao acaso, ocorra liberação de uma quantidade insuficiente de mosquitos (o que resultaria numa falha da disseminação da *Wolbachia* em campo), ou em excesso (o que causaria um incômodo desnecessário aos moradores das áreas selecionadas, comprometendo o apoio dado ao projeto previamente às liberações) (Garcia et al. 2016).

1.4.2. Probabilidade de sobrevivência diária (PDS)

Outra informação relevante que pode ser obtida por estudos de MSR, é a probabilidade de sobrevivência diária (PDS) de fêmeas de *Ae. aegypti*. Tal parâmetro é comumente estudado em condições de laboratório, gerando curvas de sobrevivência ao longo do tempo de observação (Briegel et al. 2001). Entretanto, está claro que um insetário é um ambiente artificial favorável para o mosquito, com disponibilidade farta de alimento, além de temperatura e umidade controladas dentre outros aspectos. Aplicando o método de MSR, podemos determinar valores de PDS para os insetos vetores diretamente em campo, seu ambiente natural. Nestes ensaios, o número de insetos capturados por dia em função do tempo pode se transformar em estimativas de PDS. Gilles (1961) foi pioneiro ao introduzir o modelo exponencial para estimar PDS em *Anopheles gambiae* na África. Desde então, esses modelos matemáticos têm sido usados em outras espécies de mosquitos (Gillies e Wilkes 1965; Reisen et al. 1978, 1980; Rodriguez et al. 1992; Constantini et al. 1996; Maciel-de-Freitas et al. 2006, 2007b). Dois modelos são usualmente empregados para estimar PDS: i) exponencial e ii) Buonaccorsi. O primeiro é um modelo prático e simples, entretanto não considera a remoção dos indivíduos adultos após a recaptura em campo (Styer et al. 2007a,b; Gilles 1961; Maciel-de-Freitas et al. 2007b). O segundo modelo, proposto por Buonaccorsi et al. (2003), é considerado uma análise mais robusta, pois permite a correção das estimativas devido à remoção dos indivíduos nas coletas efetuadas nos dias anteriores.

A PDS de mosquitos é um número que varia entre 0 e 1 (quanto mais próximo de 1, mais tempo a fêmea sobrevive em campo) e está diretamente relacionada com a longevidade, que é o número de dias em que os mosquitos, tendo a PDS previamente calculada, sobreviveriam (Niebylski e Craig 1994). Após estimarmos a PDS, também podemos, por exemplo, elevar este valor à potência de 10 (período extrínseco médio de incubação do DENV em *Ae. aegypti* (Salazar

et al. 2007) para calcular o percentual de fêmeas sobreviventes após 10 dias no campo, ou seja, quantas delas podem vir a se tornar infectantes e transmitir o dengue.

Além disso, torna-se importante o cálculo de PDS em estratégias de controle que liberam linhagens de laboratório de mosquitos em campo, como no controle biológico usando-se mosquitos transgênicos (1.13.1) ou com *Wolbachia* (item 1.13.4). Nestes casos, a PDS é usada como um parâmetro para estimar a performance dos insetos liberados, possibilitando ainda uma comparação com indivíduos selvagens. Em geral, estratégias que liberam mosquitos com PDS baixos, ou seja, com uma baixa performance, necessitam liberar um número maior de insetos para compensar essa baixa performance, ou podem até serem inviabilizadas (Nguyen et al. 2015; Garcia et al. 2016).

1.4.3. Inferência bayesiana

Os estimadores supracitados (como Lincoln e Fisher-Ford) são usualmente considerados de forma determinística, ou seja, não consideram incerteza associada aos valores observados nos experimentos e, por isso, apresentam um único valor de saída. São mais simples, porém tratam importantes parâmetros como taxas de sobrevivência e de captura como valores absolutos dentro de uma distribuição. Por exemplo, no caso do Índice de Lincoln, determinado a partir de estudos de MSR com mosquitos, é esperado que a estimativa de abundância seja imprecisa, por causa de múltiplos fatores como a eficiência (ou falta de) das armadilhas utilizadas, condições climáticas, número de armadilhas e outros.

A inferência Bayesiana é um tipo de inferência estatística que envolve cálculos matemáticos mais complexos, mas geram estimativas do tamanho da população juntamente com

medidas de incerteza (Service 1993). Portanto, sua utilização traz potenciais vantagens para as análises de experimentos de MSR e, por isso, vem sendo cada vez mais utilizada em ecologia (Royle et al. 2009, 2011; Royle e Dorazio 2012; Hancock et al. 2016; Dorazio e Karanth 2017). Nesta nova abordagem, são adicionados componentes importantes aos modelos, como por exemplo, a probabilidade de captura, além de explorar a distribuição espacial dos animais. Assim sendo, posteriormente, trabalhos de MSR com mosquitos utilizaram inferência Bayesiana para realizar estimativas mais refinadas de abundância, sobrevivência, recrutamento, entre outros aspectos biológicos e ecológicos por vezes pouco estudados (Villela et al. 2015, 2017).

1.5) Controle vetorial

A transmissão vetorial de arbovírus envolve um ciclo que engloba diretamente três componentes: o inseto vetor, o hospedeiro vertebrado e o vírus, governados pelas condições ambientais. Apesar de grandes esforços das pesquisas científicas, ainda não estão disponíveis vacinas eficazes em larga escala para as principais arboviroses (DENV, CHIKV e ZIKV), e mesmo para febre amarela (YFV) a disponibilidade pode ser insuficiente em momentos de grandes epidemias. Além disso, não há drogas específicas que sejam capazes de bloquear a transmissão ou suprimir totalmente suas manifestações clínicas. Por isso, estratégias cujo foco é o vetor, o mosquito *Ae. aegypti*, são atualmente as principais ferramentas para o controle destas doenças (San Martín et al. 2010).

O controle vetorial é definido como qualquer medida efetuada contra o mosquito, que tenha como objetivo prevenir a infecção mediante a redução da transmissão de patógenos. Para isto, são realizadas ações que visam diminuir o tamanho populacional de *Ae. aegypti*, que podem ser

direcionadas tanto aos criadouros, com as formas imaturas, quanto aos mosquitos adultos. Atualmente, as estratégias recomendadas pelo Ministério da Saúde (MS) que são utilizadas em larga escala no Brasil abrangem medidas de controle mecânico/físico, químico e biológico (Braga e Valle 2007b; MS 2014a).

1.5.1. Controle mecânico/físico

Este tipo de controle é realizado principalmente pelo manejo ambiental. As principais atividades focam na eliminação, proteção ou destinação adequada de criadouros. Esse tipo de controle também inclui outras medidas eficazes como utilização de telas em portas e janelas, coleta adequada de lixo, melhoramento das condições sanitárias, fornecimento regular de água encanada, entre outros (Braga e Valle 2007b; MS 2009). É um tipo de controle que pode ser otimizado com a sensibilização da população em campanhas como a dos "10 minutos contra o *Aedes*", desenvolvida por pesquisadores do Instituto Oswaldo Cruz (http://www.ioc.fiocruz.br/dengue/textos/10minutos.html).

1.5.2. Controle biológico

O controle biológico é feito pela utilização de organismos, ou de produtos gerados por estes, que agem diretamente em uma população do inseto vetor. Vírus, bactérias, protozoários, nematódeos, fungos e diversos predadores do organismo alvo podem ser utilizados neste tipo de controle (Rozendaal 1997). O controle biológico é visto como uma alternativa ao uso de inseticidas químicos, com menor potencial de gerar danos ao meio ambiente. No caso dos mosquitos, a

bactéria *Bacillus thuringiensis israelensis* (*Bti*) é recomendada pela Organização Mundial da Saúde (OMS) para uso em água potável e tem se mostrado um potente biolarvicida contra o *Ae. aegypti* (Chavasse e Yap 1997; Mittal 2003; Araújo et al. 2013). Recentemente, este biolarvicida começou a ser produzido comercialmente pela Fiocruz em uma formulação mais estável (www.denguetech.com.br), evitando problemas operacionais anteriormente enfrentados no país (Braga e Valle 2007b).

Outro tipo de controle biológico de *Ae. aegypti*, no momento em teste em campo, é a bactéria *Wolbachia*, que, embora não cause a morte do mosquito, inibe a transmissão dos patógenos por ele transmitidos, com enfoque para os vírus dengue, chikungunya e Zika (Moreira et al. 2009a; Hoffmann et al. 2011; Dutra et al. 2016). Detalhes sobre o uso desta ferramenta estão descritos no item 1.13.4.

1.5.3. Controle químico

Este tipo de controle é efetuado com uso de inseticidas químicos e tem uma história de mais de 60 anos no âmbito do controle de insetos. Devido ao seu importante papel no combate a pragas agrícolas e insetos vetores, os inseticidas permanecem até os tempos atuais como uma ferramenta essencial em todo o mundo (Rose 2001). Inseticidas são compostos que, quando aplicados direta ou indiretamente sobre os insetos, em concentrações adequadas, causam sua morte ou afetam o seu desenvolvimento. Portanto, o uso de inseticidas impacta na densidade populacional da espécie alvo, causando uma supressão momentânea após sua aplicação. Este tipo de controle pode ser altamente eficiente quando implementado de maneira adequada. Porém, a falta de qualificação de operadores, inseticidas adulterados ou de má qualidade, uso excessivo e

resistência a inseticidas (item 1.9) são alguns dos fatores que podem afetar sua eficácia em campo (Karunamoorthi e Sabesan 2013).

Até início de 1930, os compostos químicos usados contra insetos se limitavam a arsênico (verde paris à base de cobre e arsênio), óleos de petróleo, nicotina, piretro e enxofre. Tais compostos apresentavam baixa persistência devido à fotossensibilidade, além de terem uma alta toxicidade para humanos (no caso dos metais pesados) (Mallet 1989; Casida e Quisad 1998). Por isso, o desenvolvimento de inseticidas eficientes e que permanecem ativos por períodos longos em campo foi um dos mais importantes avanços no controle de insetos no século XX. O primeiro inseticida orgânico sintético de destaque foi o dicloro-difenil-tricloroetano (DDT), um organoclorado desenvolvido durante a Segunda Guerra Mundial (Braga e Valle 2007b). Após a descoberta do DDT deu-se início a "Era química", com o desenvolvimento de outros inseticidas sintéticos que, desde então, têm sido rotineiramente empregados em campo.

Atualmente, os principais inseticidas utilizados no controle químico de vetores são neurotóxicos, ou "clássicos", que atuam no Sistema Nervoso Central (SNC) e os reguladores do crescimento de insetos (IGR), que atuam sobre o desenvolvimento dos insetos (OMS 2009, WHOPES 2017).

1.6) Inseticidas neutoróxicos

São substâncias que agem nos neurônios (células do SNC) dos insetos que tem como alvo principal importantes moléculas, como o canal de sódio dependente de voltagem (Na_V) e a enzima acetilcolinesterase (AChE). A ação dos inseticidas neurotóxicos é rápida, pois impacta na transmissão de impulsos elétricos, tornando-os incessantes. Assim, o SNC será continuamente

estimulado, levando o inseto à paralisia e culminando em sua morte. Os principais inseticidas deste grupo pertencem às classes dos Organoclorados, Organofosforados, Carbamatos e Piretroides (Ware e Whitacre 2004; Braga e Valle 2007b).

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1.6.1. Organoclorados (OC)

A classe dos OC inclui principalmente o DDT, provavelmente a substância química mais notória do século passado. Em 1939, o químico suíço Paul Muller descobriu a propriedade inseticida do DDT, o que lhe rendeu o prêmio Nobel de Medicina pela ampla utilização deste composto no controle de vetores da malária, febre amarela e tifo (Ware e Whitacre 2004). Na época, este OC era visto como uma revolução no controle de insetos, pois permanecia ativo no ambiente por longos períodos de tempo, apresentava um baixo custo e tinha um amplo espectro de ação (Rozendaal 1997). Inseticidas do tipo DDT agem no SNC, especificamente no canal de sódio dependente de voltagem (Na_V) dos neurônios dos insetos, mantendo-os abertos (mesmo mecanismo que os PIs), o que culmina na morte do inseto (Ware e Whitacre 2004).

Embora o DDT seja considerado até hoje um potente inseticida para controle de insetos vetores e pragas, a descoberta de seus efeitos negativos ao ambiente e a bioacumulação em tecidos dos animais levou a sua proibição em vários países (Carson 1962; Ware e Whitacre 2004). No Brasil, seu uso foi proibido gradativamente: primeiramente em 1985, na agricultura; em 1997, no controle de vetores; em 1998, foi finalmente banido para todos os fins (D'Amato et al. 2002; Guimarães et al. 2007). Hoje em dia, o uso do DDT ainda é indicado pela OMS em casos específicos, como no controle da malária, principalmente em regiões hiperendêmicas na África (OMS 2011).

1.6.2. Organofosforados (OP)

Inseticidas OP, genericamente, compartilham o elemento fósforo em sua estrutura química. Nesse grupo são encontrados compostos com uma grande variedade de combinações de carbono, hidrogênio, oxigênio, fósforo, enxofre e nitrogênio (Ware e Whitacre 2004). Suas propriedades inseticidas foram primeiramente observadas na Alemanha durante a Segunda Guerra Mundial, em estudos realizados com gases que atuam no SNC, como sarin (Stoddart 1979; OMS 1997). Assim como outros inseticidas neurotóxicos, os OP agem no SNC dos insetos. Entretanto, inibem especialmente a AChE, enzima que degrada o neurotransmissor acetilcolina (mesmo mecanismo que os CA). O grupo fosfato dos OP ataca o grupo éster da AChE e as fosforila, tornando a enzima irreversivelmente inativada. Com isso, acetilcolina se acumula nas junções nervosas (sinapses), impedindo a interrupção da propagação do impulso elétrico, culminando com a morte do inseto (Braga e Valle 2007b).

Os OP foram primeiramente utilizados contra pragas agrícolas e passaram a ser usados no controle de vetores em Saúde Pública, principalmente após a detecção de resistência aos OC (OMS 1997). Esta classe de inseticidas apresenta vantagens em seu uso por sua ampla eficiência, ser biodegradável e não se acumular nos tecidos (Ware e Whitacre 2004; Braga e Valle 2007b). Exemplos de compostos desta classe usados no controle do *Ae. aegypti* são o temephos (larvicida) e o malathion (adulticida).

1.6.3. Carbamatos (CA)

Os carbamatos são inseticidas derivados do ácido carbâmico e sua comercialização teve início por volta dos anos 1960. Os inseticidas desta classe têm o mecanismo de ação semelhante aos OP; atuam na AChE, embora neste caso a inibição seja reversível (reação de carbamilação). Estes compostos têm como seus principais representantes o carbaril e o propoxur. Na Saúde Pública, a utilização dos CA tem sido considerada por apresentar baixa persistência no ambiente, assim como baixa toxicidade para mamíferos e amplo espectro de ação contra insetos. Seu uso é recomendado principalmente em áreas onde há resistência aos OC e PI (em regiões na África), especialmente no controle da malária (OMS 1997; Ware e Whitacre 2004; Braga e Valle 2007b).

1.6.4. Piretroides (PI)

Os PI compõem uma classe de inseticida sintéticos análogos das piretrinas, substância extraída de plantas do gênero *Chrysanthemum*, da família Asteraceae (Casida 1980). Suas propriedades inseticidas são derivadas de ésteres e ácidos piretroicos, que são altamente lipofílicos e penetram rapidamente no organismo dos insetos, agindo no seu SNC (Reigart e Roberts 1999). As piretrinas naturais foram utilizadas por muitos anos como inseticidas, mas como apresentam uma elevada fotossensibilidade, sua eficiência é limitada em campo. Tal fato estimulou o desenvolvimento dos PI sintéticos atuais, que são compostos com maior estabilidade e potencial inseticida. Na década de 1970, após sua formulação sintética, os PI passaram a ser utilizados amplamente na agricultura e no controle de insetos vetores (Beaty e Marquardt 1996).

Inseticidas desta classe atuam no canal de sódio regulado por voltagem (Na_V), das membranas dos axônios, mudando sua conformação e mantendo-os abertos (ação similar ao DDT). Sua ação nos insetos é bem rápida, causando paralisia imediata e morte. Tal efeito, quase que

instantâneo e característico desta classe, é chamado de “knockdown” (Braga e Valle 2007b; Nkya et al. 2013).

Os PI são considerados inseticidas muito eficientes quando comparados a outros inseticidas por possuírem um amplo espectro de atividade, eficiência em baixa dose, baixo poder residual no ambiente e ação rápida, além de serem biodegradáveis. Embora não sejam nocivos para mamíferos, em animais aquáticos como peixes, os PI podem apresentar alta toxicidade (Ware 2000; Braga e Valle 2007b). Por esse motivo, raramente são empregados diretamente na água como larvicidas no controle de vetores.

A importância desta classe de inseticidas no controle de mosquitos adultos é inquestionável. É a única classe recomendada pela OMS para uso em mosquiteiros impregnados no controle da malária (OMS 2015), além de ser a classe mais utilizada contra os *Ae. aegypti* adultos, por aplicação espacial e residual (OMS 2016). Alguns exemplos de compostos PI são a deltametrina, cipermetrina, permetrina e lambda-cialotrina.

1.7) Reguladores do crescimento de Insetos (IGR)

Os reguladores do crescimento de insetos, mais conhecidos pela sigla em inglês IGR (*Insect Growth Regulator*), são considerados uma alternativa ao uso de inseticidas neurotóxicos clássicos por atuarem sobre alvos distintos. Por isso, muitas vezes, substituem-se estes compostos em casos onde há detecção de resistência em campo. Diferente dos neurotóxicos que apresentam ação rápida, os IGR agem de forma lenta no inseto, modificando principalmente a fisiologia e morfologia e, consequentemente, interferindo no seu desenvolvimento e reprodução (Graf 1993; Fournet et al. 1997). Atualmente, os IGRs são utilizados como larvicidas no controle de *Ae.*

aegypti, principalmente os grupos dos inibidores de síntese de quitina - ISQ (como o diflubenzuron e novaluron); e análogos de hormônio juvenil – AHJ (como o pyriproxyfen). O primeiro interfere na produção de quitina pelos insetos e a segunda age no sistema endócrino.

Tabela 1. Principais inseticidas utilizados no controle de insetos vetores e seus respectivos alvos ou efeitos.

Inseticidas	Classes	Alvo ou efeito
Neurotóxicos (ação rápida)	**Organoclorados (OC)** ex: DDT	Canal de Sódio (Na_V)
	Organofosforados (OP) ex: temephos, malathion	Acetilcolinesterase (AChE)
	Carbamatos (CA) ex: propoxur	Acetilcolinesterase (AChE)
	Piretroides (PI) ex: deltametrina	Canal de Sódio (Na_V)
IGRs (ação lenta)	**Análogo do hormônio juvenil (AHJ)** ex: pyriproxyfen	Inibe a muda para estágio adulto
	Inibidores de síntese de quitina (ISQ) ex: diflubenzuron	Interfere na síntese da quitina

1.8) Histórico recente do uso de inseticidas contra o *Ae. aegypti* no Brasil

De 1967 até 1999, a única classe de inseticidas usada no controle de *Ae. aegypti* no Brasil foi a dos OP, tanto no combate às larvas quanto aos adultos. Seu uso foi intensificado depois dos surtos de dengue ocorridos a partir de 1986. O larvicida temephos (OP), na época o único produto recomendado pela OMS para uso em água potável (Chavasse e Yap 1997), foi o principal composto utilizado durante todo este período. Além do temephos, adulticidas OP também eram utilizados nesta época, como o malathion e o fenitrothion (Braga e Valle 2007b).

A partir de 1999, em consequência do excessivo uso de OP, foram detectadas diversas populações brasileiras de *Ae. aegypti* resistentes a esta classe de inseticidas (Lima et al. 2003; Macoris et al. 2003, 2007; Braga et al. 2004; Montella et al. 2007; Lima et al. 2011). Por esse motivo, em 2001, o MS recomendou a interrupção do uso dos adulticidas OP e iniciou o uso público dos PI. Desde então, os PI se tornaram os adulticidas mais utilizados em campo. Isso porque além do uso governamental, por aplicação residual e ultra baixo volume (UBV), campanhas que estimulam o uso doméstico são comumente encontradas na mídia, em especial em meses epidêmicos. Adicionalmente, empresas privadas contratadas por condomínio e associação de moradores continuam aplicando PI via UBV em áreas privadas. Neste contexto, apenas um ano após a implementação de PI já foram detectadas alterações no status de susceptibilidade dos *Ae. aegypti* adultos de várias localidades a esta classe de inseticidas (da-Cunha et al. 2005; Martins et al. 2009a). Por isso, a partir de 2009, o Brasil passou a adotar novamente o uso de malathion (OP) para o controle de adultos (MS 2009; Fiocruz 2011). Entretanto, esta transição mostrou-se bem complexa e lenta no país, sendo os PI usados até hoje, em campo, por empresas e no uso doméstico (Bellinato et al. 2016; Garcia et al. 2017a).

Para larvicidas, como já citado anteriormente, o temephos (OP) foi o principal composto usado em campo até o diagnóstico de resistência em *Ae. aegypti* brasileiros, em 1999. A partir deste ano, ocorreu uma tentativa de substituição deste composto pelo biolarvicida *Bti*. Entretanto, suas formulações disponíveis na época apresentavam uma baixa persistência em campo, acarretando problemas operacionais e limitações para sua utilização (Mittal 2003; Braga e Valle 2007b). Assim sendo, somente em 2009 os IGRs foram incorporados na rotina de controle de larvas de *Ae. aegypti* no Brasil (MS 2009), logo após a sua aprovação para uso em água potável (OMS 2009).

Os primeiros IGRs a serem usados foram os ISQ, como o diflubenzuron (MS 2009), alterando em 2014 para os AHJ, como o pyriproxyfen (MS 2014b). Atualmente, pretende-se manter uma rotação de larvicidas IGR a cada 3-4 anos, alternando-se o uso de composto com diferentes mecanismos de ação. O objetivo deste manejo é tornar o controle químico sustentável em longo prazo, retardando a disseminação da resistência a inseticidas em função de uma intensa e constante pressão seletiva realizada por apenas um princípio ativo. Excepcionalmente, não é possível realizar a rotação de compostos com adulticidas, visto que só duas classes são disponíveis para uso, PI e OP. Sendo que para PI boa parte das populações brasileiras de *Ae. aegypti* já se encontram resistentes, restando assim, apenas o malathion OP para ser usado com eficiência.

É importante ressaltar que, numa mesma região, paralelamente ao controle químico realizado para *Ae. aegypti*, pode também ocorrer a aplicação de inseticidas que têm como alvo outras espécies de insetos vetores, por exemplo para o controle da leishmaniose e da malária (uso de PI ou CA por aplicação residual). Portanto, embora seja difícil estimar, é possível que ocorra uma interferência deste tipo de atividade em relação à resistência a inseticidas em *Ae. aegypti*.

Neste contexto, considerando-se o histórico do uso de inseticidas nos últimos anos, é notório que a resistência em populações de *Ae. aegypti* vem impactando diretamente na escolha dos compostos químicos empregados em campo. Com isso, torna-se necessário, por vezes, a mudança de compostos químicos empregados no Brasil, tanto adulticidas quanto larvicidas (Figura 4).

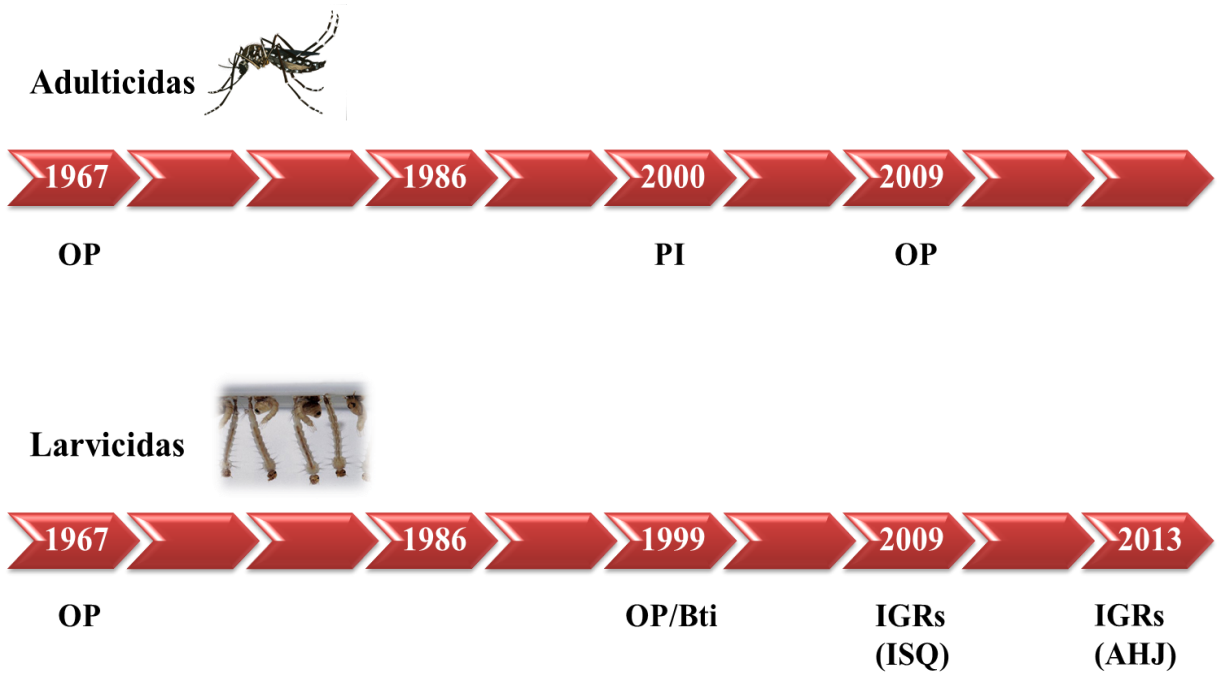

Figura 4. Linha do tempo retratando o histórico recente de aplicação de inseticidas no Brasil para uso no controle de adultos e larvas de *Aedes aegypti*.

1.9) Resistência a inseticidas

A resistência a inseticidas é um fenômeno que advém da variabilidade genética natural em populações selvagens de insetos. Essas diferenças genéticas permitem que indivíduos com mutações vantajosas, relacionadas ao fenótipo de resistência, possuam maior probabilidade de sobreviver a tratamentos com inseticidas. Ou seja, em um ambiente com aplicação de inseticidas, indivíduos resistentes contribuem com uma prole mais numerosa que aqueles indivíduos suscetíveis, resultando no aumento da frequência do gene que confere resistência nas próximas gerações (Mallet 1989; Beaty e Marquardt 1996). Após sucessivos ciclos de aplicação de inseticidas, a frequência de indivíduos resistentes tende a aumentar até níveis em que a eficácia do inseticida se torne comprometida (ffrench-Constant 2006).

A resistência a inseticida é definida como a habilidade de uma população de insetos em sobreviver a doses de inseticidas que seriam letais para a maioria dos indivíduos de uma população susceptível da mesma espécie (Beaty e Marquardt 1996). Em outras palavras, a resistência pode ser compreendida como um processo de "mudança genotípica" acelerada de uma população, em resposta a uma intensa pressão seletiva exercida pelo inseticida (Braga e Valle 2007b). Seu desenvolvimento está diretamente relacionado com fatores genéticos (frequência e dominância dos alelos R, número de alelos R e custo no *fitness*), biológicos e ecológicos (tempo de geração, número de indivíduos por prole e migração), além de também sofrer influência de práticas operacionais inadequadas (como por exemplo, aplicação excessiva, formulação incorreta, falha na rotação de compostos, seleções prévias de resistência por outros químicos) (Ferrari 1996; Sarwar e Salman 2015).

Os primeiros relatos sobre casos de resistência a inseticidas iniciaram-se por volta dos anos 1940, em conjunto com a introdução dos inseticidas sintéticos em larga escala no mercado. Desde então, a resistência vem aumentando em consonância com a aplicação excessiva de compostos (Mallet 1989). Atualmente, a resistência em insetos foi detectada para praticamente todas as classes de inseticidas, como OC, OP, CA e PI (Sarwar e Salman 2015).

Particularmente para insetos vetores, a resistência a inseticidas estaria relacionada com um aumento na capacidade vetorial (CV) (item 1.3) (McCarrol et al. 2000), por impedir que um grande número, ou a totalidade, de indivíduos expostos a um inseticida venha a óbito pela ação do químico utilizado (Marcombe et al. 2011; Macoris et al. 2014). Dessa maneira, pouco se diminui a densidade populacional do vetor e, portanto, pouco se altera o risco de transmissão de patógenos (McCarrol et al. 2000; Rivero et al. 2010). Ademais, a resistência a inseticidas gera um enorme

problema para a Saúde Pública, visto que limita a quantidade de compostos químicos disponíveis para uso em campo (OMS 1998).

1.10) Mecanismos de resistência

Os mecanismos responsáveis pela resistência a inseticidas resultam de algumas modificações genéticas que podem afetar características comportamentais ou fisiológicas dos insetos (Brogdon e Mcallister 1998). Entretanto, estes mecanismos não são específicos e geralmente podem causar uma resistência cruzada e/ou múltipla. A primeira ocorre quando um mesmo mecanismo confere resistência a mais de um composto químico (por exemplo, alterações no Na_V, alvo dos PI e DDT, que podem causar resistência a vários compostos destas classes). Já a segunda, a resistência múltipla, acontece quando vários mecanismos atuam em conjunto e conferem resistência a mais de um composto químico (por exemplo, aumento na atividade de enzimas detoxificantes que respondem a vários tipos de inseticidas) (Braga e Valle 2007b).

Nesse contexto, a compreensão sobre a resistência e seus mecanismos envolvidos torna-se imprescindível para subsidiar o manejo racional do controle químico de vetores, auxiliando na escolha do composto ideal a ser empregado em campo (Ferrari 1996). Abaixo, serão descritos brevemente os mecanismos de resistência classificados como resistência comportamental e fisiológica:

1.10.1. Resistência comportamental

Indivíduos com habilidade de evitar o contato com o inseticida são selecionados em uma população de insetos, caracterizando uma resistência comportamental (Forattini 1962; Ferrari 1996). Neste tipo de resistência pode ocorrer uma redução da proporção de mosquitos que descansam nas paredes das casas após realizarem o repasto sanguíneo, evitando assim a exposição ao inseticida residual (Lokwood et al. 1984; Hemingway et al. 2004). A resistência comportamental ainda pode incluir alterações específicas nos hábitos alimentares dos insetos, com mudanças: i) de endofagia para exofagia; ii) no horário de picada; e iii) de antropofagia para zoofagia. Na literatura, este mecanismo de resistência é mais descrito em mosquitos do gênero *Anopheles*, transmissores do *Plasmodium*, em resposta ao uso de inseticidas no interior de habitações, em mosquiteiros impregnados e aplicações residuais em paredes (Sougoufara et al. 2017).

1.10.2. Resistência fisiológica

Neste caso, a resistência ocorre devido a mudanças fisiológicas, que envolvem um ou mais mecanismos que podem atuar por redução da penetração, alterações no sítio alvo e aumento na atividade das enzimas detoxificantes. Esses mecanismos resultam de alterações genéticas, quantitativas e/ou qualitativas, como amplificação gênica, expressão gênica alterada e mudanças estruturais das moléculas (Hemingway et al. 2004; Li et al. 2007).

1.10.2.1) Redução da penetração do inseticida

A resistência a inseticidas pode ocorrer devido ao espessamento da cutícula do inseto, que diminui a permeabilidade e, consequentemente, reduz a taxa de penetração de compostos químicos

(Georghiou 1994; Ferrari 1996). Este mecanismo é pouco estudado em mosquitos, principalmente *Ae. aegypti*, e os processos fisiológicos ou moleculares envolvidos ainda não estão muito bem elucidados.

1.10.2.2) Resistência metabólica

A resistência metabólica ocorre em função de uma maior atividade das enzimas responsáveis pelo metabolismo de xenobióticos, acarretando em uma maior eficiência na detoxificação do inseticida pelo organismo do inseto. Ou seja, promove uma rápida inativação e eliminação do inseticida, impedindo assim que este alcance seu sítio de ação no SNC. Três classes de enzimas, quando alteradas, são responsáveis pela resistência metabólica: glutationa-S-transferases (GST), oxidases de função Múltipla (MFO) e esterases (ESTs) (Brogdon e McAllister 1998; Hemingway e Ranson 2000). Estas enzimas fazem parte de superfamílias gênicas, cada qual composta de dezenas de genes. Embora possua especificidade por um determinado substrato, uma mesma classe de enzimas pode detoxificar mais de um grupo de inseticidas, conferindo a chamada resistência cruzada (Ranson et al. 2002; Montella et al. 2012).

1.10.2.3) Resistência por alteração no sítio-alvo dos inseticidas

Este tipo de mecanismo ocorre por alterações na molécula-alvo do inseticida, resultando em menor sensibilidade aos compostos (Hemingway et al. 2004; Perry et al. 2011). Os alvos dos inseticidas neurotóxicos são moléculas que exercem funções essenciais na fisiologia da transmissão do impulso nervoso, tendo estruturas bem conservadas ao longo da evolução. Assim sendo, poucas alterações estruturais são permitidas sem afetar a viabilidade do inseto (ffrench-Constant et al. 1998). Na literatura, já foram descritos diferentes mecanismos de resistência por

alteração no sítio-alvo em insetos, como mutações na acetilcolinesterase, AchE - 1 (gene *ace-1*) e no canal de sódio regulado por voltagem (gene Na_V) (Hemingway e Ranson 2000).

Mutações no *ace-1* foram descritas em diversos insetos resistentes a OP e CA, inclusive em mosquitos dos gêneros *Anopheles* e *Culex* (Weil et al. 2004; Hemingway et al. 2004; Cassanelli et al. 2006; Labbé et al. 2007; Alout e Weil 2008). Entretanto, nenhuma mutação no gene *ace-1* foi descrita em *Ae. aegypti* e, até então, parece não estar associada à resistência a inseticidas nesta espécie. Em *Ae. aegypti*, o mecanismo mais relevante relacionado à resistência por alteração de sítio alvo é o causado por mutações pontuais no gane Na_V. Estas mutações são comumente conhecidas como mutações *kdr* (do inglês, *knockdown resistance*) e estão relacionadas diretamente com a resistência a PI e OC (tipo DDT) (Soderlund e Knipple 2003).

1.11) Canais de sódio e mutações *knockdown resistance* (*kdr*)

O canal de sódio regulado por voltagem (Na_V) é uma proteína transmembranar que permite o influxo de íons sódio nas células excitáveis (neurônios, miócitos e células endócrinas). Esta molécula é responsável por gerar o potencial de ação, possibilitando assim a transmissão do impulso nervoso. Por ser primordial na fisiologia dos animais, o Na_V é alvo não só de inseticidas, mas também de uma variedade de outras neurotoxinas, incluindo as que ocorrem na natureza em plantas e drogas terapêuticas (Wing et al. 2005; Du et al. 2016). Estruturalmente, o Na_V é uma molécula constituída por quatro domínios homólogos (I-IV), cada um destes contendo seis subunidades em alfa-hélice (S1-S6) e um loop entre os segmentos S5 e S6 (Figura 5).

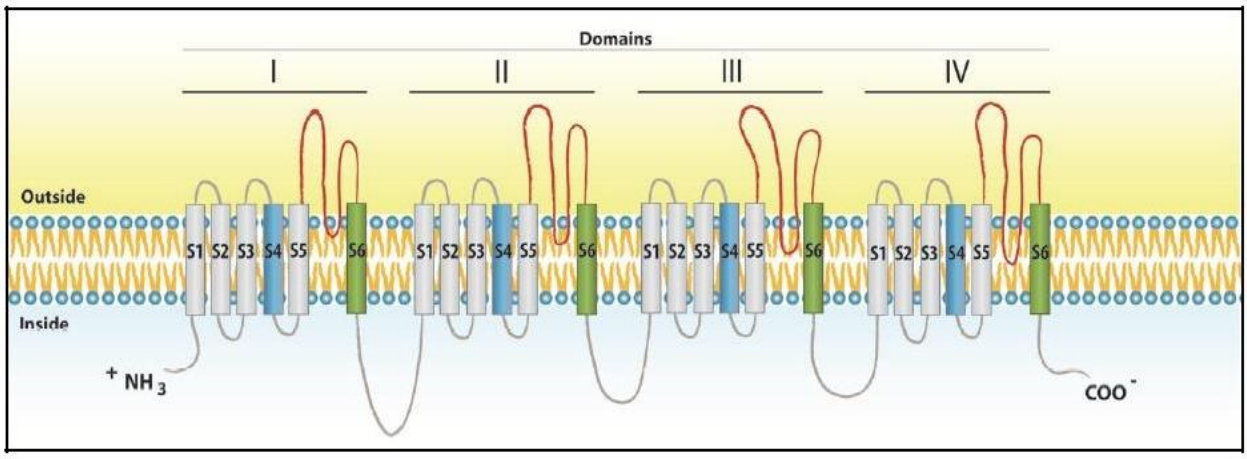

Figura 5. Esquema representativo do Na_v inserido na membrana celular, mostrando seus quatro domínios homólogos (I-IV), cada um com seis segmentos hidrofóbicos (S1-S6). Em azul, segmentos S4, responsáveis pela percepção de alteração da voltagem e, em verde, segmento S6, responsáveis pela cinética do poro. Figura retirada de Martins e Valle (2011).

Inseticidas DDT e PI agem ligando-se a subunidades dos canais de sódio, modificando e mantendo-os abertos por mais tempo, promovendo um aumento do fluxo de sódio para o interior da membrana e a manutenção da fase de despolarização. Consequentemente, são desencadeados potenciais de ação incessantes quando a despolarização atinge o limite, levando os insetos à morte por hiperexcitação (Du et al. 2016). Modelos indicam que os inseticidas se ligam especificamente à cavidade delimitada pela sequência que une os segmentos S4-S5 do domínio II e pelas hélices IIS5 e IIIS6, acessível a inseticidas lipofílicos. Alguns aminoácidos pertencentes a essas hélices não são conservados entre os insetos e os outros organismos, o que poderia ser responsável pela especificidade dos efeitos dos PI sobre os insetos (O'Reilly et al. 2006; Martins e Valle 2011).

A primeira mutação no Na_v, relacionada com a resistência a inseticidas, foi descrita em *Musca domestica* logo após o início da utilização do DDT, em 1950. Esses insetos, quando expostos ao DDT, apresentavam paralisia momentânea, seguida por recuperação completa dos

movimentos, fenótipo que passou a ser chamado de *kdr*, originando o nome da mutação (Busvine 1951). Em seguida, após o início do uso de inseticidas da classe dos PI, que compartilham o mesmo sítio alvo do DDT (Na_V), outras espécies de insetos também foram descritas com fenótipo *kdr* (Fine 1961; Hemingway e Ranson 2000).

A mutação *kdr* pode reduzir a sensibilidade ao inseticida, em geral, da ordem de 10-40 vezes. Mas, nos casos onde ocorrem duas ou mais mutações, esta redução pode ser de mais de 100 vezes (Soderlund 2008; Scott 2016). Geralmente, as mutações *kdr* têm caráter recessivo e podem ser mantidas em níveis baixos nas populações, em espécimes heterozigotos (Milani 1954; Milani e Travaglino 1957; Davies et al. 2007). Entretanto, podem ser rapidamente selecionadas em casos de forte pressão seletiva (Garcia et al. 2009; Linss et al. 2014). Já foram identificadas mais de 50 mutações *kdr* pontuais, associadas com a resistência a inseticidas, em diferentes espécies de artrópodes. Algumas dessas mutações são encontradas no mesmo sítio em mais de uma espécie de insetos, embora outras sejam encontradas em uma espécie apenas (Du et al. 2016).

A mutação *kdr* mais comum ocorre no segmento IIS6, na posição 1014 (posição referente ao gene Na_V da *M. domestica*) e consiste de substituição de uma leucina por uma fenilalanina, chamada de mutação "clássica" Leu1014Phe (Smith et al. 1997). Pelo menos 14 espécies já foram descritas com essa mesma mutação, em sítio homólogo (Davies et al. 2007). Já se identificaram outras alterações, incluindo a substituição de leucina por outros aminoácidos e mutações em outras posições do Na_V de diferentes insetos (Martinez-Torres et al. 1998; Chandre et al. 1998; Du et al. 2016).

Em *Ae. aegypti*, não há registro de mutações na posição "clássica" 1014. Contudo, outras mutações relacionadas com o fenótipo *kdr* foram detectadas nesta espécie (Tabela 2) (Du et al.

2016). No Brasil, estudos apontam que as mutações Phe1534Cys (sozinha) e Val1016Ile (em conjunto com a Phe1534Cys) contribuem consideravelmente para a resistência a PI (Martins et al. 2009a; Brito et al. 2013; Linss et al. 2014; Bellinato et al. 2016). Trabalhos de monitoramento no Brasil destacaram que estas mutações estão se espalhando e aumentando sua frequência de forma muito rápida, assim como foi observado no México, nos últimos 15 anos (Garcia et al. 2009; Linss et al. 2014). Além das mutações Phe1534Cys e Val1016Ile, ainda existe em populações de *Ae. aegypti* brasileiras a mutação Ile1011Met, entretanto sua relação com a resistência a PI ainda não está clara (Lima et al. 2011; Martins et al. 2013)

Tabela 2. Exemplo de mutações *kdr* associadas à resistência a inseticidas em *Aedes aegypti* (Du et al. 2016; Smith et al. 2016).

Mutações *kdr*	**Países ou Regiões**
Ile1011Met	Brasil
Ser989Pro	Tailândia
Ile1011Val	Tailândia
Val1016Gly	Sudeste asiático
Val1016Ile	Países da América Latina
Leu982Tri	Vietnã
Phe1534Cys	Sudeste Asiático, Índia, Brasil, Venezuela
Val1016Ile + Phe1534Cys	Venezuela, Brasil, Grand Cayman

Val1016Gly + Ser989Pro	Sudeste asiático e China
Val1016Gly + Phe1534Cys	Cingapura
Ser989Pro + Val1016Gly + Phe1534Cys	Sudeste asiático

1.12) Custo evolutivo da resistência a inseticidas

Há indícios de que populações de insetos resistentes a inseticidas frequentemente diferem de populações sensíveis em relação aos componentes biológicos de aptidão (Kliot e Ghanim 2012). A aptidão dos indivíduos, também chamado de performance ou *fitness*, refere-se à contribuição com sua prole (ou genes) para a próxima geração. Assim, também é chamado de sucesso reprodutivo e está associado às variações genéticas sobre as quais a seleção natural atua (Ridley 2006). Não existe um consenso universal sobre o termo em questão, mas, normalmente, engloba a sobrevivência e fecundidade dos organismos. Entretanto, deve-se mencionar que outros parâmetros biológicos também estão relacionados com uma boa performance, como tempo de desenvolvimento, taxa de crescimento, tamanho corporal, entre outros, que podem atuar tanto sozinhos quanto em conjunto.

Na resistência a inseticidas, efeitos no *fitness* podem ocorrer devido ao impacto na fisiologia gerado por alterações na molécula alvo dos compostos químicos (por exemplo, mutações *kdr* no Na_V) (Brito et al. 2013). Ainda, tais efeitos podem ocorrer em função de alocação energética para produção e/ou manutenção dos mecanismos associados com a detoxificação. Neste caso ocorreria um balanço (*trade-off*) entre a resistência e características biológicas relacionadas ao

fitness (Kliot e Ghanim 2012). Todavia, como a resistência a inseticidas, em geral, tem caráter multifatorial, onde diferentes mecanismos atuam em conjunto, os efeitos no *fitness* podem ser somados, comprometendo ainda mais a performance do vetor (Berticat et al. 2008; Rivero et al. 2010).

Nesse contexto, populações de insetos resistentes evidenciam uma desvantagem reprodutiva (custo no *fitness*) que pode ser notada principalmente na ausência de pressão de seleção, em ambientes livres de inseticidas. Assim, a frequência de indivíduos resistentes tende a diminuir em função do tempo (Crow 1957; Carrière et al. 1994; Brito et al. 2013). Em outras palavras, a resistência a inseticidas só seria vantajosa em ambiente onde ocorre exposição constante ao inseticida. Entretanto, deve-se destacar que, devido ao custo no *fitness* causado pela resistência, raramente os genes que conferem resistência se fixam nas populações (Kliot e Ghanim 2012).

Identificar e caracterizar o custo no *fitness* gerado pela resistência pode ser uma grande vantagem no âmbito de um programa de controle de vetores, como, por exemplo, para se realizar um manejo racional e limitar a propagação de indivíduos resistentes. Quanto maior esse custo, mais lentamente os indivíduos resistentes se espalham na população. Custos no *fitness* associados à resistência a inseticidas têm sido relatados em insetos pertencentes a diferentes ordens (Kliot e Ghanim 2012), inclusive em *Ae. aegypti* (Belinato et al. 2012; Brito et al. 2013). Dentre os parâmetros que podem ser afetados devido à resistência estão principalmente o tempo de desenvolvimento larval, capacidade e frequência de realização de repasto sanguíneo, atividade locomotora, longevidade, fecundidade e fertilidade dos ovos (Belinato et al. 2012; Kliot e Ghanim 2012; Brito et al. 2013; David et al. 2017).

1.13) Novas alternativas de controle de *Ae. aegypti*

O controle do *Ae. aegypti* tem se constituído num enorme desafio na Saúde Pública. Apesar dos grandes esforços nos últimos anos, não se tem alcançado o sucesso do controle deste vetor. No Brasil, destaca-se que a infestação do mosquito tem apresentado expansão contínua no território, com disseminação ativa ou passiva para todas as regiões do país (Braga e Valle 2007b). Ademais, o aumento da ocorrência de surtos epidêmicos por novos arbovírus e reemergência de sorotipos de DENV vêm colocando o país em uma situação alarmante, com a possibilidade de sobreposição de epidemias de diferentes arboviroses. Ainda se conclui que as estratégias de controle utilizadas estão fragilizadas e com enfoque, predominante, no uso de inseticidas químicos. Neste contexto, devido à disseminação de alelos que conferem resistência aos inseticidas em *Ae. aegypti*, o emprego desta ferramenta pode se tornar cada vez menos efetivo no controle vetorial. Além disso, aspectos relacionados a problemas de infraestrutura das cidades, tais como baixas coberturas na coleta de lixo e intermitência no abastecimento de água, são fatores que comprometem a efetividade dos métodos tradicionais de controle do *Ae. aegypti* (Araújo et al 2015).

Diante deste quadro, a busca por estratégias de controle mais eficientes tem tido grande destaque atualmente, com diversas tecnologias sendo desenvolvidas como alternativas no controle do *Ae. aegypti*. Tais estratégias utilizam formas de atuação distintas das apresentadas pelo controle "tradicional" até então utilizado, por exemplo, como uso dos próprios mosquitos como dispersores de inseticidas, de novos agentes de controle biológico e mosquitos transgênicos, inclusive considerando-se combinações entre essas novas técnicas (Araújo et al. 2015; Zara et al. 2016).

1.13.1) Mosquitos transgênicos

Pesquisas no campo do controle de insetos vetores apontam para uma nova perspectiva com a manipulação genética, que vem mostrando um potencial de interferência na transmissão de patógenos, como arbovírus. Este tipo de estratégia pode visar tanto à supressão quanto à substituição das populações do mosquito. Porém, as linhagens de mosquitos transgênicos que são refratários a infecções por patógenos, cujo objetivo final é a substituição populacional em campo, estão ainda em teste em laboratório (Fraz et al. 2006; Jupatanakul et al. 2017). Estas linhagens também podem ser desenvolvidas pela técnica de para-transgênese, que consiste na transformação de bactérias simbiontes para que estas expressem, no organismo do vetor, gene de bloqueio contra os patógenos. Neste caso, o vetor se infectaria com a bactéria transformada e esta bloquearia o desenvolvimento dos vírus ou protozoários no inseto (Dotson et al. 2003; Favia et al. 2008; Crotti et al. 2010). Até o presente momento, estas estratégias não estão, em sua grande maioria, em fase de teste em campo, sendo as pesquisas concentradas no laboratório.

No entanto, a técnica utilizando mosquitos transgênicos mais cotada atualmente, esta já em teste em campo, visa a reduzir a densidade populacional de *Ae. aegypti* por meio da liberação de mosquitos machos carreando genes letais, que são capazes de provocar a morte da prole das fêmeas por eles copuladas. A OX513A, produzida pela empresa britânica Oxitec, é a principal linhagem de mosquitos transgênicos utilizada e recebeu aprovação técnica da Comissão Técnica Nacional de Biossegurança (CTNBio) para liberação comercial no Brasil (Carvalho et al. 2014; Zara et al. 2016). A partir de 2010, testes foram implementados em duas áreas na Bahia e em São Paulo (Piracicaba), para avaliação dos riscos e da efetividade desta estratégia. Resultados preliminares mostraram que, após a liberação dos mosquitos machos transgênicos em Juazeiro-BA, teria havido redução de 80% a 95% da população de *Ae. aegypti* (Carvalho et al. 2015). Esta técnica, no entanto, possui algumas desvantagens, como a necessidade de sexagem dos mosquitos durante a criação

em massa para soltura, além de requerer uma liberação constante de mosquitos no meio ambiente para ser eficiente em longo prazo (Zara et al. 2016). A princípio, a interrupção das liberações semanais de machos OX513A seria capaz de manter a densidade de mosquitos baixa apenas por algumas semanas. Invariavelmente, mosquitos selvagens poderiam recolonizar esse ambiente, oriundos de bairros/cidades vizinhas, reestabelecendo a infestação tratada.

1.13.2) Mosquitos irradiados

Outra alternativa de controle vetorial é a esterilização de insetos por irradiação (*sterile insect technique* – SIT). Nesta técnica, os insetos machos são expostos a uma dose mínima de raios gama ou raios X para provocar rearranjos cromossômicos aleatórios e causar sua esterilização. Assim, esta ferramenta objetiva a liberação em massa de machos estéreis para que ocorra, em campo, o acasalamento com fêmeas nativas, culminando em uma diminuição do potencial reprodutivo das fêmeas e, consequentemente, contribuindo para a supressão da população de vetores (Ferreira et al. 2008; Alphey et al. 2010; Zara et al. 2016). O controle via SIT já obteve resultados positivos na agricultura, por exemplo, no controle da mosca do melão (*Bactrocera cucurbitae*) (Koyama et al. 2004), e na Saúde Pública. Em insetos vetores, deve-se destacar a redução de 99% da população de *Anopheles albimanus*, vetor da malária, em uma área em El Salvador (Lofgren et al. 1974; Benedict e Robinson 2003). Embora eficiente, a técnica apresenta desvantagens como as comentadas no item acima para mosquitos transgênicos, pois são necessárias constantes liberações de mosquitos irradiados em campo, além da realização laboriosa da sexagem em laboratório antes das liberações (Zara et al. 2016).

1.13.3) Mosquitos disseminadores de larvicidas

A estratégia consiste em atrair as fêmeas do *Ae. aegypti* até pequenos recipientes, chamados de "estações de disseminação", tratados com o larvicida pyryproxifen, um AHJ (item 1.7). Nas estações de disseminação, as micropartículas do pyryproxifen em pó grudam no corpo do mosquito, que as visitam, e são carreadas por eles até outros criadouros. Quando as fêmeas pousarem nesses últimos reservatórios para ovipor, as partículas do inseticida seriam deixadas por elas na água e, assim, os reservatórios passariam a ser letais para as larvas dos mosquitos (Itoh et al 1994; Devine et al. 2009). No Brasil, um teste em campo utilizando esta técnica foi realizado na cidade de Manacapuru, Amazonas, onde resultados promissores foram demonstrados, com uma redução na emergência de fêmeas do vetor em até 98% (Abad-Franch et al. 2017). Uma desvantagem desta técnica é que requer uma formulação de inseticidas com concentrações ideais, em micropartículas. Por isso, até o momento, somente um tipo de larvicida tem sido empregado, o que facilitaria a seleção de populações de mosquitos resistentes (Zara et al. 2016).

1.13.4) *Wolbachia*

A *Wolbachia* é uma bactéria simbionte gram-negativa, obrigatoriamente intracelular, que infecta naturalmente artrópodes, como insetos (Werren et al. 1995, Jeyaprakash e Hoy 2000), aracnídeos (Breeuwer e Jacobs 1996), crustáceos (Gotoh et al. 2003) e os isópodes (Werren et al. 2008). Além disso, são também encontradas em nematoides (Bandi et al. 1998, 2001). Especialmente em insetos, esta bactéria está presente em até 60% das espécies no Brasil (de Oliveira et al. 2015) e no mundo (Hilgenboecker et al. 2008). Infecções naturais por *Wolbachia* são relativamente frequentes em mosquitos, incluindo muitas espécies presentes em nosso país,

como *Culex quinquefasciatus*, *Aedes fluviatilis* (Ricci et al. 2002) e *Aedes albopictus* (Noor et al. 2015). Entretanto, importantes mosquitos vetores como os do gênero *Anopheles* e o *Ae. aegypti* raramente são encontrados naturalmente infectados por esta bactéria na natureza (de-Oliveira e Moreira 2012; Baldini et al. 2014; Coon et al. 2016).

O primeiro relato da *Wolbachia* ocorreu nos tecidos reprodutivos de *Culex pipiens*, por Hertig e Wolbach, em 1924, sendo a espécie denominada *Wolbachia pipientis* (Herting 1936; Werren 1997). Para assegurar sua propagação e estabelecimento na natureza, esta bactéria manipula a reprodução do hospedeiro para garantir a sua transmissão vertical (da fêmea infectada para a prole) (Sinkins et al. 1997). Em muitos insetos infectados pela *Wolbachia*, inclusive em mosquitos, ocorre também um fenômeno conhecido como incompatibilidade citoplasmática - IC (o cruzamento de um macho infectado com uma fêmea sem a bactéria gera uma prole inviável). Nestes casos, a *Wolbachia* é passada para a prole sempre que a fêmea tiver a bactéria, favorecendo a disseminação da mesma em campo (Figura 6) (Werren et al. 2008). Além disso, outras formas de manipulação reprodutiva podem ser observadas em diferentes artrópodes, como feminização, morte de machos e partenogênese (Figura 6) (Werren et al. 2008).

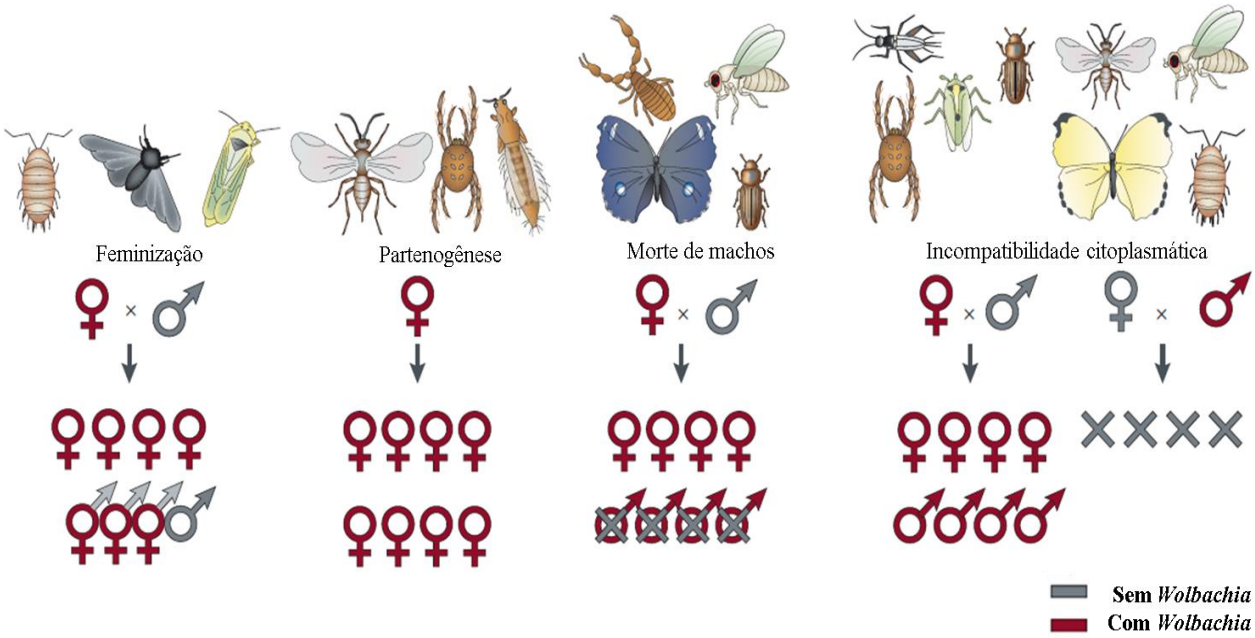

Figura 6. Possíveis cruzamentos na natureza entre insetos infectados e não infectados pela *Wolbachia* (Figura adaptada de Werren et al. 2008).

1.13.4.1) Uso da *Wolbachia* como estratégia auto-limitante

Esta estratégia tem como objetivo final a supressão populacional, alcançada por meio da liberação apenas de insetos machos contendo *Wolbachia* em campo. Conhecida como "técnica do inseto incompatível" (do termo em inglês, *Incompatible insect technique* - IIT), este método se beneficia do fenótipo da IC causado pela bactéria (Werren et al. 2008). Neste caso, os machos liberados agem como machos estéreis, pois sua cópula com fêmeas selvagens resulta em prole inviável, de forma semelhante à estratégia dos transgênicos ou SIT (descritas no item 1.13.1 e 1.13.2). Assim como comentado para as técnicas citadas acima, a IIT tem uma ação momentânea, sendo necessárias liberações constantes de mosquitos machos em campo, e em número elevado, para que a estratégia tenha êxito a médio/longo prazo. Deve-se destacar que esta metodologia já

obteve sucesso em campo na Saúde Pública para mosquitos do gênero *Culex* (Laven 1967; Zabalou et al 2004). Atualmente, a técnica do IIT vem sendo aplicada para controle de *Culex*, no continente asiático (Atyame et al. 2011; Chen et al. 2013), e do *Ae. albopictus* nos Estados Unidos (www.mosquitomate.com) e na China (www.wolbachia.cn).

1.13.4.2) Uso da *Wolbachia* como estratégia autossustentável

O uso da *Wolbachia* como ferramenta autossustentável iniciou-se quando foi demonstrado que a bactéria causava significativa redução na capacidade vetorial de insetos. A partir daí, foi questão de tempo até que essa estratégia fosse testada em mosquitos de importância médica. Nesse contexto, foi criado na Austrália o Programa "Eliminate Dengue" (www.eliminatedengue.com), projeto internacional financiado pela "Bill & Melinda Gates Foundation", baseado na utilização da *Wolbachia* para o controle de arboviroses. A ideia principal desta estratégia é que ocorra a substituição da população selvagem de *Ae. aegypti* (suscetível a infecções por arbovírus, e, portanto, eficientes vetores) por mosquitos com a *Wolbachia* (que tem sua capacidade vetorial reduzida) e, desta forma, diminuir a transmissão de arbovírus em campo (Figura 7).

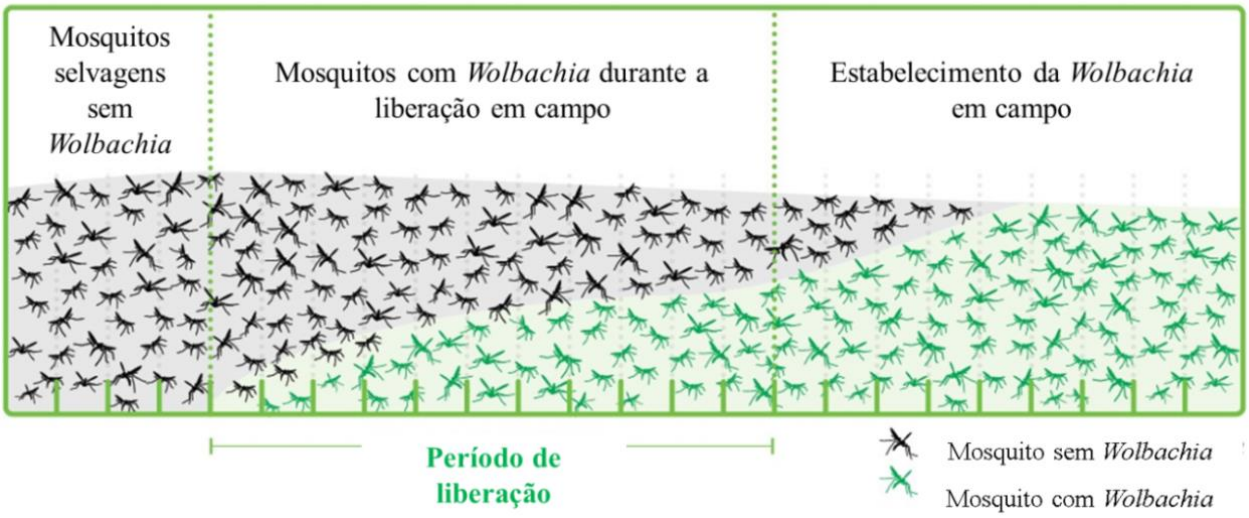

Figura 7. Demonstração esquemática de uma provável invasão da *Wolbachia* em populações nativas de *Ae. aegypti*. Após sucessivas semanas de liberação (10- 30 semanas), ocorre a substituição da população selvagem por mosquitos com a bactéria (Figura adaptada de www.eliminatedengue.com).

Os primeiros passos para o desenvolvimento da *Wolbachia* como ferramenta autossustentável de controle ocorreram em laboratório. Seu papel para este fim começou a ser desenhado a partir de resultados promissores obtidos em uma série de estudos com moscas do gênero *Drosophila*, infectadas naturalmente com *Wolbachia* (cepa *w*MelPop). Os estudos demonstraram que além da bactéria ter causado significativa redução na longevidade destas moscas (McMeniman et al. 2009), ela também ofereceu a proteção contra infecções por diferentes vírus RNA (Teixeira et al. 2008; Hedges et al 2008). Logo em seguida, explorou-se a ideia de se utilizarem os efeitos causados pela infecção com *Wolbachia* em mosquitos de importância médica, como o *Ae. aegypti*, pois os tornariam insetos com uma baixa capacidade vetorial, para diminuir a transmissão de patógenos em campo. Entretanto, uma vez que o *Ae. aegypti* não apresenta infecção

natural por *Wolbachia*, era necessário realizar uma transifecção em laboratório para inserção desta bactéria neste vetor.

Após a inserção, por microinjeção, da cepa *w*MelPop de *Wolbachia* oriundas de *D. melanogaster* em ovos de *Ae. aegypti*, deu-se início às experimentações que também demonstraram, assim como observado nas moscas, redução na longevidade do vetor em cerca de 50% (McMeniman et al. 2009). Deste modo, a ideia inicial do Programa "Eliminate Dengue" era causar mortalidade precoce nos mosquitos e, assim, diminuir o número de insetos capazes de sobreviver ao PIE de arbovírus em campo (McMeniman et al. 2009). Posteriormente, descobriu-se que os mosquitos com *w*MelPop também eram capazes de bloquear integralmente ou reduzir drasticamente a carga parasitária de vírus dengue e chikungunya, do protozoário *Plasmodium gallinaceum* e do nematoide *Brugia pahangi* (Moreira et al. 2009; Kambris et al. 2009).

Após essas constatações, o uso da *Wolbachia* no Programa "Eliminate Dengue" foi ampliado, sendo adotada tanto a ideia da mortalidade precoce quanto a de bloqueio de infecções por arbovírus. Entretanto, além de oferecer longevidade reduzida e bloqueio integral ao DENV, a cepa *w*MelPop também é capaz de comprometer severamente outros aspectos do *fitness*, como diminuição da resistência à dessecação de seus ovos e alteração de hábitos alimentares dos mosquitos. Assim sendo, a baixa performance do *Ae. aegypti* infectado pela cepa *w*MelPop na natureza foi posteriormente relacionada ao fracasso da invasão em campo da *Wolbachia* em áreas-testes localizadas na Austrália e no Vietnam (Nguyen et al. 2015).

Diante deste quadro, outra cepa de *Wolbachia* foi testada em laboratório em seguida. A cepa *w*Mel, também originária de *Drosophila*, bloqueou o DENV em cerca de 75-85% dos mosquitos, mas desta vez sem alterar muito a biologia do vetor (Walker et al. 2011). Neste sentido,

do ponto de vista epidemiológico, uma epidemia de dengue poderia ser evitada mesmo com um 'vazamento' de 25% dos mosquitos, ou seja, mesmo se o bloqueio de infecção por DENV ocorresse em cerca de 75% dos espécimes do vetor, como é o caso com a cepa *w*Mel. Isto porque, nestas condições, a 'taxa de reprodução' (R0) se manteria abaixo de 1,0, ou seja, com indicação de decréscimo na curva epidêmica (Ferguson et al. 2015). Além disso, posteriormente, foi demonstrado em *Ae. aegypti* com a cepa *w*Mel o bloqueio para os vírus da febre amarela, Zika e chikungunya, com níveis até maiores que os observados para DENV (van den Hurk et al. 2012; Aliota et al. 2016; Caragata et al. 2016; Dutra et al. 2016). Esses resultados apontaram para o potencial uso da cepa *w*Mel para liberações em campo, uma vez que ela bloqueiaria diferentes arbovírus e, ao mesmo tempo, produz efeitos pouco perceptíveis no *fitness* do vetor.

Depois das etapas em laboratório, *Ae. aegypti* infectados com *w*Mel seguiram para testes de disseminação (invasão) em campo (Fig. 7). Inicialmente, após 10-20 semanas consecutivas de liberação de mosquitos com *Wolbachia*, duas áreas-teste na Austrália confirmaram a invasão de *Ae. aegypti* infectados com a cepa *w*Mel sobre a população selvagem (Hoffmann et al. 2011; Frentiu et al. 2014), o que incentivou a expansão do Programa "Eliminate dengue" para outros países (Vietnã, Indonésia, Índia, Brasil e Colômbia - www.eliminatedengue.com). Atualmente, além da Austrália, liberações bem-sucedidas de *Wolbachia* em áreas-piloto já ocorreram Indonésia, Vietnã, Brasil e Colômbia. Neste momento estão sendo realizadas expansões significativas nestes países.

É importante mencionar que o controle biológico utilizando a *Wolbachia* apresenta uma importante vantagem frente às outras estratégias, tanto as "tradicionais" quanto as novas, por ser potencialmente autossustentável em longo prazo. Uma vez que a bactéria tenha se estabelecido em campo, e que tenha ocorrido com sucesso a substituição populacional, não seriam necessárias

novas liberações nas áreas (Hoffmann et al. 2011; Frentiu et al. 2014). Portanto, é uma metodologia que não teria ação apenas momentânea durante a sua implementação, mas promoveria uma proteção contínua após a invasão da bactéria em campo (Zara et al. 2016).

1.13.4.3) Fatores que afetam a invasão da *Wolbachia*

Durante as liberações de *Ae. aegypti* com *Wolbachia*, cujo objetivo final é a substituição da população selvagem do vetor, os mosquitos liberados podem ser desafiados com alguns fatores específicos que comprometam a invasão da bactéria em campo. Exemplos na literatura ressaltam a densidade da população selvagem, o *fitness* da linhagem liberada e a temperatura como fatores relevantes que devem ser considerados pelo potencial de interferência na implementação desta estratégia. Portanto, estas condições podem variar nas diferentes áreas e países.

Dado que a invasão da *Wolbachia* só acontece quando a frequência da bactéria ultrapassa um limiar em relação aos insetos selvagens (Turelli e Hoffmann 1991), o tamanho da população selvagem é um fator chave que deve ser considerado ao se iniciar liberações de mosquitos com *Wolbachia* em campo. Portanto, uma grande população nativa requer um número maior de mosquitos com a bactéria a liberar e/ou exige mais semanas de solturas, tornando o processo de disseminação da bactéria mais duradouro (Hancock et al. 2016; Garcia et al. 2016).

Além disso, estudos recentes, em laboratório, demonstraram que altas temperaturas podem afetar a interação *Wolbachia-Ae. aegypti* levando a uma diminuição na densidade da bactéria, podendo até resultar em sua perda (Ulrich et al. 2016; Ross et al. 2017). Isso afetaria diretamente o potencial de supressão da infecção por arbovírus, pelo fato deste bloqueio ser dependente da

densidade da bactéria no organismo do mosquito. Assim, altas densidades da *Wolbachia* nos tecidos resultam em bloqueios mais eficientes (Ferguson et al. 2015; Joubert et al. 2016). Além disso, a temperatura elevada também impactou na transmissão materna e na incompatibilidade citoplasmática (Ross et al. 2016), fatores essenciais para o estabelecimento da bactéria em campo.

Por fim, um dos fatores mais importantes para a *Wolbachia* ser bem sucedida em campo é a viabilidade (*fitness*) da linhagem utilizada nas liberações. Os mosquitos que carregam a bactéria devem ser bons competidores em campo. Portanto, não podem apresentar um alto custo no *fitness*, pois isso os deixaria em desvantagem frente aos mosquitos selvagens. Como comentado anteriormente, tal constatação foi comprovada com as liberações de *Ae. aegypti* com a cepa *w*MelPop em campo, cuja infecção gera um alto comprometimento do *fitness* do inseto. Linhagens de *Ae. aegypti* infectados com esta cepa de *Wolbachia* não foram capazes de invadir a população selvagem e se estabelecer em campo (Nguyen et al 2015). Entretanto, após estudos, descobriu-se que embora a *w*MelPop tenha colapsado em campo, liberações de machos e fêmeas, com esta cepa, causam uma supressão da população selvagem, fato que pode ser aproveitado futuramente no controle (Ritchie et al. 2015; Suh et al. 2017).

Perante estes resultados de campo, mudou-se permanentemente a cepa de *Wolbachia* usada para substituir a população selvagem. A cepa *w*Melpop foi descartada e, a *w*Mel, que não compromete tanto o *fitness*, passou a ser utilizada em todos os países pertencentes ao Programa. Daí em diante, aspectos da viabilidade de linhagens com *Wolbachia* começaram a ser mais estudados, como, por exemplo, questões geradas por fatores intrínsecos do "background" genético das populações de *Ae. aegypti* de diferentes países e regiões. Além disso, atualmente, ocorre uma busca por outras cepas de *Wolbachia* que possam ser mais eficientes que a *w*Mel, ou seja, cepas mais estáveis em temperaturas elevadas, com baixo custo no *fitness* do mosquitos e que tenham

maior velocidade de disseminação em campo. Neste contexto, deve-se destacar uma cepa de *Ae. albopictus*, a *w*AlbB, que estudos recentemente vem mostrando resultados promissores em *Ae. aegypti* (Axford et al. 2016; Ross et al 2016).

Portanto, para avaliar se a *Wolbachia* será capaz de invadir e se estabelecer em populações de *Ae. aegypti* selvagens é necessário considerar o mais holisticamente possivel diferentes aspectos relacionados aos mosquitos nativos que irão receber a bactéria, aos fatores ambientais, e ao "background" genético e ao *fitness* da linhagem com *Wolbachia* que será liberada em campo. Esta tese explora alguns destes fatores, especificamente: (i) densidade da população selvagem; e (ii) resistência a inseticidas. Considerando-os tanto de forma independente, sem considerar as liberações da *Wolbachia*, quanto em conjuto, investigando em tempo real, durante as liberações da bactéria no Rio de Janeiro. Neste estudo, abordamos pela primeira vez na literatura a resistência/uso de inseticidas como sendo um importante fator para a invasão da *Wolbachia* em campo. Por fim, discutimos como estes fatores afetaram a implementação da estratégia no Rio de Janeiro, Brasil.

2. Justificativa

Atualmente, o Brasil se encontra em situação crítica no que diz respeito à transmissão de arbovírus. O vetor *Aedes aegypti* está disseminado por todo país, promovendo uma tríplice epidemia de arboviroses, com surtos de dengue, chikungunya e Zika em grandes centros urbanos. Isto nos alerta para as fragilidades das estratégias de controle utilizadas até então, que não foram suficientes para frear e impedir a disseminação do vetor e nem a reemergência/expansão dos arbovírus nas últimas décadas. Ademais, sabe-se que o controle vetorial tem um grande enfoque no uso de inseticidas. E, devido à disseminação de alelos que conferem resistência aos inseticidas, o emprego desta ferramenta pode se tornar cada vez menos efetivo em campo.

Assim sendo, torna-se improtelável o desenvolvimento de novas estratégias de controle de arboviroses na busca por um cenário mais brando nos próximos anos, no Brasil e no mundo. Neste contexto, a bactéria *Wolbachia* tem sido testada como controle biológico de arboviroses em alguns países. Liberações de *Ae. aegypti* com a bactéria foram bem-sucedidas na Austrália, Indonésia e Vietnã. Nestes países, a *Wolbachia* foi capaz de invadir e substituir as populações nativas de *Ae. aegypti*, em áreas-teste. O Brasil iniciou suas liberações em setembro de 2014. Entretanto, particularidades de nosso país, como altas densidades do vetor e a presença de populações nativas do *Ae. aegypti* resistentes a inseticidas são questões determinantes que podem afetar a disseminação da *Wolbachia* em campo. Situações semelhantes podem se repetir em outras regiões que implementem este projeto. Portanto, o objetivo desta tese foi estudar aspectos ecológicos e genéticos ligados às primeiras solturas de *Ae. aegypti* com a bactéria *Wolbachia* na América Latina, ocorridas no Brasil.

3. Objetivos

3.1. Objetivo geral

Estudar o efeito da resistência a inseticidas e da densidade populacional de *Aedes aegypti* na disseminação da *Wolbachia* em populações nativas do vetor.

3.2. Objetivos específicos

- Estudar a resistência a inseticidas, seus mecanismos, e seu impacto no *fitness* em populações brasileiras de *Ae. aegypti*.

- Estimar tamanho populacional e taxa de sobrevivência de *Ae. aegypti* (com ou sem *Wolbachia*) incorporando novas análises em experimentos de marcação, soltura e recaptura (MSR).

- Investigar como o *status* de resistência aos inseticidas piretroides, em populações nativas e em mosquitos com *Wolbachia*, pode afetar a invasão da bactéria no campo.

- Produzir uma linhagem de mosquitos *Ae. aegypti* com *Wolbachia* e alelos de resistência apta à invasão no campo.

4. Resultados

Os resultados desta tese são apresentados em forma de artigos, totalizando oito artigos, que são divididos em três capítulos, conforme os temas abordados.

O **capítulo I** apresenta os **artigos 1, 2 e 3** que são relacionados com o tema "Resistência a inseticidas, seus mecanismos, e custo no *fitness* em populações brasileiras de *Aedes aegypti*". O **artigo 1** descreve sobre o monitoramento da resistência a inseticidas de quatro populações de *Ae. aegypti*, de diferentes regiões do país, ao longo do ano de 2010. Neste período, quantificamos a resistência aos principais inseticidas utilizados em campo na época, os larvicidas temephos (OP) e diflubenzuron (IGR) e o adulticida deltametrina (PI). Os mecanismos de resistência por alterações metabólicas e por mutações *kdr* também foram investigados neste estudo. No **artigo 2**, analisamos o *fitness* das populações estudadas no artigo anterior, estimando em laboratório parâmetros como tempo de desenvolvimento larval, fertilidade, fecundidade, longevidade e sobrevivência ao jejum, e ainda fazemos uma correlação com os níveis de resistência encontrados. Por fim, o **artigo 3** descreve a distribuição de duas mutações *kdr* , Val1016Ile e Phe1534Cys, em populações brasileiras de *Ae.* aegypti, onde identificamos dois diferentes alelos, chamados de R1 (mutação somente no sítio 1534) e R2 (mutação no sítio 1534 + 1016), relacionadas a resistência a PI.

O **capítulo II** apresenta os **artigos 4 e 5** que abordam o tema "Estimando a densidade populacional e sobrevivência diária de *Ae. aegypti* em experimentos de marcação soltura e recaptura (MSR)". O **artigo 4** apresenta um novo modelo bayesiano hierárquico para estimar densidade populacional de *Ae. aegypti* em experimentos de MSR. Utilizando esse modelo nós comparamos a distribuição espacial dos mosquitos, densidade populacional e sobrevivência diária,

durante o inverno e o verão, em dois experimentos de MSR em um bairro do Rio de Janeiro. No **artigo 5**, comparamos múltiplos métodos quantitativos para MSR que permitem estimar abundância, sobrevivência de mosquitos marcados e não marcados (se diferente), incluindo, ainda, modelos que consideram a taxa de recrutamento. Neste trabalho, são realizadas simulações para avaliar a precisão das estimativas, variando parâmetros como o número de indivíduos liberados, abundância, sobrevivência e eficiência de captura.

Por fim, o **capítulo III** apresenta os **artigos 6, 7 e 8** referentes ao tema "Implementação da estratégia *Wolbachia* no Brasil". O **artigo 6**, é uma análise dos dados empíricos da primeira liberação de mosquitos com *Wolbachia* no Rio de Janeiro, em 2014, onde a própria bactéria foi usada como um marcador em campo. Foram estimados parâmetros populacionais como densidade de mosquitos selvagens e sobrevivência diária de mosquitos com *Wolbachia* no Rio. Neste trabalho, tivemos a oportunidade de estimar diretamente a sobrevivência diária dos mosquitos infectados com *Wolbachia* pela primeira vez em campo por meio de MSR. No **artigo 7,** em preparação**,** são apresentados os resultados das liberações da *Wolbachia* no país. Neste trabalho demonstramos a falha da invasão da *Wolbachia* na primeira soltura do Rio de Janeiro, no bairro de Tubiacanga/Ilha do Governador, e apontamos indícios de que a resistência e o uso de inseticidas foram fatores determinantes que comprometeram a disseminação da bactéria em campo. Neste mesmo artigo, mostramos como este problema foi corrigido, por meio da realização de um novo retrocruzamento para a obtenção de uma nova linhagem de *Ae. aegypti* com *Wolbachia*, com níveis de resistência similares aos encontrados em mosquitos do Rio. Após o estabelecimento desta linhagem em laboratório, novamente foram liberados mosquitos com a *Wolbachia* em campo e os resultados desta invasão também são apresentados no **artigo 7**. Em nosso último texto, o **artigo 8**, em preparação, buscamos investigar mais a fundo, por modelagem matemática e simulações,

algumas hipóteses que contemplam a resistência e do uso de inseticidas na disseminação da *Wolbachia*. Para tal, estudamos, por simulações, os efeitos exercidos pela liberação de mosquitos resistentes ou susceptíveis a PI na disseminação da *Wolbachia* em áreas do Rio de Janeiro, baseando-nos em nossos dados empíricos. Investigamos também a frequência de alelos de susceptibilidade e resistência a inseticidas a partir de diferentes cenários entomológicos, variando o nível de aplicação de inseticidas em campo. Assim, descrevemos diferentes cenários relacionados ao sucesso ou a falha na invasão da bactéria quando consideramos uso/resistência a inseticidas em campo durante as liberações de *Wolbachia*.

Capítulo I:

Resistência a inseticidas, seus mecanismos, e custo no *fitness* em populações brasileiras de *Aedes aegypti*

Justificativa

Os inseticidas, principalmente os neurotóxicos, ainda são uma importante ferramenta no controle do vetor *Ae. aegypti* em todo o mundo. O uso racional destes compostos tem como função postergar e evitar a disseminação de alelos de resistência a inseticidas em populações de insetos, de modo a tornar esta estratégia sustentável em longo prazo. Entretanto, o uso indiscriminado de inseticidas tem selecionado populações de *Ae. aegypti* resistentes em todo o mundo, tornando cada vez menos efetivo o controle químico vetorial. Além disso, trabalhos mais recentes, em laboratório, demonstraram que a resistência a inseticidas pode causar um custo na aptidão (*fitness*). Identificar e caracterizar este custo no *fitness* pode ser importante tanto para a transmissão de patógenos em campo quanto para o manejo da resistência a inseticidas. Neste contexto, estudos sobre a resistência a inseticidas, seus mecanismos biológicos envolvidos, e a avaliação do impacto da resistência no *fitness* dos mosquitos, são necessários para um melhor entendimento sobre a atual situação do controle vetorial em nosso país, além de auxiliar decisões futuras sobre quais inseticidas devemos empregar em campo. Ademais, a resistência/uso de inseticidas mostrou-se um importante fator a ser considerado na implementação da nova estratégia de controle biológico de arboviroses que utiliza a bactéria *Wolbachia*.

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

The impact of insecticide applications on the dynamics of resistance: The case of four *Aedes aegypti* populations from different Brazilian regions

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OPEN ACCESS

Citation: Garcia GdA, David MR, Martins AdJ, Maciel-de-Freitas R, Linss JGB, Araújo SC, et al. (2018) The impact of insecticide applications on the dynamics of resistance: The case of four *Aedes aegypti* populations from different Brazilian regions. PLoS Negl Trop Dis 12(2): e0006227. https://doi.org/10.1371/journal.pntd.0006227

Editor: Pattamaporn Kittayapong, Faculty of Science, Mahidol University, THAILAND

Received: June 1, 2017

Accepted: January 9, 2018

Published: February 12, 2018

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by the Programa Nacional de Controle da Dengue/ Ministério da Saúde (PNCD/MS), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) and the Instituto Nacional de Ciência e

Abstract

Background

In the tropics, the utilization of insecticides is still an important strategy for controlling *Aedes aegypti*, the principle vector of dengue, chikungunya and Zika viruses. However, increasing insecticide resistance in *Ae. aegypti* populations might hinder insecticide efficacy on a long-term basis. It will be important to understand the dynamics and evolution of insecticide resistance by assessing its frequency and the mechanisms by which it occurs.

Methodology/Principal findings

The insecticide resistance status of four Brazilian *Ae. aegypti* populations was monitored. Quantitative bioassays with the major insecticides employed in the country was performed: the adulticide deltamethrin (a pyrethroid—PY) and the larvicides, temephos (an organophosphate) and diflubenzuron (a chitin synthesis inhibitor). Temephos resistance was detected in all populations although exhibiting a slight decrease over time probably due to the interruption of field use. All vector populations were susceptible to diflubenzuron, recently introduced in the country to control *Ae. aegypti*. Resistance against deltamethrin was extremely high in three populations. Molecular assays investigated substitutions in the voltage gated sodium channel (Na_V), the PY target site, at positions 1011, 1016 and 1534. Elevated frequencies of substitutions Val1016Ile and Phe1534Cys related to high PY resistance levels were identified. Biochemical assays detected alterations in the activities of two detoxifying enzyme classes related to metabolic resistance, glutathion-S-transferases and esterases. The results obtained were evaluated in the context of both recent insecticide use and the records of dengue incidence in each locality.

Tecnologia-Entomologia Molecular (INCT-EM). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Conclusions/Significance

The four *Ae. aegypti* populations evaluated were resistant to the neurotoxic insecticides, temephos and deltamethrin. However, they were still susceptible to diflubenzuron. A probable correlation between adult insect resistance to PY and the domestic application of insecticides is discussed, pointing to the need for awareness measures regarding the correct utilization by citizens. This work aims to contribute to the efficient and rational management of *Ae. aegypti* control of both larvae and adults.

Author summary

Among the pathogens transmitted by *Aedes aegypti*, dengue virus is the most important due to the number of people affected or at risk and the high rate of mortality worldwide. The confirmation that *Ae. aegypti* is also the vector of Zika, chikungunya and urban yellow fever poses serious consequences for public health, pointing to the need of reevaluating current vector control strategies. Although there is growing recognition of the importance of social participation and community engagement to prevent high levels of infestation, insecticides are considered important vector control tools. Nevertheless, the massive and indiscriminate adoption of insecticides to control larvae and adults contributes to resistance spread. In particular, the domestic use of adulticides, especially in epidemic seasons, is assumed to induce high levels of resistance in *Ae. aegypti* populations. However, the consequences of insecticide interruption upon the resistance of field populations has been less investigated. We evaluated, in four Brazilian regions over one year, the dynamics of dengue vector population resistance to the principal insecticides used in the country. The main resistance mechanisms were also investigated. Data are discussed taking into account the potential relationship among dengue outbreaks, public and private chemical control and insecticide resistance.

Introduction

The mosquito *Aedes aegypti* is the main vector of dengue virus, an arbovirus of major importance worldwide. Among the Americas, Brazil is the country most affected by this pathogen [1], considered hyper-endemic, since the four serotypes, DENV-1 to DENV-4, circulate [2]. Recently, two other arboviruses transmitted by *Ae. aegypti* have been spreading rapidly, chikungunya (CHIKV) and Zika (ZIKV). CHIK was introduced to America through the Caribbean and its presence was confirmed in Brazil in June 2014 [3]. ZIKV was introduced to the American continent from Northeast Brazil [4]. ZIKV was associated with several neurological disorders, including cases of microcephaly and other developmental alterations in newborns from mothers infected during pregnancy. This suspicion, later confirmed, launched the announcement of a Public Health Emergency of International Concern [5] that persisted until the end of 2016 [6].

Vectorial transmission of arboviruses depends upon three components, the host (in this case, humans), the virus and the vector. Despite intense efforts of biomedical research, when DENV, CHIK and ZIKV are considered, neither effective vaccines for large scale use nor specific drugs, able to block clinical manifestations, are yet available on the market. Thus strategies that focus on the control of mosquito vectors are currently the main tools against these

health problems [1]. From a formal point of view, 'information, education and social communication' are a key component of the Brazilian dengue vector control program [7]. However in practice, insecticides play a very important role regarding control actions, from the perspective of both public managers and general society [8].

Ae. aegypti control in Brazil employs insecticides against larvae and adults. Larvicides are applied in households, 4 to 6 times a year, during visits of control agents, ideally only in water containers that cannot be discarded. In contrast, ultra low volume applications of adulticides do not have a preventive function. In spite of indiscriminate domestic use, these products are employed by health personnel only to block outbreaks in epidemic seasons or at strategic points, aiming to reduce the adult populations. One must be aware that Brazil follows WHO guidelines in order to decide which insecticides are employed in public health. In addition, when larvicides are considered for *Ae. aegypti* control, only products approved for drinking water are allowed [9,10].

For a long time, the temephos organophosphate (OP) was the sole larvicide available against *Ae. aegypti*. In Brazil, use of this OP started in 1967 when the vector was reintroduced in the country [11]. Due to the dengue epidemic in 1986 [12], its use was intensified [8]. From 2009 on, the spread of temephos resistant *Ae. aegypti* populations relegated this OP to a secondary choice of larvicide in the country [13], the recommendation, by WHO Pesticide Evaluation Scheme (WHOPES), of the availability of other products for use in drinking water containers [10], also contributing to this decision. In 2009, the substitution of OP larvicides by Insect Growth Regulators (IGR) began. The first IGR adopted on a national scale was diflubenzuron, a chitin synthesis inhibitor (CSI) [14,15]. Ultimately, it was agreed that a rotation scheme, in principle every four years, would be adopted for larvicides [16].

Until 2001, in addition to the temephos larvicide function, other OPs were used in conjunction for the control of adults. The above mentioned resistance of larvae to temephos induced the adoption of a different approach, consisting of distinct class insecticides against both larvae and adults. The aim in this case was to delay resistance development by varying selection pressure, now exerted with different products in distinct stages of the mosquito life cycle. With this strategy the Brazilian Ministry of Health (MoH) expected to preserve the few products that were still effective. That year, the OPs were replaced by pyrethroids (PY) for adult control. However, a rapid spread of *Ae. aegypti* resistance to PY ensued, due mainly to mutations in the target site, the Na_V [17, 18]. Therefore since 2009, the MoH initiated the implementation of the OP malathion, the only non-PY adulticide recommended by WHO [9].

Brazil is a country of continental dimensions and, despite general MoH guidelines, there are local decisions, deriving both from public managers and the private initiative, that exert distinct pressures on vector populations. In addition, taking into account the varied genetic backgrounds of *Ae. aegypti* from different locations, it is appropriate to assume that distinct resistance profiles and mechanisms can be selected in different geographic regions. This multiplicity of scenarios justifies the importance of monitoring the insecticide resistance dynamics of natural vector populations, whether they are exposed to the pressure of a given insecticide or to its interruption in the field.

In the present study, we evaluated the dynamics of resistance of *Ae. aegypti* populations of four distinct Brazilian regions over the course of one year. The chief insecticides employed by the National Dengue Control Program (PNCD) were considered. Two of the major associated mechanisms, metabolic and target site resistance, were also investigated. In the first case, the activity of different classes of detoxifying enzymes was quantified. The target site for PY was analyzed with molecular assays. Several insecticide resistance mechanisms are potentially effective against distinct insecticides simultaneously, belonging or not to the same class, a phenomenon known as cross-resistance. Therefore, the identification of resistance mechanisms

involved in each specific situation, together with the possibility of using alternative insecticides bearing different modes of action (such as the use of a CSI to replace OP) is important in vector control programs. Furthermore, comparison of insecticide resistance levels and resistance mechanisms with the local history of chemical control can contribute to a rational management of resistance and, consequently, preservation of the insecticides still available.

Materials and methods

Ethics statements

The use of anesthetized mice to blood feed mosquitoes was authorized by Fiocruz Ethical Committee for Animal Use (CEUA P-0498/08 and CEUA L-0007/09)

Study areas

Dengue is endemic throughout Brazil, except for the southernmost region. Four midsized tropical cities, each one in a different region, all with representative climate regimes and significant chronicles of dengue cases, were chosen (Fig 1, more details in [19]):

Santarém, Pará (PA) State, North Region. 2˚26'35"S, 54˚42'29"W. This city, located in the Amazon region, bears an elevated humidity, high precipitation indices and temperatures ranging from 23 to 33˚C. Santarém has a demographic density of 12.9 inhabitants/km^2 [20].

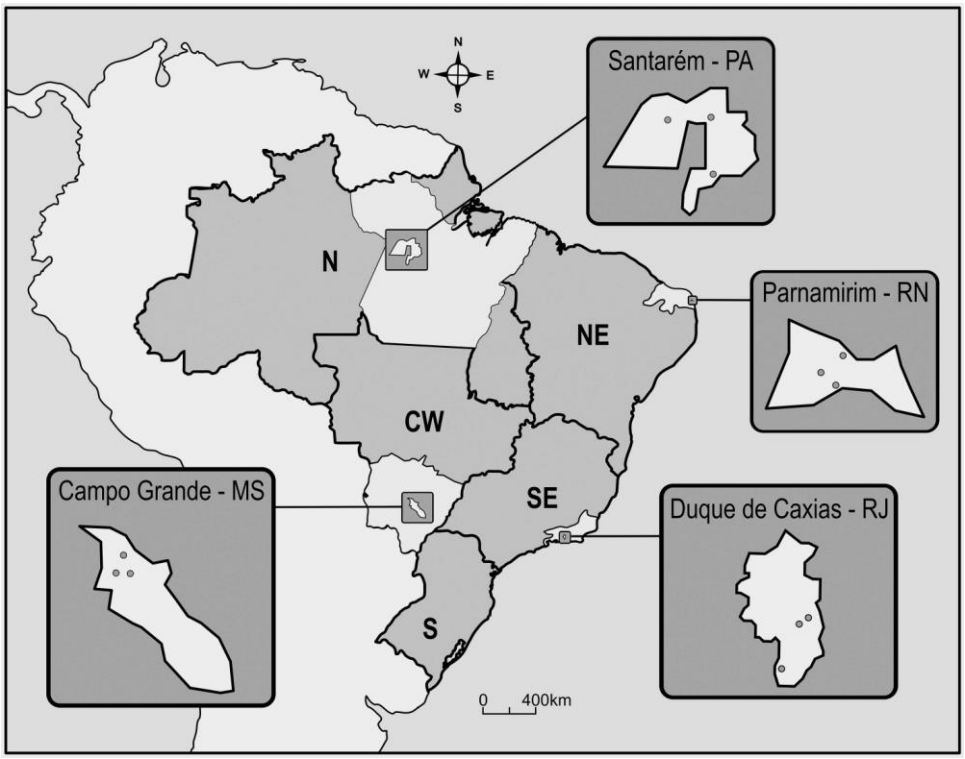

Fig 1. Map of Brazil showing the location of the four municipalities evaluated. In the course of one year, in each municipality, *Ae. aegypti* eggs were gathered in the field monthly, in three 1 km^2 areas, as indicated by grey circles. Letters beside city names account for the respective States: Santarém—Pará (PA), Parnamirim—Rio Grande do Norte (RN), Duque de Caxias—Rio de Janeiro (RJ) and Campo Grande—Mato Grosso do Sul (MS). References to the different Brazilian Regions are placed directly on the map: N—North, NE—Northeast, SE—Southeast, S—South, CW—Central-West.

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Parnamirim, Rio Grande do Norte (RN) State, Northeast Region. 5˚54'56"S,35˚15'46"W. Parnamirim has a milder and dryer climate compared to Santarém. Temperatures vary from 22 to 29˚C and the city has 1,858 inhabitants /km^2 [21].

Duque de Caxias, Rio de Janeiro (RJ) State, Southeast Region. 22˚47'08"S, 43˚18'42"W. Similar to Parnamirim, it is a densely populated city, with 1,828 inhabitants/ km^2 [22]. Temperature differences among seasons are more pronounced compared to the North and Northeast Regions. During summer, heavy precipitation and flooding often occur.

Campo Grande, Mato Grosso do Sul (MS) State, Central-West Region. 20˚26'34"S, 54˚38'47"W. Of all cities in this study, Campo Grande is located most above sea level, roughly at 600 meters altitude. Among the four study areas here presented, the highest temperature and precipitation amplitudes were registered in Campo Grande. Winter is particularly dry and cold in this city, with a demographic density of 97 inhabitants/ km^2 [23].

Field collection of eggs and *Ae. aegypti* samples to establish colonies

Ovitraps were used for monthly egg collection [24], in three 1 km^2 areas in each of the four municipalities (Fig 1). Egg collection was initiated in November 2009 (Duque de Caxias), December 2009 (Parnamirim and Campo Grande) and March 2010 (Santarém) proceeding for 12 months. In each 1 km^2 area, 120 ovitraps were installed. In the laboratory, eggs of the parental generation collected in the field were reared until the adult stage, and specimens were identified up to the species level. *Ae. aegypti* parental adults always corresponded to at least 90% of field samples, and these mosquitoes were used to search for *kdr* mutations (see below). For each municipality, on four occasions roughly at three month intervals, F1 colonies were established in order to perform bioassays and biochemical tests. As depicted in S1 Table, the number of adult females starting the colonies was always over 500. Except for Parnamirim, 18–20 months after the last egg collection, a new field sample was obtained for each locality, and the F1 derived specimens were submitted to a temephos dose-response assay, as indicated below.

Mosquito rearing

Synchronously reared F1 L3 instar larvae or 1–3 day old adult females were used for bioassays with, respectively, larvicide or adulticide compounds (see below). Rearing was performed essentially as described by Bellinato et al. (2016) [25]. The Rockefeller ("Rock") strain was adopted both as an internal quality control of all assays and an insecticide susceptible reference lineage [26]. For each insecticide and each field population, as well as for Rockefeller, effective doses (ED_{50} and ED_{95}) were obtained by probit analysis with the aid of Polo-PC software [27]. Resistance ratios (RR) were then calculated by dividing the ED of field populations by that of the corresponding Rock. RR_{95} was used to compare all bioassays in accordance with the Brazilian MoH guidelines [28].

Larvae bioassays

Quantitative bioassays were employed to evaluate the susceptibility status of field *Ae. aegypti* populations against the OP temephos and the CSI diflubenzuron. Eight to ten insecticide concentrations, varying from 0.006 to 0.072 mg/L for temephos and 1.0 to 5.5 μg/L for diflubenzuron, were used per assay. For each insecticide concentration in each assay, four replicates, each one with 20 or 10 larvae were exposed to temephos or diflubenzuron, respectively. This corresponds to a total of 640–800 larvae per temephos assay and to 320–400 larvae in the case of diflubenzuron. Each assay was repeated at least three times on different days, mortality varying between 10 and 95% [29, 30]. Results were registered 24 hours after temephos exposure. In

the case of diflubenzuron, according to protocols standardized previously [25, 31, 32, 33] the bioassays were followed until adult emergence of all control specimens, not exposed to the CSI.

Adult bioassays

Quantification of adult resistance to the deltamethrin PY was also performed through dose-response assays, adhering to methodology adapted from the original WHO protocol, with insecticide impregnated papers [34]. Up to 10 different deltamethrin concentrations were used per assay, varying between 2.1 and 109.6 mg/m^2, depending upon the susceptibility status of the field sample under test. Assays were repeated at least three times on different days. In all cases, three replicates with 15 to 20 adult females each were used.

Biochemical assays

Quantification of enzyme activities potentially involved in insecticide detoxification was performed in agreement with a standardized biochemical procedure [35, 36]. In all cases, 80 to 120 non-blood-fed young females (up to 24 hours after emergence), stored at -80˚C, were individually analyzed. For each female, the following enzyme activities were quantified: glutathione-S-transferase (GST), esterase (EST) and mixed function oxidase (MFO). Three substrates were employed for EST: α- and β-naphtyl and ρ-nitrophenyl acetates, accounting respectively, for activities named α-EST, β-EST and ρNPA-EST.

According to former protocols, the 99 percentile of the susceptible control strain Rockefeller (p99Rock) was calculated for each enzyme class. Field population data were classified as follows: enzyme activity of any given population was considered unaltered when 0–15% specimens remained beyond p99Rock; values between 15 and 50% and above 50% were classified as altered or highly altered, respectively [35, 36].

Molecular assays

Allele-specific PCR was applied to investigate the presence of the Ile1011Met, Val1016Ile and Phe1534Cys mutations in the PY target site, Na_V. Adults of the parental generation derived from monthly field collected eggs were used for evaluation of 1016 allelic frequencies. The other positions, 1011 and 1534, were investigated in the first and last months of the first year interval. In all cases, genomic DNA was extracted from 30 individual adult males of each field sample. If no substitutions were detected at the three positions in all samplings of a population, 30 additional specimens were submitted to evaluation. This was done in order to obtain more accurate measures of *kdr* frequencies. Males were recruited in order to avoid the risk of contamination with spermathecae in the case inseminated females were used. The methodology described elsewhere [37, 38] was followed.

Results

Bioassays with larvae: Temephos

Biological assays identified resistance to temephos in all the *Ae. aegypti* populations evaluated throughout the study period (Fig 2, S2 Table). Duque de Caxias presented the highest RR_{95} levels (between 9.8 and 16.3) and Campo Grande, the lowest (3.6 to 7.9). Nevertheless, a temephos resistance decay trend was observed in all cases in the period (2009–2012) although the rate of decay was different among populations. In the course of the study, temephos RR_{95} decreased up to 50% in mosquitoes from Campo Grande, 40% in Duque de Caxias, 30% in Santarém and 15% in Parnamirim. This result is compatible with the withdrawal of temephos

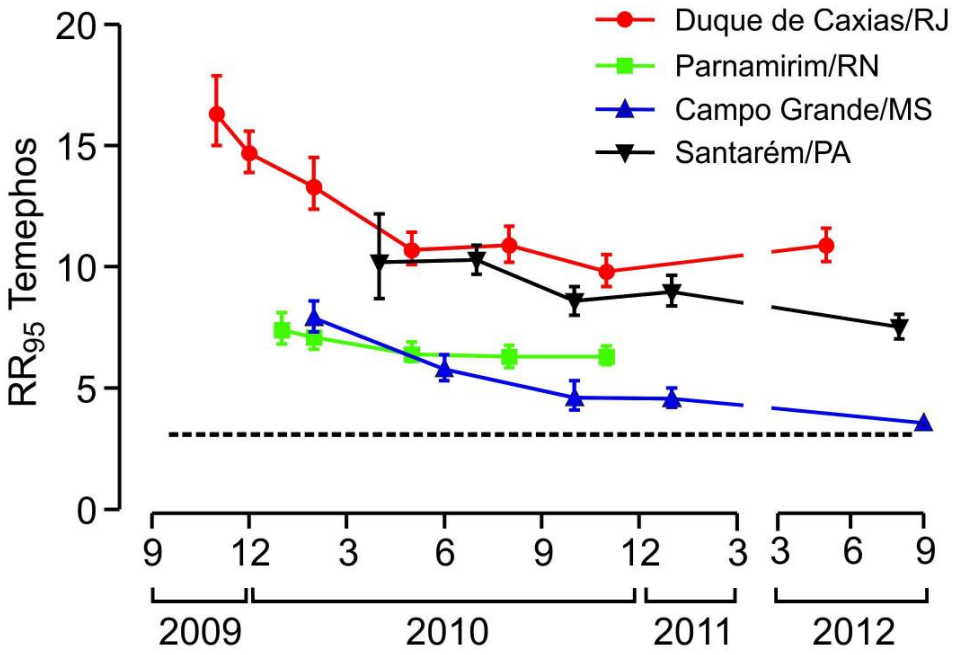

Fig 2. Temporal evaluation of temephos susceptibility status of four Brazilian *Ae. aegypti* populations. Each point indicates the Resistance Ratio (RR_{95}) and the 95% confidence interval. The dashed line, at RR_{95} = 3.0, points to the threshold that triggers interruption of temephos application, according to the Brazilian Health Ministry [28].

https://doi.org/10.1371/journal.pntd.0006227.g002

in the four municipalities, as reported by each Municipal Health Secretariat. Despite the decrease in temephos resistance, the RR_{95} always remained above 3.0. This value corresponds to the threshold defined by the MoH, above which temephos interruption is recommended [28]. It was also evident that slopes obtained for field populations were always lower than those for Rockefeller (S2 Table), pointing to a higher heterogeneity compared to the control strain.

Bioassays with larvae: Diflubenzuron

Emergence inhibition of adults (EI) was the parameter evaluated in the dose-response tests of larvae exposed to diflubenzuron. Subtle variations in the effective doses were noted throughout the analyses of all populations with no apparent trend (Fig 3, S3 Table). Diflubenzuron RR_{95} always remained below 3.0 when compared to the Rockefeller strain, indicating susceptibility of field populations to this IGR. Moreover, slopes of the evaluated populations were always higher than the Rockefeller strain (S3 Table), suggesting, unlike results for temephos, a greater homogeneity of these field populations in relation to diflubenzuron susceptibility.

Bioassays with adults: Deltamethrin

The deltamethrin RR_{95} was extremely elevated in all municipalities, always higher than 10 (S4 Table). Excluding Parnamirim, where deltamethrin RR_{95} ranged between 10.1 and 14.3, all other municipalities were above 35. It is noteworthy that in Campo Grande, for example, the lowest value obtained was 58.2. No trend in RR was noted during the study period, neither a tendency for decrease nor increase. Adult bioassay results are separate for each population, simultaneously with the dengue incidence in the intervals evaluated (Fig 4). In two locations, Duque de Caxias and Campo Grande, the highest RR values were in the period corresponding to the highest dengue incidence. In particular, the numbers of dengue cases in Campo Grande

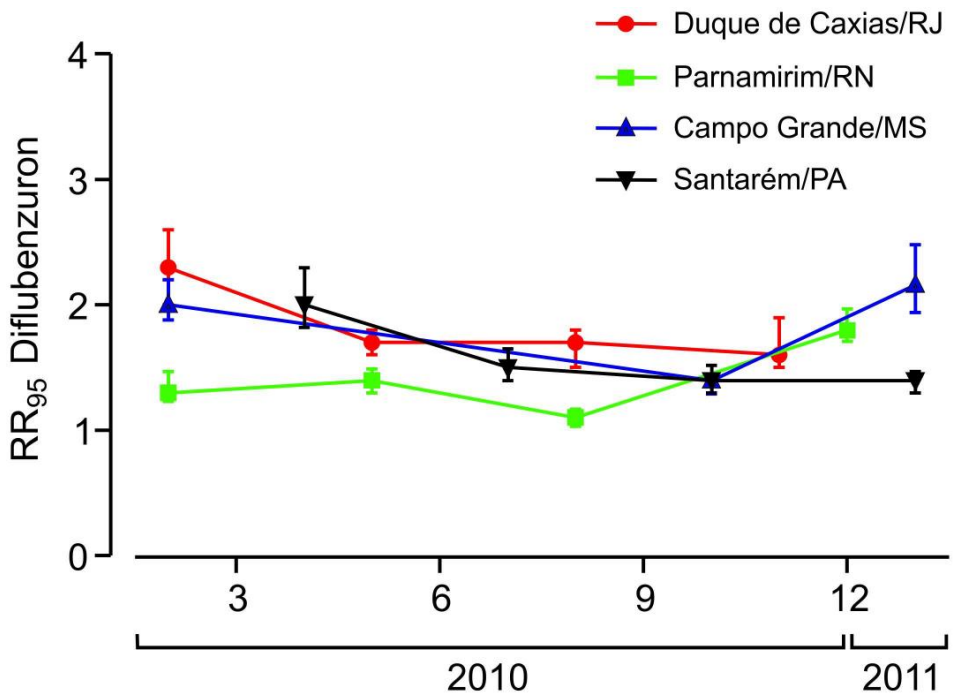

Fig 3. Temporal evaluation of diflubenzuron susceptibility status of four Brazilian *Ae. aegypti* populations. Each point indicates the Resistance Ratio (RR_{95}) and the 95% confidence interval.

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were compatible with an explosive outbreak. Adulticide applications by the municipal health agents in each locality were also included in Fig 4. In this case, only the applications carried out in the study areas (and not in the entire municipality) are shown. Both the intensity and frequency of adult chemical control by the Municipal Health Secretariats varied widely. The intense use of deltamethrin by health agents in Duque de Caxias (much more than the amount recommended by the MoH, see Discussion) should be noted, as well as the use of malathion, a non-PY adulticide, in Campo Grande precisely the municipality where the largest dengue incidence was concomitant with the highest recorded deltamethrin RR_{95}. In general, as was the case with temephos and deduced from the slope values, heterogeneity of field populations was higher than that of the Rockefeller strain regarding deltamethrin status.

Biochemical assays: Metabolic resistance

Adult females were submitted to biochemical assays, disclosing changes in all classes of detoxifying enzymes (Table 1). GST and EST were the most affected activities. However, concerning esterases, greater alterations were observed with the "ρNPA" substrate. The MFO enzymes were the least altered in all populations. Only DQC and PNM populations presented GST and EST altered activities in all evaluated samples. In Campo Grande, no ρNPA-EST alteration was detected, and Santarém was the population with the least changes in the detoxifying enzymes.

Molecular assays: The PY target site

The presence and frequency of the Val1016Ile mutation in the *Ae. aegypti* Na_V was investigated monthly in all field populations (Fig 5). Additionally, quantification of two other *Aa*Na_V mutations, Phe1534Cys and Ile1011Met, was performed in samples collected in the first and last

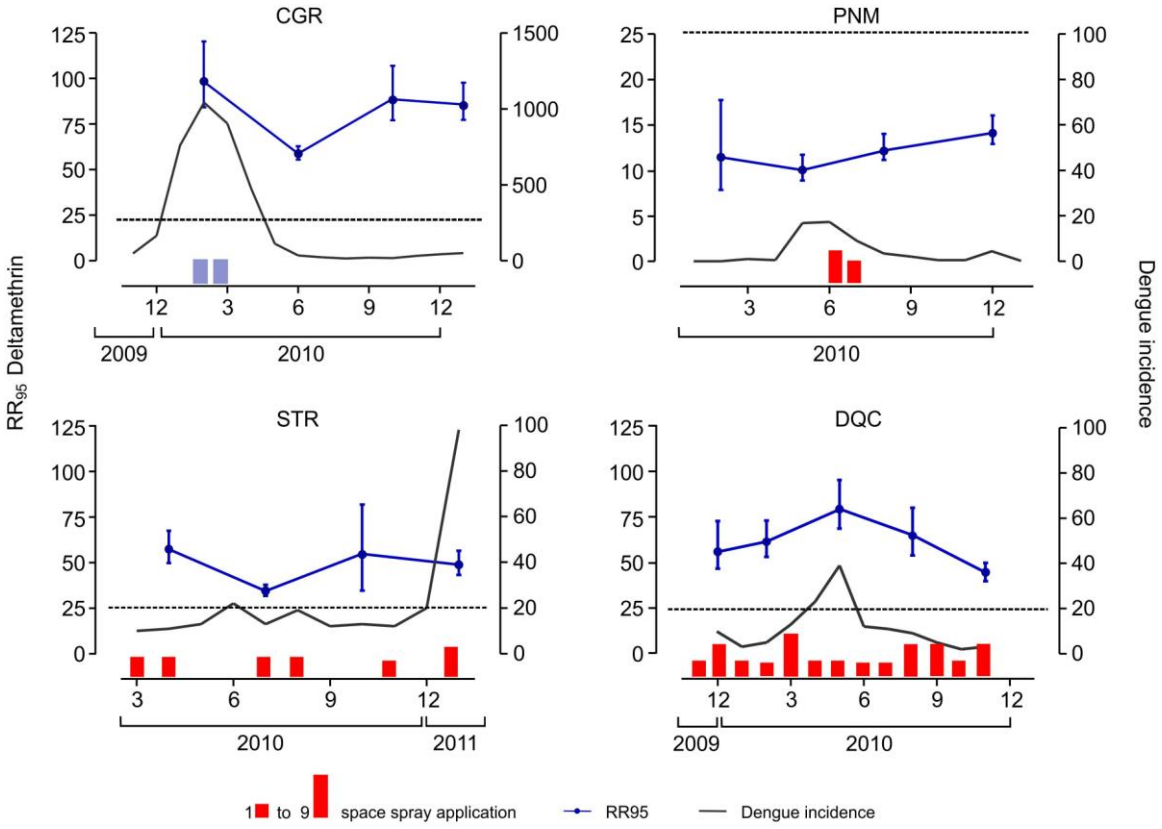

Fig 4. Temporal evaluation of deltamethrin susceptibility/resistance status of adult females from four Brazilian *Ae. aegypti* populations: Campo Grande/MS (CGR), Parnamirim/RN (PNM), Santarém/PA (STR) and Duque de Caxias/RJ (DQC). Each point indicates the Resistance Ratio (RR_{95}) and the 95% confidence interval. Bars represent the number of deltamethrin (red) and malathion (blue) space spray applications conducted monthly in each municipality, varying between 1 and 9. The dashed line refers to an arbitrary value of 25.0, included in the figure to facilitate visualization of differences in RR among populations (note that y-axis scale, referring to deltamethrin RR_{95}, is different for PNM population). Grey lines point to the dengue incidence in the period, expressed in number of cases/100,000 inhabitants. Note that the y-axis scale (Dengue incidence) of the CGR population was altered.

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months of evaluation for each population (Table 2). While the relation of the Val1016Ile and Phe1534Cys mutations with PY resistance is well documented [18, 37], the status of the Ile1011Met substitution [39] is still controversial (see Discussion). Substitutions at positions 1016 and 1534 are recessive, i.e., resistance to PY is expressed only in homozygosis [40]. Regarding the third position, there is evidence that the Ile1011Met mutation can be used as a marker of an early duplication event in this species, occurring in the wild type and susceptible genotype. Hence, decrease in the rate of the Ile1011Met substitution should occur in parallel with an increase of the more recent *kdr* mutants in the 1534 and 1016 positions [38].

Fig 5 shows, for the four populations evaluated, the *kdr* 1016Ile allelic frequency and the genotypic frequency of the *kdr* homozygotes at this position (1016Ile/Ile). For two populations, Duque de Caxias and Campo Grande, rates of the 1016Ile substitution were high throughout the evaluation period, allelic frequencies always above 70%. In these localities, in contrast to the low frequencies in the Ile1011Met mutation, allelic frequencies of the Phe1534Cys substitution were also high (Table 2). The substantial dissemination of these *kdr* mutations in Duque de Caxias and Campo Grande endorsed the high levels of PY resistance previously detected (Fig 5).

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Table 1. Activity alterations of enzymes related to metabolic resistance in adult specimens of four Brazilian *Ae. aegypti* populations.

Population	Period	MFO	α-EST	β-EST	ρNPA-EST	GST
Duque de Caxias/RJ	Feb/10		38	17	62	81
	May//10	10			57	33
	Aug/10	21	45	24	22	46
	Nov/10	15	24	7	26	34
Parnamirim/RN	Feb/10		12	6	34	80
	May//10	14			43	44
	Aug/10	14	39	36	7	27
	Dec/10	5	31	13	23	70
Campo Grande/MS	Feb/10	3	5	14	14	76
	Jun/10	18			7	35
	Oct/10	3	27	44	1	30
	Jan/11	10	8	34	4	16
Santarém/PA	Abr/10	7	0	11	11	48
	Jul/10	31			20	16
	Oct/10	2	3	15	9	16
	Jan/11	14	3	9	3	3

According to criteria defined previously [35, 36], enzyme activities were classified as unaltered (green), altered (yellow) and extremely altered (pink) if the rate of individuals above the 99 percentile for Rockefeller strain was below 15, between 15 and 50 and above 50%, respectively.

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In contrast in Parnamirim, mutations at 1016 and 1534 sites are present, notwithstanding at a low frequency. Accordingly in this population, the highest Ile1011Met frequencies were present (Table 2, see Discussion). No homozygous *kdr* specimens at position 1016 were detected in this population (Fig 5, Table 2), and allelic *kdr* 1016Ile rates were always below 10%. Accordingly, deltamethrin RR levels in Parnamirin, although high, were much lower than those observed in the other three populations (Fig 5). Regarding position 1534, approximately 13% of *kdr* homozygotes were found, suggesting participation of the PY target site mechanism in the resistance of this population to deltamethrin.

In spite of the high deltamethrin resistance levels in Santarém (Fig 4, S4 Table), the 1016Ile mutation was not apparent in any mosquito from this population (Fig 5). However, the *kdr* 1534Cys allelic frequencies were high, 94% in the first month of collection and 100% at the end of the work. In parallel, the Ile1011Met frequencies were the lowest, reaching zero in the last evaluation (Table 2).

Discussion

In Brazil, the monitoring of insecticide resistance in *Ae. aegypti* populations assists the rational management of chemical control. We accompanied, over the course of one year, the dynamics of resistance of four field populations belonging to different geographical scenarios and with distinct vector control policies. The quantification of resistance levels together with major resistance mechanisms with respect to the temephos and diflubenzuron larvicides as well as the deltamethrin adulticide, all employed in the control of this vector on a national scale, was considered (S5 Table). The results are discussed taking into account the previous insecticide use and dengue cases in each locality.

In Brazil, since 1967 until recently, temephos was the only larvicide adopted by the public health services for the control of *Ae. aegypti*. We confirmed that all populations evaluated in the present study were resistant to this OP. Our results are in accordance with prior reports

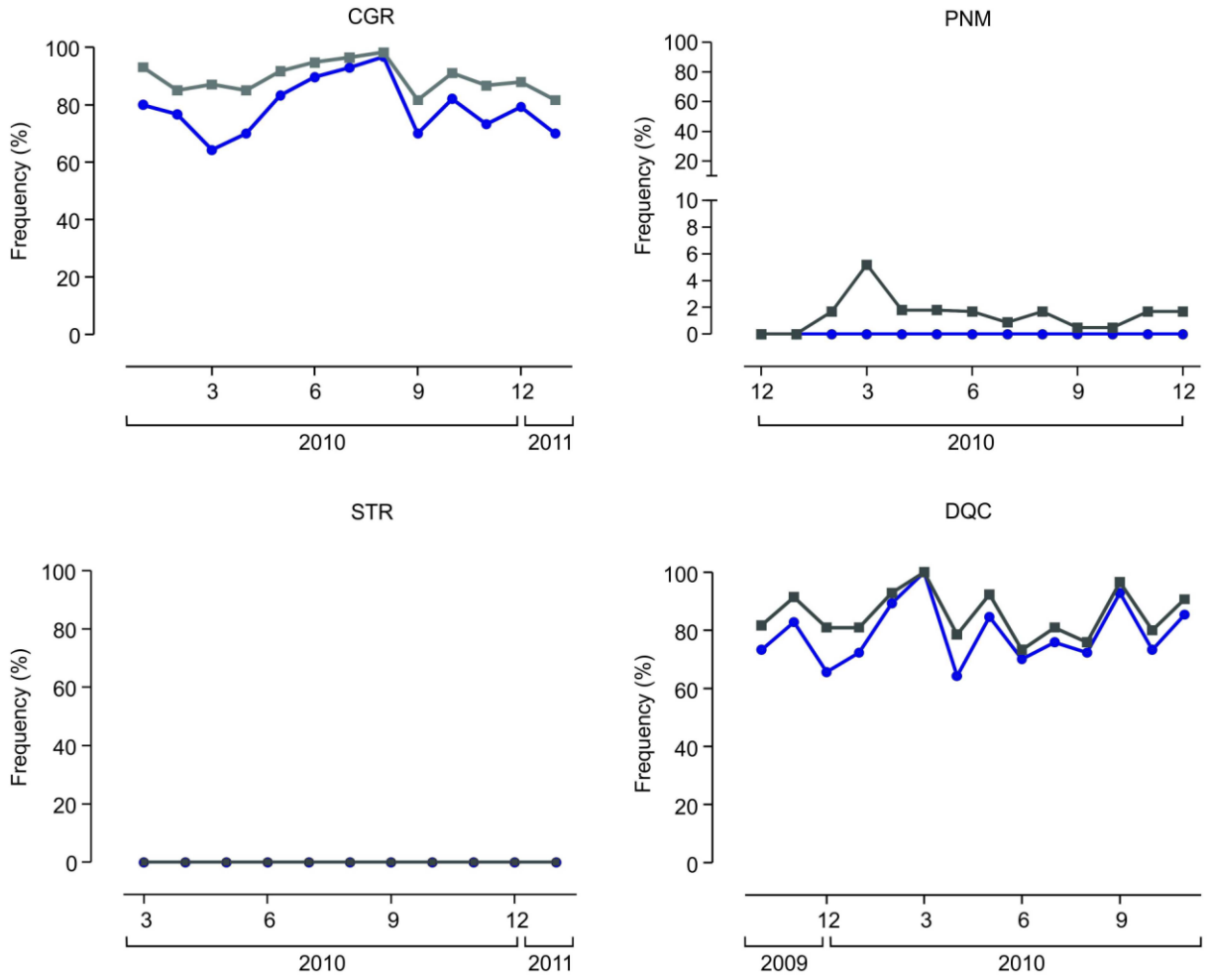

Fig 5. Allelic (grey) and genotypic (blue) frequencies of the *Ae. aegypti kdr* Val1016Ile substitution in the Na_V. Populations names were abbreviated as in Fig 4. Note that the y-axis scale of the Parnamirim (PNM) population was altered in order to enable a better visualization of frequency variations.

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Table 2. Allelic and genotypic frequencies of specific *Ae. aegypti* Na_V alleles at positions 1016, 1534 and 1011, shown separately, at the first and the last periods of field evaluation.

Population	Period	1016		1534		1011	
		%Ile	%Ile/Ile	%Cys	%Cys/Cys	%Met	%Met/Ile
Duque de Caxias/RJ	Feb/10	92.9	89.3	ND	ND	3.3	6.7
	Nov/10	90.7	85.2	98.2	96.4	1.7	3.4
Parnamirim/RN	Mar/10	5.2	0.0	33.3	10.0	41.7	83.3
	Dec/10	1.7	0.0	35.0	13.0	43.3	86.7
Campo Grande/MS	Mar/10	87.1	64.3	88.0	76.0	13.5	26.9
	Dec/10	87.9	79.3	98.3	96.7	1.8	3.6
Santarém/PA	Mar/10	0.0	0.0	94.4	88.9	5.2	10.3
	Jan/11	0.0	0.0	100.0	100.0	0.0	0.0

ND = not determined

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[13, 25, 41] and even with MoH data which in 2009 already pointed to temephos susceptibility alterations in 90% of the evaluated Brazilian populations [42]. Resistance to OPs has also been observed throughout Latin America, with reports in several countries such as Colombia, Mexico, Cuba, Martinique and Argentina [43–50].

As stated by the Brazilian MoH, recommendations of *Ae. aegypti* chemical control management in the country are based on RR_{95} values. In the case of temephos, suspension is indicated when RR_{95} is above 3.0 [28]. In our study, we detected RR_{95} for temephos between 3.6 and 16.3, the highest values in Duque de Caxias and Santarém. Despite the widespread resistance to temephos, in all municipalities a tendency for resistance ratios to decrease was observed during the evaluation period, attributed to the interruption of temephos utilization in the studied areas. However, resistance levels decreased slowly and the temephos RR_{95} of mosquito populations from all localities remained above the susceptibility threshold value, preventing the reutilization of this OP.

Parnamirim, one of the municipalities evaluated here, is located in the metropolitan region of Natal, the capital of RN in the Northeast Region. In accordance with our results, data from the MoH also point to a decrease in *Ae. aegypti* temephos resistance levels in Natal, after replacement by *Bti* in 2005. The RR_{95} was 18.6 in 2004 [36] and was reduced to 8.2 in 2007 [28]. In addition, Lima et al. (2011) [51] observed a decrease in temephos resistance in *Ae. aegypti* from Juazeiro do Norte, State of Ceará, also in the Northeast Region, after its discontinuation, temephos RR_{95} declining around 30% in six years (from 10.4 in 2003 to 7.4 in 2009). This same work registered an increase of temephos RR_{95} in two locations, Crato and Barbalha, that maintained temephos during this same period, from 7.5 to 30.0 in Barbalha and from 9.0 to 192.7 in Crato. Wirth and Georghiou (1999) [52] also reported, in *Ae. aegypti* from Tortola, a small Caribbean island, a decrease in temephos resistance ten years after application interruption in the field. RR_{90}, was 46.8 in 1985 [53] and declined to 6.3 in 1995/6 [54].

The *Ae. aegypti* resistance status to the inhibitors of chitin synthesis (CSI), another class of larvicides recently introduced in the country, was also quantified. Taking into account the same cutoff established for temephos (RR_{95} = 3.0), diflubenzuron data point to susceptibility of all evaluated populations, confirming previous results obtained in the country for the dengue vector [25, 30, 55]. The recent introduction in Brazil of CSI compounds against *Ae. aegypti*, together with their distinct mechanism of action regarding conventional insecticides, contributes to the low resistance rates in the evaluated populations.

In Brazil, except for the State of São Paulo, PY has only been adopted as an adulticide since 2000–2001, after dissemination of resistance to OP in *Ae. aegypti* populations was confirmed [56]. This decision was made as a management strategy to expose larva and adult stages to compounds with different action mechanisms. However, mosquito field samples collected shortly afterwards (2002–2003) already exhibited signs of PY resistance [57]. Since then, PY resistance has been detected in several regions of the country [18, 25, 51, 58, 59]. Resistance to deltamethrin was extremely high in all populations studied here. Parnamirim exhibited the lowest RR_{95} levels, despite the magnitude, between 10.1 and 14.3. For the remaining populations, deltamethrin RR_{95} was always above 35.

In Brazil, adulticides are not enlisted by public health managers as infestation prevention tools. Such products are used in attempts to block outbreaks or at strategic points such as airports and other potential vector entry points. According to the MoH, ultralow volume applications of adulticides should not exceed 5–7 times in the course of a year, in general during epidemic periods and in very specific situations and places [7]. However, a survey of the insecticide spatial applications against *Ae. aegypti* in the studied localities (Fig 4) revealed a great variation and even an uncontrolled use of these products by local public managers. Many differences were detected among municipalities in adulticide applications, both in frequency and

number. In several situations MoH recommendations were far exceeded, and up to nine applications have been registered in one single month. Despite this, the PY resistance levels during the study could not be temporally correlated to the 'public' spatial applications of adulticides in each locality. In two populations, Campo Grande and Duque de Caxias, the highest deltamethrin RR_{95} levels were registered precisely during periods of intense dengue transmission. It is worth mentioning that the Brazilian MoH considers that incidence rates beyond 300 dengue cases / 100,000 inhabitants are high [60]. In Campo Grande, in particular, the greatest dengue epidemic ever faced occurred during the period of our study (see Fig 4), when it also presented the highest deltamethrin resistance levels detected throughout the study. However, in this locality deltamethrin was not applied during this outbreak, the adulticide employed being the malathion OP. Our hypothesis is that arbovirus outbreaks cause a collective panic in the local population with a consequent pursuit towards individual protection and control measures. As a result there is a great and uncontrolled rise in the domestic use of PY products that are commercially available. This situation has a direct effect on the elevated PY resistance during epidemic outbreaks [8, 25, 61].

Although the variety of insecticides for public health use is limited, chemical management is still a relevant component of vector control programs. This combination leads to the rapid selection of resistant populations together with the exhaustion of available insecticides, often resulting in control impairment. However, in many situations the suspension of one specific insecticide by a given vector control program does not necessarily lead to its real field interruption due to its intensified domestic use at each new outbreak as well as continued availability of some products in the retail marked. In addition, the lack of integration of the different vector borne disease control programs cannot be neglected.

A significant participation of alterations in the Na_V, the PY target site in the central nervous system, in the resistance to this class of insecticides was apparent. The Val1016Ile mutation has been previously related to PY resistance in Brazilian *Ae. aegypti* populations [18, 37] as well as those of other Latin American countries [62–65]. For this reason its frequency was monitored monthly. Later, the 1534Cys allele was identified in several places throughout the country, even in the absence of the 1016Ile *kdr* allele [37]. We then opted to investigate its frequency together with substitutions in the 1011 position, also potentially interfering with the Na_V. Besides Brazil, the Phe1534Cys mutation is related to PY resistance in several other localities, such as the Cayman Islands and Thailand [66–67]. In our present study, we always found the 1534Cys mutant allele frequencies higher than those of the 1016Ile *kdr* allele (Table 2), a situation that corroborates previous evidences that the Val1016Ile substitution takes place after the *kdr* mutation at position 1534, in a genetic background already containing the 1534Cys mutation [37, 65, 66, 67]. Regarding the Ile1011Met mutation, there are indications that this substitution can be used as a diagnostic of a Na_V duplication event [38] with an unclear relationship with PY resistance. The higher frequencies of 1011Met were evident in populations where the *kdr* 1016Ile and 1534Cys were lower (Table 2).

Duque de Caxias and Campo Grande displayed extremely high levels of resistance to deltamethrin and also very high frequencies of *kdr* mutations at positions 1016 and 1534. In Nova Iguaçu, a municipality contiguous to Duque de Caxias, the 1016Ile mutation was not detected in 2003. However in 2008, the allelic frequency of 1016Ile was 62.5%, two years later peaking at 95% [18, 38]. A rapid increase in the 1016Ile allele was also observed in Campo Grande, frequency of 31.8% in 2008 [18] increasing to values above 85% in 2010 (Table 2), a situation suggestive of a rapid spread of this mutation in the region. The extremely high levels of PY resistance in Duque de Caxias and Campo Grande probably result from the combined effect of both mutations, 1016Ile and 1534Cys, as already described elsewhere [25, 37, 66]. In contrast, as expected, the 1011Met mutation frequencies were low in both municipalities.

In the populations of Duque de Caxias and Campo Grande, the rapid increase in the frequency of the *kdr* allele 1016Ile is indicative of a strong selective pressure. A fast increase in the 1016Ile mutation frequency was also observed in Mexico. Until 1999 this substitution had not been detected, however, it was already high in 2008. In 2011, frequencies above 90% of the 1016Ile allele were reported in three regions of the country, suggesting imminent fixation of this *kdr* allele [68, 69]. There is evidence that identification of PY resistance through laboratory assays may indicate impairment of spatial applications in the field [49]. In addition, similar to what was observed in the field, laboratory selection with PY of six Mexican *Ae. aegypti* populations in the course of five generations resulted in the increase of up to three-fold in the frequencies of the 1016Ile allele [70].

Although *Ae. aegypti* from Santarém possessed high rates of deltamethrin resistance (RR_{95} between 35.0 and 60.0), the 1016Ile *kdr* mutation was not detected and the 1011Met mutation frequency was very low, reaching zero, in these mosquitoes. Notwithstanding, 90–100% specimens were homozygous for the 1534Cys *kdr* mutation. Parnamirim, in the Northeast Region, presented the lowest deltamethrin RR as well as the lowest *kdr* 1016Ile and 1534Cys frequencies. In particular, the 1016Ile allele remained below 10% throughout the study. It is worth mentioning that mutations in this position had not been detected in NE Brazil in surveys prior to 2010 [18]. Later identification of 1016Ile in mosquitoes from Crato and Juazeiro do Norte, both in the State of Ceará [51], suggests their recent arrival in this Region. The restricted use of PY in the field by Parnamirim local managers, together with the absence of dengue outbreaks in the period with probable reduced domestic use of insecticides, are probably the basis of the comparatively lower PY resistance levels in this locality, as well as their limited variation throughout the study. It is noteworthy that the highest frequencies of the 1011Met mutation appeared in Parnamirim, which is in agreement with previous evidence linking this mutation to a susceptible Na_V haplotype [38]. Our results agree with data reported recently, relating high levels of pyrethroid resistance to multiple Na_V mutations, a common situation in Latin America *Ae. aegypti* populations [71, 72]

Evaluation of metabolic resistance was achieved with biochemical tests quantifying the activity of the main classes of detoxifying enzymes. Although this methodology applied in vector population monitoring routine has the potential to reflect the general dynamics of resistance, we learned with its known limitations that it is not always possible to establish precise correlations between biologic and biochemical assays for each evaluated population at a given moment [36]. Herein, we attempted to compare the metabolic changes of adult females mainly with resistance to the adulticide deltamethrin. As expected, although there was no strict temporal correlation between the levels of PY resistance and the intensity of the metabolic changes for each population, of the three enzyme classes evaluated, GST and EST (and especially ρNPA-EST) were strongly altered while MFO was the least affected class. Regarding MFO, our data, as well as those from other *Ae. aegypti* Brazilian populations, differ from other countries whose PY resistance levels tend to correlate with MFO profile alterations [25,36,73]. Still, these results corroborate previous studies that related PY resistance in Brazilian field populations with increased GST and ρNPA-EST activities [36, 74].

Santarém was the population with the lowest contribution of metabolic mechanisms to resistance levels, while Duque de Caxias and Parnamirim were the most affected. Alterations of detoxifying enzymes were identified as the main mechanism of PY resistance in Parnamirim taking into account the low frequency of *kdr* mutations in this population. Different from the other three populations, deltamethrin resistance levels in Parnamirim were consistently lower, also corroborating the strong contribution of PY target site alterations to the intensity of resistance to this class of insecticides, a situation already reported previously [40]. The influence of mutations in the PY target site on elevated resistance levels was confirmed with Santarém

mosquitoes, whose high resistance ratios are parallel to the high frequency of the 1534Cys *kdr* allele although the detoxifying enzymes in this population were the least altered in the study. In agreement with this situation, the population of Campo Grande also revealed high levels of PY resistance and high frequencies of *kdr* mutations, while persistent changes in metabolic resistance were only detected for GST enzymes.

Regarding resistance mechanisms, data indicate that different vector populations find different solutions to counteract the challenge represented by insecticides. This is attributed to the multifactorial nature of metabolic resistance as well as the abovementioned limitations of the biochemical methodology employed (which quantifies general activities and not molecular species) [36].

According to Moyes et al. (2017) [72], there is currently plenty of evidence of resistance to the two main classes of insecticides employed all over the world, PY and the OP temephos. In particular, as data related to Latin America abundantly show, high resistance levels are common in the continent. Nonetheless, our results present a trend towards a slow decrease in *Ae. aegypti* resistance to temephos since discontinuation of this OP larvicide in the field started in 2009. The CSI susceptible levels are probably a consequence of the recent introduction in the *Ae. aegypti* control routine in the country. In contrast, extremely high and disseminated PY resistance levels were noted, indicating a significant participation of the domestic use of this class of compounds in the selection pressure of Brazilian vector populations (S5 Table). The exacerbated domestic use of PY insecticides is seasonal, occurring mainly during outbreaks, and it can be accompanied by the seasonal elevation of resistance levels. Finally, it was possible to highlight the limitations of chemical control as the main methodology for *Ae. aegypti* control, taking into account both larvae and adults. There is growing evidence of the need for joint actions with other types of methodologies, social mobilization, mechanical control and biological complementary alternatives. We believe that the adoption of insecticides in a rational way is a strategy to complement other types of controls.

Supporting information

S1 Table. Total of *Ae. aegypti* adults obtained after field collection of eggs. The *Ae. aegypti* rate related to total *Aedes* specimens and the number of male and female *Ae. aegypti* mosquitoes are also shown. Only data from samples used to generate colonies are presented. *Ae. aegypti* colonies were employed to evaluate resistance and resistance mechanisms.
(DOC)

S2 Table. Details of temephos bioassays with *Ae. aegypti* larvae. Results generated by probit analysis.
(DOC)

S3 Table. Details of diflubenzuron bioassays with *Ae. aegypti* larvae. Results generated by probit analysis.
(DOC)

S4 Table. Details of deltamethrin bioassays with *Ae. aegypti* adults. Results generated by probit analysis.
(DOC)

S5 Table. Temporal evaluation of the susceptibility status of four Brazilian *Ae. aegypti* populations to the main insecticides employed by the Brazilian Dengue Control Program.
(DOC)

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Acknowledgments

To the teams of the Health Secretaries of each one of the municipalities and States involved in the study: Santarém/PA, Parnamirim/RN, Duque de Caxias/RJ and Campo Grande/MS. To all the public health personnel that worked with us in each municipality. To the technical assistance of professionals from the Laboratório de Fisiologia e Controle de Artrópodes Vetores, in particular, Valdice Paula, Rafael Guedes and Gilberto Couto Alcântara. To Heloisa Maria Nogueira Diniz for help with the figures. English review and revision by Mitchell Raymond Lishon, Native of Chicago, Illinois, U.S.A. U.C.L.A, 1969.

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References

1. San Martín JL, Brathwaite O, Zambrano B, Solórzano JO, Bouckenooghe A, Dayan GH, et al. The epidemiology of dengue in the Americas over the last three decades: a worrisome reality. Am J Trop Med Hyg. 2010; 82(1): 128–135. https://doi.org/10.4269/ajtmh.2010.09-0346 PMID: 20065008
2. Fares RC, Souza KP, Añez G, Rios M. Epidemiological scenario of dengue in Brazil. Biomed Res Int. 2015; 2015: 321873. https://doi.org/10.1155/2015/321873 PMID: 26413514
3. Azevedo RdS, Oliveira CS, Vasconcelos PF. Chikungunya risk for Brazil. Rev Saude Publica. 2015; 49: 58. https://doi.org/10.1590/S0034-8910.2015049006219 PMID: 26398876
4. Brasil, Ministério da Saúde. Boletim epidemiológico,2015 [Internet]. http://portalarquivos.saude.gov.br/images/pdf/2015/agosto/26/2015-020-publica——o.pdf.
5. World Health Organization. WHO Director-General summarizes the outcome of the Emergency Committee regarding clusters of microcephaly and Guillain-Barré syndrome. Geneva, 2016 [Internet]. http://www.who.int/mediacentre/news/statements/2016/emergency-committee-zika-microcephaly/en/.
6. World Health Organization. Fifth meeting of the Emergency Committee under the International Health Regulations (2005) regarding microcephaly, other neurological disorders and Zika virus. Geneva, 2016 [Internet]. http://www.who.int/mediacentre/news/statements/2016/zika-fifth-ec/en/.
7. Brasil, Diretrizes Nacionais Para a Prevenção e Controle de Epidemias de Dengue, Normas e Manuais Técnicos, Série A, Ministério da Saúde, Secretaria de Vigilância em Saúde, Brasília, 1st edition, 2009.

8. Valle D, Belinato TA, Martins AJ. Controle químico de *Aedes aegypti*. Resistencia a inseticidas e alternativas. In: Valle D, Pimenta DN, Cunha VR, editors. Dengue: Teorias e Praticas. Editora Fiocruz, Rio de Janeiro, Brazil, 2015. pp 93–126.
9. World Health Organization. Recommended insecticides for space spraying. Geneva, 2012 [Internet]. http://www.who.int/whopes/Insecticides_for_space_spraying_Jul_2012.pdf.
10. World Health Organization. WHOPES-Recommended compounds and formulations for control of mosquito larvae. Geneva, 2013 [Internet]. http://www.who.int/whopes/Mosquito_Larvicides_25_Oct_2013.pdf.
11. Braga IA, San Martin JL. Histórico do controle de *Aedes aegypti*. In: Valle D, Pimenta DN, Cunha VR, editors. Dengue: Teorias e Praticas. Editora Fiocruz, Rio de Janeiro, Brazil, 2015. pp 61–73.
12. Schatzmayr HG, Nogueira RMR, Rosa APAT. An outbreak of dengue virus at Rio de Janeiro—1986. Mem Inst Oswaldo Cruz. 1986; 81(2): 245–246. PMID: 3587006
13. Chediak M G Pimenta F Jr, Coelho GE, Braga IA, Lima JB, Cavalcante KR, et al. Spatial and temporal country-wide survey of temephos resistance in Brazilian populations of *Aedes aegypti*. Mem Inst Oswaldo Cruz. 2016; 111(5): 311–321. https://doi.org/10.1590/0074-02760150409 PMID: 27143489
14. Brasil, Ministério da Saúde. Nota Técnica N.° 204/ 2008 CGPNCD/DIGES/SVS/MS. Manejo da resistência de Aedes aegypti a inseticidas. Secretaria de Vigilância em Saúde, Brasília, 2008.
15. Brasil, Ministério da Saúde. Nota técnica n° 015/2009 CGPNCD/DIGES/SVS/MS. Uso do larvicida diflubenzuron (PM25%) para controle de Aedes aegypti. Secretaria de Vigilância em Saúde, Brasília. 2009.
16. Brasil, II Seminário Internacional para Avaliações de Controle Químico de Aedes aegypti no Brasil, Programa Nacional de Controle da Dengue, Secretaria de Vigilância em Saúde, Ministério da Saúde, Rio de Janeiro, 2012.
17. Brasil, Nota Tecnica 2/2011/IOC-Fiocruz/Diretoria. Recomendacao técnica sobre a interrupcao do uso de inseticidas piretroides no controle do Aedes aegypti no Brasil, Instituto Oswaldo Cruz, Rio de Janeiro, 2011.
18. Martins AJ, Lima JB, Peixoto AA, Valle D. Frequency of Val1016Ile mutation in the voltage-gated sodium channel gene of *Aedes aegypti* Brazilian populations. Trop Med Int Health. 2009; 14: 1351–1355. https://doi.org/10.1111/j.1365-3156.2009.02378.x PMID: 19735371
19. Codeço CT, Lima AW, Araújo SC, Lima JB, Maciel-de-Freitas R, Honório NA, et al. Surveillance of *Aedes aegypti*: comparison of house index with four alternative traps. PLoS Negl Trop Dis. 2015 Feb 10. 9(2): e0003475. https://doi.org/10.1371/journal.pntd.0003475 PMID: 25668559
20. Prefeitura Municipal de Santarém. Informações municipais de Santarém, SEMMA_CIAM, 2013 [Internet] http://www.santarem.pa.gov.br/pagina.asp?id_pagina=6.
21. Portal da Prefeitura de Parnamirim. Portal de transparência, Estatísticas e mapas, 2015 [Internet]. http://www.parnamirim.rn.gov.br/mapas.jsp.
22. Instituto Brasileiro de Geografia e Estatística—IBGE, 2010 [Internet. http://cidades.ibge.gov.br/xtras/perfil.php?codmun=330170.
23. Instituto Brasileiro de Geografia e Estatística—IBGE, 2010 [Internet]. http://cidades.ibge.gov.br/xtras/perfil.php?lang=&codmun=500270&search=mato-grosso-do-sul|campo-grande.
24. Braga IA, Gomes AD, Nelson M, Mello RD, Bergamaschi DP, De Sousa JM. Comparative study between larval surveys and ovitraps to monitor population of *Aedes aegypti*. Rev Soc Bras Med Trop. 2000; 33: 347–353. PMID: 10936947
25. Bellinato DF, Viana-Medeiros PF, Araújo SC, Martins AJ, Lima JB, Valle D. Resistance status to the insecticides temephos, deltamethrin, and diflubenzuron in Brazilian *Aedes aegypti* populations. Biomed Res Int. 2016; 2016: 8603263. https://doi.org/10.1155/2016/8603263 PMID: 27419140
26. Kuno G. Early history of laboratory breeding of *Aedes aegypti* (Diptera: Culicidae) focusing on the origins and use of selected strains. Review. J Med Entomol. 2010; 47: 957–971. PMID: 21175042
27. Raymond M. Presentation d'une programme d'analyse log probit pour microordinateur. Cahiers Sér Ent Parasitol. 1985; 22: 117–121.
28. Brasil, Ministério da Saúde. Reunião técnica para discutir status de resistência de Aedes aegypti Secretaria de Vigilância em Saúde, Rio de Janeiro, 2006.
29. World Health Organization. Instructions for determining the susceptibility or resistance of mosquito larvae to insecticides. Geneva: World Health Organization.1981.
30. Martins AJ, Belinato TA, Lima JB, Valle D. Chitin synthesis inhibitor effect on *Aedes aegypti* populations susceptible and resistant to organophosphate temephos. Pest Manag Sci. 2008; 64:676–680. https://doi.org/10.1002/ps.1547 PMID: 18257034

31. Braga IA, Mello CB, Peixoto AA, Valle D. Evaluation of methoprene effect on *Aedes aegypti* (Diptera: culicidae) development in laboratory conditions. Mem Inst Oswaldo Cruz. 2005 Jul; 100(4): 435–40. https://doi.org//S0074-02762005000400016 PMID: 16113894

32. Braga IA, Mello CB, Montella IR, Lima JBP, Martins AJ, Viana-Medeiros PF, Valle D. Effectiveness of methoprene, an Insect Growth Regulator, against temephos-resistant *Aedes aegypti* populations from different Brazilian localities, under laboratory conditions. J Med Entomol. 2005; 42(5): 830–837. PMID: 16363168

33. Belinato TA, Martins AJ, Lima JBP, Valle D. Effect of triflumuron, a chitin synthesis inhibitor, on *Aedes aegypti, Aedes albopictus* and *Culex quinquefasciatus* under laboratory conditions. Parasit Vectors. 2013 Apr 4; 6: 83. https://doi.org/10.1186/1756-3305-6-83 PMID: 23557173

34. World Health Organization. Pesticides and their application for the control of vectors and pests of public health importance. 6th Ed WHO/CDS/NTD/WHOPES/GCDPP/2006.1. Geneva, 2006.

35. Valle D, Montella IR, Ribeiro RA, Viana-Medeiros PF, Martins AJ, Lima JBP. Quantification methodology for enzyme activity related to insecticide resistance in Aedes aegypti, Fundação Oswaldo Cruz and Secretaria de Vigilância em Saúde, Ministério da Saúde, Rio de Janeiro and Distrito Federal, Brazil, 2006.

36. Montella IR, Martins AJ, Viana-Medeiros PF, Lima JB, Braga IA, Valle D. Insecticide resistance mechanisms of Brazilian *Aedes aegypti* populations from 2001 to 2004. Am J Trop Med Hyg. 2007 Sep; 77(3): 467–477. PMID: 17827362

37. Linss JG, Brito LP, Garcia GA, Araki AS, Bruno RV, Lima JB, et al. Distribution and dissemination of the Val1016Ile and Phe1534Cys Kdr mutations in *Aedes aegypti* Brazilian natural populations. Parasit Vectors. 2014 Jan 15; 7: 25. https://doi.org/10.1186/1756-3305-7-25 PMID: 24428880

38. Martins AJ, Brito LP, Linss JG, Rivas GB, Machado R, Bruno RV, et al. Evidence for gene duplication in the voltage-gated sodium channel gene of *Aedes aegypti*. Evol Med Public Health. 2013 Jan; 2013(1): 148–160. https://doi.org/10.1093/emph/eot012 PMID: 24481195

39. Martins AJ, Lins RM, Linss JG, Peixoto AA, Valle D. Voltage-gated sodium channel polymorphism and metabolic resistance in pyrethroid-resistant *Aedes aegypti* from Brazil. Am J Trop Med Hyg. 2009 Jul; 81(1): 108–115. PMID: 19556575

40. Brito LP, Linss JGB, Lima-Camara TN, Belinato TA, Peixoto AA, Lima JBP, et al. Assessing the effects of *Aedes aegypti* kdr mutations on pyrethroid resistance and its fitness cost. PLoS ONE. 2013 Apr 8; 8 (4):e60878. https://doi.org/10.1371/journal.pone.0060878 PMID: 23593337

41. Lima JB, Da-Cunha MP, Da Silva RC, Galardo AK, Soares Sda S, Braga IA, et al. Resistance of *Aedes aegypti* to organophosphates in several municipalities in the State of Rio de Janeiro and Espírito Santo, Brazil. Am J Trop Med Hyg. 2003 Mar; 68(3): 329–333. PMID: 12685640

42. Brasil, Ministério da Saúde. Reunião para discutir e avaliar os resultados do monitoramento do Aedes aegypti a inseticidas do período 2008 a 2009. Secretaria de Vigilância em Saúde, Fortaleza/CE. 2009.

43. Mazzari MB. Revisión del estado actual de la resistencia en *Aedes aegypti* a insecticidas utilizados en salud pública. Boletín de la dirección de malariología y saneamiento ambiental—Colombia. 1995; 35: 90–95.

44. Suarez MF, Gonzales R, Morales CA. Temephos resistance to *Aedes aegypti* in Cali, Colombia. Am J Trop Med Hyg. 1988; 55: 257.

45. Rodríguez MM, Bisset J, Ruiz M, Soca A. Cross-resistance to pyrethroid and organophosphorus insecticides induced by selection with temephos in *Aedes aegypti* (Diptera: Culicidae) from Cuba. J Med Entomol. 2002; 39: 882–888. https://doi.org/10.1603/0022-2585-39.6.882 PMID: 12495187

46. Biber PA, Duenas JR, Almeida FL, Gardenal CN, Almirón WR. Laboratory evaluation of susceptibility of natural subpopulations of *Aedes aegypti* larvae to temephos. J Am Mosq Control Assoc. 2006; 22: 408–411. https://doi.org/10.2987/8756-971X(2006)22[408:LEOSON]2.0.CO;2 PMID: 17067038

47. Flores AE, Grajales JS, Salas IF, Garcia GP, Becerra MH, Lozano S, et al. Mechanisms of insecticide resistance in field populations of *Aedes aegypti* (L.) from Quintana Roo, Southern Mexico. J Am Mosq Control Assoc. 2006; 22: 672–677. https://doi.org/10.2987/8756-971X(2006)22[672:MOIRIF]2.0.CO;2 PMID: 17304936

48. Ocampo CB, Salazar-Terreros MJ, Mina NJ, McAllister J, Brogdon W. Insecticide resistance status of *Aedes aegypti* in 10 localities in Colômbia. ActaTropica. 2011; 118: 37–44.

49. Marcombe S, Darriet F, Tolosa M, Agnew P, Duchon S, Etienne M, et al. Pyrethroid resistance reduces the efficacy of space sprays for dengue control on the island of Martinique (Caribbean). PloS Negl Trop Dis. 2011; 5: e1202. https://doi.org/10.1371/journal.pntd.0001202 PMID: 21713017

50. Marcombe S, Mathieu RB, Pocquet N, Riaz M-A, Poupardin R, Sélior S, et al. Insecticide resistance in the dengue vector *Aedes aegypti* from Martinique: distribution, mechanisms and relations with

environmental factors. PLoS One. 2012; 7: e30989. https://doi.org/10.1371/journal.pone.0030989 PMID: 22363529

51. Lima EP, Paiva MH, De Araújo AP, Da Silva EV, Da Silva UM, De Oliveira LN, et al. Insecticide resistance in *Aedes aegypti* populations from Ceará, Brazil. Parasit Vectors. 2011; 12: 5–16.

52. Wirth MC, Georghiou GP. Selection and characterization of temephos resistance in a population of *Aedes aegypti* from Tortola, British Virgin Islands. J Am Mosq Control Assoc. 1999; 15: 315–320. PMID: 10480122

53. Georghiou GP, Wirth M, Tran H, Saume F, Knudsen AB. Potential for organophosphate resistance in *Aedes aegypti* (Diptera: Culicidae) in the Caribbean area and neighboring countries. J Med Entomol. 1987; 24: 290–294. PMID: 3585923

54. Rawlins SC. Spatial distribution of insecticide resistance in Caribbean populations of *Aedes aegypti* and its significance. Rev Panam Salud Pub. 1998; 4: 243–251.

55. Fontoura NG, Bellinato DF, Valle D, Lima JBP. The efficacy of a chitin synthesis inhibitor against field populations of organophosphate-resistant *Aedes aegypti* in Brazil. Mem Inst Oswaldo Cruz. 2012; 107: 387–395. PMID: 22510835

56. Brasil, Ministério da Saúde. Reunião técnica para discutir status de resistência de Aedes aegypti e definir estratégias a serem implantadas para monitoramento da resistência no Brasil. Fundação Nacional de Saúde, Brasília, 1999.

57. da-Cunha MP, Lima JBP, Brogdon WG, Moya GE, Valle D. Monitoring of resistance to the pyrethroid cypermethrin in Brazilian *Aedes aegypti* (Diptera: Culicidae) populations collected between 2001 and 2003. Mem Inst Oswaldo Cruz. 2005; 100: 441–444. https://doi.org//S0074-02762005000400017 PMID: 16113895

58. Luna JED, Martins MF, Anjos AF, Kuwabara EF, Navarro-Silva MA. Susceptibility of *Aedes aegypti* to temephos and cypermethrin insecticides, Brazil. Rev Saude Publica. 2004; 38: 842–843.

59. Macoris ML, Andrighetti MT, Otrera VC, Carvalho LR, Caldas Júnior AL, Brogdon WG. Association of insecticide use and alteration on *Aedes aegypti* susceptibility status. Mem Inst Oswaldo Cruz. 2007; 102: 895–900. PMID: 18209926

60. Teixeira MG, Costa MCN, Barreto ML, Barreto FR. Epidemilogia da dengue. In: Valle D, Pimenta DN, Cunha VR, editors. Dengue: Teorias e Praticas. Editora Fiocruz, Rio de Janeiro, Brazil, 2015. pp. 293–315.

61. Maciel-de-Freitas R, Avedanho FC, Santos R, Sylvestre G, Araújo SC, Lima JB, et al.Undesirable consequences of insecticide resistance following *Aedes aegypti* control activities due to a dengue outbreak. PLoS One. 2014 Mar 27; 9(3): e92424. https://doi.org/10.1371/journal.pone.0092424 PMID: 24676277

62. Saavedra-Rodriguez K, Urdaneta-Marquez L, Rajatileka S, Moulton M, Flores AE, Fernandez-Salas I, et al. A mutation in the voltage-gated sodium channel gene associated with pyrethroid resistance in Latin American *Aedes aegypti*. Insect Mol Biol. 2007; 16: 785–798. https://doi.org/10.1111/j.1365-2583.2007.00774.x PMID: 18093007

63. Marcombe S, Poupardin R, Darriet F, Reynaud S, Bonnet J, Strode C, et al. Exploring the molecular basis of insecticide resistance in the dengue vector *Aedes aegypti*: a case study in Martinique Island (French West Indies). BMC Genomics. 2009; 10: 494. https://doi.org/10.1186/1471-2164-10-494 PMID: 19857255

64. Dusfour I, Zorrilla P, Guidez A, Issaly J, Girod R, Guillaumot L, et al. Deltamethrin resistance mechanisms in *Aedes aegypti* populations from three French overseas territories worldwide. PLoS Negl Trop Dis. 2015 Nov 20; 9(11): e0004226. https://doi.org/10.1371/journal.pntd.0004226 PMID: 26588076

65. Ponce-García G, Del Río-Galvan S, Barrera R, Saavedra-Rodriguez K, Villanueva-Segura K, Felix G, et al. Knockdown resistance mutations in *Aedes aegypti* (Diptera: Culicidae) From Puerto Rico. J Med Entomol. 2016 Nov; 53(6): 1410–1414. https://doi.org/10.1093/jme/tjw115 PMID: 27493252

66. Harris AF, Rajatileka S, Ranson H. Pyrethroid resistance in *Aedes aegypti* from Grand Cayman. Am J Trop Med Hyg. 2010; 83: 277–284. https://doi.org/10.4269/ajtmh.2010.09-0623 PMID: 20682868

67. Yanola J, Somboon P, Walton C, Nachaiwieng W, Somwang P, Prapanthadara LA. High-throughput assays for detection of the F1534C mutation in the voltage-gated sodium channel gene in permethrin-resistant *Aedes aegypti* and the distribution of this mutation throughout Thailand. Trop Med Int Health. 2011; 16: 501–509. https://doi.org/10.1111/j.1365-3156.2011.02725.x PMID: 21342372

68. García GP, Flores AE, Fernández-Salas I, Saavedra-Rodríguez K, Reyes-Solis G, Lozano-Fuentes S. Recent rapid rise of a permethrin knockdown resistance allele in *Aedes aegypti* in Mexico. PloS Negl Trop Dis. 2009; 3: e531. https://doi.org/10.1371/journal.pntd.0000531 PMID: 19829709

69. Siller Q, Ponce G, Lozano S, Flores AE. Update on the frequency of Ile1016 mutation in voltage-gated sodium channel gene of *Aedes aegypti* in Mexico. J Am Mosq Control Assoc. 2011; 27: 357–362. https://doi.org/10.2987/11-6149.1 PMID: 22329266

70. Saavedra-Rodriguez K, Suarez AF, Salas IF, Strodes C, Ranson H, Hemingay J, et al. Transcription of detoxification genes after permethrin selection in the mosquito *Aedes aegypti*. Insect Mol Biol. 2011; 21: 61–77. https://doi.org/10.1111/j.1365-2583.2011.01113.x PMID: 22032702

71. Smith LB, Kasai S, Scott JG. Pyrethroid resistance in *Aedes aegypti* and *Aedes albopictus*: Important mosquito vectors of human diseases. Pestic Biochem Physiol. 2016; 133: 1–12. https://doi.org/10.1016/j.pestbp.2016.03.005 PMID: 27742355

72. Moyes CL, Vontas J, Martins AJ, Ng LC, Koou SY, Dusfour I, et al. Contemporary status of insecticide resistance in the major *Aedes vectors* of arboviruses infecting humans. PLoS Negl Trop Dis. 2017 Jul 20; 11(7):e0005625. https://doi.org/10.1371/journal.pntd.0005625 PMID: 28727779

73. Pethuan S, Jirakanjanakit N, Saengtharatip S, Chareonviriyaphap T, Kaewpa D, Rongnoparut P. Biochemical studies of insecticide resistance in Aedes aegypti and Aedes albopictus (Diptera: Culicidae) in Thailand. Trop Biomed. 2007 Jun; 24(1): 7–15. PMID: 17568372

74. Viana-Medeiros PF. Investigação sobre mecanismos de resistência em larvas e adultos de Aedes aegypti, Linnaeus, 1762. [M.Sc. Thesis]. Instituto Oswaldo Cruz/Fiocruz. 2011. https://www.arca.fiocruz.br/bitstream/icict/5537/1/priscila_medeiros_ioc_mest_2011.pdf.

Artigo 2

(Submetido para PLoS Neglected and Tropical Diseases)

Association of insecticide resistance with fitness traits in four field *Aedes aegypti* populations from Brazil

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Abstract

Background: Chemical control is still a major strategy to restrain mosquito vector populations and avoid arbovirus outbreaks. However, insecticide overuse poses a high selective pressure, favoring the increase of insecticide resistance levels in wild mosquitoes. In an insecticide free environment, a fitness cost is expected in resistant insects when compared to susceptible counterparts. This study investigates whether insecticide resistance to an organophosphate (temephos) and a pyrethroid (deltamethrin) is associated with fitness traits in four *Aedes aegypti* wild populations sampled every three months during one year.

Methodology/Principal Findings: We measured development time from larvae to adult, female survival, wing length, fecundity and adult resistance to starvation in field insecticide resistant *A. aegypti* populations as well as in susceptible Rockefeller mosquitoes. These results were confronted with resistance levels to temephos and deltamethrin and presumable resistance mechanisms (*kdr* mutation frequency and the relative activity of detoxifying enzymes). Larval development of field mosquito populations was equal or slower than Rockefeller, except for one population in which ~ 40% of the specimens developed faster than the susceptible strain. In addition, *A. aegypti* samples with higher levels of detoxifying enzyme activity tended to delay in the immature stages. Females from all the four wild resistant populations survived significantly less than Rockefeller. No differences were evidenced in starvation tolerance, wing length and fecundity.

Conclusions/Significance: Overall, field resistant mosquitoes seemed to have a fitness disadvantage when compared with the Rockefeller strain. Longer larval development time in the samples with the highest activity of detoxification enzymes might represent a potential fitness cost of metabolic resistance. The overproduction of detoxifying enzymes may cause an energy trade-off which can affect energy allocation and ultimately, basic demands of insect biology. The extent of fitness cost due to insecticide resistance is valuable information to delay the evolution of resistance in wild vector populations.

Author Summary

In evolution, the term *fitness* refers to the degree to which an organism contributes with its offspring (or genes) to the next generation. In other words, it represents a definition of reproductive success in a certain environment and is associated with the genetic variations on which natural selection operates. There is no universal measurement of individual fitness but it usually incorporates both survivorship and fecundity. Nevertheless, there are many other biological traits that confer fitness, either alone or in combination, such as development time, age of maturity, growth rate, adult body size and mating success. Besides, fitness can be also influenced by ecological features, responding to environmental variables such as temperature, humidity or food availability. Insecticide resistance alleles confer a great selective advantage to mosquitoes under insecticide applications, but keeping resistance mechanisms in insecticide free environments might cause a fitness cost since energetic resources are reallocated from other life history traits. We correlated several biological parameters of four *Aedes aegypti* field populations with the resistance levels to the major insecticides employed in Brazil until recently, in order to detect potential resistance fitness costs. Knowledge about insecticide resistance evolution must be considered in vector control and resistance management programs in order to avoid the loss of effectiveness of the chemical control tools currently available.

Introduction

Arbovirus transmission has become one of the major global public health issues in the past decades. Dengue virus (DENV) is estimated to affect annually about 390 million people worldwide [1], causing more illness and death than any other virus transmitted by arthropods. In 2013-14, chikungunya virus (CHIKV) was introduced into the Caribbean and Latin America rapidly

spreading throughout this region and infecting ~450,000 people in 2016 [2]. Zika virus (ZIKV) is believed to have invaded South America from French Polynesia in the same period [3,4], causing ~215,000 infections in Brazil [5]. This virus is associated to congenital disease and neurological complications such as Guillan-Barré syndrome, with major disabilities [6,7]. All evidences so far point that DENV, CHIKV and ZIKV are primarily transmitted by the bite of infected *Aedes aegypti* mosquitoes [8,9].

Once there is currently no vaccine widely available for DENV, CHIKV or ZIKV, disease prevention relies exclusively upon actions directed toward the insect vector. Today, control efforts focus essentially on maintaining mosquito populations below a critical threshold thus avoiding arboviruses outbreaks [10]. Among the vector control approaches available, chemical control is still a major strategy in endemic areas. However, insecticide overuse poses a selective pressure on wild mosquito populations, favoring the increase in the frequency of insecticide resistance alleles, a situation that gradually impairs chemical control effectiveness. Insecticide resistance alleles confer a great selective advantage whenever chemical control is prioritized over vector mechanical control, the consequence in general being a quick increase in field mosquito population resistance levels [11,12]. Nevertheless, resistance alleles are rarely detected in high frequencies in these insect populations when insecticides are absent from the environment. Therefore, an association between the frequency of resistance alleles and fitness costs is expected [13,14].

Some hypotheses can explain possible negative fitness effects of resistance, which are: (i) large phenotypic changes (e.g. insecticide resistance) can often be deleterious within the context of the previous environment, i.e. without insecticide [14]; (ii) the production and maintenance of resistance mechanisms may reallocate energetic resources from other life history traits, e.g. egg production and/or survival [15] and (iii) changes in the insecticide-target nervous system proteins

may result in fitness disadvantage in insecticide resistant mosquitoes [16]. These phenomena have been observed for agricultural pests and disease vectors [17–20], including *A. aegypti* [21–23].

Knowledge of the evolutionary cost of insecticide resistance in *A. aegypti* populations is essential to manage insecticide resistance dissemination. Studies aiming to investigate resistance cost often repeatedly backcross resistant insects with those susceptible [24–26] or artificially select a sample of a field population by exposition to insecticide for several generations [21,23,27]. However, it is challenging to estimate the fitness cost of resistant field populations as changes in mosquito life-history traits can be easily influenced and confused by a variety of other genetically determined characteristics beyond those directly related to insecticide resistance [28,29].

Resistance levels are expected to vary over time according to insecticide selective pressures [11,12]. In this scenario, samples from the same mosquito populations collected over time could reveal *A. aegypti* life-history trait variation in accordance with insecticide resistance fluctuation. Therefore, we investigated how insecticide resistance to an organophosphate (temephos) and a pyrethroid (deltamethrin) associated with fitness traits in four *A. aegypti* wild populations sampled four times over one year.

Materials and Methods

Mosquito populations and field collection

Laboratory tests were conducted with the F1 generation of different Brazilian *A. aegypti* populations. Four midsized, Brazilian cities from different regions were chosen for mosquito collection to represent different landscapes, demography, climatic conditions as well as epidemiological and entomological histories (Table 1). For each city, four samplings were performed during one year with an interval of 3 months. For each of the 16 samplings (four localities and four collections), the parental generation was collected with 360 ovitraps baited with

hay infusion [30] placed in peridomestic, shaded environments and arranged in three 1 km² areas per city, located around 10 Km apart from each other. In the laboratory, eggs were reared until the adult stage when specimens were species level identified. At least 500 *A. aegypti* adult females were enlisted to initiate laboratory colonies from each sampling. Thus, we believe our sampling procedure was representative of the natural genetic diversity of each population. The Rockefeller strain, laboratory established around 1935, was adopted both as an internal control of all fitness assays and an insecticide susceptible reference lineage [31].

Table 1. Cities of mosquito collection as well as climatic, demographic and epidemiological characteristics.

Location	Region	Geographical Coordinates	Annual temperature range[a] (°C)	Annual precipitation range[a] (mm)	Demographic density (inhabitants/km^2)[b]	Dengue incidence (cases/100,000 inhabitants[c])	Period of mosquito collection
Campo Grande, Mato Grosso do Sul (MS)	Central-West	20°26'34"S, 54°38'47"W	9.9 to 29.9	34 to 234	97	3,557	Feb, Jun, Oct-2010 and Jan-2011
Duque de Caxias, Rio de Janeiro (RJ)	Southeast	22°47'08"S, 43°18'42"W	17.8 to 29.3	43 a 171	1,828	123.7	Feb, May, Aug and Nov- 2010
Parnamirim, Rio Grande do Norte (RN)	Northeast	5°54'56"S, 35°15'46"W	22 to 29	19 to 212	1,858	55.3	Feb, May, Aug and Dec-2010
Santarém, Pará (PA)	North (Amazon region)	2°26'35"S, 54°42'29"W	23 to 33	33 a 388	12.9	230.7	Apr, Jul, Oct-2010 and Jan-2011

[a] Based on daily average temperature during mosquito collection.

[b] Data obtained from [32–34].

[c] During each complete mosquito collection period [35].

Insecticide resistance levels and resistance mechanisms

The resistance status to the organophosphate temephos and the pyrethroid deltamethrin, as well as the investigation of resistance mechanisms were reported [36]. Briefly, field *A. aegypti* larvae (F1) were submitted to quantitative bioassays to evaluate the temephos susceptibility status [37]. Quantification of adult resistance to deltamethrin was also performed through dose-response assays with females with a WHO adapted methodology [38]. The activity of enzymes potentially involved in insecticide detoxification (glutathione-S-transferase - GST, esterases - EST and mixed function oxidases - MFO) were quantified in adult females as in [39] and Montella *et al.* [40]. In order to evaluate alterations in the detoxification pathway, the 99^{th} percentile of the susceptible control strain Rockefeller (p99Rock) was calculated for the activity of each enzyme class. Afterwards, for each field sample, the average percentage of specimens that remained above p99Rock for GST, ESTs and MFOs activity (referred as 'global metabolic alteration', Table 2) was adopted as a proxy for metabolic resistance.

Allele-specific PCR was conducted to investigate the presence of the Val1016Ile, Phe1534Cys and Ile1011Met mutations in the pyrethroid target site, the voltage gated sodium channel (Na_V), in adult males from the parental generation [41,42].

Table 2. Temephos and deltamethrin resistance ratios (RR_{95}) and main related mechanisms detected in four Brazilian *A. aegypti* populations. The allelic frequencies of the *kdr* mutation Val1016Ile (pyrethroid target site) and global alteration levels of detoxification enzymes are also shown.

Municipality/ State	**Sample**	**RR_{95} Temephos[a]**	**RR_{95} Deltamethrin[a]**	**Allelic frequency of Na_v Val1016Ile[a,b]**	**Global metabolic alteration (%) [a,c]**
Campo Grande/MS	Feb-2010	7.9	97.8	0.860	22.4
	Jun-2010	5.8	58.2	0.973	20.0
	Oct-2010	4.6	88.3	0.911	21.0
	Jan-2011	4.6	85.5	0.817	14.0
Duque de Caxias/RJ	Feb-2010	13.3	61.3	0.929	49.5
	May-2010	10.7	79.4	0.923	33.3
	Aug-2010	10.9	64.4	0.759	31.6
	Nov/2010	9.8	44.5	0.907	21.2
Parnamirim/RN	Feb-2010	7.1	11.6	0.017	33.0
	May-2010	6.4	10.1	0.000	33.7
	Aug-2010	6.3	12.4	0.017	24.6
	Dec-2010	6.3	14.3	0.017	28.4
Santarém/PA	Apr-2010	10.3	57.8	0.000	15.4
	Jul-2010	10.3	35.1	0.000	22.3
	Oct-2010	8.6	55.1	0.000	9.0
	Jan-2011	9.0	49.3	0.000	6.4

[a] Data originally reported by [36].

[b] The *kdr* Val1016Ile mutation was the only one investigated in all mosquito samples. Results for the Phe1534Cys and Ile1011Met mutations [36] are mentioned in the text.

[c] For each population sample, the average percentage of field specimens above p99Rock for GST, ESTs and MFOs activity was calculated.

Fitness assays

Five parameters were assumed to investigate the potential fitness cost associated with insecticide resistance. Assays were conducted on four occasions for the mosquitoes collected in each city with the same samples submitted to the insecticide resistance bioassays. Rockefeller mosquitoes were always evaluated simultaneously as a referential of insecticide susceptibility and laboratory rearing. In all cases, specimens were kept in B.O.D. incubators at 27.6 ± 0.6°C and 70±10% RH, both parameters verified twice a day.

Immature development time, starvation tolerance and wing length. The immature development time refers to the period, in hours, elapsed from egg hatching to adult emergence. For each sampling, we individually reared 120 F1 field-derived and 36 Rockefeller specimens, the larvae monitored three times a day (08:00, 12:00 and 17:00). The assays were in 12-well tissue culture plates. Each well was filled with 4 ml of dechlorinated water receiving 100 µL of a dry yeast suspension daily (Prolev, Recife, PE), containing 0.04 mg on days 01 and 02, 0.08 mg on day 03, 0.16 mg on day 04, 0.32 mg on day 05, 0.64 mg on day 06 and 0.32 mg for the remaining days until the pupae stage [43]. The pupae were then individually transferred to cylindrical plastic tubes (6.5 cm height, 2.5 cm diameter) in which they emerged as adults.

Adult mosquitoes received wet moistened cotton swabs without any nutrient to avoid death by dehydration. Starvation tolerance (i.e. survival without any nutrition) was monitored

twice a day (08:00 and 17:00) up to mosquito death, when sex and wing length were registered.

Due to the variation in Rockefeller immature development assays despite identical rearing conditions, each field sampling was normalized by its corresponding Rockefeller specimens to enable comparison among different experimental groups. Normalization procedure is detailed in the S1 Text. Thus, values generated for the experimental groups, called 'scaled time values', are a measure of how much each field population development time deviates from its corresponding Rockefeller referential. Values above and below 1.0 indicate, respectively, slower or faster development than the Rockefeller strain. Statistics were calculated on these scaled time values which we refer to as 'scaled developmental time'.

Female survival and fecundity. Pools of 100 larvae were reared in plastic basins filled with 1 L of dechlorinated water. Larvae received 500 mg of dry yeast (Prolev, Recife, PE, Brazil) on day 1 and 250 mg on day 4, after which pupae were transferred to cardboard cages. Following 3-4 days after adult emergence, 60 females from each sampling and 10 Rockefeller females were randomly selected and individually transferred to labeled cylindrical plastic vials (6.5 cm height, 2.5 cm diameter), covered by mosquito netting. A moistened cotton swab overlaid with filter paper on the bottom of the vials served as substrate for oviposition. Mosquitoes received 10% sugar solution *ad libitum*, except during each 24-hour period prior to the blood meals weekly offered from an anesthetized mouse. Three to four days after blood-feeding, filter papers were substituted and checked for eggs which were counted. Mosquito survival was scored daily until the 60th day.

Statistical analysis. The biological parameters were compared with insecticide resistance levels (RR_{95}) and with presumable resistance mechanisms in order to investigate

potential fitness costs of insecticide resistance in the *A. aegypti* mosquitoes. Wing length and starvation tolerance were analyzed using female data. Fecundity was the number of eggs laid in the first week of monitoring considering females that laid at least one egg. For resistance mechanisms, the parameters considered were the allelic frequency of the *kdr* Val1016Ile mutation in the pyrethroid Na_V target site and the 'global metabolic alteration', values depicted in Table 2. In the case of pyrethroid target site resistance, only the mutation at position 1016 could be tested since it was the only one evaluated in all samplings.

In all cases, the median was employed since the evaluated parameters indicated non-normal distribution (Shapiro-Wilk test, scaled development time: $W = 0.91$, p-value < 0.01; starvation tolerance: $W = 0.82$, p-value < 0.01; wing length: $W = 0.95$, p-value < 0.01; adult female survival: $W = 0.97$, p-value < 0.01; fecundity: $W = 0.93$, p-value < 0.01). Scaled development time was described as the percentage of values that remained below and above the Rockefeller counterpart. Starvation tolerance and adult female survival were compared with Kaplan-Meier curves and log-rank tests. Wing length and fecundity were compared with the Kruskal-Wallis test. All the p-values were corrected for multiple comparisons by the Bonferroni method.

Firstly, field mosquito populations were contrasted with the reference Rockefeller strain. Secondly, the fitness parameters were plotted against the temephos/deltamethrin resistance levels and presumable resistance mechanisms within each mosquito population (i.e. from the same city), thus contrasting mosquitoes with a similar genetic background and potentially the same insecticide history and resistance mechanisms. We also investigated global associations between fitness and insecticide resistance mechanisms. The 16 sampling assays were analyzed together with Spearman correlation analyses. All the graphics and analyses were carried out with the statistical software R 3.2.3 [44].

Ethical issues

The use of anesthetized mice to blood feed mosquitoes was authorized by Fiocruz Ethical Committee for Animal Use (CEUA P-0498/08 and CEUA L-0007/09) following the National guidelines for the scientific use of animals disposed by the Law 11.794/2008.

Results

Comparison among the different field mosquito populations and Rockefeller

Median scaled development time varied from 1 (Campo Grande) to 1.07 (Duque de Caxias). In no case was the larval development of field populations faster than that of Rockefeller (Figure 1A). In general, almost all scaled development time values were equal or above 1.0, the exception being the population of Campo Grande for which ~40% of the insects developed faster than Rockefeller (Figure 1A). Regarding starvation tolerance, male mosquitoes survived significantly longer than females in all the 16 samplings (Log rank χ^2 = 724; p-value < 0.01) (Figure 1B). The female median starvation tolerance also varied among populations (Figure 1C). Median starvation tolerance varied from 72 hours (Duque de Caxias and Parnamirim) to 96 hours (Santarém). Comparing female starvation data of field populations with Rockefeller revealed a lower tolerance for Campo Grande mosquitoes (Log rank: χ^2 = 14.4, p-value < 0.01) (Figure 1C). The median female wing length varied from 2.35mm (Duque de Caxias) to 2.20 mm (Parnamirim). The median wing size of Duque de Caxias females was considered larger than those of Rockefeller females (Kruskal-Wallis χ^2 = 19.05, p-value < 0.01) (Figure 1D).

Median adult female survival varied from 25 (Duque de Caxias) to 40 days (Rockefeller). Females from all four field populations survived significantly less than Rockefeller (Log-rank *vs.* Santarém: $\chi^2 = 10.1$, p-value < 0.01; log-rank *vs.* Parnamirim: $\chi^2 = 6.9$, p-value < 0.05; log-rank *vs.* Duque de Caxias: $\chi^2 = 28.4$, p-value < 0.01; log-rank *vs.* Campo Grande: $\chi^2 = 25.2$, p-value < 0.01) (Figure 1E). Considering the first week of monitoring, 60% of Rockefeller mosquitoes laid at least one egg in average, while this percentage varied between 28.2 and 48.3% in field populations. The median number of eggs per female varied from 80 (Duque de Caxias) to 104 (Santarém). No differences in fecundity were detected among the four field *A. aegypti* populations analyzed and Rockefeller (statistics not shown, Kruskal-Wallis p-value > 0.05) (Figure 1F).

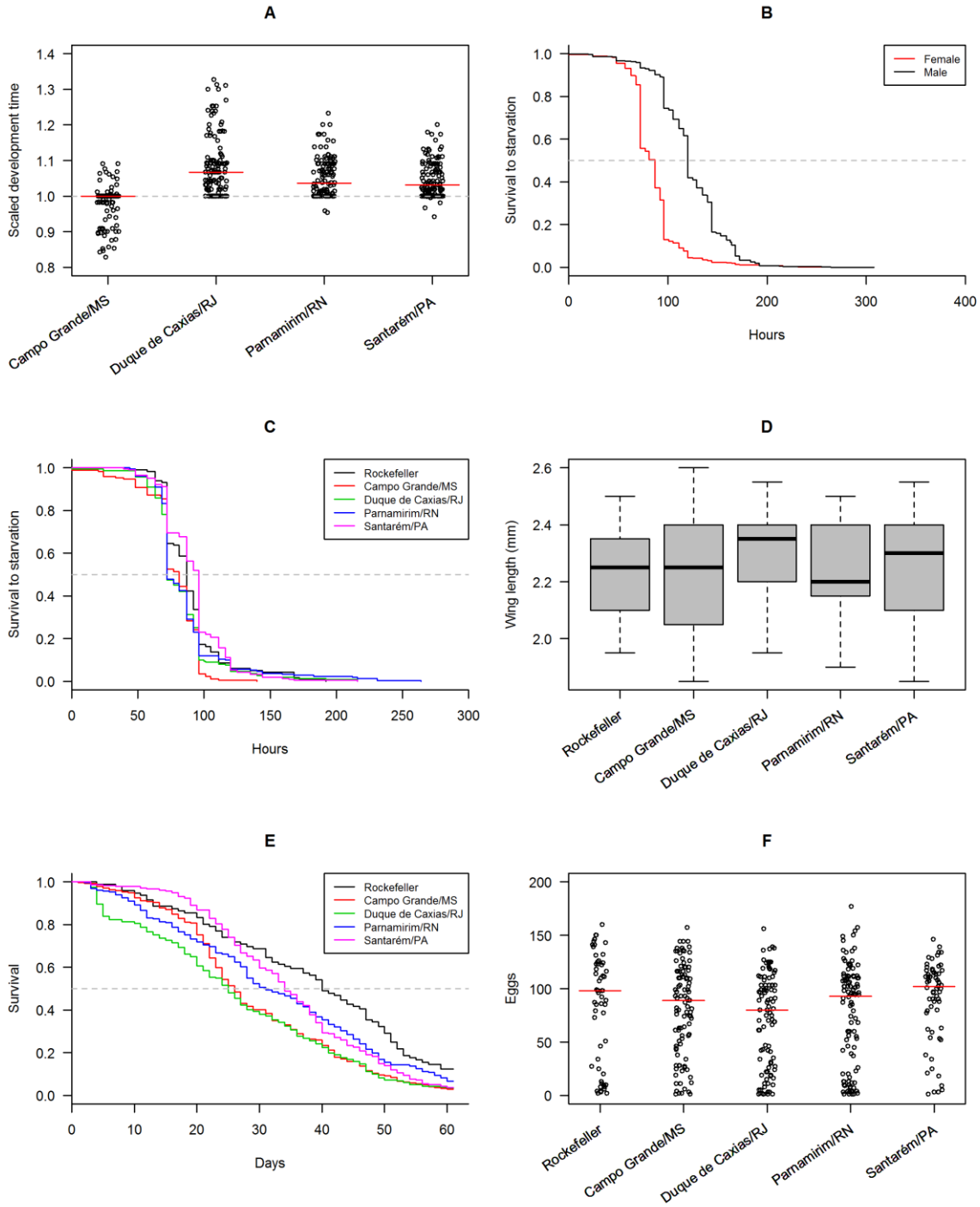

Figure 1. Fitness parameters of four *Aedes aegypti* field populations: scaled development time (A), general male and female starvation time (B), female starvation time of each population (C), female wing length (D), female survival (E) and fecundity (F).

Mosquito biology versus insecticide resistance variation

Next, we confronted the parameters of mosquito biology with insecticide resistance levels (i.e. RR_{95}) and presumable resistance mechanisms (i.e. the allelic frequency of the Val1016Ile mutation in the Na_V and global metabolic alteration). As displayed in Table 2, resistance to temephos presented a consistent dynamic in all mosquito populations analyzed, tending to decrease in all localities. Meanwhile, resistance to the deltamethrin adulticide exhibited extremely high levels [36]. In no case were there clear associations among the fitness parameters evaluated (scaled development time, starvation tolerance, wing length, adult female survival and fecundity) and the temephos or deltamethrin resistance ratios or mechanisms (statistics not shown). Fitness parameters *versus* RR_{95}, global metabolic alteration and the allelic frequency of the Val1016Ile mutation within populations are graphically represented in the S2 Appendix.

No significant correlations were observed between the biological parameters measured and the allelic frequencies of the Val1016Ile mutation in the Na_V (Spearman correlation analysis, p-value > 0.05) (Figure 2). On the other hand, the scaled development time demonstrated a moderate positive significant correlation with the global metabolic alteration (Spearman's rank correlation = 0.52, S = 327.51, p-value < 0.05) (Figure 3A). Besides, although the metabolic profile was not significantly correlated with any other biological parameter, the sample with the highest metabolic alteration (Duque de Caxias Feb/10, with 49.5 of mean metabolic alteration, Table 2) presented the lowest median values of longevity and fecundity (Figures 1E,F and 3D, E).

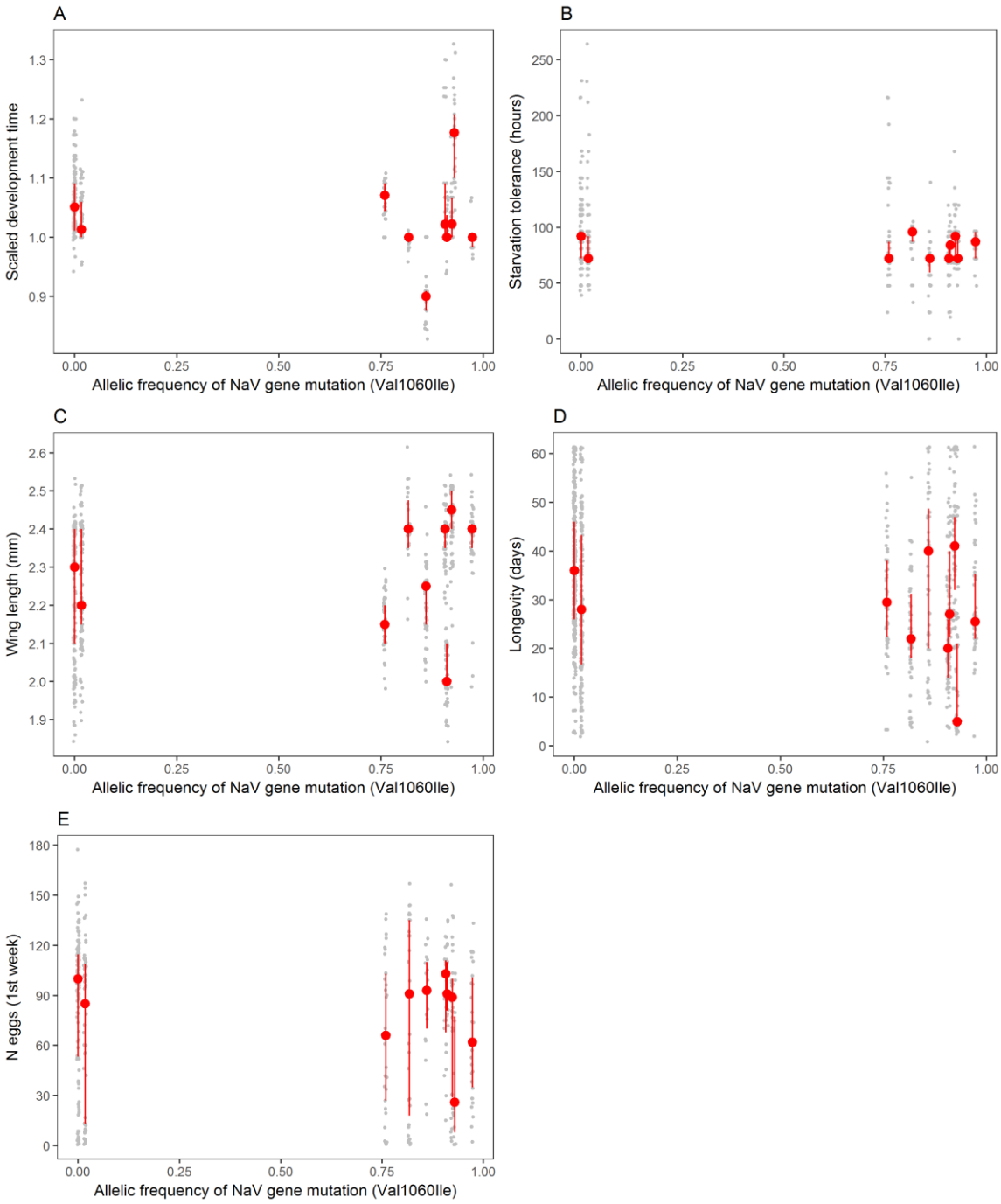

Figure 2. The allelic frequency of the Val1016Ile mutation in the pyrethroid Na_V target site alteration versus scaled development time (A), female starvation tolerance (B), female wing length (C), female adult survival (D) and fecundity (E) of *Aedes aegypti* field populations. Red circles and vertical lines correspond to the median and interquartile range, respectively.

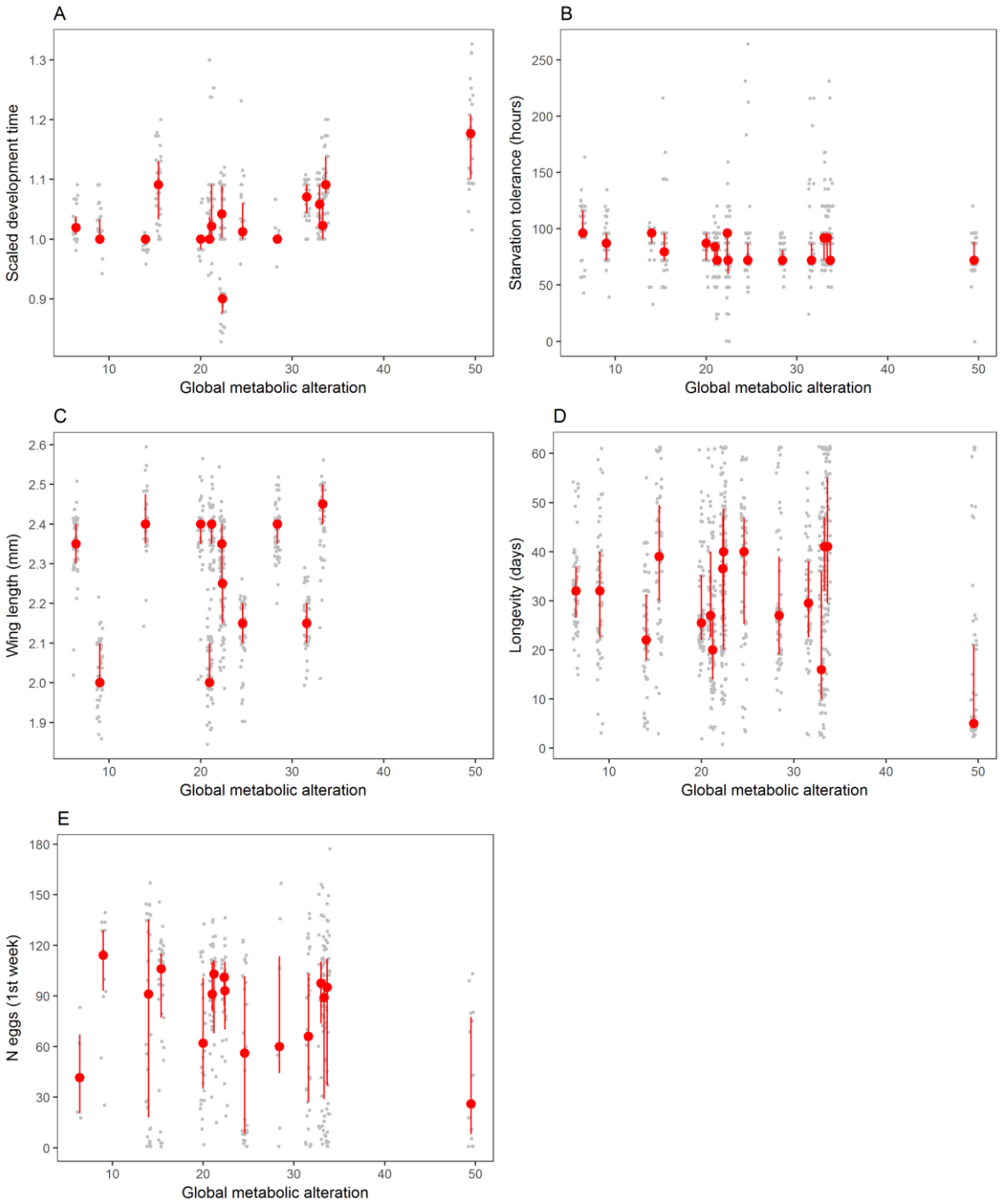

Figure 3. The global metabolic alteration versus scaled development time (A), female starvation tolerance (B), female wing length (C), female adult survival (D) and fecundity (E) of *Aedes aegypti* field populations. Red circles and vertical lines correspond to the median and interquartile range, respectively.

Discussion

In the absence of insecticide applications, resistance alleles can result in energetic costs or fitness disadvantages in comparison with its susceptible counterparts. Attempts to register resistance costs in general select insects in the laboratory for increased resistance or perform backcrosses with laboratory strains to produce lineages differing only in the resistance traits [24–27]. This approach supposedly enables more accurate measures of fitness differences related specifically to insecticide resistance rather than other genetic differences. However, it suffers from an intense inbreeding and the consequent loss of genetic variability which may not reflect insecticide resistance features in the field (reviewed by [45]). In contrast, field populations represent a more realistic situation but exhibit variable genetic backgrounds as the outcome of local adaptation. In this case, differences among susceptible and resistant mosquitoes might be assigned to other characteristics beyond insecticide resistance alleles. Herein, we had the opportunity to compare four *A. aegypti* resistant field populations with a susceptible laboratory strain and also correlate resistance and its potentially associated fitness costs on several occasions over the course of one year, a situation that is roughly equivalent to the comparison of populations with the same general genetic background submitted to insecticide selection pressure under field conditions.

The larval stage is crucial to the adult fitness as the energetic resources gained through larval stage shape several traits of adult mosquitoes directly related to vectorial capacity [43,46]. Additionally, mosquito larvae are more vulnerable to predation, insecticide application and habitat loss than adults. Overall, field resistant mosquitoes seemed to have a slight fitness disadvantage when compared with Rockefeller. Larval development delay was noted in Duque de Caxias and eventually in Parnamirim and Santarém mosquitoes compared with the susceptible strain. In addition, pooling in a single analysis all the 16 sampling assays,

there was a tendency of increasing larval development time in those samples with the highest activity of detoxification enzymes which might represent a potential fitness cost of metabolic resistance.

Survival is another important component of mosquito fitness since higher longevity increases the number of blood meals and subsequently the size and lifetime number of egg batches laid [47]. Although it was not possible to assign fitness impairment to any specific insecticide class, due to multiple resistance status in the evaluated populations, all field derived adult females exhibited a lower survival in comparison to Rockefeller. Duque de Caxias had the lowest development and survival rates and presented the highest global metabolic alteration. Metabolic resistance is usually achieved by the increased production of enzymes such as glutathione-S-transferases, esterases and mixed function oxidases [48]. The overproduction of these detoxifying enzymes requires that a significant part of insect energy resources be redirected to the machinery related to the metabolism and excretion of the xenobiotic, like the insecticide. This effect, known as energy trade-off, is at the expense of other physiological aspects of the organism, which may reflect negatively on the basic demands of its biology [49]. In particular, this is expected when resistance to organophosphates, such as temephos, and esterases are involved. In insects, esterases have almost no catalytic activity against organophosphates. In this case, esterases act sequestering the insecticide, a phenomenon that requires the production of large amounts of esterase molecules [50]. This situation which has a strong potential impact on vector viability seems to have occurred precisely with *A. aegypti* from Duque de Caxias. Not only do these mosquitoes present the highest average global metabolic alterations, but also the highest average changes when only esterases are considered [36].

Martins *et al.* [21] investigating a series of life-trait parameters in five field *A. aegypti* populations (F1 generation), verified that when RR_{95} to temephos was higher than 40, a delay in larval development together with a reduction of both adult longevity and fecundity occurred compared to Rockefeller mosquitoes. Diniz *et al.* [23] also reported a delay in larval development, decreased longevity and a reduced fecundity in a field population strongly resistant to temephos (RR_{95} > 200). These above described resistant levels are notably higher than those for the populations dealt with here (temephos RR_{95} 4.6 -13.3). However, there also was a decrease in blood meal acceptance, amount of ingested blood, number of eggs laid and rate of inseminated females in field populations with lower temephos RR_{95}, 7.4 to 19.2 compared to the Rockefeller mosquitoes [22].

The Rockefeller strain was adopted as an insecticide susceptible reference lineage despite lab establishment for around 80 years [31]. Thus, it is possible that the perceived advantage of Rockefeller mosquitoes over those of the field is derived from their laboratory adaptation instead of the absence of an insecticide resistance energy cost. Hence, we circumvented this issue by confronting samples collected over a year, assuming that fitness costs would be more intense as resistance increased. However, when each population was evaluated separately, it was not possible to identify fitness cost changes associated with insecticide resistance alterations, i.e. loss of biotic potential when resistance increased and vice-versa. In order to avoid the intense inbreeding and loss of genetic variability, we evaluated the F1 generation without any artificial insecticide selection. Therefore, we depended on the natural variation of resistance levels among samples to search for fitness costs oscillations. In the field populations evaluated here, variations of temephos RR_{95} not higher than 1.26 fold were evident while deltamethrin RR_{95} varied 1.59 fold, at maximum. Laboratory insecticide selection experiments can produce control/susceptible and resistance

strains with very different levels of insecticide resistance [21,23,27,51]. Thus, it is possible that the natural fluctuation in RR_{95} levels was insufficient to cause detectable fitness differences among mosquito samples.

Ae. aegypti resistance to temephos is supposedly acquired essentially through metabolic mechanisms since there are no reports of alterations in acetylcholinesterase, the organophosphate target site, in field populations of this species [27,40,52]. The use of temephos in Brazil was reduced by the Health Ministry from 1999 until 2009 when it was interrupted throughout the country [53]. Theoretically, the absence of insecticide selective pressure would reduce the resistance levels by a rate dependent upon the biological resistance cost [13,54]. Reversion of organophosphate resistance has already been observed for *Culex pipiens* [55,56] and *Drosophila melanogaster* [57]. A temephos resistance reduction trend was noted in the four *A. aegypti* populations investigated here which is probably the outcome of a lower fitness of resistant insects in the absence of this insecticide [36]. The non-attendance of resistance fitness cost in the laboratory may be a consequence of measuring biological traits under optimal conditions, which is a different situation compared to the stressful environment faced by insects in nature [45]. The nutritional status, for example, has been shown to significantly influence the presence and the magnitude of insecticide resistance fitness costs in cockroaches [22].

Regarding pyrethroid resistance, all populations with the exception of Parnamirim exhibited high resistance ratios to deltamethrin (Table 2, [36]), until recently the principal adulticide for *A. aegypti* control in Brazil by public managers. Since pyrethroids are freely available in the retail market, they continue to be a major insecticide class for domestic use. Overall, deltamethrin resistance did not disclose any temporal trend throughout the one-year sampling period. However, resistance rates varied according to the timing and intensity of

dengue outbreaks, corroborating the impact of the domestic chemical control of this urban vector on its resistance status [36]. Changes in the Na_v proteins significantly contribute to pyrethroid resistance in several arthropod species [58,59], including *A. aegypti* [60]. Theoretically, mutations in the target sites of neurotoxic insecticides can lead to high levels of resistance which are rapidly selected in the presence of such chemicals [12,49]. Accordingly, *kdr* mutations in the Na_v genes were detected in high frequencies in all populations, except Parnamirim, precisely the population displaying the lowest deltamethrin resistance levels [36]. Notwithstanding, neither of these specific resistance parameters presented a connection with those of evaluated fitness.

The fitness costs of at least one *A. aegypti kdr* mutation has already been assessed after introducing the Val1016Ile mutation into the Rockefeller susceptible genetic background. A delay in larval development together with reduction in female insemination rate and number of eggs laid were noted. In addition, the allelic frequency of the Val1016Ile mutation rapidly dropped after 15 generations without any insecticide exposure, further suggesting an associated fitness cost of this mutation [26]. Two out of the four *A. aegypti* populations analyzed (Duque de Caxias and Campo Grande) presented high Val1016Ile mutation frequencies in all samples (Table 1, [36]). This lack of *kdr* frequency variation over the one-year period is probably related to the absence of detectable fitness differences among field mosquito samples. In opposition, the Val1016Ile mutation was absent, or present at very low levels, in the remaining populations, Santarém and Parnamirim, in the whole period (Table 1, [36]). Accordingly, there were no correlations with biological parameters. Since Santarém and Parnamirim mosquitoes are also resistant to pyrethroids, it is reasonable to assume that other pyrethroid resistance mechanisms are present. The *kdr* Phe1534Cys

mutation, present at high frequencies in Santarém but not in Parnamirim mosquitoes [36], was also correlated to the high deltamethrin RR_{95} levels in this population [42].

Finally, the knowledge about the insecticide resistance evolutionary process in arthropods must be applied in resistance management programs to avoid the loss of effectiveness of the chemical control tools currently available. The occurrence and the magnitude of fitness costs can determine the rate of resistance evolution in field populations as well as the pace to return to a susceptible status after insecticides are removed from the environment [13,54]. Furthermore, insecticide resistance might entail changes in arthropod biology which can influence the rates of infection, development and transmission of pathogens harbored by several species of insects [20].

Acknowledgements

To the teams of the Health Secretaries of each one of the municipalities and States involved in the study: Santarém/PA, Parnamirim/RN, Duque de Caxias/RJ and Campo Grande/MS. To all the public health personnel that worked with us in each municipality. To the technical assistance of professionals from the Laboratório de Mosquitos Transmissores de Hematozoários and Laboratório de Fisiologia e Controle de Artrópodes Vetores, in particular, Valdice Paula, Rafael Guedes and Gilberto Couto Alcântar. To Cláudia Codeço and Cláudio Struchiner for statistical analysis support. English review and revision by Mitchell Raymond Lishon, Native of Chicago, Illinois, U.S.A. U.C.L.A, 1969.

References

1. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, et al. The global distribution and burden of dengue. Nature. Nature Publishing Group; 2013;496: 504–507. doi:10.1038/nature12060

2. Pan American Health Organization. Number of reported cases of chikungunya fever in the Americas, by country or territory 2016. In: Chikungunya: PAHO/WHO Data, Maps and Statistics [Internet]. 2017 [cited 17 Apr 2017].

3. Campos G, Bandeira A, Sardi S. Zika virus outbreak, Bahia Brazil. Emerg Infect Dis. 2015;21: 1881. doi:10.32301/eid2110.150847

4. Zanluca C, De Melo VCA, Mosimann ALP, Dos Santos GIV, dos Santos CND, Luz K. First report of autochthonous transmission of Zika virus in Brazil. Mem Inst Oswaldo Cruz. 2015;110: 569–572. doi:10.1590/0074-02760150192

5. Secretaria de Vigilância em Saúde - Ministério da Saúde. Monitoramento dos casos de dengue, febre de chikungunya e febre pelo vírus Zika até a Semana Epidemiológica 52, 2016. Bol Epidemiológico. 2017;48: 1–11. Available: http://portalarquivos.saude.gov.br/images/pdf/2017/abril/06/2017-002-Monitoramento-dos-casos-de-dengue--febre-de-chikungunya-e-febre-pelo-v--rus-Zika-ate-a-Semana-Epidemiologica-52--2016.pdf

6. Oehler E, Watrin L, Larre P, Leparc-Goffart I, Lastère S, Valour F, Baudouin L, Mallet HP, Musso DGF. Zika virus infection complicated by Guillain-Barré syndrome – case report, French Polynesia, December 2013. Eur Cent Dis Prev Control. 2014;1: 7–9. doi:10.2807/1560-7917.ES2014.19.9.20720

7. Calvet G, Aguiar RS, Melo ASO, Sampaio SA, de Filippis I, Fabri A, et al. Detection and sequencing of Zika virus from amniotic fluid of fetuses with microcephaly in Brazil: a case study. Lancet Infect Dis. 2016;16: 653–660. doi:10.1016/S1473-3099(16)00095-5

8. Vega-Rúa A, Zouache K, Girod R, Failloux A-B, Lourenço-de-Oliveira R. High level of vector competence of *Aedes aegypti* and *Aedes albopictus* from ten American countries as a crucial factor in the spread of chikungunya virus. J Virol. 2014;88: 6294–306. doi:10.1128/JVI.00370-14

9. Fernandes RS, Campos SS, Ferreira-de-Brito A, Miranda RM de, Barbosa da Silva KA, Castro MG de, et al. *Culex quinquefasciatus* from Rio de Janeiro is not competent to transmit the local Zika virus. PLoS Negl Trop Dis. 2016;10: 1–13. doi:10.1371/journal.pntd.0004993

10. Focks DA. A review of entomological sampling methods and indicators for dengue vectors. Geneva: World Health Organization; 2003.

11. Mallet J. The evolution of insecticide resistance: Have the insects won? Trends Ecol Evol. 1989;4: 336–40. doi:10.1016/0169-5347(89)90088-8

12. García GP, Flores AE, Fernández-Salas I, Saavedra-Rodríguez K, Reyes-Solis G, Lozano-Fuentes S, et al. Recent rapid rise of a permethrin knock down resistance allele in *Aedes aegypti* in México. PLoS Negl Trop Dis. 2009;3. doi:10.1371/journal.pntd.0000531

13. Crow JF. Genetics of insecticide resistance to chemicals. Annu Rev Entomol. 1957; 227–246.

14. Coustau C, Chevillon C, ffrench-Constant R. Resistance to xenobiotics and parasites: can we count the cost? Trends Ecol Evol. 2000;15: 378–383.

15. Chevillon C, Raymond M, Guillemaud T, Lenormand T, Pasteur N. Population genetics of insecticide resistance in the mosquito *Culex pipiens*. Biol J Linn Soc. 1999;68: 147–157. doi:10.1111/j.1095-8312.1999.tb01163.x

16. Hoffmann F, Fournier D, Spierer P. Minigene rescues acetylcholinesterase lethal mutations in *Drosophila melanogaster*. J Mol Biol. 1992;223: 17–22. doi:10.1016/0022-2836(92)90710-2

17. Carriere Y, Deland J-P, Roff D, Vincent C. Life-History costs associated with the evolution of insecticide resistance. Proc R Soc B Biol Sci. 1994;258: 35–40. doi:10.1098/rspb.1994.0138

18. Berticat C, Boquien G, Raymond M, Chevillon C. Insecticide resistance genes induce a mating competition cost in *Culex pipiens* mosquitoes. Genet Res. 2002;79: 41–7.

19. Bourguet D, Guillemaud T, Chevillon C, Raymond M. Fitness costs of insecticide resistance in natural breeding sites of the mosquito *Culex pipiens*. Evol Int J Org Evol. 2004;58: 128–135.

20. Rivero A, Vézilier J, Weill M, Read AF, Gandon S. Insecticide control of vector-borne diseases: when is insecticide resistance a problem? PLoS Pathog. 2010;6: e1001000. doi:10.1371/journal.ppat.1001000

21. Martins AJ, Ribeiro CDEM, Bellinato DF, Peixoto AA, Valle D, Lima JBP. Effect of insecticide resistance on development, longevity and reproduction of field or laboratory selected *Aedes aegypti* populations. PLoS One. 2012;7: e31889. doi:10.1371/journal.pone.0031889

22. Belinato TA, Martins AJ, Valle D. Fitness evaluation of two Brazilian *Aedes aegypti* field populations with distinct levels of resistance to the organophosphate temephos. 2012;107: 916–922.

23. Diniz DFA, de Melo-Santos MA, dos Santos EM, Beserra EB, Helvecio E, de Carvalho-Leandro D, et al. Fitness cost in field and laboratory *Aedes aegypti* populations associated with resistance to the insecticide temephos. Parasit Vectors; 2015;8: 662. doi:10.1186/s13071-015-1276-5

24. Berticat C, Bonnet J, Duchon S, Agnew P, Weill M, Corbel V. Costs and benefits of multiple resistance to insecticides for *Culex quinquefasciatus* mosquitoes. BMC Evol Biol. 2008;8: 104. doi:10.1186/1471-2148-8-104

25. Djogbénou L, Noel V, Agnew P. Costs of insensitive acetylcholinesterase insecticide resistance for the malaria vector *Anopheles gambiae* homozygous for the G119S mutation. Malar J. 2010;9: 12. doi:10.1186/1475-2875-9-12

26. Brito LP, Linss JGB, Lima-Camara TN, Belinato TA, Peixoto AA, Lima JBP, et al. Assessing the effects of *Aedes aegypti* kdr mutations on pyrethroid resistance and its fitness cost. PLoS One. 2013;8. doi:10.1371/journal.pone.0060878

27. Melo-Santos MV, Varjal-Melo JJM, Araújo P, Gomes TCS, Paiva MHS, Regis LN, et al. Resistance to the organophosphate temephos: mechanisms, evolution and reversion in an *Aedes aegypti* laboratory strain from Brazil. Acta Trop. 2010;113: 180–9. doi:10.1016/j.actatropica.2009.10.015

28. Leisnham PT, Sala LM, Juliano SA. Geographic variation in adult survival and reproductive tactics of the mosquito *Aedes albopictus*. J Med Entomol. 2008;45: 210–221. doi:10.1038/jid.2014.371

29. Suman DS, Tikar SN, Mendki MJ, Sukumaran D, Agrawal OP, Parashar BD, et al. Variations in life tables of geographically isolated strains of the mosquito *Culex quinquefasciatus*. Med Vet Entomol. 2011;25: 276–88. doi:10.1111/j.1365-2915.2010.00939.x

30. Fay RW, Eliason D. A preferred oviposition site as a surveillance method for *Aedes aegypti*. Mosquito News. 1966; 26: 531–535.

31. Kuno G. Early history of laboratory breeding of *Aedes aegypti* (Diptera: Culicidae) focusing on the origins and use of selected strains. J Med Entomol. 2010;47: 957–971. doi:10.1603/ME10152

32. Prefeitura Municipal de Santarém. Informações municipais de Santarém, SEMMA_CIAM [Internet]. 2013 [cited 17 Apr 2017]. Available: http://www.santarem.pa.gov.br/arquivosdb/basico1/0.668764001357580532__informacoes_2.pdf

33. Portal da Prefeitura de Parnamirim. Portal de transparência, Estatísticas e mapas [Internet]. 2015 [cited 17 Apr 2017]. Available: http://www.parnamirim.rn.gov.br/mapas.jsp

34. Instituto Brasileiro de Geografia e Estatística - IBGE. Cidades [Internet]. 2010 [cited 1 Jan 2010]. Available: http://cidades.ibge.gov.br/xtras/home.php?lang=

35. Ministério da Saúde. Sistema de Informação de Agravos de Notificação (SINAN) [Internet]. [cited 12 Feb 2012]. Available: http://dtr2004.saude.gov.br/sinanweb/

36. Garcia GA, David MR, Araújo SC, Lima JBP, Martins AJ, Maciel-de-Freitas R, et al. The impact of insecticide applications on the dynamics of resistance: the case of four *Aedes aegypti* populations from different Brazilian regions. Submitt to PLOS Neglected Trop Dis.

37. World Health Organization. Division of Vector Biology and Control. Instructions for determining the susceptibility or resistance of mosquito larvae to insecticides. Geneva: World Health Organization; 1981.

38. Maciel-de-Freitas R, Avendanho FC, Santos R, Sylvestre G, Araújo SC, Lima JBP, et al. Undesirable consequences of insecticide resistance following *Aedes aegypti* control activities due to a dengue outbreak. PLoS One. 2014;9. doi:10.1371/journal.pone.0092424

39. Valle D, Montella IR, Ribeiro RA, Viana-Medeiros PF, Martins AJ. Metodologia para

quantificação de atividade de enzimas relacionadas com a resistência a inseticidas em *Aedes aegypti*/Quantification methodology for enzyme activity related to insecticide resistance in *Aedes aegypti*. Fundação Oswaldo Cruz and Secretaria de Vigilância em Saúde, Ministério da Saúde, Rio de Janeiro and Distrito Federal, Brazil; 2006.

40. Montella IR, Martins AJ, Viana-Medeiros PF, Lima JBP, Braga IA, Valle D. Insecticide resistance mechanisms of Brazilian *Aedes aegypti* populations from 2001 to 2004. Am J Trop Med Hyg. 2007;77: 467–477. doi:77/3/467 [pii]

41. Martins AJ, Brito LP, Linss JGB, Rivas GB, Machado R, Bruno RV, et al. Evidence for gene duplication in the voltage-gated sodium channel gene of *Aedes aegypti*. Evol Med Public Heal. 2013; 148–160. doi:10.1093/emph/eot012

42. Linss JGB, Brito LP, Garcia GA, Araki AS, Bruno RV, Lima JBP, et al. Distribution and dissemination of the Val1016Ile and Phe1534Cys Kdr mutations in *Aedes aegypti* Brazilian natural populations. Parasites & Vectors. 2014;7: 25. doi:10.1186/1756-3305-7-25

43. Tun-Lin W, Burkot TR, Kay BH. Effects of temperature and larval diet on development rates and survival of the dengue vector *Aedes aegypti* in north Queensland, Australia. Med Vet Entomol. 2000;14: 31–7. Available: http://www.ncbi.nlm.nih.gov/pubmed/10759309

44. R Development Core Team R. R: A Language and Environment for Statistical Computing [Internet]. Team RDC, editor. R Foundation for Statistical Computing. R Foundation for Statistical Computing; 2011. p. 409. doi:10.1007/978-3-540-74686-7

45. Kliot A, Ghanim M. Fitness costs associated with insecticide resistance. Pest Manag Sci. 2012;68: 1431–7. doi:10.1002/ps.3395

46. Nasci RS. The size of emerging and host-seeking *Aedes aegypti* and the relation of size to blood-feeding success in the field. J Am Mosq Control Assoc. 1986;2: 61–62. doi:10.1101/gr.3715005

47. Charlwood JD. May the force be with you: measuring mosquito fitness in the field. Ecol Asp Appl Genet Modif Mosquitoes. 2003;2: 47–62.

48. Hemingway J, Hawkes NJ, McCarroll L, Ranson H. The molecular basis of insecticide resistance in mosquitoes. Insect Biochem Mol Biol. 2004;34: 653–65. doi:10.1016/j.ibmb.2004.03.018

49. Roush RT, Mckenzie JA. Ecological Genetics of Insecticide and Acaricide Resistance. Annu Rev Entomol. 1987;32: 361–380.

50. Montella IR, Schama R, Valle D. The classification of esterases: An important gene family involved in insecticide resistance - A review. Mem Inst Oswaldo Cruz. 2012;107: 437–449. doi:10.1590/S0074-02762012000400001

51. Saavedra-Rodriguez K, Urdaneta-Marquez L, Rajatileka S, Moulton M, Flores a E, Fernandez-Salas I, et al. A mutation in the voltage-gated sodium channel gene associated

with pyrethroid resistance in Latin American *Aedes aegypti*. Insect Mol Biol. 2007;16: 785–98. doi:10.1111/j.1365-2583.2007.00774.x

52. Marcombe S, Poupardin R, Darriet F, Reynaud S, Bonnet J, Strode C, et al. Exploring the molecular basis of insecticide resistance in the dengue vector *Aedes aegypti*: a case study in Martinique Island (French West Indies). BMC Genomics. 2009;10: 494. doi:10.1186/1471-2164-10-494

53. Bellinato DF, Viana-Medeiros PF, Araújo SC, Martins AJ, Lima JBP, Valle D, et al. Resistance status to the insecticides temephos, deltamethrin, and diflubenzuron in Brazilian *Aedes aegypti* populations. Biomed Res Int. 2016; 2016: 1–12. doi:10.1155/2016/8603263

54. Schechtman H, Souza MO. Costly inheritance and the persistence of insecticide resistance in *Aedes aegypti* populations. PLoS One. 2015;10: 1–22. doi:10.1371/journal.pone.0123961

55. Chevillon C, Bourguet D, Rousset F, Pasteur N, Raymond M. Pleiotropy of adaptive changes in populations: comparisons among insecticide resistance genes in *Culex pipiens*. Genet Res. 1997;70: 195–203.

56. Lenormand T, Bourguet D, Guillemaud T, Raymond M. Tracking the evolution of insecticide resistance in the mosquito *Culex pipiens*. Nature. 1999;400: 861–4. doi:10.1038/23685

57. Miyo T, Akai S, Oguma Y. Seasonal fluctuation in susceptibility to insecticides within natural populations of *Drosophila melanogaster*: empirical observations of fitness costs of insecticide resistance. Genes Genet Syst. 2000;75: 97–104.

58. Busvine JR. Mechanism of resistance to insecticide in houseflies. Nature. 1951;168: 193–195. doi:10.1038/168193a0

59. Martinez-Torres D, Chandre F, Williamson MS, Darriet F, Bergé JB, Devonshire L, et al. Molecular characterization of pyrethroid knockdown resistance (kdr) in the major malaria vector *Anopheles gambiae* s.s. Insect Mol Biol. 1998;7: 179–84.

60. Severson DW, Anthony NM, Andreev O, Ffrench-Constant RH. Molecular mapping of insecticide resistance genes in the yellow fever mosquito (*Aedes aegypti*). J Hered. 1997;88: 520–524.

Supporting Information Legends

S1 Text. Development time normalization procedure. Each mosquito field sample was normalized by its corresponding Rockefeller specimens to enable comparison among different experimental groups.

Due to the great variation in immature development assays, including those with the Rockefeller larvae, each evaluated sampling was normalized as follows:

(a) the experimental groups, consisting of only the specimens that survived to the adult stage (out of the 120 tested), were ranked in ascending order of development time, the same was done with the corresponding Rockefeller insects (from an initial sample of 36);

Development time of field mosquitoes (hours)*	Development time of corresponding Rockefeller mosquitoes (hours)*
216	216
221	220
236	225
240	240
245	
249	
260	
308	
388	
404	

* Illustrative example, not real data.

(b) data from the experimental group were divided into N subgroups respecting the increasing order, N the number of Rockefeller larvae that completed development, each subgroup differing by at most one specimen; example:

N from field population = 10

N from Rockefeller = 4

10 ÷ 4 = 2 and there were 2 left over. So, we will have 2 groups with 2 specimens and 2 groups with 3 specimens (indicated by different colors in the table below). The left overs were always distributed in the last groups.

Development time of field mosquitoes (hours)*	Development time of corresponding Rockefeller mosquitoes (hours)*
192	216
198	220
236	225
240	240
245	
249	
260	
308	
388	
404	

(c) the mean development time of each experimental subgroup was divided by the corresponding Rockefeller value, considering the ranking order (indicated by different colors in the table below);

Development time of field mosquitoes (hours)*	Mean of subgroups	Development time of corresponding Rockefeller mosquitoes (hours)*	Scaled development time
192	195	216	0.90
198			
236	238	220	1.08
240			
245	251.3		
249		225	1.11
260			
308	366.6		
388		240	1.53
404			

Thus, the values generated for the experimental groups, called 'scaled time values', are a measure of how much each field population development time deviates from its corresponding Rockefeller referential. Values above and below 1.0 indicate, respectively, slower or faster development than the Rockefeller strain.

S2 Appendix. Graphical representation of fitness parameters versus RR_{95}, global metabolic alteration and the allelic frequency of the Val1016Ile mutation within field mosquito populations.

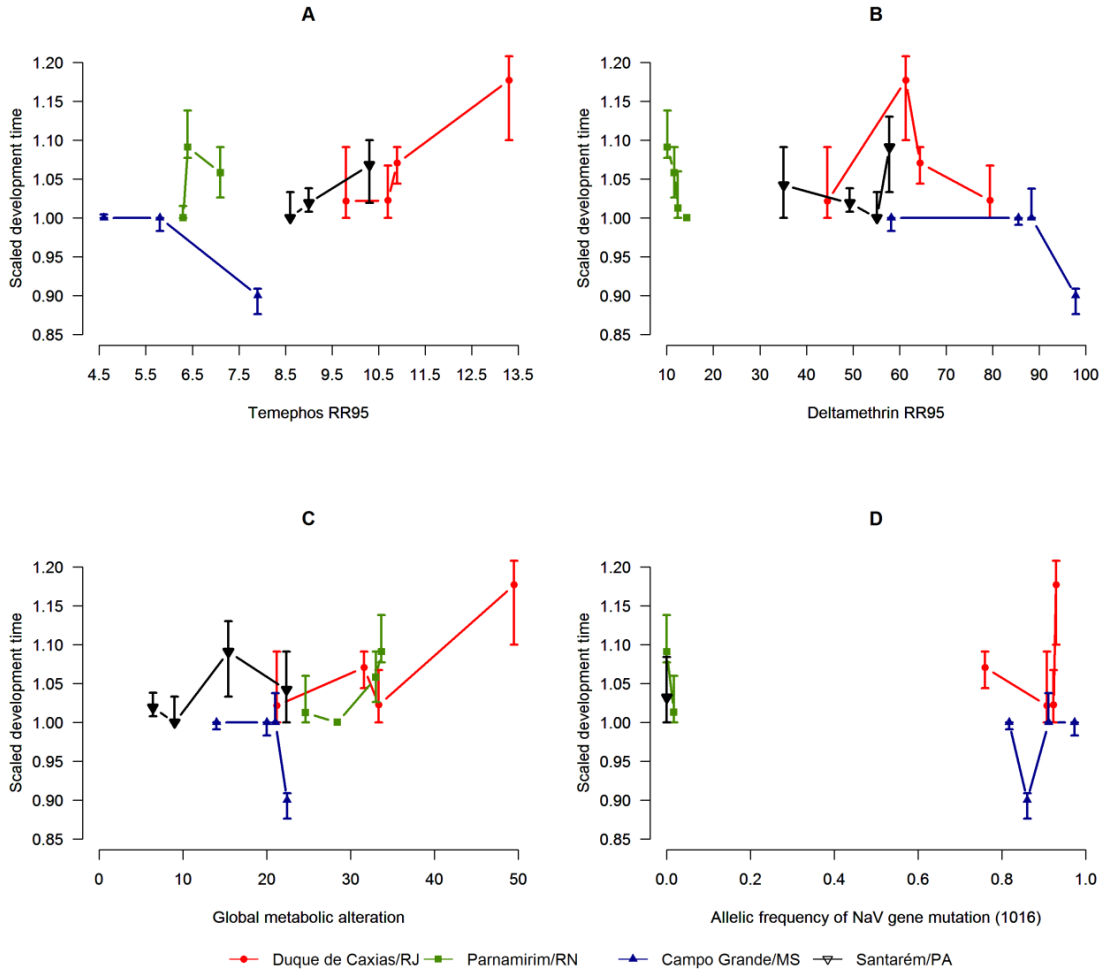

Figure S1. Scaled development time versus RR95 to temephos (A), RR95 to deltamethrin (B), global metabolic alteration (C) and allelic frequency of the Val1016Ile mutation in the pyrethroid NaV target site (D).

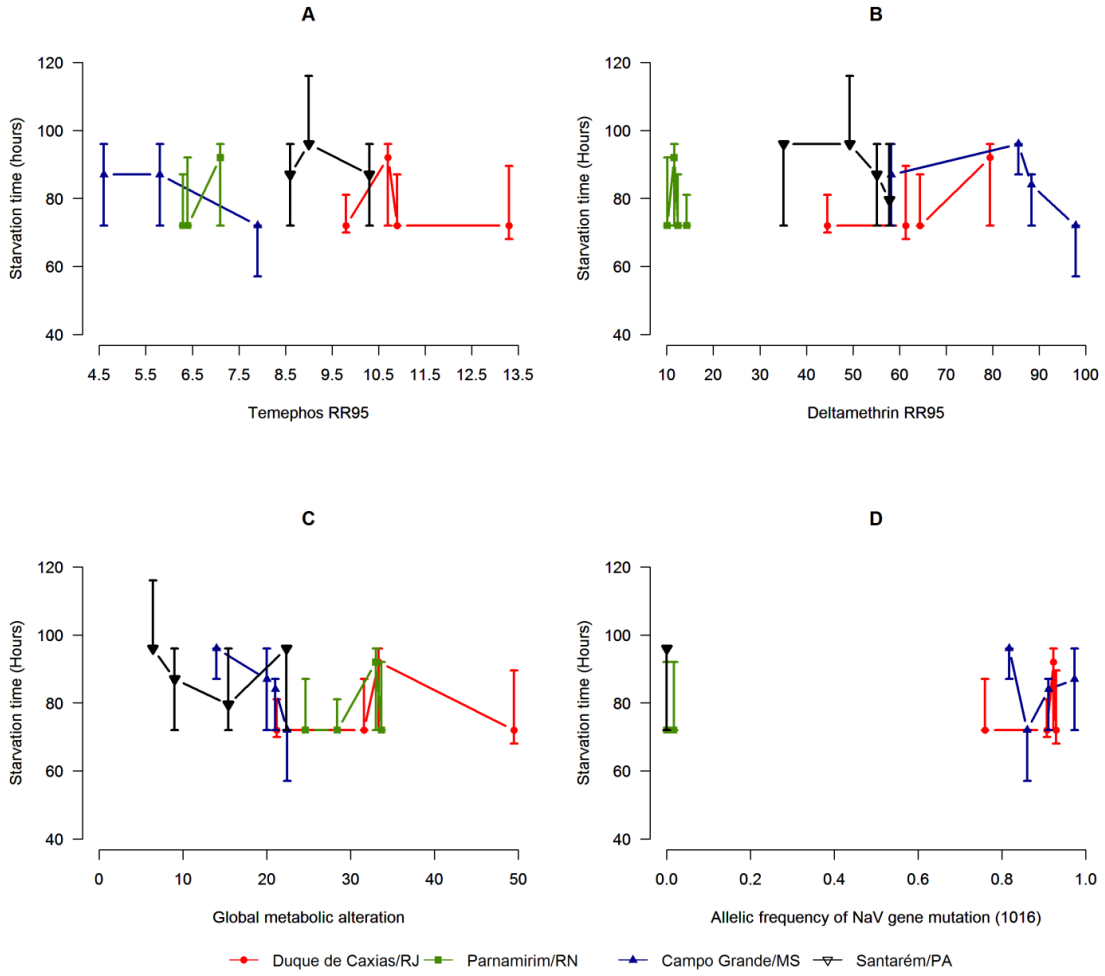

Figure S2. Female starvation tolerance versus RR95 to temephos (A), RR95 to deltamethrin (B), global metabolic alteration (C) and allelic frequency of the Val1016Ile mutation in the pyrethroid NaV target site (D).

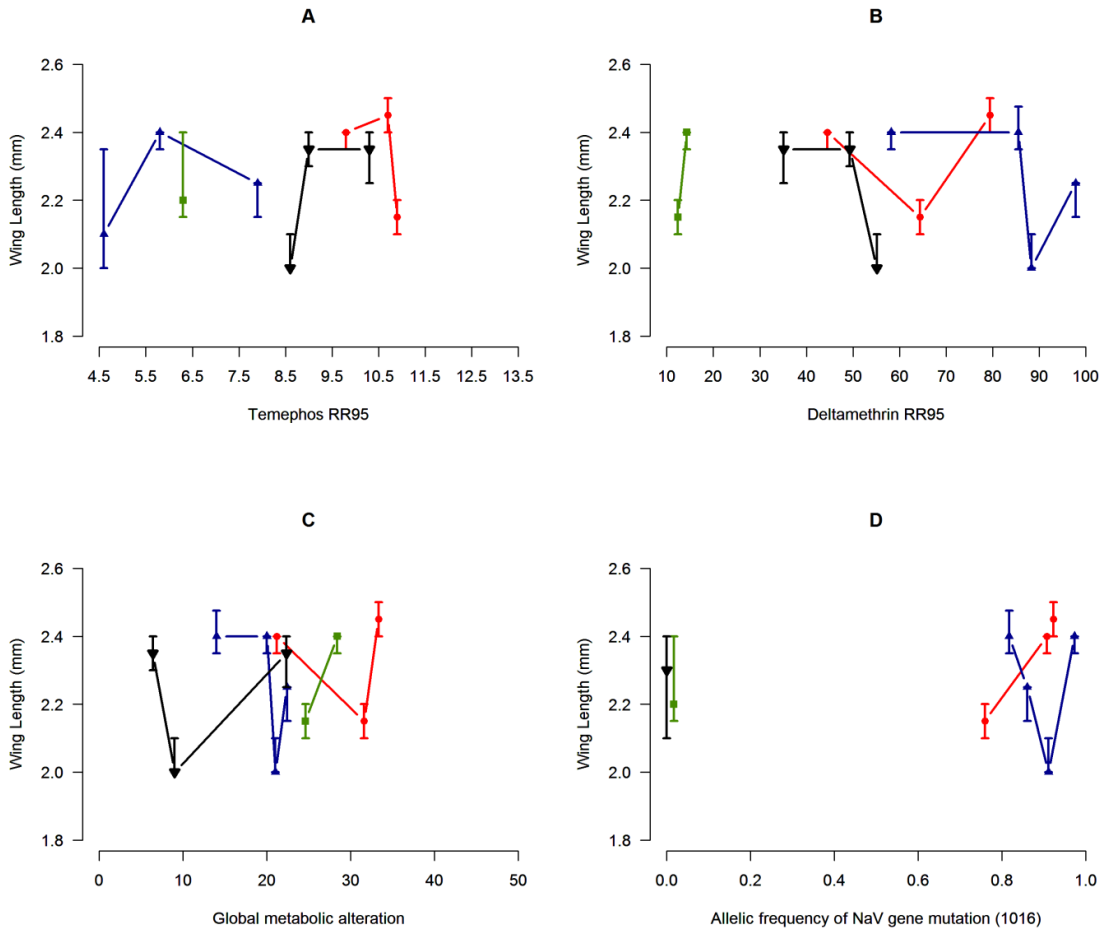

Figure S3. Female wing length versus RR95 to temephos (A), RR95 to deltamethrin (B), global metabolic alteration (C) and allelic frequency of the Val1016Ile mutation in the pyrethroid NaV target site (D).

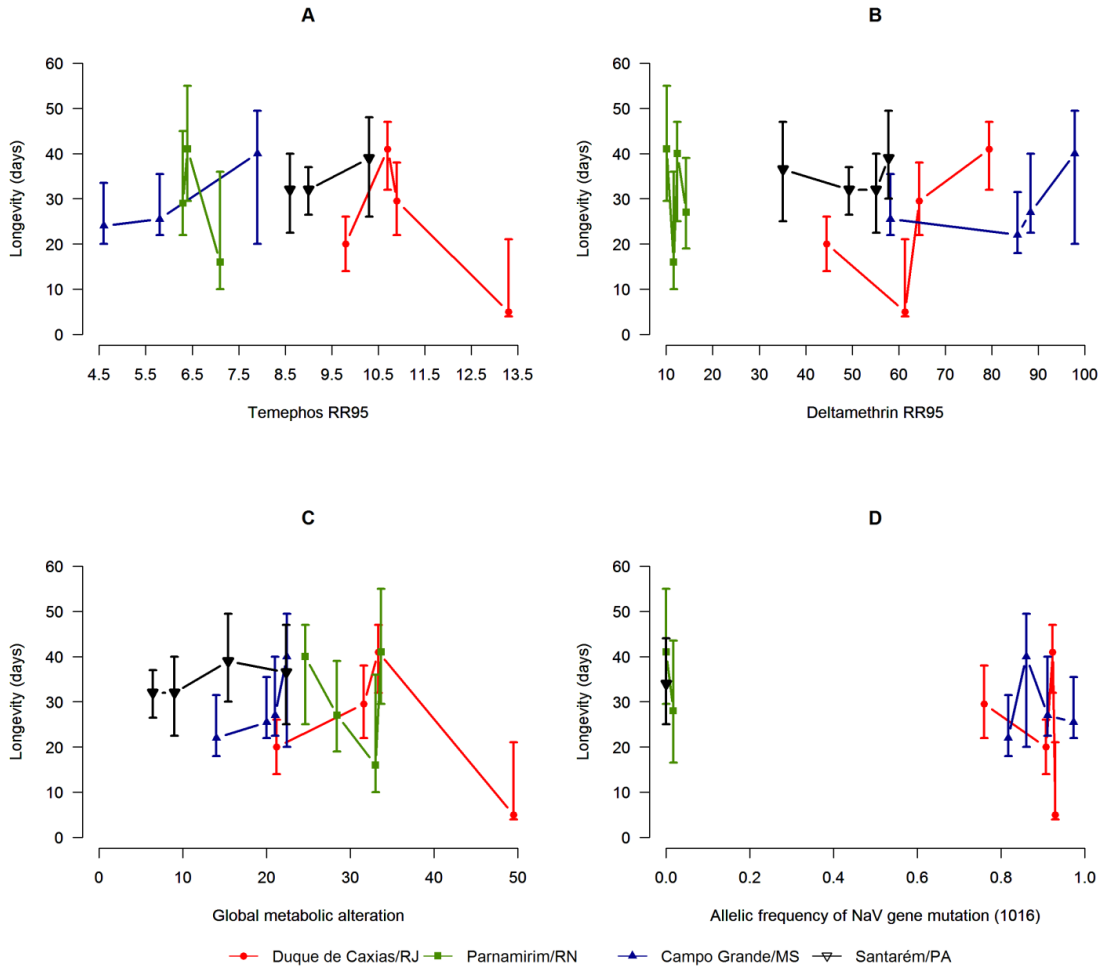

Figure S4. Female adult survival versus RR95 to temephos (A), RR95 to deltamethrin (B), global metabolic alteration (C) and allelic frequency of the Val1016Ile mutation in the pyrethroid NaV target site (D).

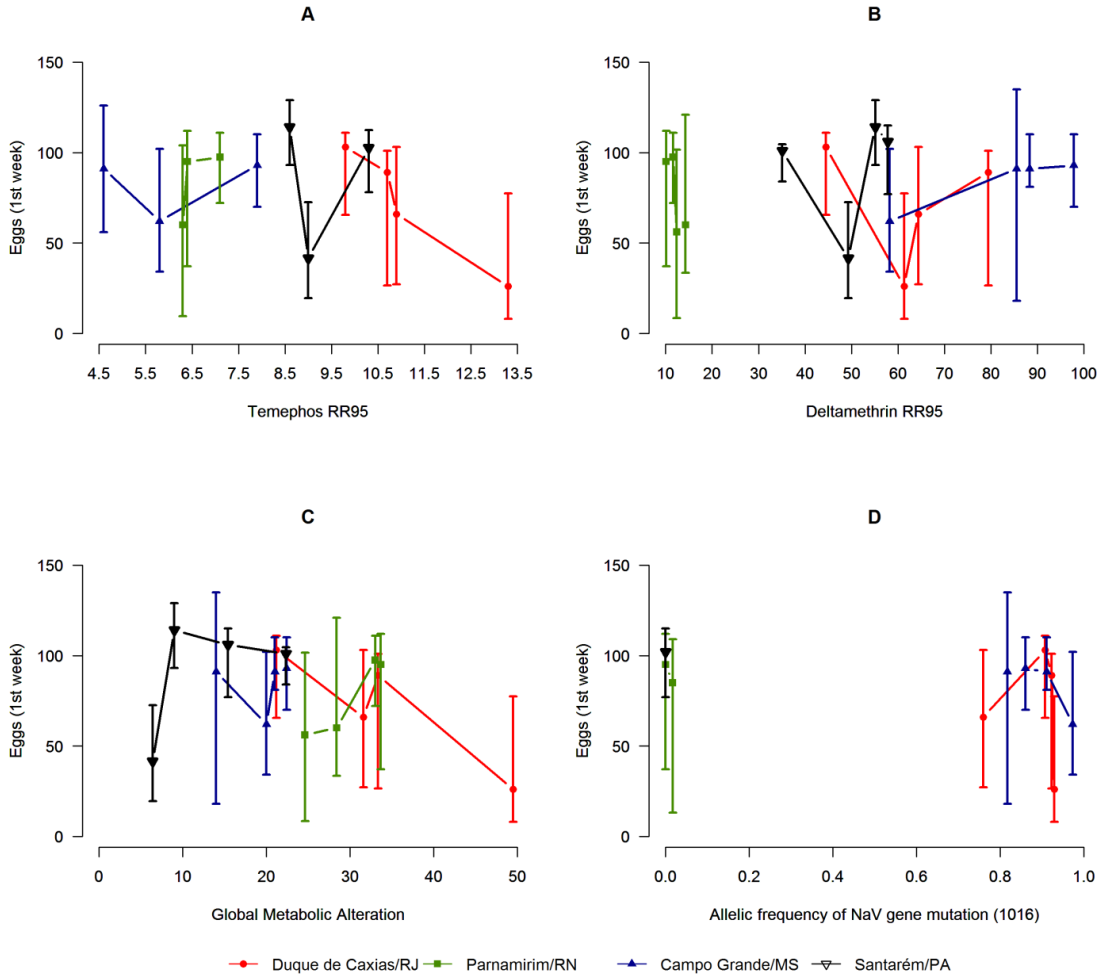

Figure S5. Fecundity versus RR95 to temephos (A), RR95 to deltamethrin (B), global metabolic alteration (C) and allelic frequency of the Val1016Ile mutation in the pyrethroid NaV target site (D).

Linss *et al. Parasites & Vectors* 2014, **7**:25
http://www.parasitesandvectors.com/content/7/1/25

Parasites & Vectors

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Distribution and dissemination of the Val1016Ile and Phe1534Cys *Kdr* mutations in *Aedes aegypti* Brazilian natural populations

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Abstract

Background: The chemical control of the mosquito *Aedes aegypti*, the major vector of dengue, is being seriously threatened due to the development of pyrethroid resistance. Substitutions in the 1016 and 1534 sites of the voltage gated sodium channel ($AaNa_V$), commonly known as *kdr* mutations, confer the mosquito with knockdown resistance. Our aim was to evaluate the allelic composition of natural populations of Brazilian *Ae. aegypti* at both *kdr* sites.

Methods: The $AaNa_V$ IIIS6 region was cloned and sequenced from three Brazilian populations. Additionally, individual mosquitoes from 30 populations throughout the country were genotyped for 1016 and 1534 sites, based in allele-specific PCR. For individual genotypes both sites were considered as a single locus.

Results: The 350 bp sequence spanning the IIIS6 region of the $AaNa_V$ gene revealed the occurrence of the *kdr* mutation Phe1534Cys in Brazil. Concerning the individual genotyping, beyond the susceptible wild-type (Na_V^S), two *kdr* alleles were identified: substitutions restricted to the 1534 position (Na_V^{R1}) or simultaneous substitutions in both 1016 and 1534 sites (Na_V^{R2}). A clear regional distribution pattern of these alleles was observed. The Na_V^{R1} *kdr* allele occurred in all localities, while Na_V^{R2} was more frequent in the Central and Southeastern localities. Locations that were sampled multiple times in the course of a decade revealed an increase in frequency of the *kdr* mutations, mainly the double mutant allele Na_V^{R2}. Recent samples also indicate that Na_V^{R2} is spreading towards the Northern region.

Conclusions: We have found that in addition to the previously reported Val1016Ile *kdr* mutation, the Phe1534Cys mutation also occurs in Brazil. Allelic composition at both sites was important to elucidate the actual distribution of *kdr* mutations throughout the country. Studies to determine gene flow and the fitness costs of these *kdr* alleles are underway and will be important to better understand the dynamics of *Ae. aegypti* pyrethroid resistance.

Keywords: *kdr* mutation, Pyrethroid resistance, Vector control, *Aedes aegypti*, Dengue in Brazil, Sodium channel

Background

Dengue is currently the most important arbovirus in the world. Dengue has spread widely in urban areas of tropical and subtropical regions during the last decades, including countries of Southeast Asia, Pacific and Latin America [1]. Between 2001–2011, almost 10 million dengue cases were reported in Latin America, almost 60% of these were registered in Brazil [2]. Dengue mortality can reach up to 5% of the confirmed infection cases. In addition, in tropical dengue endemic countries a loss of 1,300 disability-adjust life years (DALYs) per million people is estimated [1]. *Aedes aegypti* is the main dengue vector throughout the world. Control of this mosquito consists primarily of the elimination of artificial and disposable water flooded larvae breeding sites and application of insecticides. The WHO Pesticide Evaluate Scheme (WHOPES) recommends ten different compounds to eliminate larvae, including neurotoxicants (organophosphates, pyrethroids and neo-cotinoids), Insect Growth Regulators (chitin synthesis inhibitors and juvenile hormone analogs), and *Bacillus*

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(like *B. thuringiensis var israelensis*) as larvicides. However, fewer formulations are recommended for the control of adult mosquitoes, mostly five pyrethroids and one organophosphate [3].

Given their rapid mode of action and low hazardous effect to the environment, compared to organophosphate insecticides, the use of pyrethroids has increased significantly in the last two decades. Nowadays, pyrethroids are widely employed in and around households, even for pet protection and mosquito control [4]. Since *Ae. aegypti* is essentially an urban mosquito, it is constantly exposed to strong pyrethroid selection. As a consequence, many *Ae. aegypti* populations worldwide are becoming resistant to this class of insecticides [5].

Pyrethroids target the transmembrane voltage gated sodium channel (Na_V) from the insect nervous system, triggering rapid convulsions followed by death, a phenomenon known as *knockdown* effect [6]. The Na_V is composed of four homologous domains (I-IV), each with six hydrophobic segments (S1-S6) [7]. Because the Na_V is a very conserved protein among invertebrates, small changes are permissive without impairing its physiological role [8]. A series of mutations have been identified in different orders of insects and acarids that affect pyrethroid susceptibility, thus being referred to as '*k*nock*d*own *r*esistance' or *kdr* mutations [9]. These *kdr* mutations may lead to conformational changes in the whole channel that maintain its physiological role but avoid insecticide action [10].

In insects, the most common *kdr* mutation is the substitution Leu/Phe in the 1014 site (numbered according to the *Musca domestica* Na_V primary sequence), followed by the Leu/Ser substitution in the same position, in *Anopheles* and *Culex* mosquitoes [11]. In the *Ae. aegypti* Na_V ($AaNa_V$), the 1014 Leu codon is encoded by CTA, rather than TTA as in *Anopheles* and *Culex* mosquitoes. This means that two substitutions would have to be simultaneously selected in the same codon in order to change Leu to Phe (TTT) or Ser (TCA) [12]. Although several mutations have been identified in natural populations at $AaNa_V$ [13], only the Val1016Ile and Phe1534Cys substitutions were clearly related to the loss of pyrethroid susceptibility [12,14]. These sites are placed respectively in the IIS6 and IIIS6 regions of the channels that are known to be involved in the interaction with pyrethroids [10]. It has been previously observed that in Latin America the 1016 Ile *kdr* is highly disseminated [12,15,16] and its frequency is rapidly increasing in localities with intense pyrethroid use, such as Brazil and Mexico [15,16]. High frequencies of 1534 Cys *kdr* were also observed in Grand Cayman and Martinique [14,17].

In the current study, we demonstrate that the1534 Cys *kdr* mutation is present in Brazil together with the 1016 Ile allele previously found. The simultaneous occurrence of both *kdr* mutations at the 1016 and 1534 was found in several localities. Spatial and temporal analysis of these alleles point to a significant role of the *kdr* mutations in pyrethroid resistance in Brazil.

Methods

Mosquito samples

Ae. aegypti used for *kdr* genotyping originated from the same samples evaluated by the Brazilian *Aedes agypti* Insecticide Resistance Monitoring Network, collected with ovitraps according to recommendations of the Brazilian Dengue Control Program [18]. Adult mosquitoes resulting from the eggs collected in the field (F0 generation) were preferentially used. However, in some cases only the following generations reared in the laboratory were available. Details regarding sampling as well as individual data from mosquitoes used for *kdr* genotyping are found in Table 1. A total of 30 localities were analyzed at least once, with AJU, SGO, MSR and VIT analyzed for two-four time-points.

Genotyping assays

Thirty individual mosquitoes from each locality were genotyped at both 1016 and 1534 positions from genomic DNA by allele-specific PCR (AS-PCR) which contains a common primer and two specific primers targeting each polymorphic site. The specificity is attained in the 3′-end, strengthened by a transition three nucleotides before [19]. Additionally, a GC-tail of different sizes was added at the 5′-end of these primers so products can be distinguished by their melting temperature (Tm) in a melting curve analysis or by electrophoresis [12,20,21]. Primer sequences are shown in Table 2. DNA extraction and amplification of the 1016 (Val/Ile) site were conducted as previously described [15]. The reaction for the 1534 (Phe/Cys) site was optimized from previous work [16,22]. In both cases, PCR was carried out with the GoTaq Green Master Mix kit (Promega), 0.5 μL of genomic DNA, 0.24 μM of the common primer, 0.12 and 0.24 μM of the specific primers (1534 Cys^{kdr} and 1534 Phe), in a total volume of 12.5 μL. Denaturing, annealing and extension conditions were, respectively, 95°C / 30″, 54°C / 40″ and 72°C / 45″, in 32 cycles. Alternatively, real-time PCR was conducted with the SYBR Green PCR Master Mix kit (LifeTechnologies/Applied Biosystems), 1 μL genomic DNA and 0.24 μM of each primer, in a total volume of 10 μL. The best conditions for denaturing, annealing and extension were respectively 95°C / 15″, 54°C /15″ and 60°C / 30″, in 33 cycles, followed by a standard melting curve stage. The amplification reaction and melting curve analyses were performed in a StepOne Plus or in a 7500 Real-time PCR system (LifeTechnologies/Applied Biosystems). DNA pools of individuals from CGR, STR

Table 1 *Aedes aegypti* populations used in this study

Code	Municipality	Locality state	Coordinates	Brazilian macroregion	Year of sampling	Generation used in the assays	Gender
AJU	Aracajú	Sergipe	10°54'AJU S, 37°04'O	Northeast	2002	F1	Males
					2006	F1	Females
					2010	F1	Females
					2012	F0	Males
APG	Aparecida de Goiânia	Goiás	16°48'S, 49°14'O	Central-west	2012	F0	Males
BEL	Belém	Pará	1°27'S, 48°30'O	North	2010	F1	Males
BVT	Boa Vista	Roraima	2°49'N, 60°40'O	North	2011	F1	Males
CAC	Caicó	Rio Grande do Norte	6°27'S, 37°05'O	Northeast	2010	F1	Females
CAS	Castanhal	Pará	1°17'S, 47°55'O	North	2011	F0	Males
CBL	Campos Belos	Goiás	13°02'S, 46°45'O	Central-west	2011	F0	Males
CGR	Campo Grande	Mato Grosso do Sul	20°26'S, 54°38'O	Central-west	2010	F0	Males
CIT	Cachoeiro do Itapemirim	Espírito Santo	20°51'S, 41°06'O	Southeast	2012	F0	Males
CLT	Colatina	Espírito Santo	19°32'S, 40°37'O	Southeast	2011	F0	Males
DQC	Duque de Caxias	Rio de Janeiro	22°47'S, 43°18'O	Southeast	2001	F3	Females
					2010	F1	Males
					2012	F0	Males
FOZ	Foz do Iguaçú	Paraná	25°32'S, 54°35'O	South	2009	F2	Females
GVD	Governador Valadares	Minas Gerais	18°50'S, 41°56'O	Southeast	2011	F1	Males
ITP	Itaperuna	Rio de Janeiro	21°12'S, 41°53'O	Southeast	2011	F2	Males
LZN	Luziânia	Goiás	16°15'S, 47°55'O	Central-west	2011	F2	Females
MRB	Marabá	Pará	5°22'S, 49°07'O	North	2011	F0	Males
MSR	Mossoró	Rio Grande do Norte	5°11'S, 37°20'O	Northeast	2009	F0	Males
					2011	F0	Males
PCR	Pacaraima	Roraima	4°25'N, 61°08'O	North	2011	F0	Males
PGT	Porangatu	Goiás	13°25'S, 49°08'O	Central-west	2012	F0	Males
PNM	Parnamirim	Rio Grande do Norte	5°54'S, 35°15'O	Northeast	2010	F0	Males
RVD	Rio Verde	Goiás	17°47'S, 50°55'O	Central-west	2011	F0	Males
SGO	São Gonçalo	Rio de Janeiro	22°49'S, 43°03'O	Southeast	2002	F2	Males
					2008	F2	Males
SIP	Santana do Ipanema	Alagoas	9°21'S, 37°14'O	Northeast	2010	F2	Maless
SMA	São Miguel do Araguaia	Goiás	13°15'S, 50°09'O	Central-west	2012	F0	Males
SRO	Santa Rosa	Rio Grande do Sul	27°52'S, 54°28'O	South	2011	F1	Males
SSO	São Simão	Goiás	18°59'S, 50°32'O	Central-west	2011	?	Males
STR	Santarém	Pará	2°26'S, 54°41'O	North	2010	F0	Males
TCR	Tucuruí	Pará	3°46'S, 49°40'O	North	2010	F0	Males
URU	Uruaçu	Goiás	14°31'S, 49°09'O	Central-west	2011	F0	Males
VIT	Vitória	Espírito Santo	20°18'S, 40°18'O	Southeast	2006	F1	Males
					2010	F0	Males

and PNM were used to amplify the region spanning the Na_V IIIS6 segment with the primers AaEx31P and AaEx31Q (Table 2), as specified elsewhere [14]. The PCR products were purified in S-400 microcolumns (GE Healthcare) according to manufacturer instructions and cloned with CloneJet PCR Cloning Kit (Thermo Scientific). The DNA sequencing was carried out in an ABI377 Sequencer with the Big Dye 3.1 Kit (Life-Technologies/Applied Biosystems). Sequence analysis was performed using the BioEdit software version 7.2.

Table 2 Primer sequences

Primer name	Sequence (5′ - 3′)	References
1016 Val^{+} (for)	$^{\#\#}$ACAAATTGTTTCCCACCCGCACCGG	[12,15]
1016 Ile^{kdr} (for)	$^{\#}$ACAAATTGTTTCCCACCCGCAC*T*GA	
1016 comom (rev)	GGATGAACCGAAATTGGACAAAAGC	
1534 Phe^{+} (for)	$^{\#}$TCTACTTTGTGTTCTTCATCAT*A*TT	[22]
1534 Cys^{kdr} (for)	$^{\#\#}$TCTACTTTGTGTTCTTCATCAT*G*TG	
1534 comom (rev)	TCTGCTCGTTGAAGTTGTCGAT	
AaEx31P (for)	TCGCGGGAGGTAAGTTATTG	[14]
AaEx31Q (rev)	GTTGATGTGCGATGGAAATG	
long 5′-tail	GCGGGCAGGGCGGCGGGGGCGGGGCC	
short 5′-tail	GCGGGC	

$^{+}$wild-type specific primer, ^{kdr}*kdr* specific primer, $^{\#}$short 5′tail attached, $^{\#\#}$long 5′tail attached.

All individuals were genotyped for both 1016 and 1534 sites. Linkage disequilibrium was tested by the online Genepop version 4.2 [23], and since the 1016 and 1534 sites are linked (see Results section), genotypic and allelic frequencies were taken as a single locus. Hardy-Weinberg equilibrium was evaluated by the classical equation [24], being the null hypothesis of equilibrium checked by a chi-square test with three or one degrees of freedom, respectively, when six or three genotypes were evidenced.

Results

Allele-specific discrimination

A 20 bp size difference, due to the 5′-GC tail of allele specific primers, enabled the easy discrimination of homozygous and heterozygous genotypes in either a polyacrylamide gel electrophoresis or in dissociation curves through real-time PCR (Figure 1). Electrophoresis revealed products of around 80 and 100 bp, respectively for Ile^{kdr} and Val^{+} (1016 reaction), and 90 and 110 bp, respectively for Phe^{+} and Cys^{kdr} (1534 reaction). The dissociation curve exhibited Tm of around 76 and 84°C, respectively for Ile^{kdr} and Val (1016 reaction), and 77 and 82°C, respectively for Phe and Cys^{kdr} (1534 reaction). The PCR conditions of annealing temperature, number of cycles and concentration of each primer were crucial to avoid unspecific amplification. All reactions were accompanied by positive controls, each one consisting of the three potential genotypes at the 1016 and 1534 positions, which were obtained by previously genotyped individuals: homozygous wild type, heterozygous, and homozygous *kdr*. As the Phe1534Cys mutation was detected for first time in Brazilian samples, we cloned and sequenced the IIIS6 region (exon 31) of the *AaNa*$_V$ gene of three genotyped populations (CGR, STR and PNM), confirming the primers' specificity. The 350 bp fragments were submitted to GenBank (accession numbers: KF527414 and KF527415, for 1534 Cys^{kdr} and 1534 Phe^{+}, respectively). Excluding the site of the 1534 *kdr* mutation (TTC/TGC), no other polymorphic site was detected relative to the sequence deposited in VectorBase (Liverpool strain).

Genotyping 1016 and 1534 *Aa*Na$_V$ sites in natural populations

Around 30 *Ae. aegypti* individuals from each one of 30 distinct Brazilian localities were genotyped for both 1016 and 1534 Na$_V$ sites, totalling 1,112 analyzed mosquitoes. Some localities were sampled two to four times within a ten-year interval. The genotypes of individual mosquitoes for both sites were first calculated independently: 1016 Val^{+}/Val^{+}, Val^{+}/Ile^{kdr} and Ile^{kdr}/Ile^{kdr}, and 1534 Phe^{+}/Phe^{+}, Phe^{+}/Cys^{kdr} and Cys^{kdr}/Cys^{kdr}. These data were used to perform a genotypic linkage disequilibrium analysis and total linkage between them was demonstrated (Fisher's method, $p < 0.001$), as expected from two sites placed in the same gene. In this sense both sites were considered as constituents of a single locus, thus evidencing the occurrence of six genotypes in individual mosquitoes (Table 3). Based on the composition of these genotypes, we concluded that three alleles were present in the evaluated samples: '1016 Val^{+} + 1534 Phe^{+}' (wild-type), '1016 Val^{+} + 1534 Cys^{kdr}' (1534 *kdr*) and '1016 Ile^{kdr} + 1534 Cys^{kdr}' (1016 *kdr* + 1534 *kdr*). Hereafter these alleles will be simply referred to as 'Na$_V^{S}$', 'Na$_V^{R1}$' and 'Na$_V^{R2}$', respectively (Figure 2). Double mutants and individuals with mutation only in the 1534 position were found (respectively, Na$_V^{R2}$ and 'Na$_V^{R1}$'); however, in no case was the 1016 *kdr* mutation observed alone, precluding the existence of a 1016 Ile^{kdr} + 1534 Phe^{+} allele in the evaluated populations. Figure 3 shows the frequencies for Na$_V^{S}$, Na$_V^{R1}$ and Na$_V^{R2}$ alleles in the most recent samples obtained from each locality. The 95% CI of the allele frequencies is shown in the Additional file 1: Table S1. According to the alleles, the genotypes were named SS, SR1, SR2, R1R1, R1R2 and R2R2. Their frequencies and the Hardy-Weinberg Equilibrium deviation test are presented in Table 3. In only seven out of 38 samplings the Hardy-Weinberg Equilibrium assumption was rejected ($p < 0.05$). No specific genotype contributed to the deviation in these seven localities.

Overall, the distribution of the three alleles differed according to the geographical region (Figure 3). In the North and Northeast Regions, the Na$_V^{R1}$ allele, mutant only at position 1534, was found in all localities, nevertheless the Na$_V^{S}$ wild-type allele was the most representative in six of the localities (BEL, CTL, MRB, CAC, SIP and PNM). The highest frequency of Na$_V^{R1}$, was found in the North: 0.750 (STR), among all populations analyzed. On the other hand, with exception of the most recent AJU (AJU2012), the Na$_V^{R2}$ double mutant allele was either absent or < 5% in the North and Northeast of Brazil. In contrast, the wild-type allele, Na$_V^{S}$, was absent from

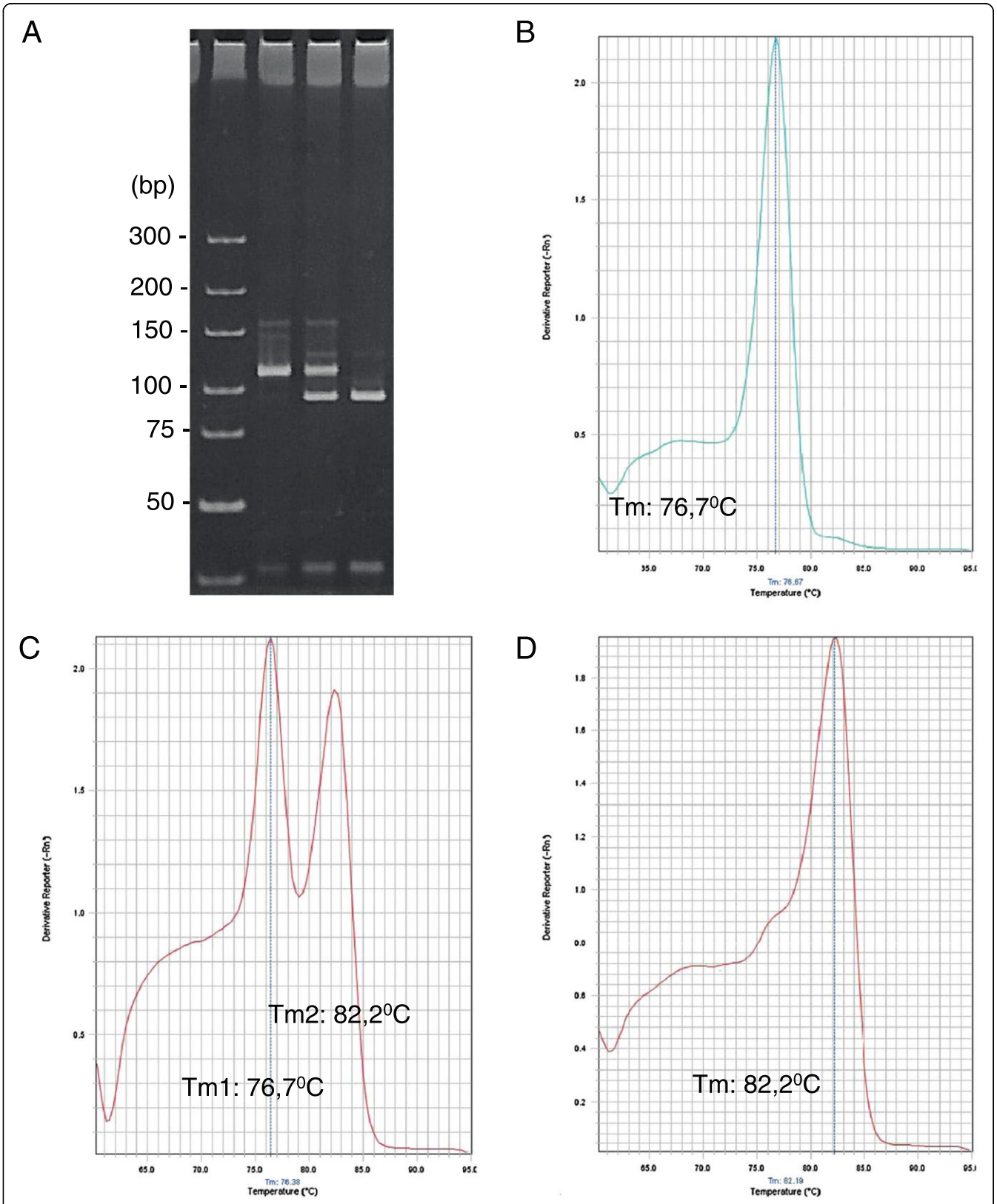

Figure 1 Allele specific PCR (AS-PCR) for genotyping *kdr* mutations in the *Aedes aegypti* voltage gated sodium channel. All panels represent reactions for the 1534 site. **(A)** Visualization of the amplicons in a 10% polyacrylamide gel electrophoresis, run under 170 V/45′ and stained with ethidium bromide (1 μg/mL). Amplicons of approximately 90 and 110 bp correspond to alleles 1534 Phe^{+} and 1534 Cys^{kdr}, respectively. DNA ladder was used as size marker (O'GeneRuler DNA Ladder, Ultra Low Range/Fermentas, 150 ng). Dissociation curve analysis in real time PCR differentiating the Phe/Phe **(B)**, Phe/Cys **(C)**, and Cys/Cys **(D)** genotypes. The Tm for the respective alleles are indicated.

Table 3 Genotype frequencies of Brazilian *Aedes aegypti* populations at the 1016 and 1534 sites of the Na_V locus

Macro-region	Population	Genpotype frequencies						Total (n)	HWE test	
		SS	SR1	SR2	R1R1	R1R2	R2R2		χ^2	p
North	PCR11	0.000	0.000	0.000	0.367	0.467	0.167	30	0.0	0.879
	BVT11	0.000	0.000	0.000	0.536	0.393	0.071	28	0.0	0.993
	CAS11	0.400	0.500	0.033	0.033	0.033	0.000	30	2.3	0.512
	BEL10	0.536	0.357	0.000	0.107	0.000	0.000	28	0.4	0.932
	STR10	0.200	0.100	0.000	0.700	0.000	0.000	30	16.1	0.000
	TCR10	0.200	0.300	0.000	0.500	0.000	0.000	30	3.5	0.062
	MRB11	0.621	0.138	0.000	0.241	0.000	0.000	29	13.3	0.000
Northeast	MSR09	0.600	0.367	0.000	0.033	0.000	0.000	30	0.2	0.660
	MSR11	0.000	0.767	0.000	0.200	0.000	0.033	30	14.9	0.002
	PNM10	0.704	0.111	0.037	0.111	0.037	0.000	27	9.3	0.025
	CAC10	0.833	0.133	0.033	0.000	0.000	0.000	30	0.0	0.998
	SIP10	0.433	0.500	0.067	0.000	0.000	0.000	30	4.7	0.199
	AJU02	1.000	0.000	0.000	0.000	0.000	0.000	30	0.0	1.000
	AJU06	0.767	0.033	0.167	0.000	0.033	0.000	30	0.3	0.955
	AJU10	0.269	0.038	0.308	0.000	0.000	0.385	26	3.6	0.306
	AJU12	0.200	0.033	0.333	0.033	0.100	0.300	30	3.4	0.338
Central-west	CBL11	0.069	0.069	0.414	0.000	0.103	0.345	29	0.5	0.918
	SMA12	0.207	0.172	0.241	0.103	0.207	0.069	29	1.2	0.750
	PGT12	0.000	0.069	0.241	0.241	0.241	0.207	29	5.9	0.115
	URU11	0.233	0.133	0.300	0.000	0.100	0.233	30	1.3	0.723
	LZN11	0.200	0.333	0.200	0.033	0.167	0.067	30	1.7	0.639
	APG12	0.000	0.207	0.207	0.138	0.241	0.207	29	2.5	0.466
	RVD11	0.103	0.034	0.241	0.069	0.241	0.310	29	2.8	0.421
	SSO11	0.000	0.133	0.033	0.200	0.233	0.400	30	7.6	0.056
	CGR10	0.000	0.033	0.100	0.000	0.267	0.600	30	1.2	0.749
Southeast	GVD11	0.000	0.033	0.200	0.267	0.067	0.433	30	18.3	0.000
	CLT11	0.067	0.333	0.300	0.000	0.100	0.200	30	9.0	0.029
	VIT06	0.267	0.100	0.333	0.000	0.033	0.267	30	2.4	0.492
	VIT10	0.000	0.067	0.100	0.000	0.000	0.833	30	2.3	0.507
	CIT12	0.000	0.069	0.138	0.103	0.172	0.517	29	3.8	0.281
	ITP11	0.148	0.111	0.259	0.074	0.074	0.333	27	5.0	0.172
	SGO02	1.000	0.000	0.000	0.000	0.000	0.000	30	0.0	1.000
	SGO08	0.192	0.231	0.308	0.115	0.115	0.038	26	1.6	0.669
	DQC01	1.000	0.000	0.000	0.000	0.000	0.000	30	0.0	1.000
	DQC10	0.000	0.033	0.067	0.100	0.067	0.733	30	13.0	0.005
	DQC12	0.000	0.033	0.000	0.000	0.433	0.533	30	5.6	0.136
South	FOZ09	0.133	0.100	0.400	0.033	0.000	0.333	30	3.6	0.311
	SRO11	0.296	0.259	0.222	0.037	0.000	0.185	27	7.4	0.059

the two northernmost localities evaluated (PCR and BVT, both in the State of Roraima), where both mutant alleles were at high frequencies. In all localities from Central-West, Southeast and South regions, all three alleles were present. The most frequent allele was the Na_V^{R2} double mutant. Exceptions were LZN, SMA, URU, SGO and SRO, where the Na_V^{S} wild-type allele was the most representative (Figure 3).

The dynamics of the genotype frequencies was analyzed in AJU, MSR, VIT and DQC. Samples from AJU were

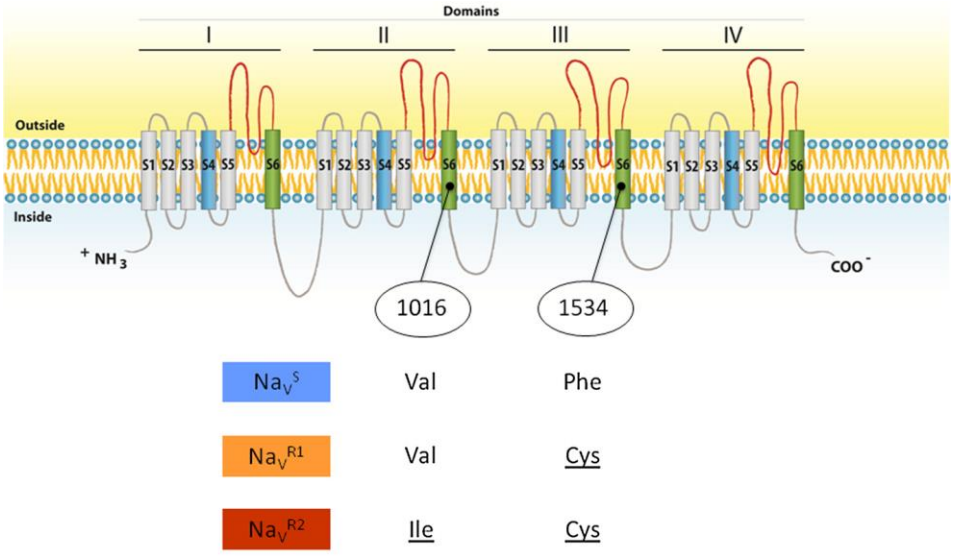

Figure 2 Voltage gated sodium channel and the 1016 and 1534 alleles found in Brazilian *Aedes aegypti* populations. The Na_V is represented with its four domains (I-IV), each with the six transmembrane segments (S1-S6). The voltage sensitive S4 and the pore forming S6 segments are colored in blue and green, respectively (scheme adapted from [9]). The 1016 and 1534 *kdr* sites in *Aedes aegypti* are indicated. Mutant amino acids are underlined.

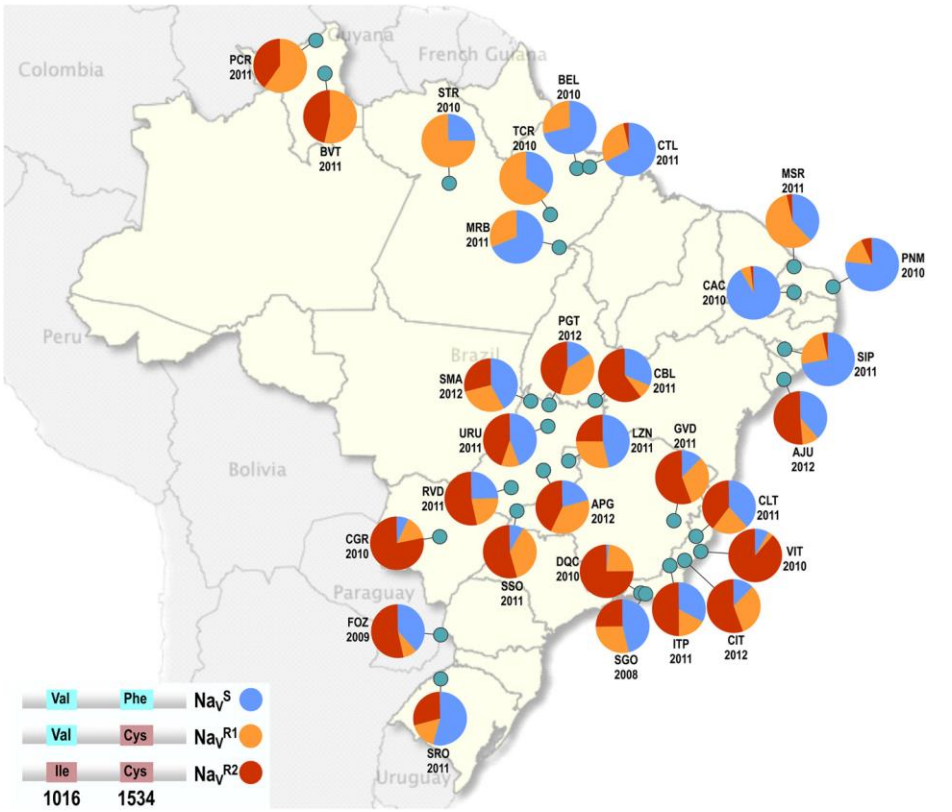

Figure 3 Distribution of the *kdr* alleles in Brazilian *Aedes aegypti* populations. For each locality, only the most recent samples evaluated are shown. Details of the localities are shown in Table 1. Alleles are represented according to the colors used in Figure 2.

collected four times in the course of a decade, between 2002 and 2012. In 2002, only the Na_V^S wild-type allele was detected. The *kdr* alleles appeared first in 2006 and the double mutant Na_V^{R2} was the most frequent allele by 2012 (Figure 4). Accordingly, the 'SS' wild-genotype progressively decayed from 100% in 2002 to 20% in 2012, when the double mutant 'R2R2' represented 30% of the individuals, and was the most frequent genotype (AJU2012, Table 3). The frequency of the Na_V^S wild-type allele also decreased in all other localities evaluated where the *kdr* alleles increased in frequency (Figure 4). Except for MSR, the Na_V^{R2} double mutant is likely to be the most favorably selected allele. It is noteworthy that in AJU, the Na_V^{R1} allele showed the larger frequency increase, probably because Na_V^{R2} must have arrived to the Northeast more recently.

Discussion

The genotyping of mutations directly related to insecticide resistance is an important surveillance tool for agricultural and sanitary purposes. Among selected mechanisms of pyrethroid resistance, *kdr* mutations in the voltage gated sodium channel (Na_V) are those that better correlate particular genotypes with insecticide resistance [25]. The increased efficiency of insecticide detoxification, known as metabolic resistance – involving super families of enzymes such as GST, esterases and especially the multi function oxidases P450 – may also confer resistance to pyrethroids. However, identification of these mechanisms is mainly based on enzymatic assays of low specificity [26] or on bioassays with synergist compounds [27], and are not clearly linked to particular genes. More recently, many successful transcriptome tools for metabolic resistance genes have emerged, pointing to a very complex and diverse scenario regarding insecticide selected genes and their pattern of expression among insect populations [28,29]. Because the metabolic resistance based selection seems to have a high fitness cost, due to reallocation of energetic resources, this mechanism is expected to induce lower resistance levels, if compared to mutations in the target site molecules [30]. This was corroborated by laboratory selection with pyrethroids in an *Ae. aegypti* lineage: increase of the 1016 Ile^{kdr} frequency was inversely proportional to the number of 'metabolic' genes differentially transcribed [29]. It was hypothesized that, in the presence of pyrethroid, *kdr* mutations are preferentially selected among other mechanisms, contributing to higher resistance levels and/or resulting in less deleterious effects.

In addition to the classical Leu1014Phe *kdr* mutation, several others have been associated with pyrethroid resistance [6]. Interactions of multiple Na_V mutations may modulate pyrethroid resistance levels. For instance, certain Na_V haplotypes, including synonymous substitutions, were found in two distinct field populations of *Culex quinquefasciatus* selected for pyrethroid resistance during 6–8 generations in the laboratory. It was suggested that some of these haplotypes were selected at an early stage of permethrin resistance and later evolved to other mutation combinations in the course of selection pressure [31]. In *Ae. aegypti*, a synonymous substitution at exon 20,

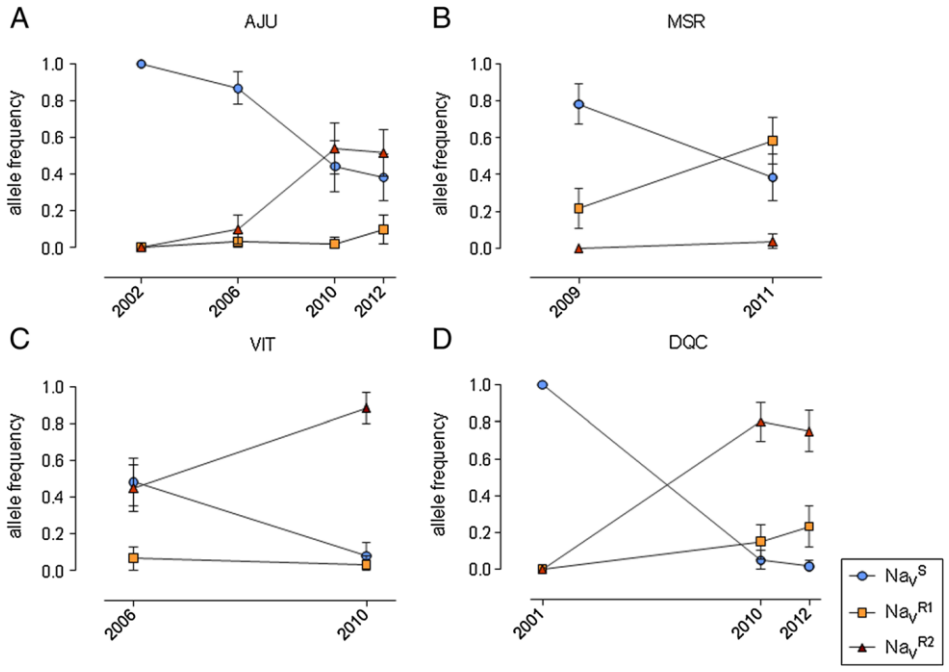

Figure 4 Time-course of *kdr* alleles frequencies in four Brazilian *Aedes aegypti* populations. Localities: **A -** Aracaju (AJU), **B -** Mossoró (MSR), **C** - Vitória (VIT) and **D** - Duque de Caxias (DCQ). Bars indicate the 95% CI of allele frequencies.

together with an extensive polymorphism in the following intron, were linked to both Ile1011Met and Val1016Ile mutations [15,32]. Additionally, a gene duplication event was recently described in the $AaNa_V$ of natural populations and in a laboratory strain selected for pyrethroid resistance [33]. Although there are at least seven different mutations described in the $AaNa_V$, only those corresponding to the 1016 and 1534 positions are clearly related to resistance; both are placed in a domain of the sodium channel that interacts directly with the pyrethroid molecule [34].

There are two mutations described in the *Aa*Na_V 1016 site, Val to Ile or Gly, respectively in Latin America [12,14,15] and in Southeast Asia [35]. In Brazil, we found no evidence of a haplotype that contains exclusively the 1016 Ile^{kdr} mutation, since it was always found together with 1534 Cys^{kdr} (Na_V^{R2} allele, herein). Nevertheless, we are aware that it is possible for a haplotype carrying the 1016 *kdr* mutation to occur in the populations examined, however, it would be present at very low frequencies. Actually, this putative allele must have occurred in two out of three *Ae. aegypti* populations from Grand Cayman, given that the 1016 Ile^{kdr} presented a higher frequency than the 1534 Cys^{kdr} substitution [14].

Differently from the 1016 position, only one substitution, Phe/Cys, was found in the 1534 site by far [14,36]. This 1534 substitution can be linked with another one. In Thailand, the 1534 Cys^{kdr} co-occurred with 1016 Gly^{kdr} and 989 Pro^{kdr} in the same molecule [22]. In that region an allele 1534 Cys^{kdr} without mutation in 1016 site (Na_V^{R1} allele, herein) seemed to be very common, since its frequency was higher than the 1016 Gly^{kdr} [35].

Here we presented the distribution of the *kdr* variants for the AaNa$_V$, considering both 1016 and 1534 sites screen from several natural Brazilian populations. We considered that once these sites are very close in the genome, reporting the allele/genotypic frequencies of each site separately would not be fully informative. However, because there are still some gaps concerning the actual role of these mutations in pyrethroid resistance, regarding whether they are acting alone or synergistically, and present in *cis* or trans mutations, we are reporting the allele frequencies of each site rather than as an haplotype. The implication of the 1016 Ile^{kdr} allele in resistance to pyrethroids was corroborated by laboratory selection, which highly increased the allelic frequency up to fixation in only five generations [29]. Accordingly, in the last decade this mutation has been rapidly spreading in natural populations from Brazil and Mexico, concomitantly with the intensification of pyrethroid usage due to the emergence of severe dengue outbreaks [15,16]. In these cases however, the co-occurrence of the 1534 Cys^{kdr} mutation has been overlooked. A recent study reported high frequencies of 1534 Cys^{kdr} in Grand Cayman [14], suggesting it is not a novel mutation in Latin America. In a recent report, nine single and two double $AaNa_V$ mutants were constructed and inserted in a *Xenopus* oocyte system in order to perform functional evaluations of these substitutions in the presence of type I or II pyrethroids [34]. The 1016 Ile^{kdr} construct did not result in sensitivity reduction, to either pyrethroid types. On the other hand, the 1534 Cys^{kdr} significantly diminished the AaNa$_V$ sensibility to type I but not to type II pyrethroids. This same substitution in the homologous *kdr* site of the cockroach Na_V exhibited similar results [37].

An *Ae. aegypti* lineage, selected for permethrin resistance in the laboratory, exhibited high frequencies of 1016 Gly^{kdr} + 1794 Tyr^{kdr} substitutions in the same molecule, which suggested a synergistic effect towards pyrethroid resistance [38]. We hypothesize that mutation in the 1016 site should be important when in synergism with other specific mutations. In Brazil, the 1534 Cys^{kdr} mutation is widespread throughout the territory. The Na_V^{R1} allele is more frequent in North/Northeast regions whereas Na_V^{R2} is more commonly present in Central/Southeast regions, generally where the highest resistance levels to pyrethroids are observed [18]. Both mutant haplotypes appear to be rapid and favorably selected in all evaluated populations. However, in the most recent samplings the Na_V^{R2} double mutant was the more frequent *kdr* allele. The exception was MSR, in the Northeast Region, where Na_V^{R2} was only recently introduced. Together these data suggest that Na_V^{R2} allele would be more advantageous for pyrethroid resistance, or impose a lower fitness cost when compared to Na_V^{R1}. We recently demonstrated that an Na_V^{R2} homozygous *Ae. aegypti* lineage, highly resistant to pyrethroids, exhibited a fitness cost in a series of life-trait parameters [39]. Further comparisons between Na_V^{R1} and Na_V^{R2} lineages will be of importance to better clarify those assumptions.

It is of note that since 2001 and up to 2009 the Brazilian Dengue Control Program employed pyrethroids in ultra-low volume applications in several municipalities as part of the effort to control the dengue vector [18]. With very few exceptions, the basis for pyrethroid selection pressure derived from national campaigns is essentially the same in the whole country. Therefore, differential selection pressures would not explain the aforementioned regionalization of the *kdr* alleles. It is likely that the current distribution of the *kdr* alleles reflects distinct *Ae. aegypti* populations that colonized the continent. Population genetics analysis of neutral loci will help us to unravel the evolutionary routes of these resistance genes.

Conclusions

In conclusion, pyrethroids are the most employed insecticides worldwide and the only chemical class presently allowed in long lasting treated materials, such as nets

and curtains [40]. Although novel control strategies are being tested in the field, such as those based on transgenic and on *Wolbachia*-infected mosquitoes [2,41,42], insecticides will certainly play an important role for yet a long time. Knowledge of the sodium channel diversity in natural populations together with the role of each allele regarding pyrethroid resistance as well as their fitness effects are crucial for preserving the effectiveness of this class of compounds as a viable tool against *Ae. aegypti*.

Additional file

Additional file 1: Table S1. *Kdr* allele frequencies of *Aedes aegypti* natural populations from Brazil. The CI95%* is under parentheses.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Conceived and designed the experiments: JGBL, LPB, AJM. Performed the experiments: JGBL, LPB, GAG. Analyzed the data: JGBL, LPB, ASA, RVB, AJM. Contributed reagents/materials/analysis tools: RVB, JBPL, DV. Wrote the paper: AJM, DV. All authors read and approved the final version of the manuscript.

Acknowledgements

We thank Dr Alexandre Afranio Peixoto for his friendship and orientation throughout this study. This work is dedicated to his memory. We also thank the DNA sequencing facility of FIOCRUZ (Plataforma de Sequenciamento/PDTIS/Fiocruz) and the Brazilian Dengue Control Program that allowed utilization of samples collected in the scope of the Brazilian *Aedes aegypti* Insecticide Resistance Monitoring Network (MoReNAa). We are grateful to Dr Andrea Gloria-Soria for critical reading the manuscript.

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Received: 12 August 2013 Accepted: 18 December 2013
Published: 15 January 2014

References

1. Guzman MG, Halstead SB, Artsob H, Buchy P, Farrar J, *et al*: **(2010) Dengue: a continuing global threat.** *Nat Rev Microbiol* 2010, **8:**S7–S16.
2. Maciel-de-Freitas R, Aguiar R, Bruno RV, Guimaraes MC, Lourenco-de-Oliveira R, *et al*: **Why do we need alternative tools to control mosquito-borne diseases in Latin America?** *Mem Inst Oswaldo Cruz* 2012, **107:**828–829.
3. WHOPES: *Pesticides and their application for the control of vectors and pests of public health importance (WHO/CDS/NTD/WHOPES/GCDPP/2006.1).* Geneva: World Health Organization; 2006.
4. Agency USEP: *Pesticides: Regulating Pesticides.* U.S. Environmental Protection Agency; 2012. http://www.epa.gov/oppsrrd1/reevaluation/pyrethroids-pyrethrins.html.
5. Ranson H, Burhani J, Lumjuan N, Black WC IV: **Insecticide resistance in dengue vectors.** *TropIKAnet* 2010, **1:**1. cited 2013-12-02], pp. 0–0. Available from: http://journal.tropika.net/scielo.php?script=sci_arttext&pid=S2078-86062010000100003&lng=en&nrm=iso. ISSN 2078–8606.
6. Dong K: **Insect sodium channels and insecticide resistance.** *Invert Neurosci* 2007, **7:**17–30.
7. Catterall WA: **From ionic currents to molecular mechanisms: the structure and function of voltage-gated sodium channels.** *Neuron* 2000, **26:**13–25.
8. ffrench-Constant RH, Pittendrigh B, Vaughan A, Anthony N: **Why are there so few resistance-associated mutations in insecticide target genes?** *Philos Trans R Soc Lond B Biol Sci* 1998, **353:**1685–1693.
9. Martins AJ, Valle D: **The pyrethroid knockdown resistance.** In *Insecticides - Basic and Other Applications.* Edited by Soloneski S, Larramendy M. Rijeka: InTech; 2012:17–38.
10. O'Reilly AO, Khambay BP, Williamson MS, Field LM, Wallace BA, *et al*: **Modelling insecticide-binding sites in the voltage-gated sodium channel.** *Biochem J* 2006, **396:**255–263.
11. Davies TG, Field LM, Usherwood PN, Williamson MS: **A comparative study of voltage-gated sodium channels in the Insecta: implications for pyrethroid resistance in Anopheline and other Neopteran species.** *Insect Mol Biol* 2007, **16:**361–375.
12. Saavedra-Rodriguez K, Urdaneta-Marquez L, Rajatileka S, Moulton M, Flores AE, *et al*: **A mutation in the voltage-gated sodium channel gene associated with pyrethroid resistance in Latin American *Aedes aegypti*.** *Insect Mol Biol* 2007, **16:**785–798.
13. Brengues C, Hawkes NJ, Chandre F, McCarroll L, Duchon S, *et al*: **Pyrethroid and DDT cross-resistance in *Aedes aegypti* is correlated with novel mutations in the voltage-gated sodium channel gene.** *Med Vet Entomol* 2003, **17:**87–94.
14. Harris AF, Rajatileka S, Ranson H: **Pyrethroid resistance in *Aedes aegypti* from Grand Cayman.** *Am J Trop Med Hyg* 2010, **83:**277–284.
15. Martins AJ, Lima JB, Peixoto AA, Valle D: **Frequency of Val1016Ile mutation in the voltage-gated sodium channel gene of Aedes aegypti Brazilian populations.** *Trop Med Int Health* 2009, **14:**1351–1355.
16. Garcia GP, Flores AE, Fernandez-Salas I, Saavedra-Rodriguez K, Reyes-Solis G, *et al*: **Recent rapid rise of a permethrin knock down resistance allele in *Aedes aegypti* in Mexico.** *PLoS Negl Trop Dis* 2009, **3:**e531.
17. Marcombe S, Mathieu RB, Pocquet N, Riaz MA, Poupardin R, *et al*: **Insecticide resistance in the dengue vector *Aedes aegypti* from Martinique: distribution, mechanisms and relations with environmental factors.** *PLoS One* 2012, **7:**e30989.
18. Montella IR, Martins AJ, Viana-Medeiros PF, Lima JB, Braga IA, *et al*: **Insecticide resistance mechanisms of Brazilian *Aedes aegypti* populations from 2001 to 2004.** *Am J Trop Med Hyg* 2007, **77:**467–477.
19. Okimoto R, Dodgson JB: **Improved PCR amplification of multiple specific alleles (PAMSA) using internally mismatched primers.** *Biotechniques* 1996, **21:**20–22. 24, 26.
20. Germer S, Higuchi R: **Single-tube genotyping without oligonucleotide probes.** *Genome Res* 1996, **9:**72–78.
21. Wang J, Chuang K, Ahluwalia M, Patel S, Umblas N, *et al*: **High-throughput SNP genotyping by single-tube PCR with Tm-shift primers.** *Biotechniques* 2005, **39:**885–893.
22. Yanola J, Somboon P, Walton C, Nachaiwieng W, Somwang P, *et al*: **High-throughput assays for detection of the F1534C mutation in the voltage-gated sodium channel gene in permethrin-resistant *Aedes aegypti* and the distribution of this mutation throughout Thailand.** *Trop Med Int Health* 2011, **16:**501–509.
23. Raymond M, Rousset F: **Genepop (Version-1.2) - population-genetics software for exact tests and ecumenicism.** *J Hered* 1995, **86:**248–249.
24. Shorrocks B: *The Genesis of Diversity.* London: Hodder and Stoughton; 1978.
25. Donnelly MJ, Corbel V, Weetman D, Wilding CS, Williamson MS, *et al*: **Does kdr genotype predict insecticide-resistance phenotype in mosquitoes?** *Trends Parasitol* 2009, **25:**213–219.
26. Valle D, Montella IR, Medeiros PFV, Ribeiro RA, Martins AJ, *et al*: *Quantification methodology for enzyme activity related to insecticide resistance in Aedes aegypti.* Ministério da Saúde/Brasil: Brasília; 2006.
27. Brogdon WG, McAllister JC: **Simplification of adult mosquito bioassays through use of time-mortality determinations in glass bottles.** *J Am Mosq Control Assoc* 1998, **14:**159–164.
28. Bariami V, Jones CM, Poupardin R, Vontas J, Ranson H: **Gene amplification, ABC transporters and cytochrome P450s: unraveling the molecular basis of pyrethroid resistance in the dengue vector, *Aedes aegypti*.** *PLoS Negl Trop Dis* 2012, **6:**e1692.
29. Saavedra-Rodriguez K, Suarez AF, Salas IF, Strode C, Ranson H, *et al*: **Transcription of detoxification genes after permethrin selection in the mosquito *Aedes aegypti*.** *Insect Mol Biol* 2012, **21:**61–77.
30. Martins AJ, Ribeiro CD, Bellinato DF, Peixoto AA, Valle D, *et al*: **Effect of insecticide resistance on development, longevity and reproduction of field or laboratory selected *Aedes aegypti* populations.** *PLoS One* 2012, **7:**e31889.

31. Xu Q, Zhang L, Li T, Zhang L, He L, *et al*: **Evolutionary adaptation of the amino acid and codon usage of the mosquito sodium channel following insecticide selection in the field mosquitoes.** *PLoS One* 2012, **7:**e47609.
32. Martins AJ, Lins RM, Linss JG, Peixoto AA, Valle D: **Voltage-gated sodium channel polymorphism and metabolic resistance in pyrethroid-resistant *Aedes aegypti* from Brazil.** *Am J Trop Med Hyg* 2009, **81:**108–115.
33. Martins AJ, Brito LP, Linss JGB, Rivas GB, Machado R, *et al*: **Evidence for gene duplication in the voltage gated sodium channel gene of *Aedes aegypti*.** *EMPH* 2013:eoto12v1–eot012.
34. Du Y, Nomura Y, Satar G, Hu Z, Nauen R, *et al*: **Molecular evidence for dual pyrethroid-receptor sites on a mosquito sodium channel.** *Proc Natl Acad Sci USA* 2013:1305118110v1–1305118110v201305118.
35. Kawada H, Higa Y, Komagata O, Kasai S, Tomita T, *et al*: **Widespread distribution of a newly found point mutation in voltage-gated sodium channel in pyrethroid-resistant *Aedes aegypti* populations in Vietnam.** *PLoS Negl Trop Dis* 2009, **3:**e527.
36. Yanola J, Somboon P, Walton C, Nachaiwieng W, Prapanthadara LA: **A novel F1552/C1552 point mutation in the *Aedes aegypti* voltage-gated sodium channel gene associated with permethrin resistance.** *Pestic Biochem Physiol* 2010, **96:**127–131.
37. Hu Z, Du Y, Nomura Y, Dong K: **A sodium channel mutation identified in *Aedes aegypti* selectively reduces cockroach sodium channel sensitivity to type I, but not type II pyrethroids.** *Insect Biochem Mol Biol* 2011, **41:**9–13.
38. Chang C, Shen WK, Wang TT, Lin YH, Hsu EL, *et al*: **A novel amino acid substitution in a voltage-gated sodium channel is associated with knock-down resistance to permethrin in *Aedes aegypti*.** *Insect Biochem Mol Biol* 2009, **39:**272–278.
39. Brito LP, Linss JG, Lima-Camara TN, Belinato TA, Peixoto AA, *et al*: **Assessing the effects of *Aedes aegypti kdr* mutations on pyrethroid resistance and its fitness cost.** *PLoS One* 2013, **8:**e60878.
40. Zaim M, Aitio A, Nakashima N: **Safety of pyrethroid-treated mosquito nets.** *Med Vet Entomol* 2000, **14:**1–5.
41. Walker T, Johnson PH, Moreira LA, Iturbe-Ormaetxe I, Frentiu FD, *et al*: **The wMel Wolbachia strain blocks dengue and invades caged Aedes aegypti populations.** *Nature* 2011, **476:**450–453.
42. Harris AF, Nimmo D, McKemey AR, Kelly N, Scaife S, *et al*: **Field performance of engineered male mosquitoes.** *Nat Biotechnol* 2011, **29:**1034–1037.

doi:10.1186/1756-3305-7-25
Cite this article as: Linss *et al.*: Distribution and dissemination of the Val1016Ile and Phe1534Cys *Kdr* mutations in *Aedes aegypti* Brazilian natural populations. *Parasites & Vectors* 2014 **7**:25.

Capítulo II:

Estimativas de densidade populacional e sobrevivência diária de *Aedes aegypti* em experimentos de marcação soltura e recaptura (MSR).

Justificativa

A capacidade vetorial de *Ae. aegypti* engloba diversos parâmetros relacionados à transmissão de patógenos em campo. Dentre estes, a abundância e a sobrevivência de mosquitos são relevantes, pois apresentam uma intensa correlação com o curso temporal e espacial de uma epidemia. Ambos os parâmetros podem ser estimados por meio de experimentos de marcação, soltura e recaptura (MSR), que auxiliam a obter um melhor entendimento sobre a dinâmica da transmissão de patógenos em campo, bem como para o planejamento e direcionamento de atividades de controle.

Além disso, estimar a abundância de *Ae. aegypti* também é importante em estratégias que utilizam a liberação de insetos em campo, como no controle biológico que usa a bactéria *Wolbachia* e mosquitos transgênicos. Nestes casos, realizar uma estimativa prévia da densidade populacional na área, antes da liberação em campo, norteia o cálculo de mosquitos a liberar, evitando assim, liberar um número inadequado de indivíduos para se atingir o objetivo.

Artigo 4

PLOS | ONE

RESEARCH ARTICLE

A Bayesian Hierarchical Model for Estimation of Abundance and Spatial Density of *Aedes aegypti*

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OPEN ACCESS

Citation: Villela DAM, Codeço CT, Figueiredo F, Garcia GA, Maciel-de-Freitas R, Struchiner CJ (2015) A Bayesian Hierarchical Model for Estimation of Abundance and Spatial Density of *Aedes aegypti*. PLoS ONE 10(4): e0123794. doi:10.1371/journal.pone.0123794

Academic Editor: Olle Terenius, Swedish University of Agricultural Sciences, SWEDEN

Received: October 13, 2014

Accepted: March 6, 2015

Published: April 23, 2015

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This study was partially supported by grants received from the Foundation for the National Institutes of Health through the Grand Challenges in Global Health initiative of the Bill and Melinda Gates Foundation, from CNPq–Brazil (PRONEX Dengue 550030/2010-7) and from FAPERJ (Rede Dengue–E26-110.977/2011). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Strategies to minimize dengue transmission commonly rely on vector control, which aims to maintain *Ae. aegypti* density below a theoretical threshold. Mosquito abundance is traditionally estimated from mark-release-recapture (MRR) experiments, which lack proper analysis regarding accurate vector spatial distribution and population density. Recently proposed strategies to control vector-borne diseases involve replacing the susceptible wild population by genetically modified individuals' refractory to the infection by the pathogen. Accurate measurements of mosquito abundance in time and space are required to optimize the success of such interventions. In this paper, we present a hierarchical probabilistic model for the estimation of population abundance and spatial distribution from typical mosquito MRR experiments, with direct application to the planning of these new control strategies. We perform a Bayesian analysis using the model and data from two MRR experiments performed in a neighborhood of Rio de Janeiro, Brazil, during both low- and high-dengue transmission seasons. The hierarchical model indicates that mosquito spatial distribution is clustered during the winter (0.99 mosquitoes/premise 95% CI: 0.80–1.23) and more homogeneous during the high abundance period (5.2 mosquitoes/premise 95% CI: 4.3–5.9). The hierarchical model also performed better than the commonly used Fisher-Ford's method, when using simulated data. The proposed model provides a formal treatment of the sources of uncertainty associated with the estimation of mosquito abundance imposed by the sampling design. Our approach is useful in strategies such as population suppression or the displacement of wild vector populations by refractory *Wolbachia*-infected mosquitoes, since the invasion dynamics have been shown to follow threshold conditions dictated by mosquito abundance. The presence of spatially distributed abundance hotspots is also formally addressed under this modeling framework and its knowledge deemed crucial to predict the fate of transmission control strategies based on the replacement of vector populations.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

Dengue fever is the most prevalent arbovirus infection in the world with 2.5 billion people living in areas under the risk of transmission [1]. The temporal and spatial pattern of dengue distribution is influenced by mosquito, human, viral and environmental factors. The abundance, survival and dispersal of its vector, the *Aedes aegypti* mosquito, are important factors to describe the ecology involving the mosquito. In particular, spatial distributions of mosquito populations at fine scales [2, 3] can help understand the impact on dengue transmission. Estimation of parameters that describe these components is still a complex challenge for field entomologists and modelers [4–7].

Knowledge about these key components is crucial [8] in guiding vector control strategies based on population suppression in areas at transmission risk. Several dengue endemic countries, including Brazil, plan their vector control strategies based on the assessment of infestation indices through larval surveys, most commonly House and Breteau Indices (HI and BI, respectively) [9, 10]. These traditional infestation indices show low correlation with adult mosquito abundance, as HI and BI do not consider container productivity and larval mortality [10, 11]. Alternatively, trapping of adults with a variety of devices has been proposed as a more efficient approach to monitor *Aedes aegypti* populations and many initiatives are already in place worldwide. Traps allow the development of more standardized protocols, provide indices faster and require less effort than the traditional searching approach. One drawback of a trap based infestation index, however, is that it is a relative measure of population density, with unit mosquito/trap. For comparative purposes, this may suffice. But there are situations when absolute measures of population abundance (unit: mosquitoes/area or mosquitoes/person) are of interest. For example, vector thresholds for transmission are defined in terms of mosquito/person [8]; population thresholds for *Aedes aegypti* + *Wolbachia* invasion is defined in mosquito/area [12, 13].

Traditionally, the estimation of animal population size is performed via experiments of mark, release and recapture (MRR). In MRR experiments, subjects are typically captured from the environment, marked either uniquely or as a cohort using a piece of identification (tags, colors etc.), released back into the environment and later recaptured, possibly multiple times. For this kind of experiment, mosquitoes are challenging subjects due to their small size and short life span, making recapturing difficult, which led to important modifications of the standard protocols, mainly, that marked mosquitoes are released and subsequently recaptured only once [7]. Models for estimating adult population size from MRR datasets, either deterministic or stochastic ones, include Lincoln, Jolly-Seber, and Fisher and Ford [6, 7, 14–16]. The Lincoln Index, due to its simplicity, is often the method of choice. However this simplicity comes at a price, since the strong assumptions required for the proper utilization of this index are difficult to meet in most field conditions, for example, that the population is closed and that there is no heterogeneity in capture rates. The Fisher—Ford method relaxes some of these assumptions allowing the loss of individuals by mortality. These models are not probabilistic and do not treat sampling uncertainty properly. The Jolly-Seber family of models is very popular due to their probabilistic framework, however they are tailored for data with multiple recaptures and fitting with typical mosquito data does not show convergence (results not shown). In this work we investigate the potential advantages to analyze MRR mosquito trials of a class of stochastic models containing an ecological component introduced by Royle *et al.* [17]. Stochastic models involve more complex mathematical calculations, but generate estimates of population size along with measures of uncertainty [7]. The model presented here was developed upon the basic structure of the model of Royle *et al.* [17] to accommodate particular aspects of the mosquito ecology and its observation. We perform Bayesian analysis using this model to estimate

abundance and spatial distribution of the population of female *Aedes aegypti* in a dengue endemic area in the city of Rio de Janeiro, Brazil. The proposed model is also tested with artificial data from a simulator of typical mosquitoes' MRR experiments.

In the next section we describe the MRR experiments carried out in the study area in Rio de Janeiro, Brazil. The description of the experiments is helpful here as it illustrates the particular aspects of a typical MRR dataset for mosquitoes. We then describe the structure of the hierarchical model, its layered components and the simulation environment. We present estimates of abundance, spatial density and survival probability of *Aedes aegypti* in the study area comparing the estimates from the proposed hierarchical model with those from the Fisher—Ford method. Subsequently, we show some statistical properties of the proposed model obtained from its application to simulated data.

Methods

Mark—Release—Recapture Experiments

Study area. We conducted mark-release-recapture studies in a 7.2 ha neighborhood called Z—10, located in the Governador Island, city of Rio de Janeiro, Brazil. Z—10 (22°52′30″*S*; 43°14′53″*W*) is an isolated suburban community, surrounded by shores and mangroves, which discourage mosquito migration through its limits. This residential area has paved streets, regular sidewalks and low-moderate vegetation coverage, with around 2,350 people living in 787 houses. Most houses have 2–3 bedrooms, regular water supply and garbage collection, lacking peridomestic areas with pools or yards.

Climate and MRR periodicity. The climate in Rio de Janeiro is characterized by a moderately dry winter (May-August) and a wet summer season (November—March), with mean temperatures of 25.1°C and 28.8°C and mean total rainfall of 46.4 mm and 132 mm, respectively. MRR experiments were conducted during nine consecutive days in September 2012 and during another period of nine days in March 2013. In Sep/2012, temperatures ranged between 22.3°C and 30.6°C (average of 28.6°C) with no rainfall. In Mar/2013, temperatures ranged from 23.1°C to 32.5°C, with an average of 29.7°C and rainfall of 0.4 mm. Air temperature and precipitation data were obtained from a meteorological station located at approximately 5 km away from the study area.

Mosquitoes. *Aedes aegypti* adults used in MRR experiments were derived from the F1 generation of eggs collected at Z—10 using 60 ovitraps filled with hay infusion. Larvae were fed with fish food (Tetramin, Tetra Sales, Blacksburg, VA) and reared according to Consoli and Lourenço-de-Oliveira [18]. After emergence, females were kept together at 25±3°C and 65±5% relative humidity (RH) and fed with sucrose solution until the time to release.

Marking and releasing. Before releasing in Sept/2012, *Ae. aegypti* adults were split into four cohorts, each one composed of 500 females marked with different colors of fluorescent dust (Day-Glo Color Corp., Cleveland, OH) and placed in small cylindrical cups (12 cm x 10 cm). Each cohort was released from a different outdoor location in Z—10. In Mar/2013, only one cohort composed of 2000 females was released in a central outdoor location of Z—10. In both occasions, mated, unfed females (4—day old) were released in the morning hours (between 8:00 AM and 9:00 AM), at approximately 1 hour after dust marking. Wind direction and speed was 139.7° and 4.7 m/s in the moment of release in Sep/2012. Meanwhile, in Mar/2013, wind direction and speed was 25.1° and 2.6 m/s.

Capturing. Dust-marked females were captured using 66 uniformly distributed BG-Sentinels traps (BioGents, Regensburg, Germany), designed to attract mosquitoes seeking a host to blood feed [19, 20]. Captures started at the following day after mosquitoes' release and was performed daily by inspection of the 66 BGs-Traps. Daily capturing stopped when no dust-

marked females were collected for 3 consecutive days. Captured mosquitoes were examined under UV light to check for the presence of fluorescent dust.

Ethics Statement. Fiocruz conducts regular research activities in the city of Rio de Janeiro, in partnership and also with permission of the Rio de Janeiro Department of Health (*Secretaria de Saúde da cidade do Rio de Janeiro*) that assist in the control of transmission of infectious diseases. Mark-release-recapture experiments were approved by Fiocruz Ethical Committee (CEP 253/04) and carried out as part of this research effort, not requiring a specific permission. Since mosquitoes are released in public open spaces such as squares, there was no need to obtain individual consent for releases. In order to recapture marked mosquitoes, inside people's dwellings, we have to obtain consent from residents to install BG-Traps and also to carry daily collections after the release of marked individuals. The use of mosquito traps for sampling *Aedes aegypti* belongs to the routine of vector surveillance in Rio de Janeiro. Due to this surveillance routine, many residents in the study area are used to have mosquito traps installed in their houses and, indeed, there were just few refusals by residents to have traps installed. Written consent was not required under the ethical committee requirements. As the effort to collect mosquitoes goes along with city surveillance, these tasks are typically done with verbal consent only and our collection effort also had verbal consent, as approved by the ethics committee, and individual consents were registered by the entomology field team in regular work files.

The release of mosquitoes does not involve directly endangered or protected species and, from our experience, it does not have any significant impact on endangered or protected species. One of our main concerns during MRR experiments is related to dengue transmission by the mosquitoes released in the field. In order to address this issue, we follow two guidelines. The first one is to not release a number of mosquitoes greater than the number of mosquitoes removed by trapping in pilot experiments and monitoring activities. Second, the procedure is to interrupt or to suspend, depending on circumstances, mosquito releases if dengue cases are notified in that neighborhood. Since both trap inspection and mosquito collection are done with the assistance of the health department in the city government, i.e., the same agency that is responsible for dengue notification, dengue cases are rapidly identified.

Hierarchical Model

The proposed model has three components. The first component is a probabilistic model describing the spatial distribution of mosquitoes in the study area, while the second component is also a probabilistic model describing the daily survival of marked and native mosquitoes. Finally, the observation model describes the sampling process.

Ecological process—center of activities. There is some evidence that mosquitoes tend to remain close to their birth location if conditions for blood feeding and ovipositing are adequate. Specialists typically agree that *Ae. aegypti* females rarely visit more than 2–3 houses during their lifetime leading to a strong spatial correlation between the distribution of immature and adults [21]. Harrington *et al.* [4] reviewed several MRR experiments carried out in different locations, different release sites (both indoors and outdoors) and experimental protocols. Overall, these studies agree on the limited dispersal behavior of *Aedes aegypti*. When release is done indoors, most of the collections occur in the same house [22, 23]. This tendency to remain in the same location (at least during the length of the experiment) can be formalized by the concept of center of activity. An individual center of activity is a central point (centroid) of the space occupied by the individual during a time interval [24]. This concept is borrowed from the works of Royle *et al.* [17, 25], in which they model the movement of tigers [17] and birds [25].

Here, there are two important claims. First, we assume that for the duration of the MRR experiments spatial density of mosquitoes can be described by the density of activity centers, as mosquitoes tend to stay within a constrained space. This is reasonable for both marked and unmarked individuals given the empirical observations as described above. Second, the marked mosquitoes are released and go through a dispersal phase. Here, we have evidence that the dispersal is fast and subsequent captures of individuals will find them already in the whereabouts of their center of activities. In the MRR experiment studied here, marked cohorts were released outdoors, in locations that stimulate dispersal towards more suitable habitats. Under stress mosquitoes can fly more than 600 m for three days to find suitable conditions [26]. Evidence for the ability to reach the whole area comes from the observation that in the first day of capturing, marked mosquitoes were already found in the most distant traps. Therefore, we distinguish between the short distance movements close to centers of activity and the long range movements realized when dispersal happens.

In the model the center of activity is given by a pair $\mathbf{s} = (s_x, s_y)$ of coordinates that describe the center of an individual area. The prior distribution for these pairs of coordinates is generally a uniform distribution. After a procedure of inference, given the observations in the MRR experiments, the distribution of center of activities is effectively evaluated as a spatial density distribution within the study area.

Ecological process—survival. *Aedes aegypti* life span ranges from 5 to 30 days [4, 27–31], making survival an important process to be considered in an experiment that lasts for 9 days. In the capture data (shown in the Results section), there is clearly a loss of marked individuals as days go by. This loss can be attributed to mortality by natural causes. In the hierarchical model, there is a component that describes daily survival probability ϕ, assumed equal across all marked individuals. For the unmarked individuals, however, under an assumption that abundance stays at a stable level for the relatively short length of an MRR study, we consider that recruitment cancels out mortality, *i.e.*, in terms of the model, $\phi = 1$ for unmarked individuals.

Observation process. In the model each trap has associated with it a probability of capturing mosquitoes as a function of the distance between the trap location, recorded in the experiment, and any point in the study area. Hence, each trap has a probability of capturing the mosquito as function of the distance between the trap location and its center of activity. Following the approach in [17], the probability to be attracted to a trap is described by a function that has parameters to be estimated as a *Generalized Linear Model* (GLM). We choose the complementary log—log function as a link function, thus we have for the probability $\pi_{i,j}$ of individual i be attracted to trap j, $cloglog(\pi_{i,j})|\mathbf{s_i} \sim \beta_0 + \beta_1 d_{i,j}$, where $d_{i,j}$ is the distance from individual i's center of activity and trap j's location, and β_0 and β_1 are parameters to be estimated.

The observation model takes into account that each individual can only be trapped once. This limitation impacts the model formulation in two main aspects. First, at each observation time point (intervals given in days and total time T) the probability of an individual being captured at a particular trap is a product of two factors: the probability $\pi_{i,j}$ of individual i to be captured at trap j, given by a function of the distance from its center of activity to the trap, and the probability to be captured at trap j, given by a categorical random variable with parameter vector given by the ratio $\frac{\pi_j}{\sum_{j=1}^{J} \pi_{i,j}}$. Once a marked individual is captured, it is removed from the study. Therefore, it cannot be observed again. In the model, removal is introduced in the survival component as a variable that indicates presence in the study. For each individual, this presence depends on its survival and also on the individual not being captured. Captures of mosquitoes at traps are described by the observation variables $y_{i,j,t}$, indicating whether a mosquito i is observed ($y_{i,j,t} = 1$) or not ($y_{i,j,t} = 0$) at trap j, $j \leq J$, at time t, $t \leq T$.

We want to estimate the number N_2 of unmarked mosquitoes in the study area using the hierarchical model in which each individual is indexed, whereas the number N_1 of marked mosquitoes released in the study is known. Without any loss of generality, the first N_1 individuals are the marked ones. We use the data augmentation technique [32] as used by Royle and Dorazio in [33] to estimate the number of unmarked mosquitoes N_2. The technique involves taking a number $M > N_1 + N_2$ by adding non-existing individuals, since we do not know N_2. The non-existing individuals will not be present in the study and will not be captured (zero values in observation). This requires another layer in the model to treat the zero–inflated component, that distinguishes zeroes from zero–inflation to zeroes due to non-observations. This component uses a set of variables w_i, $1 \leq i \leq M$, $M > N_1 + N_2$, each of which describes whether a particular individual i is effectively in the study population (either marked or unmarked population). Finally, this technique permits us to have abundance as an indirect measure obtained from an inference procedure.

The model is described in detail in S1 Text, including a table that describes the model and each of the components.

Estimation and Bayesian Inference

Inference is performed via analysis of Monte–Carlo Markov Chain (MCMC) simulations. The abundance, *i.e.*, the number N_2 of unmarked individuals is estimated as a latent variable:

$$N_2 = \sum_{i=N_1+1}^{M} w_i. \tag{1}$$

Since the estimation of variables is performed using multiple runs of MCMC simulations, results are generally given by statistical measures that include mean, median, standard deviation and credibility intervals.

The spatial density of mosquitoes is found using the posterior samples from MCMC simulation and constructing a grid over the study area. The count of individuals in each of the grid cells is found by the sum of center of activities inside each of them, similar to counts performed by Royle *et al.* in [17].

In order to find estimates, we use JAGS [34], a Bayesian analysis tool that implements MCMC through Gibbs sampling and uses a model specification very similar to another popular tool, WinBUGS. The JAGS model is included in S1 Text. The prior distribution for survival probability ϕ, defined in the [0, 1] interval, places higher weight on values closer to one, while also weakening extreme values in the [0, 1]—interval. This distribution is in accordance with empirical estimates of the survival rates of *Aedes aegypti* [35].

Fisher—Ford method. We also find estimates of abundance using Fisher—Ford method [36], which has been used in MRR analyses and is closely related to the Lincoln index (MRR on estimating abundance of *Aedes albopictus* by Cianci *et al.* [6] and *Anopheles gambiae* by Baber *et al.* [37]). The abundance estimator derives from the same argument of the Lincoln index, including an adjustment that takes into account the survival probability ϕ for marked individuals:

$$\hat{N}_2 = \frac{n_{tot}\phi^t N_1}{m_{tot}} - \phi^t N_1,$$

where n_{tot} and m_{tot} are, respectively, the numbers of unmarked and marked individuals, captured at time t.

Bailey in [38] propose a bias correction in the case of small number of captures (typically below 20): $\hat{N}_2 = \frac{(n_{tot}+1)\phi^t(N_1+1)}{m_{tot}+1} - \phi^t(N_1+1)$. We take a bootstrap approach in order to find a confidence interval for the Fisher—Ford index, using a number R_s of re–sampling.

Simulation. To assess the efficiency of the MRR experiment in various scenarios, an Individual-based model (IBM) was developed to simulate the dispersal and capture of marked mosquito cohorts, as a computational tool, written in the Perl programming language, that mimics the field experiments. Using this type of simulation environment it is possible to observe actual emergent phenomena from simple rules and it has been gaining attention in theoretical ecology since the last two decades [39–41].

The simulator is implemented by constructing classes that define objects for each agent, including agents for mosquitoes and traps, and it also defines a square area for the environment. For each mosquito, dispersal can be considered either random or towards a randomly chosen center of activity (sampled from a uniform distribution). Traps can be positioned at random locations, or at points comprising a centralized grid. In simulations we have the possibility of varying the area around which the trap attracts mosquitoes and also possibility to define the probability that describes a Bernoulli random variable to indicate mosquito capture at traps.

The output of the simulator is given by tables discriminating trap and mosquito data as well as a capture history file typical for MRR analysis programs, such as the program MARK [42]. Full descriptions for the objects, data structures and validation are provided in S2 Text. The code (open-source license—GNU PGL v3) is available from https://launchpad.net/mmrrsim.

Results

Field data from MRR experiments

Table 1 shows the number of daily captures observed in each of the mark–release–recapture experiments, conducted in Sept 2012 (ST1) and March 2013 (ST2). Data are shown separated by each of the cohorts (marking color) and the number of captures of unmarked mosquitoes is also shown. The counts of marked individuals generally decrease over time. For marked individuals the capture ratio is visibly higher in the days immediately subsequent to releasing in the field and has a decreasing trend along with time. The number of captures of unmarked individuals, however, tends to remain at a constant level. For the experiment in Sept. 2012, the ratio between total number of captures per number of released individuals varied from 6% (green cohort) to 13.4% (blue cohort), and including all cohorts the return capture ratio is 9.2%. For

Table 1. Numbers on captures obtained from the two MRR experiments conducted in the Z—10 region in Rio de Janeiro, Brazil. ST1 refers to the study realized in Sept. 2012 and ST2 to the study realized in March 2013. For ST1, we present data on each of the four cohorts as indicated by the marked colors. For each of the studies, unmarked refers to the number of unmarked individuals captured.

Cohort	Num. Released	Day1	Day2	Day3	Day4	Day5	Day6	Day7	Day8	Total	Ratio
ST1–b	500	19	14	7	9	9	4	3	2	67	0.134
ST1–p	500	27	11	4	2	1	2	5	0	52	0.104
ST1–y	500	10	10	3	4	3	1	1	3	35	0.07
ST1–g	500	9	7	3	5	4	0	0	2	30	0.06
ST1–unmarked	–	15	22	12	17	36	14	25	21	162	–
ST2–b	2000	52	26	20	23	4	5	13	8	151	0.076
ST2–unmarked	–	119	92	95	64	93	114	110	99	786	–

doi:10.1371/journal.pone.0123794.t001

Table 2. Estimates of abundance for the populations of female *Aedes aegypti* in Z—10, Rio de Janeiro, in Sept 2012 (ST1) and March 2013 (ST2), according to the hierarchical model and the Fisher-Ford model. Estimation realized using samples from 16000 iterations using data from both studies. The hierarchical model results are obtained using JAGS after running 10000 iterations per chain (first 2000 were discarded) in two Markov chain simulations. Fisher—Ford estimates are obtained using a bootstrap approach (re-sampling $R_s = 1000$). For the Fisher—Ford estimation the survival rate is assumed to be $\phi = 0.8$.

	Hierarchical Model				Fisher—Ford	
Cohort	Mean	Std. dev.	Median	95% CI	Mean	95% CI
ST1—b	667	63	669	548–783	467	392–592
ST1—b+p	660	83	652	520–851	670	480–920
ST1—b+p+y	743	83	738	593–914	730	607–977
ST1—b+p+y+g	782	84	778	633–966	822	666–996
ST2	4118	319	4142	3423–4666	4768	3185–9198

doi:10.1371/journal.pone.0123794.t002

the experiment in March 2013, the capture ratio is 7.6%. Datasets are available as supporting information (S1 Dataset and S2 Dataset).

In September 2012, we also measured size of the female wings from both populations (lab–reared and wild ones). Lab-reared *Ae. aegypti* females had 2.92 mm ± 0.085 mm (mean ± SD), meanwhile wild mosquitoes in this period had 2.74 mm ± 0.12 mm.

Analysis of field data

To study the impact of increasing sample size on the abundance estimation, data from ST1 was pooled by using a separate cohort, defined by the marking color, or a combination of cohorts: cohort b ($N_1 = 500$), cohorts b+p ($N_1 = 1000$), cohorts b+p+y ($N_1 = 1500$) and cohorts b+ p+ y +g ($N_1 = 2000$).

Table 2 summarizes the pooled data and provides the abundance estimates found using the hierarchical model and the Fisher—Ford method. Fig 1 and Table 2 show the *Aedes aegypti* abundance estimates, according to both models.

Analysis using the hierarchical model provides an abundance estimate of approximately 700 female mosquitoes in Z—10 in September 2012, during the low transmission season, with point estimates ranging from 660 to 782, depending on the pooled data. The 95% credible interval varied from 548 to 966 and was the narrowest when the unpooled data (ST1-b) was used, and the widest of all when all cohorts were analyzed together. This might be due to the lowest overall capture ratio when pooling all four cohorts (about 9%) and also due to variation between cohorts. In March 2013 (ST2), during the high transmission season, the estimated abundance of *Aedes aegypti* was four times higher than in the low transmission period, with approximately 4100 female mosquitoes in Z—10. The credible interval (3423 – 4666) is wider than in the low abundance period, but the coefficient of variation is smaller (0.30 for ST2; 0.35 for ST1-b and 0.42 for ST1-b+p+y+g.).

The Fisher—Ford model tended to agree with the hierarchical model (Table 2 and Fig 1). The exception is for the ST1-b data, where the Fisher—Ford point estimate of mosquito abundance is considerably underestimated compared to the other method. Overall, the hierarchical model produced more precise estimates than the ones produced using Fisher—Ford method. This is evident observing the high abundance data (ST2) where the Fisher—Ford confidence interval is 5 times greater than the hierarchical model.

Analysis using the hierarchical model provides an estimate of the spatial distribution of mosquitoes that is not possible using the Fisher—Ford method. Figs 2 and 3 show the

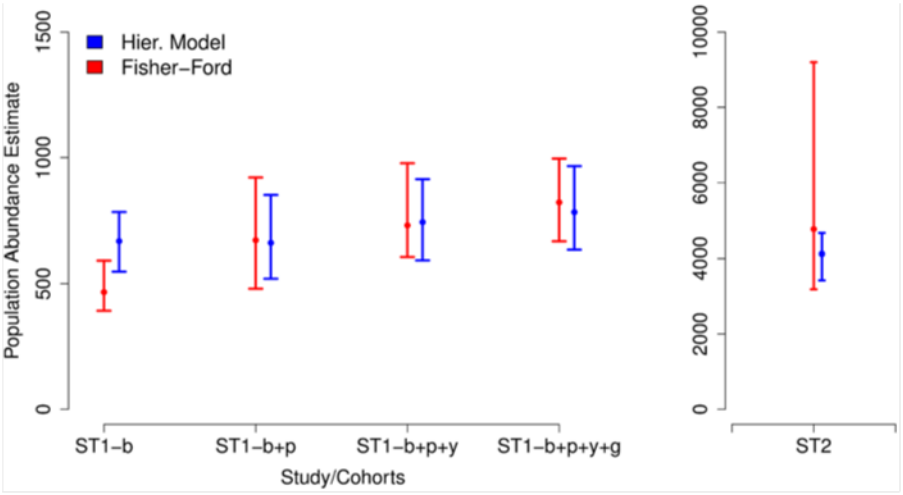

Fig 1. Results from estimation using data from field studies. Intervals colored in solid red indicate Fisher—Ford estimates. Intervals colored in solid blue indicate estimates from the hierarchical model. Labels ST1 and ST2 indicate studies conducted in September 2012 and March 2013, respectively. The September 2012 study had 4 cohorts given by 4 different colors: blue (b), pink (p), yellow (y) and green (g). Labels ST1 also include which cohorts were used in the analysis by grouping cohorts ({b}, {b+p}, {b+p+y}, { b+p+y+g}). Estimates of population abundance are shown along with both *y*–axes (different scales), the one on the left side for study ST1 and on the right side for study ST2.

doi:10.1371/journal.pone.0123794.g001

estimated spatial distribution of *Aedes aegypti* in Z—10 in Sept. 2012 (ST1) and March 2013 (ST2), respectively. To facilitate interpretation Figs 2 and 3 have on the left–hand side images of the study area superimposed with a bubble representation of the capture counts per trap and on the right–hand side the posterior spatial density according to the hierarchical model. First, the difference between scales should be noted. In September, local mosquito density peaks at 1.42 mosquitoes per 100 m^2 while in March, the ceiling is at 5.4 mosquitoes per 100 m^2. During the high abundance period, mosquito abundance is concentrated in the center—south region.

The survival probabilities for the marked individuals, as estimated by analysis from the hierarchical model, are shown in Table 3. Estimates (mean values) of survival probabilities are expectedly higher for cohort combinations whose capture ratios are also higher.

The posterior distributions of abundance and survival probability after analysis of data from both experiments are found in S1 Text.

Analysis of simulation data

To further compare and understand the behavior of the hierarchical and Fisher—Ford estimates, artificial data from simulated scenarios were analyzed. Table 4 and Fig 4 shows the results obtained from simulations done by varying the population size of marked and unmarked individuals *i.e.*, (N_1, N_2): (200, 300), (300, 500), (400, 700), (500, 900), (2000, 3000). Besides the population size, we also compared scenarios with two attraction areas, defined as the radial spatial coverage of each trap. The larger the attraction area, the more attractive the trap is and the higher the chance of capturing mosquitoes. This is a parameter that is very difficult to measure in the field as it depends on the microenvironment and on the trap features. All simulations were done using the concept of center of activity (AC mode in simulator). Table 4 shows the number of captures and the mean and other statistics of the estimated abundance for each simulated scenario. Fig 4 shows the results graphically. In all scenarios, the credible intervals

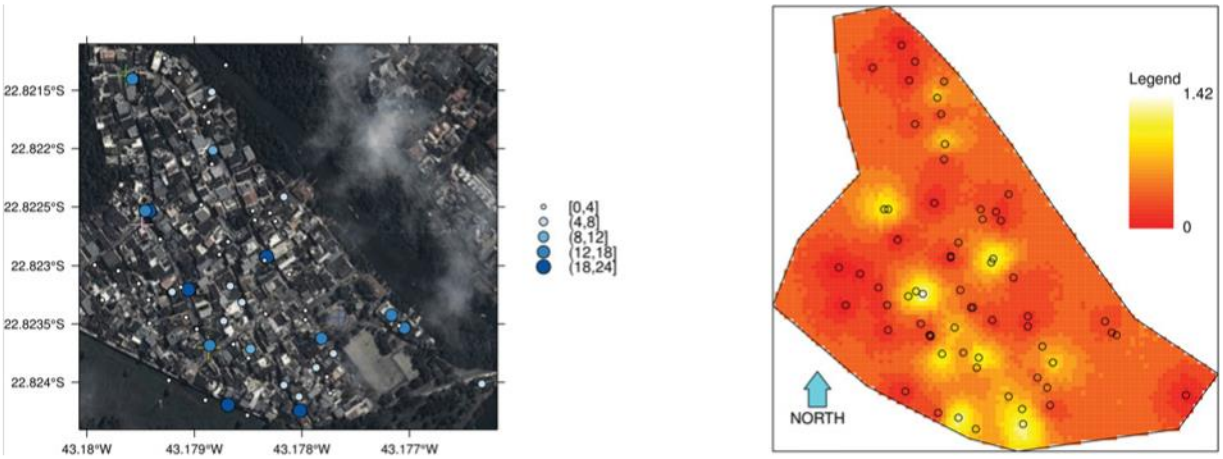

Fig 2. Spatial distribution of individuals in the Z—10 area in Rio de Janeiro, Brazil, in September 2012. Results for ST1 (Sept 2012) obtained with 16,000 iterations (8,000 iterations in each of two chains after a 2,000 burn-in period). Results from analysis using all cohort of marked individuals. Circles indicate trap locations. Bubbles as shown in the maps on the left–hand side indicate the counts of mosquitoes trapped in the MRR experiments. The release points of marked mosquitoes are depicted by crosses, each of which appear in the color of its respective cohort. The spatial density unit is number of mosquitoes per 100 m^2.

doi:10.1371/journal.pone.0123794.g002

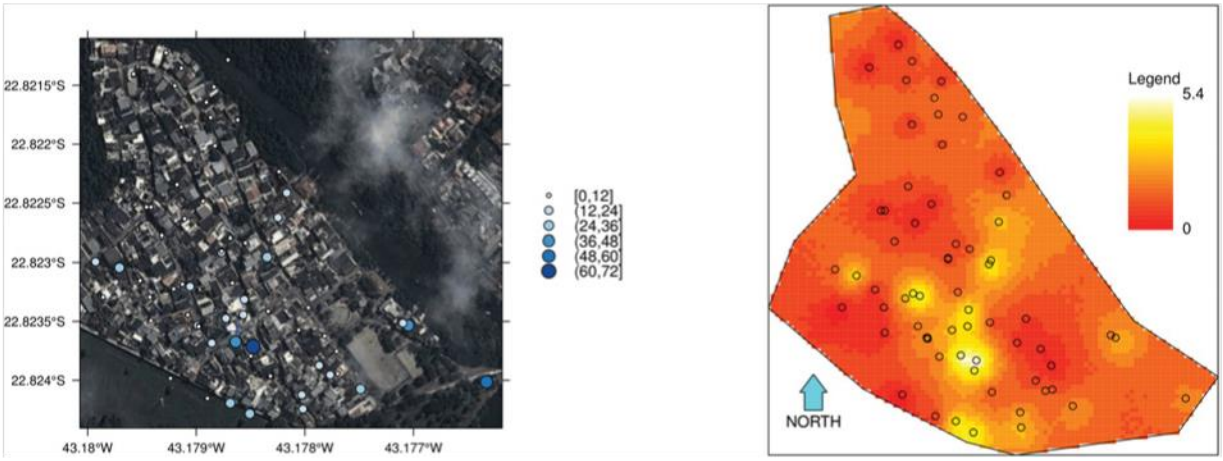

Fig 3. Spatial distribution of individuals in the Z—10 area in Rio de Janeiro, Brazil, in March 2013. Results obtained with 20,000 iterations (10,000 iterations in each of two chains after a 10,000 burn-in period). Results from analysis using the cohort of individuals marked in blue. Circles indicate trap locations. Bubbles as shown in the maps on the left–hand side indicate the counts of mosquitoes trapped in the MRR experiments. A cross indicates the point of releases of marked mosquitoes. The spatial density unit is number of mosquitoes per 100 m^2.

doi:10.1371/journal.pone.0123794.g003

Table 3. Estimates of survival probability for marked population of *Ae. aegypti* released in Z—10, Rio de Janeiro, according to the hierarchical model. Estimation realized using samples from 16000 iterations using data from both studies. The hierarchical model results are obtained using JAGS after running 10000 iterations per chain (first 2000 were discarded) in two Markov chain simulations. Fisher—Ford estimates are obtained using a bootstrap approach (re-sampling R_s = 1000).

	Hierarchical Model			
Cohort	Mean	Std. dev.	Median	95% CI
ST1—b	0.82	0.03	0.82	0.75–0.89
ST1—b + p	0.77	0.03	0.76	0.70–0.83
ST1—b + p + y	0.76	0.03	0.76	0.71–0.82
ST1—b + p + y + g	0.75	0.03	0.76	0.71–0.80
ST2	0.80	0.02	0.80	0.75–0.84

doi:10.1371/journal.pone.0123794.t003

produced from the hierarchical model included the true value. The same was not true for the Fisher—Ford method, which tended to underestimate the true abundance. For the case of a larger basin attraction area the actual numbers N_2 of unmarked mosquitoes are within the intervals given by the hierarchical model, even though the capture ratio of marked individuals is small, approximately 10%. The estimated values found for survival probability and posterior distributions of abundance are found in S1 Text.

Discussion

Recently proposed strategies [43] to control vector-borne diseases could either reduce the number of individual vector mosquitoes (population suppression) or reduce the competence of these vectors in transmitting the pathogen by replacing the susceptible wild population by genetically modified individuals refractory to the infection by the pathogen (population

Table 4. Abundance estimation in simulated scenarios, using the hierarchical and the Fisher-Ford models. The total number of iterations was 12000 for each of 2 Markov chains. The first 3000 iterations are discarded as burn-in interval. The area of attraction is of size 5 (b5) and also 8 (b8), probability p = 0.5. The number of traps is J = 64. For the Fisher—Ford estimates a number of R_s = 1000 re-sampling was used and the daily survival probability was ϕ = 0.8, same value used in the simulations. For the case (200, 300) with a small attraction area the sample size (2) is very small and no Fisher—Ford estimates are reported. The notation (m,u) refers to the quantity (number of captures, capture ratios) for marked and unmarked mosquitoes, respectively.

Simulated scenarios			Hierarchical Model				Fisher—Ford	
simulation	# captures (m,u)	capture ratio	Mean	Std. dev.	Median	95% CI	Mean	95% CI
b5-h / (200, 300)	(5, 29)	(0.03, 0.10)	323	85	329	152–470	–	–
b5-h / (300, 500)	(14, 48)	(0.05, 0.10)	386	128	372	169–642	217	152–332
b5-h / (400, 700)	(17, 74)	(0.04, 0.11)	583	176	566	295–953	435	246–552
b5-h / (500, 900)	(21, 85)	(0.04, 0.09)	705	185	686	414–1116	473	350–658
b8-h / (200, 300)	(17, 64)	(0.09, 0.21)	363	88	360	202–546	199	124–316
b8-h / (300, 500)	(36, 104)	(0.12, 0.21)	501	109	491	322–736	263	172–339
b8-h / (400, 700)	(41, 167)	(0.10, 0.24)	963	164	973	612–1234	539	328–811
b8-h / (500, 900)	(46, 205)	(0.09, 0.23)	818	166	790	570–1237	731	546–961
b8-h / (2000, 3000)	(186, 645)	(0.09, 0.21)	2780	286	2755	2229–3399	2895	2253–4961

doi:10.1371/journal.pone.0123794.t004

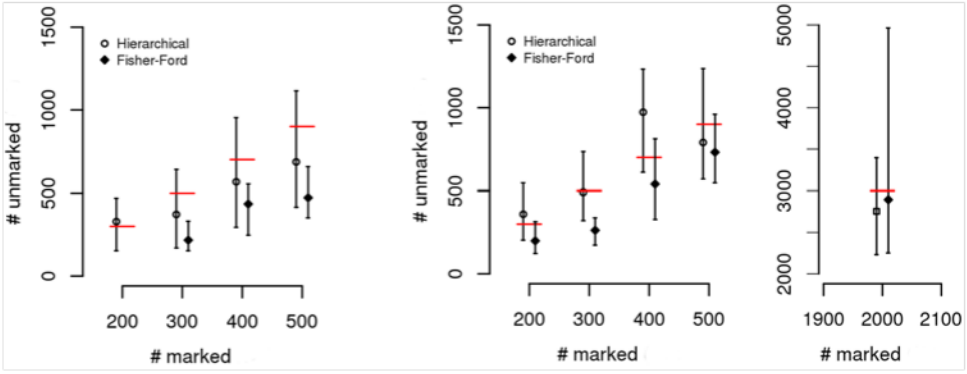

Fig 4. Estimates obtained from MRR data in the simulated scenarios. The estimated values for abundance are shown along the *y*–axis (for all cases in the *y*–axis on the left–hand side, except for the case $N_2 = 3000$ shown along the *y*–axis on the right–hand side), whereas each case is shown along the *x*-axis, described by the number (known value of) of unmarked individuals used in the simulation. The plot on the left–hand side shows results for a small basin of attraction, whereas on the right–hand side results are shown for traps' basins of attraction that have radiuses 60% greater. Red lines indicate the numbers of unmarked mosquitoes that were used in the simulations for each of the configurations.

doi:10.1371/journal.pone.0123794.g004

replacement). Evaluation of the potential success of such interventions requires detailed knowledge of the dynamics of the mosquito population, including its abundance in time and space [8]. In this paper, we present a hierarchical probabilistic model for the estimation of female *Aedes aegypti* abundance from MRR studies. The knowledge of mosquito density from estimation can help in control strategies, at least at a planning phase. The model performs well under simulation conditions and provides more precise estimates than the ones given by the Fisher—Ford method. The proposed model expands previous contributions by Royle *et al.* [17] in adding a survival component and a trap component that models the life span of individuals during MRR experiments and the trapping limitations that make difficult multiple capture events for each of the study individuals. We apply the concept of centers of activity present in [17, 25] to the distribution of a mosquito population within a given area. Clusters of human dengue cases have been identified and associated with certain locations in the community, like neighbor's homes, local meeting or gathering places and attributed primarily to variation in *Ae. aegypti* population density [44, 45]. The underlying mechanism leading to a positive association between human and mosquito infections at the level of individual houses and neighboring residences [46] is yet not entirely explained and one might speculate that this pattern expresses an indirect evidence of the presence of center of activities for the vector.

This model can be extended in order to account for difference in life–history components, for instance, if using cohorts of different origins. This could be the case of releasing cohorts of mosquitoes especially prepared for control interventions, such as *Wolbachia*–infected mosquitoes. Other extension possibilities include recruitment and survival of unmarked mosquitoes and also influence of gonotrophic cycle in the centers of activity. Such extensions would permit to study important biological relations but would also require more elaborated design in the field experiments to gather data, and by consequence, an added cost of complexity in the analysis.

The method was illustrated with data collected from Z—10, a study area located in a dengue endemic area. According to the hierarchical model's results, Z—10 had approximately 700 female mosquitoes in the late winter-early spring of 2012, corresponding to a measure of 0.99 mosquitoes/premise (95% CI: 0.80 – 1.23 mosquitoes/premise) or 0.33 mosquitoes/person

(95% CI: 0.27 – 0.41 mosquitoes/person). This corresponds to the period of low dengue transmission. In March 2013, during the high transmission season, mosquito abundance jumped to 5.2 mosquitoes/premise (95% CI: 4.3– 5.9 mosquitoes/premise) or 1.8 mosquitoes/person (95% CI: 1.5 – 2.0 mosquitoes/person). The estimated daily survival probability was similar in both seasons, despite the difference in temperature, being between 0.71 and 0.80. This provides an estimate of average life span of 7.5 to 9 days (adding the four days in the laboratory) and is comparable to other values presented in the literature [35]. The analysis using the hierarchical model also provides an estimation of the pattern of spatial distribution of the mosquito population. One can see that in the low transmission season (Sept 2012), there are several hotspots distributed throughout the area, but each one holds an average of approximately 1 mosquito per 100 m^2. In the high transmission season, local mosquito abundance has a fourfold increase in some areas, and the highest abundance is concentrated in the southern part of the neighborhood. Considering that this is the area with more human movement, the presence of hotspots represents a critical situation for virus invasion and dissemination in this community.

The possibility of estimating spatial density of the population of *Aedes aegypti* is an advantage over methods that consider homogeneous distribution of mosquitoes, such as Lincoln, and Fisher—Ford. The Fisher—Ford method takes survival into account, which is quite important in the case of mosquito populations that have high mortality rates. It is, however, inherently sensitive to a good estimate of survival probability, since an error introduced in the survival parameter might underestimate or overestimate the abundance as a consequence. The attempt at using another capture–recapture method, the Jolly–Seber model, in a package implemented by Laake *et al.* [47] that uses Markov chain Monte–Carlo was not successful because of lack of convergence, due to the insufficient data (low capture ratio, single capture per individual). Such results, taken in a pre–intervention phase, integrated with other tools might be useful in control policies for limiting the spread of the vector.

Aedes aegypti larvae were raised using standard rearing protocols, which include low intra- or inter- specific competition, low variations in temperature and high amount of resources. Thus, it is expected that the wing size of lab-reared mosquitoes would be higher when compared to wild mosquitoes, as shown in the results. This would clearly influence dispersal as size has been shown to impact dispersal of individuals, i.e., small females disperse further than larger individuals [27]. However, also in [27], size had no significant impact on survival rates.

Methods for estimating *Aedes aegypti* absolute abundance, other than methods using MRR data, have been proposed in the literature. Williams *et al.* [48] and Jeffery *et al.* [49] used data from comprehensive quantitative pupae surveys to estimate absolute population abundances using life-table models. Since pupal mortality is low, one can compute the mosquito standing crop from the number of containers in the area, number of pupae per container, and adult survival. The latter is generally taken from published MRR studies. The underlying assumptions are that pupal production and survival are stable over time, which rarely hold for more than a few days. An even more detailed life-table model is the classical CIMSIM [50] which requires as input, meteorological, demographic and container availability data. A stochastic model with more than one hundred parameters is required to convert input data into estimates of mosquito abundance [43]. Despite the success of this approach in some settings [50], its large scale application might have drawbacks, especially in areas where the availability and quality of containers varies temporally and/or spatially.

Vector density and survival are key parameters that enter the expression of vectorial capacity $V = \frac{ma^2p^v}{-\log p}$, where m is the density of mosquitoes per humans, a is the biting rate, v is the average incubation period for the pathogen, and p is the survival probability [8]. This expression has guided past control efforts, such as the use of insecticides, and remains the primary

benchmark against which new strategies are compared. The methods proposed here make more efficient use of data collected in the field than under current approaches familiar to entomologists and surveillance systems. These models make explicit the sources of uncertainty by allowing the estimation of credibility intervals that accompany each estimate. By casting the estimation process under a formal statistical framework, one can benefit from well established processes of model building which consist of the entertainment of several possible sensible models, parameter estimation in these models, hypothesis testing and deviance analysis, examination of residuals, graphical considerations including displays of the observed and fitted values, and model selection by means of information criteria. This procedure does not guarantee the identification of the "true" model but provides clear steps in model validation through the quantification of uncertainty and the identification of deviations from the premises that entered the formulation of the model. Such formal framework will certainly impact positively on the evaluation process of modern complex intervention strategies that intend to suppress or replace vector populations.

Supporting Information

S1 Text. Hierarchical Model. Description of the components of the hierarchical model, JAGS code, and results including posterior distributions.
(PDF)

S2 Text. Simulation Tool. Description of the Individual Based Model and the computational tool that generates data simulating Mark–Release–Recapture experiments.
(PDF)

S1 Dataset. Data from Experiment ST1. CSV file that contains data obtained from MRR experiment in September 2012 in the Z10 area in the city of Rio de Janeiro: cohorts and coordinates of capture points (traps) and day of collection.
(CSV)

S2 Dataset. Data from Experiment ST2. CSV file that contains data obtained from MRR experiment in March 2013 in the Z10 area in the city of Rio de Janeiro: cohorts and coordinates of capture points (traps) and day of collection.
(CSV)

Acknowledgments

We would like to thank the anonymous reviewers whose comments helped us to improve the quality of this paper.

Author Contributions

Conceived and designed the experiments: DAMV CTC FF GAG RM CJS. Performed the experiments: GAG RM. Analyzed the data: DAMV CTC FF GAG RM CJS. Contributed reagents/materials/analysis tools: DAMV CTC FF RM CJS. Wrote the paper: DAMV CTC FF GAG RM CJS.

References

1. Halstead SB (2008) Dengue virus-mosquito interactions. Annu Rev Entomol 53: 273–291. PMID: 17803458
2. Olanratmanee P, Kittayapong P, Chansang C, Hoffmann AA, Weeks AR, Endersby NM (2013) Population genetic structure of *Aedes (Stegomyia) aegypti* (L.) at a micro-spatial scale in Thailand: implications

for a dengue suppression strategy. PLOS Neglected Tropical Diseases 7 (1) doi: 10.1371/journal.pntd.0001913 PMID: 23326609

3. Perkins TA, Scott TW, Le Menach A, Smith DL (2013) Heterogeneity, mixing, and the spatial scales of mosquito-borne pathogen transmission PLOS Computational Biology 9 (12) doi: 10.1371/journal.pcbi.1003327 PMID: 24348223
4. Harrington LC, Scott TW, Lerdthusnee K, Coleman RC, Costero A, Clark GG, et al. (2005) Dispersal of the dengue vector *Aedes aegypti* within and between rural communities. The American Journal of Tropical Medicine and Hygiene 72: 209–220. PMID: 15741559
5. Buonaccorsi JP, Harrington LC, Edman JD (2003) Estimation and comparison of mosquito survival rates with release-recapture-removal data. Journal of Medical Entomology 40: 6–17. doi: 10.1603/0022-2585-40.1.6 PMID: 12597647
6. Cianci D, Van Den Broek J, Caputo B, Marini F, Torre AD, Heesterbeek H, et al. (2013) Estimating mosquito population size from mark-release-recapture data. Journal of Medical Entomology 50: 533–542. doi: 10.1603/ME12126 PMID: 23802447
7. Service MW (1993) Mosquito—Ecology Field Sampling Methods. Chapman & Hall.
8. Smith DL, Battle KE, Hay SI, Barker CM, Scott TW, McKenzie FE (2012) Ross, Mac-Donald, and a theory for the dynamics and control of mosquito-transmitted pathogens. PLoS pathogens 8: e1002588. doi: 10.1371/journal.ppat.1002588 PMID: 22496640
9. Focks DA, Chadee DD (1997) Pupal survey: an epidemiologically significant surveillance method for *Aedes aegypti*: an example using data from Trinidad. The American Journal of Tropical Medicine and Hygiene 56: 159–167. PMID: 9080874
10. Focks DA (2003) A review of entomological sampling methods and indicators for dengue vectors. Geneva: WHO.
11. Focks DA, Brenner RJ, Hayes J, Daniels E (2000) Transmission thresholds for dengue in terms of *Aedes aegypti* pupae per person with discussion of their utility in source reduction efforts. American Journal of Tropical Medicine and Hygiene 62: 11–18. PMID: 10761719
12. Keeling M, Jiggins F, Read J (2003) The invasion and coexistence of competing Wolbachia strains. Heredity 91: 382–388. doi: 10.1038/sj.hdy.6800343 PMID: 14512953
13. Barton NH, Turelli M (2011) Spatial waves of advance with bistable dynamics:cytoplasmic and genetic analogues of Allee effects. Am Nat 178: E48–E75.
14. Costantini C, Li SG, Torre AD, Sagnon N, Coluzzi M, Taylor CE (1996) Density, survival and dispersal of *Anopheles gambiae* complex mosquitoes in a West African Sudan savanna village. Medical and Veterinary Entomology 10: 203–219. doi: 10.1111/j.1365-2915.1996.tb00733.x PMID: 8887330
15. Maciel-de Freitas R, Eiras ÁE, Lourenço-de Oliveira R (2008) Calculating the survival rate and estimated population density of gravid *Aedes aegypti* (*Diptera: Culicidae*) in Rio de Janeiro, Brazil. Cadernos de Saúde Pública 24: 2747–2754. doi: 10.1590/S0102-311X2008001200003 PMID: 19082265
16. Touré YT, Dolo G, Petrarca V, Dao A, Carnahan J, Taylor CE (1998) Mark–release-recapture experiments with *Anopheles gambiae sl* in Banambani Village, Mali, to determine population size and structure. Medical and Veterinary Entomology 12: 74–83. doi: 10.1046/j.1365-2915.1998.00071.x PMID: 9513942
17. Royle JA, Karanth KU, Gopalaswamy AM, Kumar NS (2009) Bayesian inference in camera trapping studies for a class of spatial capture-recapture models. Ecology 90: 3233–3244. doi: 10.1890/08-1481.1 PMID: 19967878
18. Consoli RA, Oliveira RLd (1994) Principais mosquitos de importância sanitária no Brasil. Fiocruz.
19. Maciel-de Freitas R, Eiras ÁE, Lourenço-de Oliveira R (2006) Field evaluation of effectiveness of the BG-sentinel, a new trap for capturing adult *Aedes aegypti* (*Diptera: Culicidae*). Memórias do Instituto Oswaldo Cruz 101: 321–325. PMID: 16862330
20. Kroeckel U, Rose A, Eiras AE, Geier M (2006) New tools for surveillance of adult yellow fever mosquitoes: comparison of trap catches with human landing rates in an urban environment. Journal of the American Mosquito Control Association 22: 229–238. doi: 10.2987/8756-971X(2006)22%5B229:NTFSOA%5D2.0.CO;2
21. Getis A, Morrison AC, Gray K, Scott TW (2010) Characteristics of the spatial pattern of the dengue vector, *Aedes aegypti*, in Iquitos, Peru. In: Perspectives on Spatial Data Analysis, Springer. pp. 203–225.
22. McDonald P (1977) Population characteristics of domestic *Aedes aegypti* (*Diptera: Culi-cidae*) in villages on the Kenya Coast i. adult survivorship and population size. Journal of Medical Entomology 14: 42–48. doi: 10.1093/jmedent/14.1.42 PMID: 903935
23. Edman J, Scott T, Costero A, Morrison A, Harrington L, Clark GG, (1998) *Aedes aegypti* (*Diptera: Culicidae*) movement influenced by availability of oviposition sites. Journal of Medical Entomology 35: 578–583. doi: 10.1093/jmedent/35.4.578 PMID: 9701948

24. Royle JA, Chandler RB, Sollman R, Gardner B (2013) Spatial Capture-Recapture. Academic Press
25. Royle JA, Kéry M, Guélat J (2011) Spatial capture-recapture models for search-encounter data. Methods in Ecology and Evolution 2: 602–611. doi: 10.1111/j.2041-210X.2011.00116.x
26. Honório NA, da Costa Silva W, Leite PJ, Gonçalves JM, Lounibos LP, Lourenço de Oliveira R (2003) Dispersal of *Aedes aegypti* and *Aedes albopictus* (*Diptera: Culicidae*) in an urban endemic dengue area in the State of Rio de Janeiro, Brazil. Mem Inst Oswaldo Cruz 98: 191–198. doi: 10.1590/S0074-02762003000200005 PMID: 12764433
27. Maciel-de Freitas R, Codeco C, Lourenço-de Oliveira R (2007) Body size-associated survival and dispersal rates of *Aedes aegypti* in Rio de Janeiro. Medical and Veterinary Entomology 21: 284–292. doi: 10.1111/j.1365-2915.2007.00694.x PMID: 17897370
28. Muir LE, Kay BH (1998) *Aedes aegypti* survival and dispersal estimated by mark-release-recapture in northern Australia. The American Journal of Tropical Medicine and Hygiene 58: 277–282. PMID: 9546403
29. David MR, Lourenço-de Oliveira R, Maciel-de-Freitas R (2009) Container productivity, daily survival rates and dispersal of *Aedes aegypti* mosquitoes in a high income dengue epidemic neighbourhood of Rio de Janeiro: presumed influence of differential urban structure on mosquito biology. Memórias do Instituto Oswaldo Cruz 104: 927–932. doi: 10.1590/S0074-02762009000600019 PMID: 19876569
30. Tsuda Y, Takagi M, Wang S, Wang Z, Tang L (2001) Movement of *Aedes aegypti* (*Diptera: Culicidae*) released in a small isolated village on Hainan Island, China. Journal of Medical Entomology 38: 93–98. doi: 10.1603/0022-2585-38.1.93 PMID: 11268697
31. Trpis M, Hausermann W (1986) Dispersal and other population parameters of *Aedes aegypti* in an African village and their possible significance in epidemiology of vector-borne diseases. The American Journal of Tropical Medicine and Hygiene 35: 1263. PMID: 3789275
32. Van Dyk DA, Meng X-L (2001) The Art of Data Augmentation. Journal of Computational and Graphical Statistics 10: 1–50. doi: 10.1198/10618600152418584
33. Royle JA, Dorazio RM (2012) Parameter-expanded data augmentation for Bayesian analysis of capture-recapture models. Journal of Ornithology 152: 521–537. doi: 10.1007/s10336-010-0619-4
34. Plummer M (2003) JAGS: A program for analysis of Bayesian graphical models using Gibbs sampling. In Proceedings of the 3rd International Workshop on Distributed Statistical Computing, Vienna.
35. Maciel-De-Freitas R, Codeco CT, Lourenço-de-Oliveira R (2007) Daily survival rates and dispersal of *Aedes aegypti* females in Rio de Janeiro, Brazil. American Journal of Tropical Medicine and Hygiene 76: 659–665. PMID: 17426166
36. Dowdeswell W, Fisher R, Ford E (1940) The quantitative study of populations in the lepidoptera *I. Polyommatus icarus* rott. Annals of Eugenics 10: 123–136. doi: 10.1111/j.1469-1809.1940.tb02242.x
37. Baber I, Keita M, Sogoba N, Konate M, Doumbia S, Traoré SF, et al. (2010) Population size and migration of *Anopheles gambiae* in the Bancoumana Region of Mali and their significance for efficient vector control. PloS ONE 5: e10270. doi: 10.1371/journal.pone.0010270 PMID: 20422013
38. Bailey NT (1952) Improvements in the interpretation of recapture data. The Journal of Animal Ecology: 120–127. doi: 10.2307/1913
39. Judson OP (1994) The rise of the individual-based model in ecology. Trends in Ecology and Evolution 9: 9–14. doi: 10.1016/0169-5347(94)90225-9 PMID: 21236754
40. Grimm V (1999) Ten years of individual-based modelling in ecology: what have we learned and what could we learn in the future? Ecological Modelling 115: 129–148. doi: 10.1016/S0304-3800(98)00188-4
41. Bonabeau E (2002) Agent-based modeling: Methods and techniques for simulating human systems. Proceedings of the National Academy of Sciences of the United States of America 99: 7280–7287. doi: 10.1073/pnas.082080899 PMID: 12011407
42. Cooch E, White G, editors (2013) Program MARK: A Gentle Introduction. 12th edition. URL http://www.phidot.org/software/mark/docs/book/.
43. Magori K, Legros M, Puente ME, Focks DA, Scott TW, Lloyd AL, et al. (2009) Skeeter Buster: a stochastic, spatially explicit modeling tool for studying *Aedes aegypti* population replacement and population suppression strategies. PLoS Neglected Tropical Diseases 3: e508. doi: 10.1371/journal.pntd.0000508 PMID: 19721700
44. Gatrell AC, Bailey TC, Diggle PJ, Rowlingson BS (1996) Spatial point pattern analysis and its application in geographical epidemiology. Trans Institute British Geographers 21:256–274 doi: 10.2307/622936
45. Morrison AC, Getis A, Santiago M, Rigua-Perez JG, Reiter P (1998) Exploratory space-time analysis of reported dengue cases during an outbreak in Florida, Puerto Rico, 1991–1992. Am J Trop Med Hyg 58:287–298 PMID: 9546405

46. Yoon IK, Getis A, Aldstadt J, Rothman AL, Tannitisupawong D, Koenraadt CJ, et al. (2012) Fine scale spatiotemporal clustering of dengue virus transmission in children and *Aedes aegypti* in rural Thai villages. PLoS Neglected Tropical Diseases 2012; 6(7):e1730. doi: 10.1371/journal.pntd.0001730

47. Laake JL, Johnson DS, Conn PB (2013) marked: an R package for maximum likelihood and Markov Chain Monte Carlo analysis of capture-recapture data. Methods in Ecology and Evolution 4: 885–890. doi: 10.1111/2041-210X.12065

48. Williams C, Johnson P, Ball T, Ritchie S (2012) Productivity and population density estimates of the dengue vector mosquito *Aedes aegypti* (*Stegomyia aegypti*) in Australia. Medical and Veterinary Entomology.

49. Jeffery JA, Yen NT, Nam VS, Hoffmann AA, Kay BH, Ryan PA (2009) Characterizing the *Aedes aegypti* population in a vietnamese village in preparation for a *Wolbachia*-based mosquito control strategy to eliminate dengue. PLoS Neglected Tropical Diseases 3: e552. doi: 10.1371/journal.pntd.0000552 PMID: 19956588

50. Williams CR, Johnson PH, Long SA, Rapley LP, Ritchie SA (2008) Rapid estimation of *Aedes aegypti* population size using simulation modeling, with a novel approach to calibration and field validation. Journal of Medical Entomology 45: 1173–1179. doi: 10.1603/0022-2585(2008)45%5B1173:REOAAP%5D2.0.CO;2 PMID: 19058645

Artigo 5

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Novel inference models for estimation of abundance, survivorship and recruitment in mosquito populations using mark-release-recapture data

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Citation: Villela DAM, Garcia GdA, Maciel-de-Freitas R (2017) Novel inference models for estimation of abundance, survivorship and recruitment in mosquito populations using mark-release-recapture data. PLoS Negl Trop Dis 11(6): e0005682. https://doi.org/10.1371/journal.pntd.0005682

Editor: Christopher M. Barker, University of California, Davis, UNITED STATES

Received: August 17, 2016

Accepted: June 7, 2017

Published: June 26, 2017

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: We are grateful to Fundação de Amparo a Pesquisa do Estado do Rio de Janeiro (DAMV - Grant 211.356/2015 and RM - Grant Grupos Emergentes - http://www.faperj.br). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Background

Experiments involving mosquito mark-release-recapture (MRR) design are helpful to determine abundance, survival and even recruitment of mosquito populations in the field. Obstacles in mosquito MRR protocols include marking limitations due to small individual size, short lifespan, low efficiency in capturing devices such as traps, and individual removal upon capture. These limitations usually make MRR analysis restricted to only abundance estimation or a combination of abundance and survivorship, and often generate a great degree of uncertainty about the estimations.

Methodology/Principal findings

We present a set of Bayesian biodemographic models designed to fit data from most common mosquito recapture experiments. Using both field data and simulations, we consider model features such as capture efficiency, survival rates, removal of individuals due to capturing, and collection of pupae. These models permit estimation of abundance, survivorship of both marked and unmarked mosquitoes, if different, and recruitment rate. We analyze the accuracy of estimates by varying the number of released individuals, abundance, survivorship, and capture efficiency in multiple simulations. These methods can stand capture efficiencies as low as usually reported but their accuracy depends on the number of released mosquitoes, abundance and survivorship. We also show that gathering pupal counts allows estimating differences in survivorship between released mosquitoes and the unmarked population.

Conclusion/Significance

These models are important both to reduce uncertainty in evaluating MMR experiments and also to help planning future MRR studies.

Competing interests: The authors have declared that no competing interests exist.

Author summary

Mosquito-borne diseases such as dengue and malaria impose a global burden with recurrent outbreaks. Recently, emergence of arboviral diseases caused by Zika and chikungunya viruses has also become a global concern. Knowledge about the ecology of mosquito populations under natural conditions may provide significant aid to help designing more effective vector control strategies. Quantitative metrics such as the abundance of mosquito populations are difficult to be measured in the field without resorting to experiments with markers. There are, however, limitations to these kinds of experiments such as short mosquito lifespan, marking limitations due to small body size, low efficiency in capturing devices such as traps, and once-only individual capture. Due to these limitations most methods estimate either only abundance or a combination of abundance and survivorship. In this work, we present statistical methods designed to estimate abundance, survivorship and recruitment using inference models and information such as counts of pupae. Results indicate that having low capture efficiencies as often observed in field assays still permits good estimation. Also, low number of released mosquitoes compromise density and survival estimations. We expect these methods to be helpful to people collecting mosquito field data and for health analysts to evaluate possible outcomes of control interventions.

Introduction

Mark-release-recapture (MRR) methods applied to study mosquito populations permit analysis of vector survival, dispersal, and abundance in natural environment. Various mosquito species, in particular of the *Aedes*, *Culex* and *Anopheles* genera, are vectors associated with persistent diseases such as dengue, filariasis and malaria and also emergent infections by chikungunya and Zika viruses. Given such medical importance, early mathematical models for malaria transmission [1,2] established the vectorial capacity as an important metric to assess epidemic risk by a mosquito population. Reliable vectorial capacity assessment requires accurate estimations of mosquito density (mosquitoes/human) and survivorship (daily survival probability). These estimates typically help to improve vector control policies and practices in endemic regions and might lead to mitigation of disease transmission [3].

By their nature, mosquito MRR experiments have important design restrictions that hinder the application of more sophisticated capture-recapture models such as the commonly known Jolly-Seber method [4]. For example: (a) individual mosquitoes are released and typically not recaptured multiple times because once collected at traps they do not survive for new releases, (b) recapture rates are low, often ranging from 5–10% [5], (c) most of the experimental designs, with notable exceptions [6], consider groups of marked individuals as cohorts due to small mosquito body size and consequent difficulty of individual marking methods and because a high number of mosquitoes are released from a few selected points, and (d) average lifespan under natural conditions is short. These limitations restrict models which consider individual markers and multiple recaptures. In early designs of capture-recapture experiments involving mosquitoes, most works used deterministic estimators such as Lincoln-Petersen and Fisher-Ford indexes to evaluate vector abundance [3,7]. Currently, deterministic models are still used mainly due to lower mathematical complexity, when compared to stochastic/Bayesian models. In the case of the Lincoln-Petersen index, the ratio between the number of marked individuals recaptured and the total insects released allows estimation of the total

abundance from the count of captures of unmarked individuals. For an MRR experiment spanning at most a dozen days, we have observations over multiple days, but only a low number of recaptures due to low capture efficiencies at traps. In the case of mosquito populations, Lincoln-Petersen abundance estimation is expectedly inaccurate, since the number of marked mosquitoes alive for trapping after a few days is significantly smaller than the number released due to a sharpened mortality across the released cohort, plus trapping on previous days [8–10]. In fact, daily captures of mosquitoes at traps typically exhibit an exponential decay largely due to mortality of marked individuals. The Fisher-Ford model [11] is another deterministic method that requires the probability of daily survival to adjust the capture ratio for the multiple estimations over time. Estimates of survival probability are possible using MRR data from the exponential decay of capture counts of marked individuals. In order to estimate abundance, Buonaccorsi *et al.* [12] consider not only the survival probability but also removal of individuals captured at traps. Recruitment in mosquito MRR experiment areas occurs either through birth or immigration. Recruitment rate estimation is possible under stable abundance, even though still challenging due to mosquito MRR limitations.

Here we build Bayesian models that leverage the concepts behind the Fisher-Ford model [11] and Buonaccorsi *et al.*'s model [12]. Moreover, we propose another two novel Bayesian approaches to estimate relevant parameters of mosquito population biology such as adult population size, survival rates and recruitment. Recruitment estimation is possible if assuming equal adult survival rates or including a component into the model that uses counts of immature individuals, typically pupae. For various mosquito species such as *Ae. aegypti* pupae are known to present low mortality and thus are likely to emerge as adult individuals [13]. Analyses using these models permit us to infer abundance, survivorship and recruitment rate using both field data and datasets obtained from simulations, when taking into account counts of immature individuals. Furthermore, our results reveal the degree of tolerance of these methods to both capture efficiency at traps and number of released mosquitoes.

Materials and methods

MRR–*Aedes aegypti*

We used the capture counts of adult females obtained from trap collections in the Z-10 neighborhood located at the city of Rio de Janeiro, Brazil, during an MRR experiment described by Villela *et al.* [10]. We used data from experiment ST2, in which a single release point (map available in Villela *et al.* [10]—supplementary files) was considered. We summed the number of trapped individuals over all traps for each day in the study. Before releases started, pupal surveys were carried out over all of the breeding sites found in the 66 premises containing an adult trap, observed in the same occasion when the trap was installed. A total of 212 larvae and 47 pupae were collected in 7 containers from 7 (11%) different dwellings. All immatures were collected in man-made containers such as plastic plant dishes and uncovered water tanks and were brought to the entomology laboratory at Fiocruz for further classification using taxonomic keys. The choice about using number of adult females is due to the use of adult traps specifically designed to attract female mosquitoes [10].

Ecological processes and experimental design

Capturing. Traps used in MRR experiments capture mosquitoes possibly using substances to attract them. Capturing, however, is not perfect as only a subset of all released mosquitoes are collected at traps due to not fully covering the experiment area. Each single trap covers a limited area over which a mosquito can be attracted to it and trapped. Both this

capability of being attracted and the probability of being captured, once attracted, are together described quantitatively here as trap capture efficiency β_0.

Survivorship. We consider survivorship only during the adult stage. There is evidence of senescence in *Aedes aegypti* mosquito, i.e. mortality rate increases with mosquito age [14]. Most models, however, describe survivorship by a single parameter. An age-dependent parameter estimated from laboratory experiments is unlikely to represent field conditions. We consider a constant probability of daily survival (PDS) during adult phase. We consider female survivorship since generally only females are released in MRR experiments and traps are designed to capture females. Factors affecting mortality include predation, lack of resources, harsh climatic conditions and use of chemical compounds. The usual quantitative measurement to describe survivorship is the probability of daily survival φ in the case of marked mosquitoes (φ_u for unmarked mosquitoes).

Recruitment. Recruitment rate b of individuals includes immigration and births. Births are clearly density dependent, whereas immigration might not be. Other factors also impact recruitment such as climate conditions, for instance water resources for breeding sites impacting adult emergence. However, birth rate will not vary significantly within a short duration of an MRR experiment. Concerning immigration, we typically assume that an MRR site is geographically restricted such that any potential flow of new mosquitoes from outside areas is neglected.

Abundance. Here abundance U is the number of females estimated for the whole area in which a MRR experiment is carried out. This abundance might also be presented as indirect quantity such as a ratio of number of females per premise.

Pupal search. Collection of immature individuals may happen before MRR experiment. Since searches are typically imperfect, we describe the efficiency of pupal search by parameter μ.

MRR data for mosquito population—Simulations

Several designs are used for mosquito MRR trials. Guerra *et al.* [15] assembled data from publicly reported mosquito MRR trials and provided a quantitative synthesis. In most of the experimental designs mosquitoes typically are not recaptured multiple times and are marked as cohorts using markers such as fluorescent dust. In MRR experiments, such as reported by Maciel-de-Freitas *et al.* [8,9] and Ritchie *et al.* [16], typically counts of recaptured mosquitoes from all cohorts (signaled by color of fluorescent dust) and counts of unmarked, captured mosquitoes are taken at each of multiple traps across an area over around a 10-day period.

We simulated multiple scenarios numerically (for instance, varying number of releases, capture probabilities and survival probabilities). Each simulation requires initial conditions and parameters such as abundance U at the beginning of the experiment, daily probabilities φ and φ_u of survival for both marked and unmarked individuals, the daily recruitment rate b. An MRR study requires a number D of days of mosquito collection at traps. Mosquito capture occurs with a given capture efficiency β_0, the number of released mosquitoes N, and the number of traps J. A pupal search in the experiment area that typically would occur in the field a day or two earlier than releasing time collects a number of pupae n_{pupae}. If the pupal search is imperfect, the number of pupae collected is given by μn_{pupae}, where μ describes the efficiency of pupal search. The simulation returns the daily numbers m_i and u_i of individuals captured at traps, both marked and unmarked ones, respectively. Table 1 shows the variables, parameters used in the simulation model and a short description.

Table 1. Model variables. List of variables used in the models and their respective descriptions.

Variables	Description
U_i	Number of unmarked individuals at time *i*. If abundance is constant over time, the index might be removed.
N	Number of marked individuals released in the field.
φ	Probability of daily survival for marked individuals.
φ_u	Probability of daily survival for unmarked individuals.
B	Daily recruitment rate.
M	Efficiency of pupal search.
P	Probability of capture at traps at a day period.
n_{pupae}	Number of pupae collected before the experiment.
m_i, $\boldsymbol{m}$	Number of marked individuals captured at day *i*, vector containing m_i
u_i,$\boldsymbol{u}$	Number of unmarked individuals captured at day *i*, vector containing u_i.
β_0	Daily capture efficiency at mosquito traps.
T	Pupal maturation time
D	Number of collection days in the MRR experiment.
$m_c = \sum_1^D m_i$	Total number of marked individuals recaptured in the experiment.
$u_c = \sum_1^D u_i$	Total number of unmarked individuals captured in the experiment.

S1 Table describes the used parameters in our simulations and our models. The number of days, number of immatures and number of traps did not vary in the simulations.

https://doi.org/10.1371/journal.pntd.0005682.t001

Multinomial Poisson inference

The inference models describe relationships between the known values, such as number N of released mosquitoes, the number of days post-release D, and observed data, such as numbers m_i and u_i of marked and unmarked mosquitos collected at traps at day i, $1 \leq i \leq D$, using parameters to be estimated.

Let $\boldsymbol{p}$ be the vector of probabilities of capture along the observation periods i, $1 \leq i \leq D$. First, we consider the number of individuals captured over the MRR experiment time as $m_c \sim Binomial(\Sigma_{i=1}^{D} p_i,\ N)$. Then, we take a multinomial distribution for the observations m_i captured at each day i: $\boldsymbol{m} \sim \text{Multinomial}(\boldsymbol{p}, m_c)$.

For a first naïve model M_0, we consider the probability p_i of capture to be only dependent on the trap capture efficiency β_0. For a second model M_S, we describe the capturing probability by a product of capture efficiency β_0 and time effects, to be estimated. Therefore, $\log(p_i) = \theta_0 + i\,\theta_1$, where the estimated capture efficiency $\beta_0 = \exp(\theta_0)$ and estimated survival probability $\varphi = \exp(\theta_1)$. Such a model is more general than model M_0, since the basic assumption in model M_0 is equivalent to assume simply that $\theta_1 = 0$, which corresponds to no mortality effects at any time i. Multiple daily estimations applying models M_0 and M_S are Bayesian counterparts to multiple values of abundance obtained by Lincoln-Petersen and Fisher-Ford estimators, respectively. For a third model M_B, we allow for removal of individuals given the daily captures at traps in a Bayesian counterpart to the model proposed by Buonaccorsi *et al.* [12]. In this case, the probability of capture is $p_i = \beta_0(1-\beta_0)^{i-1}\varphi^i$, for marked individuals and $p_i = \beta_0(1-\beta_0)^{i-1}$, for unmarked individuals. For unmarked individuals, this model does not permit estimation of probability of daily survival φ_u. In this case, the underlying assumption is that over a short period of time, typically few days, recruitment is equal to mortality.

The observed number u_i of unmarked individuals collected at traps is modeled as $u_i \sim \text{Poisson}(U\,p_i)$, for models M_0, M_S, and M_B, where the abundance number U is to be estimated. We use a prior distribution for abundance $U \sim Gamma(0.001, 0.001)$. We also have prior distribution for capture efficiency $\beta_0 \sim Beta(2,4)$ and for probability of daily survival of marked individuals $\varphi = Beta(4,2)$, which are lightly informative distributions, concentrating most mass at values close to 0 in the case of capture efficiency and close to 1 in the case of probability of daily survival.

Multinomial Poisson models with recruitment

We build two other models that include a recruitment component, including one considering the number of pupae collected from experiments before releasing mosquitoes. We build these models using relationships also described for model M_B, *i.e.*, having survivorship equal along with the experiment days and also accounting for removal of individuals. We also consider for both models the number of individuals captured over the MRR experiment time as $m_c \sim Binomial(\Sigma_{i=1}^{D} p_i,\ N)$. Then, we take a multinomial distribution for the observations m_i captured at each day i: $\boldsymbol{m} \sim \text{Multinomial}(\boldsymbol{p}, m_c)$.

We define model M_{RSU} for which we assume survival of unmarked individuals equal to the one of marked individuals, i.e., essentially $\varphi_u = \varphi$. Therefore, over a short period of time such as an MRR experiment duration, recruitment should occur at rate that maintains population at a constant level. The number of unmarked individuals at each time period, i.e. at risk of being trapped, is the sum of a number U_i of surviving individuals from start of the experiment and the total number V_i of recruited individuals. In the model a number of mosquitoes given by a recruitment rate b enter the experiment at each time interval. Therefore, from time *i-1* to time *i* the number of mosquitoes should increase by rate $b_i = b\beta_0(1-\beta_0)^{i-1}\varphi^i$. We have the same vector of probability capture described for model M_B, $p_i = \beta_0(1-\beta_0)^{i-1}\varphi^i$. In model M_{RSU}, the sum of remaining individuals is a latent variable given by $U_i \sim Poisson(U(1-\beta_0)^{i-1}\varphi^i)$ and the number of recruited individuals is another latent variable given by $V_i \sim Poisson(b\Sigma_{j=1}^{i}(1-\beta_0)^{j-1}\varphi^j)$.

We define model M_{RP} distinguishing the probabilities of daily survival of unmarked and marked mosquitoes, in order to estimate parameter φ_u. We describe the number of immature collected before the experiment to be $n_{pupae} \sim Binomial(f_a(1-\varphi_u),\tau\ U/s)$, where f_a is a factor that describes how extensive is the immature search, τ is the pupal maturation time and s is the fraction of the targeted group in the mosquito population. Very commonly, the purpose is to estimate the abundance of female mosquitoes. Here, pupal maturation time is $\tau = 2$ days and the fraction of female mosquitoes is $s = 0.5$ [3]. Factor f_a represents an adjustment since the immature search typically covers only a fraction of the area surveyed, or alternatively a fraction of the number of premises. We have the remaining and recruited individuals assessed in the same way, but survivorship for unmarked individuals is given by φ_u: capture counts of surviving individuals $U_i \sim Poisson(U\,(1-\alpha)^{i-1}\varphi_u{}^i)$ and recruitment quantities $V_i \sim Poisson(b\Sigma_{j=1}^{i}(1-\beta_0)^{j-1}\varphi_u{}^j)$.

For both models M_{RSU} and M_{RP}, the observed number of individuals is given by $u_i \sim Binomial(\beta_0,\ U_i + V_i)$. We use a prior distribution for abundance $U \sim Gamma(0.001, 0.001)$, for capture efficiency $\beta_0 \sim Beta(2,4)$ and for probability of daily survival of individuals $\varphi = Beta(4,2)$ and $\varphi_u = Beta(4,2)$, where appropriate, and for basic recruitment rate $b \sim Lognormal(10, 0.25)$.

As a reference, Table 2 describes the assumptions behind each of these models and which estimators can be extracted from them.

Table 2. Description of models M_0, M_S, M_B, M_{RSU}, and M_{RP}. Models are built using observed data and different assumptions. Depending on observed data, each model permits distinct parameters to be estimated. Some of these models are closely related to other methods proposed in the literature as shown in the counterpart model column.

Bayesian models	Description					Estimation
	Number of recaptures	Survivorship	Removal of individuals	Number of pupae	Counterpart model	
M_0	Yes	-	-	-	Lincoln-Petersen estimator	Abundance,
M_S	Yes	Yes	-	-	Fisher- Ford estimator [11]	Abundance, survival
M_B	Yes	Yes	Yes	-	Buonaccorsi et al. [12]	Abundance, survivorship
M_{RSU}	Yes	Yes	Yes	-	-	Abundance, survivorship, recruitment
M_{RP}	Yes	Yes	Yes	Yes	-	Abundance, survivorship (marked and unmarked), recruitment

https://doi.org/10.1371/journal.pntd.0005682.t002

Computational platform

We implemented the simulation tool using the R platform [17]. We also wrote description models using the WinBUGS language [18] for the statistical models (M_0, M_S, M_B, M_{RSU}, M_{RP}). We analyze the simulation data via Monte-Carlo Markov chain simulations (MCMC), by running 3 separate chains, 360,000 iterations during each of the chains, with a 320,000 burn-in period. These numbers sufficed for good convergence except otherwise noted within our results. We use R to load the simulation data and streamline pre-processed data via package R2JAGS [19] into JAGS [20], the selected tool for MCMC analysis. Output from MCMC analysis permits us to obtain samples of the posterior distribution, and as a result, mean and median values, as well as credibility intervals (CI). In S1 Text we present boxes that contain the description of our models prepared for JAGS tool. Our scripts for simulation and analysis are publicly available at https://github.com/DVMath/MosqCapRecap.

Results

Estimation of abundance, survivorship and recruitment using field data

We estimated abundance of *Aedes aegypti* mosquito population in the Z-10 neighborhood in Rio de Janeiro from inference analysis using models M_0, M_S, M_B, M_{RSU}, and M_{RP}. Results from using model M_0 reveal a much larger abundance (Fig 1A). Indeed, an overestimation is expected, since this model does not consider either survival estimation or removal of individuals. Estimation from the posterior distribution results average values of abundance in the releasing day that are 3,326 (95% CI: 2,794–3,944), 2,875 (95% CI: 2,171–3,676), 1,636 (95% CI: 1,152–2,345), and 2,143 (95% CI: 1,574–2,890) female mosquitoes, from analysis using models M_S, M_B, M_{RSU}, M_{RP}, respectively. Probability of daily survival from posterior distributions obtained from analyses of models M_B, M_{RSU}, and M_{RP} were very similar (Fig 1B). In the case of model M_{RP} the mean probability of daily survival was 0.77 (95% CI: 0.72–0.83). The mean recruiting rate was estimated at 530 mosquitoes per day (95% CI: 383–701) for model M_{RP}. Since the method is sensitive to the number of pupae collected in the field, we estimate abundance using model M_{RP} considering various alternative possibilities such as a twofold, half and a quarter of the collected number of pupae (Fig 1C). The last two possibilities (half and quarter of the collected number) result in smaller abundance estimations, when considering the collected number to be the closest to the number of pupae in the area. By contrast, a larger recruiting rate is expected if the real number of pupae to be collected is twofold.

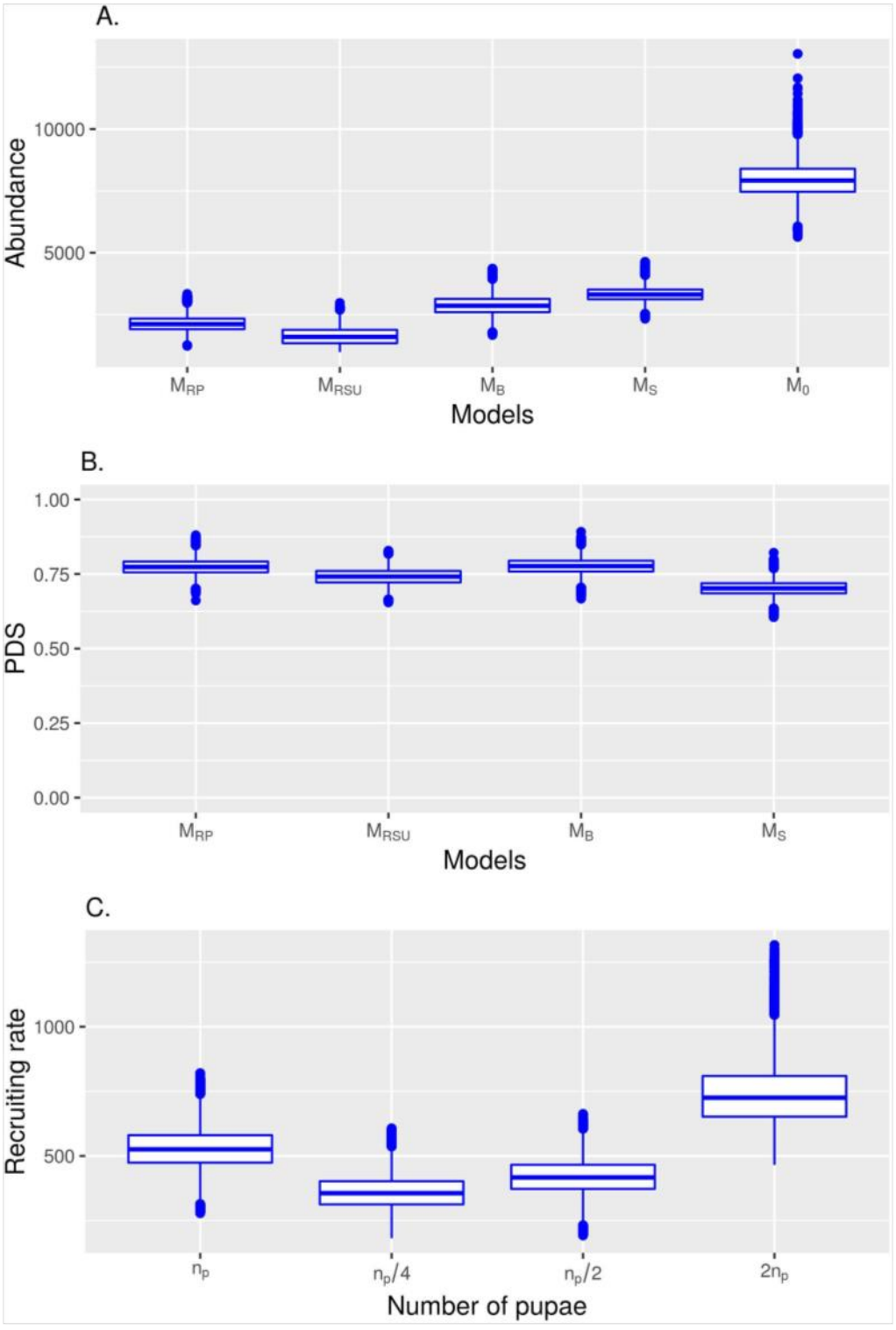

Fig 1. Estimation of abundance, survivorship, and recruiting rate in a study area in the city of Rio de Janeiro, Brazil. (A) Abundance of mosquitoes (number of females in the Z-10 area). (B) Probability of daily survival. (C) Recruiting rate, where n_{pupae} is the number of pupae collected. Outlier values are shown by points.

https://doi.org/10.1371/journal.pntd.0005682.g001

Table 3. Abundance estimates from simulated data. The simulation study number refers to the study identifier (S1 Table). Results (means and credibility intervals) are shown at thousands for clarity purposes. An asterisk (*) indicates whether the credibility interval contains the assumed abundance value (shown at first column).

Simulation Study	Abundance (input value)	Abundance mean value estimates at thousands (cred. intervals)				
		M_{RP}	M_{RSU}	M_B	M_S	M_0
1	4,000	4.2 (3.6–5.1)*	3.6 (3.0–4.2)*	3.4 (2.8–4.1)*	2.5 (2.0–2.9)	8.4 (7.6–9.3)
2	4,000	3.3 (2.5–4.3)*	2.4 (1.9–3.1)	2.7 (1.9–3.7)	1.5 (1.1–2.0)	5.8 (5.0–6.7)
3	4,000	3.9 (3.2–4.9)*	3.1 (2.6–3.7)	3.1 (2.4–3.7)	2.1 (1.7–2.6)	7.0 (6.2–7.9)
4	4,000	4.4 (3.5–5.5)*	3.2 (2.6–3.9)	3.9 (3.0–4.8)*	2.6 (2.1–3.1)	7.8 (6.9–8.8)
5	4,000	3.8 (3.3–4.3)*	3.5 (3.0–4.1)*	3.0 (2.5–3.4)	2.3 (2.0–2.7)	8.7 (7.9–9.5)
6	4,000	4.2 (3.6–4.8)*	3.7 (3.2–4.3)*	3.5 (3.0–4.0)*	2.8 (2.4–3.2)	9.1 (8.4–9.9)
7	2,000	2.3 (2.0–2.7)*	1.9 (1.6–2.3)*	2.2 (1.9–2.7)*	1.7 (1.4–2.1)*	6.9 (6.2–7.7)
8	8,000	6.5 (5.5–7.7)	6.1 (5.3–7.0)	4.6 (3.8–5.5)	3.3 (2.8–3.9)	11.7 (10.7–12.8)
9	6,000	5.3 (4.5–6.2)*	4.7 (4.0–5.4)	4.1 (3.4–4.9)	3.0 (2.5–3.5)	10.1 (9.2–11.1)
10	4,000	3.5 (2.9–4.1)*	3.0 (2.5–3.6)	2.6 (2.1–3.1)	2.0 (1.7–2.3)	7.1 (6.4–7.9)
11	4,000	2.7 (1.9–4.1)*	2.1 (1.5–2.8)	2.2 (1.3–3.3)	1.0 (0.7–1.5)	4.6 (3.8–5.5)*
12	4,000	3.9 (2.8–5.3)*	2.4 (1.8–3.1)	3.2 (2.2–4.8)*	1.6 (1.2–2.2)	4.8 (4.1–5.7)
13	4,000	3.2 (2.3–4.4)*	2.5 (1.9–3.2)	2.3 (1.6–3.3)	1.4 (1.0–1.8)	5.7 (4.9–6.7)
14	10,000	8.6 (7.1–10.0)	7.4 (6.5–8.4)	5.5 (4.6–6.6)	4.2 (3.6–4.8)	12.0 (11.0–13.1)
15	4,000	4.0 (3.2–4.9)*	3.3 (2.7–3.9)	3.4 (2.7–4.3)*	2.4 (2.0–2.9)	8.1 (7.3–9.1)
16	4,000	3.7 (3.3–4.3)*	3.6 (3.2–4.1)*	2.7 (2.3–3.1)	2.1 (1.8–2.4)	8.6 (7.9–9.4)
17	4,000	4.6 (4.0–5.2)*	4.4 (3.8–4.9)*	3.4 (3.0–3.9)	2.6 (2.3–3.0)	10.1 (9.2–10.9)
18	4,000	3.6 (3.1–4.3)*	3.0 (2.5–3.5)	2.9 (2.4–3.5)	2.2 (1.8–2.6)	6.6 (6.0–7.3)
19	4,000	4.8 (4.1–5.5)	3.4 (2.9–4.0)	3.1 (2.6–3.6)	2.4 (2.0–2.8)	5.5 (5.0–6.1)
20	12,000	9.4 (8.0–10.7)	9.0 (8.0–10.1)	6.2 (5.1–7.3)	4.6 (4.0–5.4)	14.6 (13.4–16.0)
21	14,000	10.8 (9.5–12.2)	9.9 (8.8–11.2)	7.4 (6.1–9.0)	5.3 (4.6–6.2)	16.1 (14.8–17.5)
22	4,000	3.8 (3.3–4.4)*	3.3 (2.8–3.8)	2.8 (2.4–3.3)	2.3 (2.0–2.6)	6.9 (6.3–7.5)
23	4,000	3.5 (3.0–3.9)	3.2 (2.8–3.6)	2.6 (2.3–3.0)	2.2 (1.9–2.5)	7.1 (6.6–7.8)
24	4,000	4.2 (3.6–4.8)*	3.5 (3.0–4.0)	2.9 (2.5–3.4)	2.4 (2.0–2.7)	5.8 (5.3–6.4)
25	4,000	3.5 (3.0–3.9)	3.0 (2.6–3.4)	2.4 (2.1–2.7)	2.0 (1.8–2.3)	5.8 (5.3–6.3)

https://doi.org/10.1371/journal.pntd.0005682.t003

Estimation of abundance, survivorship and recruitment using simulated datasets

Table 3 contains results obtained from multiple simulation experiments using models M_0, M_S, M_B, M_{RSU}, M_{RP}. Model M_{RP} included the assumed input values of abundance within credibility intervals in 16 simulation studies (indicated by the number of asterisks in the M_{RP} column). For assumed values of abundance of 8,000 mosquitoes and above, model M_{RP} underestimated the abundance. For values of probability of daily survival of unmarked individuals less than 0.8, model M_{RP} resulted in either overestimation (study 19) or underestimation (studies 23 and 25).

Comparing assumed input values for study 1 and its estimations in Table 4, all parameters were estimated close to the assumed values and the 95% credibility intervals indeed contain these assumed values. Analysis by model M_{RP} results in abundance of 4,220 mosquitoes (95% CI: 3,572–5,067) for an assumed abundance value of 4,000 mosquitoes. We also estimated probability of daily survival (PDS) for unmarked at 0.86 and marked individuals at 0.77 and recruitment rate 624 individuals/day. Analysis from model M_{RSU} reveals an estimation of a 95% credibility interval also containing the abundance value for simulation study 1. The probability of daily survival, however, is wrongly estimated due to the assumption of equal survival

Table 4. Results for all parameters from analysis via MCMC using the described models and simulation dataset # 1. Simulated data were obtained using parameter values in the first line (Input value). Results from analysis running MCMC simulations (3 chains, 360,000 iterations, 320,000 burn-in period) are shown in the subsequent lines (Estimation). Mean values and credibility intervals (95%) are obtained from posterior output samples. An asterisk (*) indicates whether the credibility interval contains the assumed input value in the simulation. Parameters not estimated due to the model limitations are signaled by a single dash (-).

Input values	Abundance	Trap capture efficiency	PDS (marked)	PDS (unmarked)	Recruitment (per day)
	$N = 4{,}000$	$\beta_0 = 0.05$	$\varphi = 0.78$	$\varphi_u = 0.85$	$b = 600$
Estimation					
M_{RP}	4,220 (3,572–5,067)*	0.049 (0.041–0.59)*	0.77 (.75-.81)*	0.86 (0.83–0.88)*	624 (503–760)*
M_{RSU}	3,573 (3,013–4,218)*	0.06 (0.05–0.07)*	0.75 (0.72–0.78)*	0.75 (0.72–0.78)	993 (865–1,140)
M_B	3,394 (2,805–4,086)*	0.05 (0.04–0.06)*	0.78 (0.75–0.81)*	-	-
M_S	2,458 (2,048–2,926)	0.06 (0.05–0.08)*	0.72 (.69–0.75)	-	-
M_0	8,402 (7,599–9,305)	0.018 (0.016–0.020)	-	-	-

https://doi.org/10.1371/journal.pntd.0005682.t004

rates for all individuals whether marked or not. Model M_B permits estimation of abundance, probability of daily survival (marked individuals) and trap capture efficiency. Estimates given by model M_B are also close to the assumed values, which are well within the 95% credibility intervals. Model M_S does not consider removal of individuals, an assumption that proves costly since it underestimated both the abundance and probability of daily survival. Model M_0 results greatly overestimate abundance due to not considering the daily survival.

Number of releases impact estimation

For simulations with at least 1000 marked mosquitoes, mean estimated abundance values are close to the assumed values, which are within the 95% credibility interval. Values below 1,000 marked mosquitoes were not quite as close to the estimation value. Also, the 95% credibility interval in these cases gets much larger as size of the released cohort decreases. Inspection of results from very low values indicates high uncertainty, as expected (Fig 2A). Fig 2B indicates that the low levels of capture counts due to relatively few release numbers prove costly to the capture efficiency estimation resulting in overestimation. As a consequence, abundance is underestimated.

Distinguishing survival from unmarked to marked individuals

Fig 3 shows results for survival probabilities under M_{RP} model considering only simulation experiments with same abundance values and release numbers, but varying probability of daily survival of marked individuals and unmarked population. Estimation of PDS for both marked cohort and unmarked cohorts are close to the input values assumed in the simulations, although in some cases for marked population the assumed values are closer to the extremes of the 95% credibility intervals.

Model with proper number of immatures permits accurate abundance estimates

If efficiency at collecting pupae is low, results from using model M_{RP} indicate estimations deviating from the assumed input values for abundance, recruitment and probability of daily survival. Fig 4 shows this pupal search efficiency at different levels equal to 25%, 50%, 75% and 100%. As expected, the ideal case (100%) is the best scenario, since estimations are close to the assumed values. For low number of released mosquitoes the estimation also gets worse due to the low capture counts.

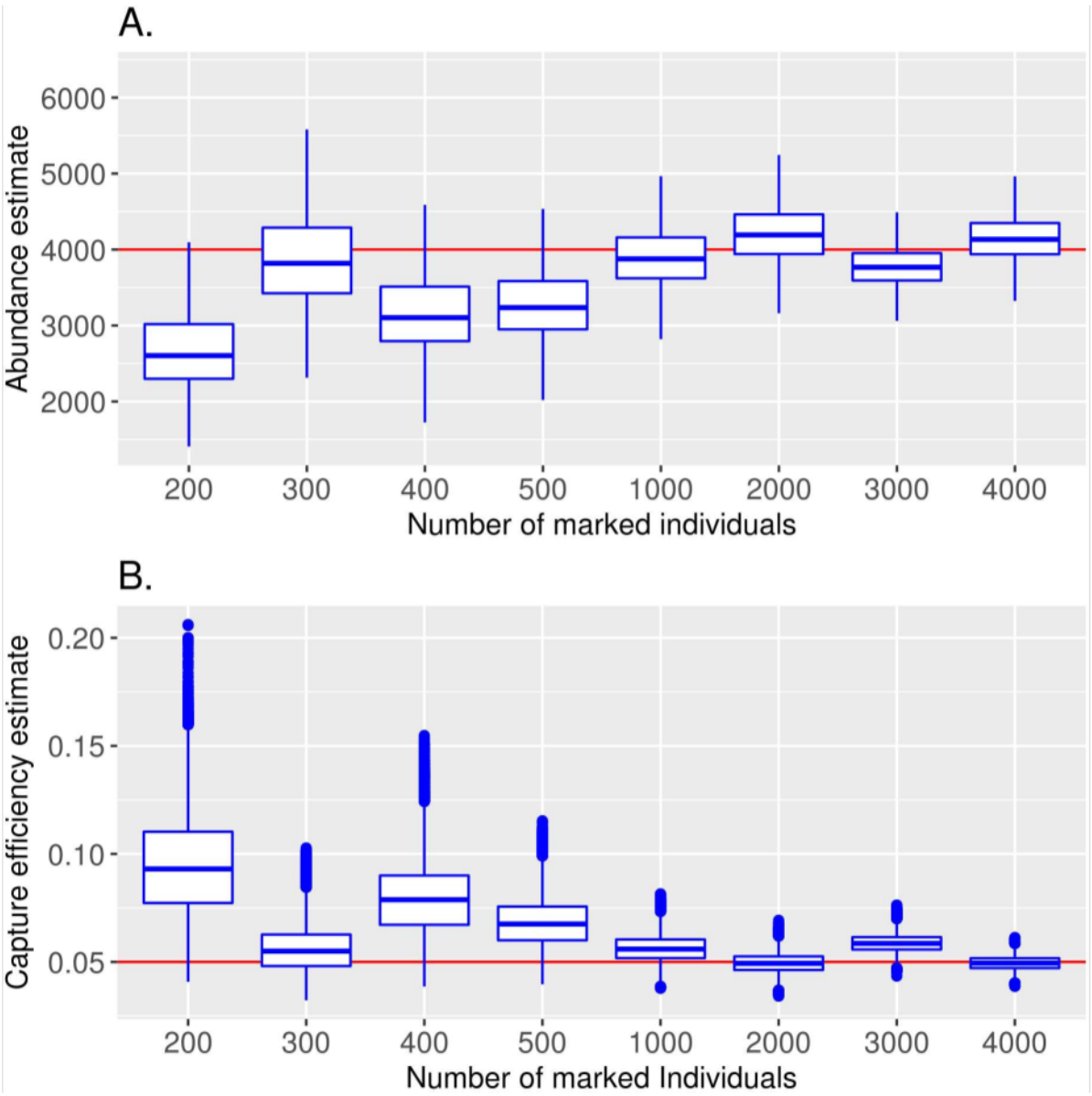

Fig 2. Impact of release numbers on parameter estimates. Results are shown for the posterior distributions (mean and 95% credibility intervals) of abundance (A) and capture effciency (B). Horizontal lines indicate the assumed input value for simulation. Points indicate outliers. Released numbers less than 1,000 reveal either mean not close to the assumed value or large 95% credibility interval/poor convergence.

https://doi.org/10.1371/journal.pntd.0005682.g002

Estimating recruitment assuming equal probability of survival among marked and unmarked individuals

Fig 5 shows the impact of distinct probabilities of daily survival between marked individuals and unmarked individuals. The cases in the middle column correspond to the assumption in the model, and assumed input values in the simulations lie within the 95% credibility intervals. In the cases where there is difference (left and right columns) between the survival of the two populations, results for recruitment rate (Chart B) in model M_{RSU} are not as close to the

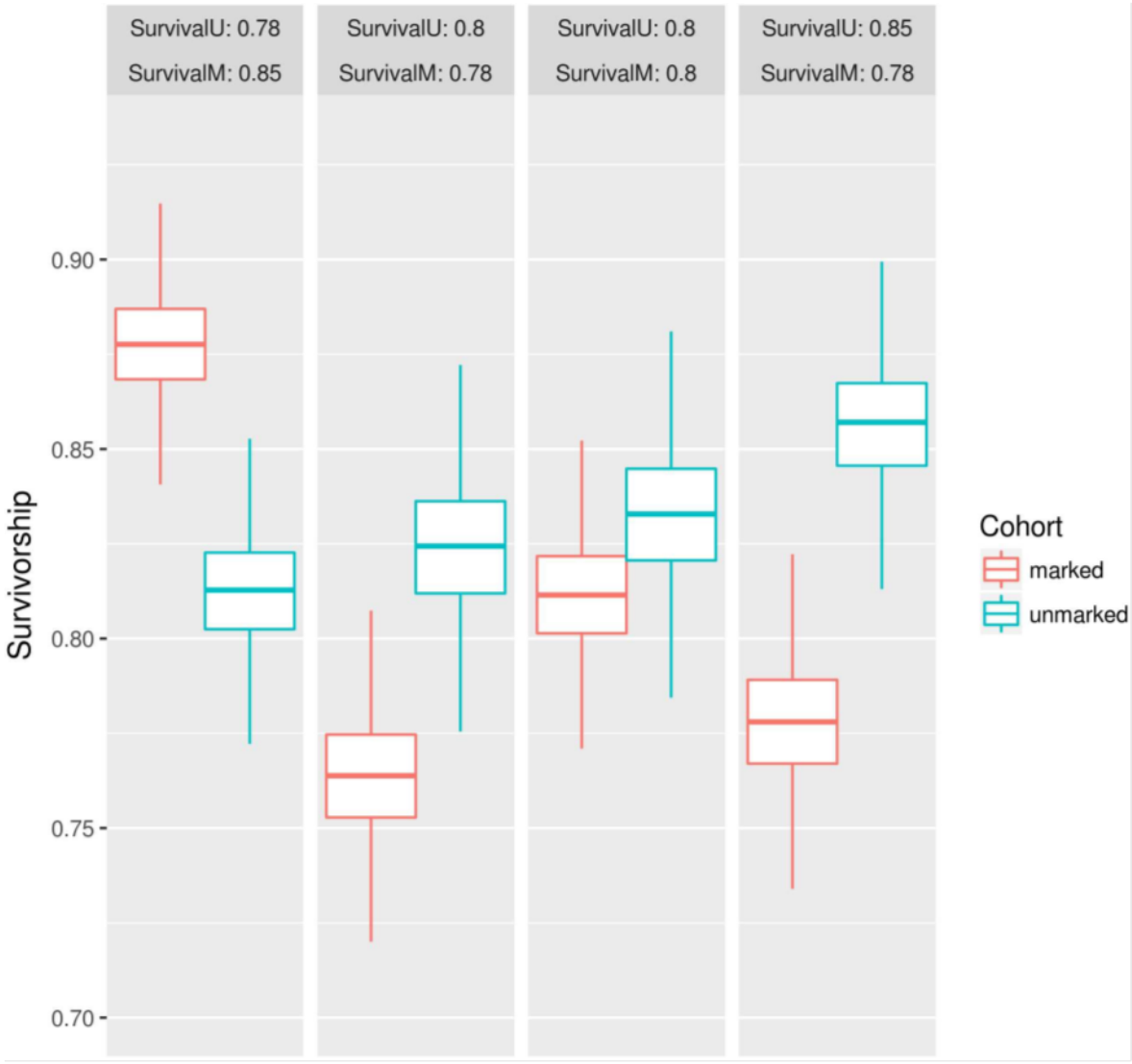

Fig 3. Estimates of probability of daily survival under M_{RP} model under distinct conditons. All simulation parameters are equal at all experiments, except for varying probability of daily survival. Abundance is 4,000 mosquitoes and 2,000 marked mosquitoes are released. Trap capture efficiency is fixed at 0.05. Results are shown for the posterior distributions (mean and 95% credibility intervals). SurvivalU indicates assumed values used for unmarked PDS, whereas SurvivalM indicates assumed values for marked PDS. Red and blue boxplots represent results for marked and unmarked cohorts, respectively.

https://doi.org/10.1371/journal.pntd.0005682.g003

expected values. Since estimation of all parameters is intertwined, abundance estimates (Chart A) also get worse.

Capture efficiency impacts estimation

In Fig 6 capture efficiency varies from 0.03 to 0.1, as we consider only simulation studies that assumed all other parameters (abundance, survival, recruitment) equal. As expected, as the capture efficiency lowers, uncertainty increases, since capture counts are low. As a consequence, 95% credibility intervals are large for capture efficiency smaller than 0.05 (5%). As a surprising effect, the estimations for capture efficiencies at 0.05, 0.08 and 0.1 do not reveal significant difference in their 95% credibility intervals.

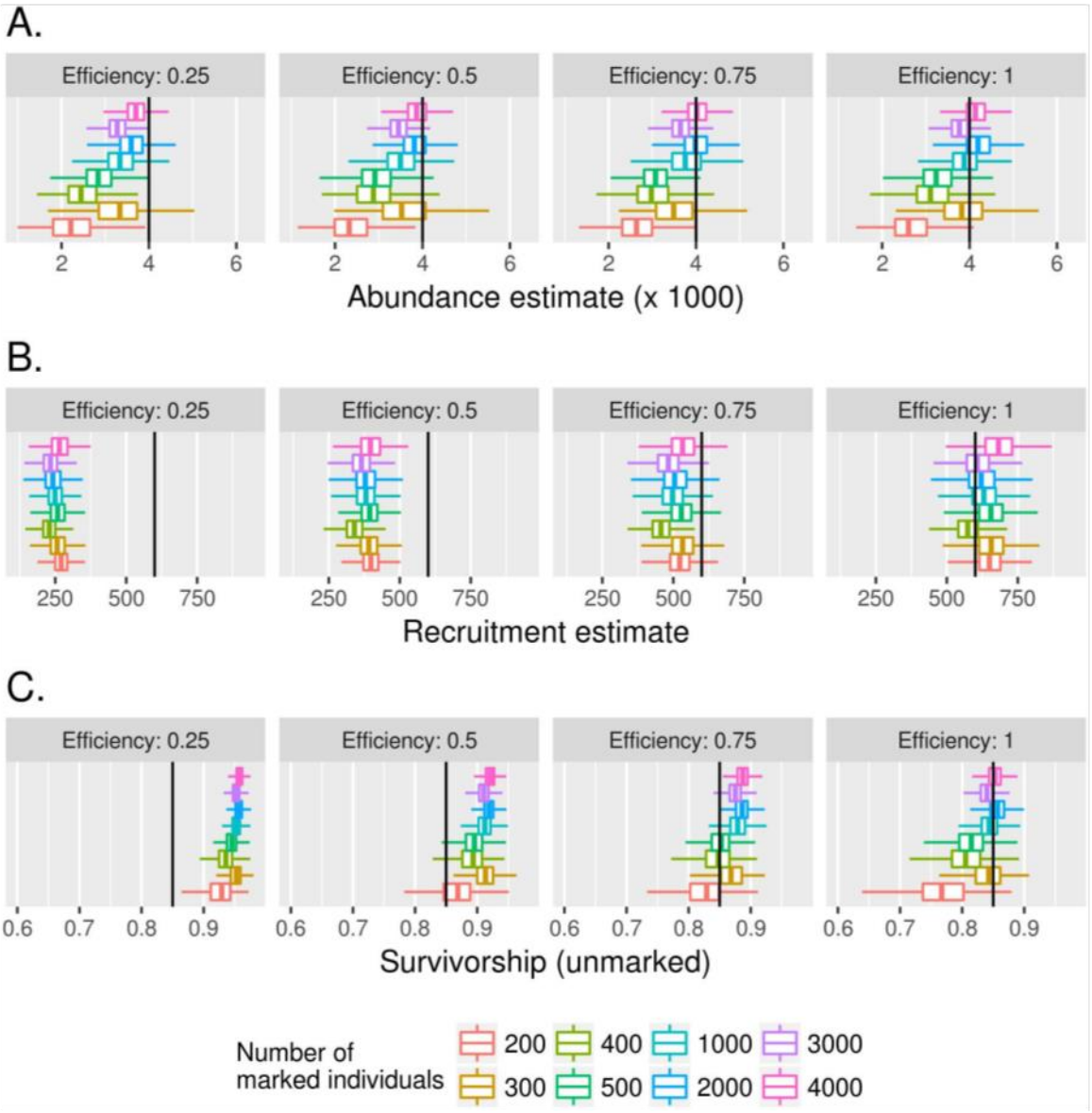

Fig 4. The effect of pupae search efficiency at observing immature counts under M_{RP} model. Here, efficiency describes how good from 0 to 1 counting the immature individuals (pupae) in the pre-MRR phase. Results are shown for the posterior distributions (mean and 95% credibility intervals). Chart A shows abundance results. Chart B indicates recruitment estimates. Chart C indicates survivorship of unmarked individuals. All estimations are intertwined and lowering efficiency causes all of them to deviate from assumed values (black bars). Colors represent the number of released individuals as shown in legend.

https://doi.org/10.1371/journal.pntd.0005682.g004

Discussion

We defined Bayesian models to estimate abundance, recruitment and probability of daily survival of mosquito populations in the field from MRR experiment data. Analyses using these models result in posterior distributions for these parameters, hence mean and 95% credibility intervals can be obtained. Moreover, counts from pupal surveys were instrumental to obtain estimated recruitment rates of the wild population. These estimates are particularly interesting since immature counting in breeding sites is one of the most common vector control approaches in countries endemic for arboviruses infections.

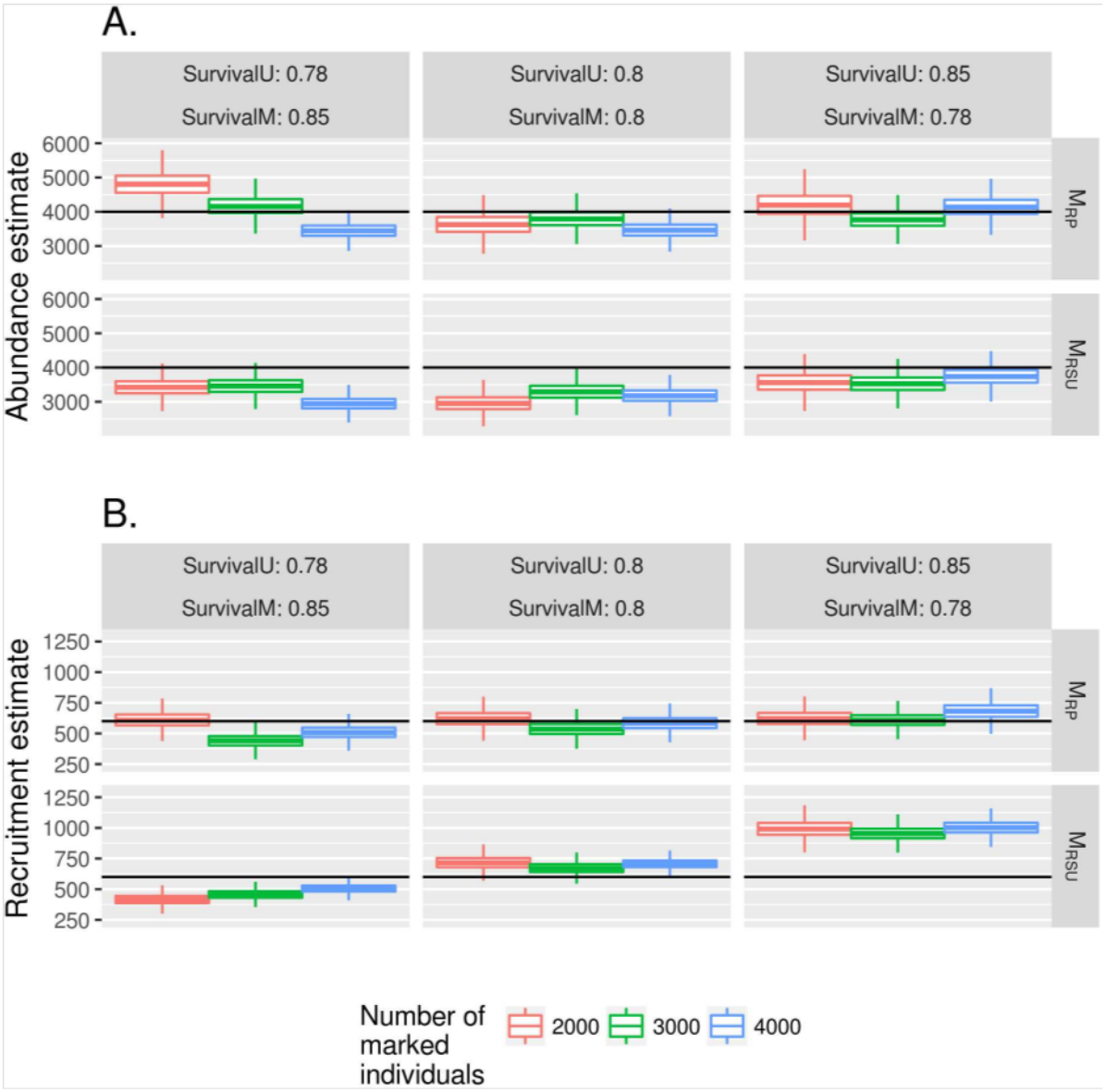

Fig 5. Distinct values of survival rates between marked population and unmarked population impact in abundance and recruitment estimates under models M_{RSU} and M_{RP}. Results are shown for the posterior distributions (mean and 95% credibility intervals). Colors represent the number of released individuals. When the absolute value of the difference between survival of unmarked and marked individuals is 0.07, estimates of either abundance (A) or recruitment and (B) are not close to the assumed value.

https://doi.org/10.1371/journal.pntd.0005682.g005

Our first set of simple Bayesian inference models is based on estimating the capture efficiency and the probability of daily survival with close relationship to existing methods used for MRR analysis. Since mosquitoes are not often individually captured multiple times (once captured they are effectively removed from the study), a Bayesian model should better describe removal of individuals not only due to mortality but also from the capture process itself. This model has close association to the method proposed by Buonaccorsi *et al.*[12]. Simpler models are defined by neglecting removal of individuals, but still assuming limited survivorship and also neglecting mortality in order to establish Bayesian counterpart models to commonly used

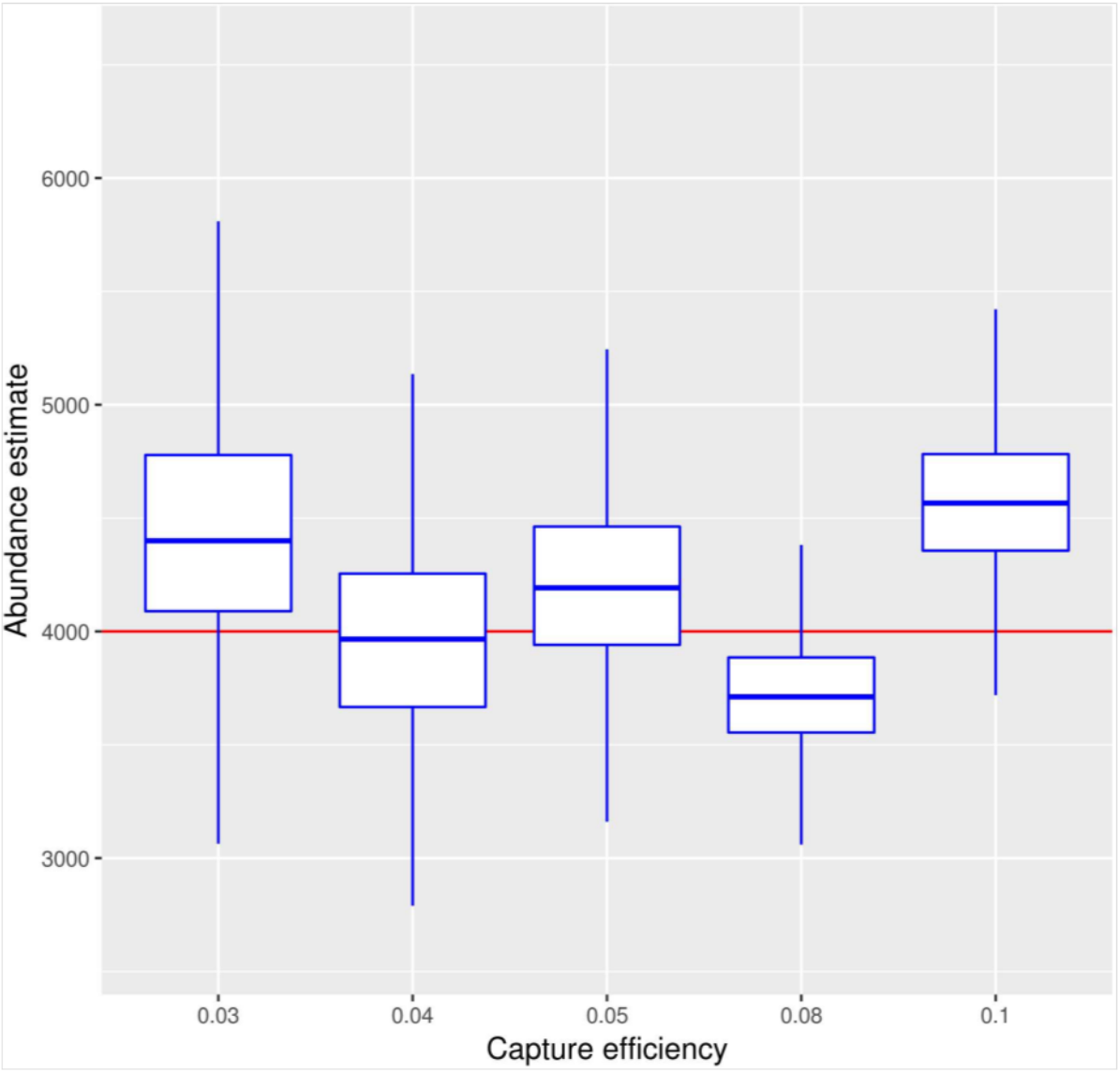

Fig 6. Capture efficiency at traps and its effect in the abundance estimates under M_{RP} model. Capture efficiency varies from values 0.03 to 0.1. Results are shown for the posterior distributions (mean and 95% credibility intervals). All other parameters (abundance, released numbers, recrutiment, survival) were equal across simulation experiments. Abundance is 4,000 mosquitoes and 2,000 mosquitoes are released. Recruitment rate is at 600 mosquitoes/day. Probabilities of daily survival is 0.85 for unmarked cohort and 0.78 for marked cohort. Surprisingly, high capture efficiency does not decrease the 95% credibility intervals significantly.

https://doi.org/10.1371/journal.pntd.0005682.g006

Fisher-Ford and Lincoln-Petersen estimators [3,11]. In the case of Lincoln, such approach is not unprecedented since Gaskell and George [21] presented a Bayesian estimation for the Lincoln index. The Bayesian method enabled by our model M_B permits inference about the capture efficiency and the probability of daily survival. However, it may not achieve accurate estimations, depending on conditions of large difference between probability survival of marked and unmarked mosquitoes, large abundance or low capture efficiency.

Estimation of recruitment becomes challenging due to the usual mosquito MRR limitations. The concept of using pupal counts for assessment of abundance has been proposed by

Focks *et al.* [22] and also advised in other works [15,23]. The estimation implicitly assumes, based on strong sampling efficiency, that pupae numbers should balance with mortality rates, for constant population sizes, therefore the pupae count is the product of the abundance and the mortality rate, but also accounting for sex ratio and the average pupating time. Since our Bayesian framework assumes priors for probability of daily survival and abundance, a description in the model for the number of pupae relating to both survival and abundance is natural, accounting for a factor that the pupae collection might not cover the whole study area. Models M_{RP} and M_{RSU} also permit to estimate recruitment, either assuming collection of pupae or not, respectively. Depending on this information, we can evaluate any potential difference between daily survival of marked and unmarked mosquitoes.

We estimated abundance, survivorship and recruitment rate of an *Aedes aegypti* population in an area in Rio de Janeiro, Brazil, from an experiment conducted in March 2013, described by Villela *et al.*[10]. The mean number per premise varied from 2.1 mosquitoes per premise (M_{RSU} model) to 4.2 mosquitoes per premise (M_S). Such twofold increase shows the importance of choosing the appropriate model to describe parameters of *Aedes aegypti* biology. As shown when using simulated datasets, analysis using model M_{RP} achieves intervals that include the simulation input value in most of the studied scenarios. Daily recruitment rate in the field was about 0.67 mosquitoes per premise in the analysis from model M_{RP}. In this case, the recruited number would be about a quarter of the total abundance. The effectiveness of vector control approaches such as targeting the most productive container or using chemical compounds (insecticides) might be evaluated based on potential changes on mosquito recruitment rates. For more effective the vector control intervention, greater decrease in recruitment rate would be expected.

Our results from analyses of simulated datasets show that these models can tolerate capture efficiencies as low as the ones observed for mosquito MRR. We also varied the abundance levels, as opposed to the released numbers, and differences in the survivorship between marked, released mosquitoes and the unmarked population. In the case of immature counts (pupae), recruitment rate can also be estimated, but we find it to be highly dependent on extensive pupal collection, which can require extensive resources in the field.

Limitations in the design of mosquito MRR studies expectedly impact estimation of abundance, survivorship and recruitment rates. First, when abundance is large, the number of released mosquitoes is critical, regardless of the method used. Also capture efficiency in regular MRR experiments is usually small, varying in the range of 5–10% [5,10]. We have shown that such rates are still acceptable, but capture efficiencies below this range lead to higher degree of uncertainty in the estimation. By contrast, to reduce credibility intervals most likely we would need a combination of higher efficiencies and multiple individual recaptures, which is very difficult to implement in the field due to trap conditions. Also, if adapting these methods to have spatial estimations, we expect effects from low capture counts, as opposed to aggregate counts. Otherwise, methods such as proposed by Villela *et al.* [10] that also involve a likelihood component are required due to distance from mosquito concentration areas to traps.

Collecting pupae in the field can be difficult due to limited accessibility to breeding sites, but we think that results from model M_{RP} should motivate getting such samples to have better estimates. Our results indicate sensitivity of recruiting rate estimates when assuming different number of pupae. Because pupal surveys may have difficult feasibility to be conducted on the routine of vector control programs, our results demonstrate that surveys with varying degrees of imperfection lead to biased estimations of abundance, recruitment and survival rates. Conversely, public health decision makers might adopt models such as M_{RP} and M_{RSU} with attention to these issues. For example, the Brazilian dengue national control program recommends

a survey 4–6 times yearly in around 10% of cities of each district of important cities to determine infestation and Breteau Indexes, plus the most productive container type across the country [24]. If at least one of these surveys, e.g. the one immediately before dengue transmission starts, has high-quality pupal surveys being conducted in blocks representatives of disease transmission over the city, estimates on vector abundance, survivorship and recruitment rate might be helpful to improve vector control efficiency by directing existing strategies towards areas in which *Ae. aegypti* population has greater vectorial capacity.

Our models estimating recruitment assume that the population stays constant during the short period of experiment time. If such assumption does not hold due to abundance fluctuations occurring as a result of changing environmental conditions, use of insecticides, or any other, we expect difficulties to get accurate estimations applying this modeling, unless the exogenous conditions can be modeled.

Simulated datasets and analyses consider typical designs used for MRR experiments involving mosquito populations of *Aedes aegypti*, a known vector of Zika, dengue and chikungunya viruses. However, these models can possibly be applied to other mosquito populations. Laboratory-reared individuals of *Aedes aegypti* used in previous field studies [8–10] had the genetic background of field mosquitoes. In this case, such designs would not necessarily imply different survival of the released mosquitoes compared to the field mosquitoes. However, daily survival estimates are essential for use of modified mosquitoes such as *Wolbachia*-carrying mosquitoes as described by Garcia *et al.* [5].

There is vast literature on MRR experiments to study ecology of wild animal species [25] (and references therein), instead of mosquito populations. Studies with mosquito MRR may benefit from more advanced techniques, including possibility of using covariates such as environmental variables, individual tagging, positional and distance effect, if overcoming important design limitations. For instance, individual marking, multiple sightings and geoposition recording has been done for estimating abundance of mammals [26]. For a few insect populations, individual marking is possible through code systems, such as applying distinct dots to the body [27], for instance by elytra puncture in beetles [28]. Krebs et al. describe density estimation of rodent population in a Canadian area, by live-trapping individuals [29]. More refined models departing from other study designs and including other variables can take elements from capture-recapture designs for populations other than mosquito ones.

Bayesian models permit us to include all parameters instead of serial parameter estimation and to use prior beliefs, if any, or vague priors in order to obtain not only mean estimations but also credibility intervals. Traditional methods require a sequence of estimations for survival and abundance, and if possible recruitment, from observed field data. Smith and McKenzie [30] demonstrated the impact on vector control strategies relying on each of the parameters of the basic reproduction number for the Ross-Macdonald model [1] for malaria. More recently the classical models for malaria have been revisited to study sensitivity in applying strategies for disease control [31,32]. Models for transmissibility of other vector-borne diseases such as Zika, dengue, and chikungunya viruses can also benefit from sensitivity analysis, if using estimated parameters describing the interaction of these pathogens and their vectors. Bayesian models reveal uncertainties that coupled with sensitivities model greatly enhances estimation of vectorial capacities. Advancing towards Bayesian models that encompass a whole set of parameters greatly enhances understanding not only of the underlying dynamics but also on sensitivity to each of the biological aspects. Such models are useful to advance on strategies of vector control that aim at reducing the vectorial capacity of mosquito populations.

Supporting information

S1 Table. Description of simulation parameters for each of the simulation studies. All experiments used a recruitment rate $b = 600$, the number of traps is $J = 64$, and the experiment time is $D = 10$ days.
(DOCX)

S1 Text. Bayesian models defined for use in R and JAGS.
(DOCX)

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Supervision: Daniel Antunes Maciel Villela, Gabriela de Azambuja Garcia.

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Writing – original draft: Daniel Antunes Maciel Villela, Gabriela de Azambuja Garcia, Rafael Maciel-de-Freitas.

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References

1. Macdonald G. The epidemiology and control of malaria. London: Oxford University Press; 1957.
2. Garrett-Jones C. The human blood index of malaria vectors in relation to epidemiological assessment. Bull World Health Organ. 1964; 30: 241. PMID: 14153413
3. Silver JB. Mosquito ecology: field sampling methods. 3rd ed. New York: Springer; 2008.
4. Seber GAF. A note on the multiple-recapture census. Biometrika. 1965; 52: 249–260. PMID: 14341277
5. de Garcia GA, dos Santos LMB, Villela DAM, Maciel-de-Freitas R. Using *Wolbachia* releases to estimate *Aedes aegypti* (Diptera: Culicidae) population size and survival. PLoS One. 2016; 11: e0160196. https://doi.org/10.1371/journal.pone.0160196 PMID: 27479050
6. Trpis M, Hausermann W. Dispersal and other population parameters of *Aedes aegypti* in an African village and their possible significance in epidemiology of vector-borne diseases. Am J Trop Med Hyg. 1986; 35: 1263–1279. PMID: 3789275

7. Costantini C, Li S-G, Torre AD, Sagnon N, Coluzzi M, Taylor CE. Density, survival and dispersal of *Anopheles gambiae* complex mosquitoes in a West African Sudan savanna village. Med Vet Entomol. 1996; 10: 203–219. PMID: 8887330

8. Maciel-de-Freitas R, Codeco C, Lourenço-de-Oliveira R. Body size-associated survival and dispersal rates of *Aedes aegypti* in Rio de Janeiro. Med Vet Entomol. 2007; 21: 284–292. https://doi.org/10.1111/j.1365-2915.2007.00694.x PMID: 17897370

9. Maciel-De-Freitas R, Codeco CT, Lourenco-de-Oliveira R. Daily survival rates and dispersal of *Aedes aegypti* females in Rio de Janeiro, Brazil. Am J Trop Med Hyg. 2007; 76: 659–665. PMID: 17426166

10. Villela DAM, Codeço CT, Figueiredo F, Garcia GA, Maciel-de-Freitas R, Struchiner CJ. A Bayesian hierarchical model for estimation of abundance and spatial density of *Aedes aegypti*. PLoS One. 2015; 10: e0123794. https://doi.org/10.1371/journal.pone.0123794 PMID: 25906323

11. Dowdeswell W, Fisher R, Ford E. The quantitative study of populations in the Lepidoptera *I. Polyommatus icarus* rott. Ann Eugen. 1940; 10: 123–136.

12. Buonaccorsi JP, Harrington LC, Edman JD. Estimation and comparison of mosquito survival rates with release-recapture-removal data. J Med Entomol. 2003; 40: 6–17. PMID: 12597647

13. Focks DA, Sackett SR, Bailey DL, Dame DA. Observations on container-breeding mosquitoes in New Orleans, Louisiana, with an estimate of the population density of *Aedes Aegypti* (L.). Am J Trop Med Hyg. 1981; 30: 1329–1335. PMID: 7325289

14. Styer LM, Carey JR, Wang J-L, Scott TW. Mosquitoes do senesce: departure from the paradigm of constant mortality. Am J Trop Med Hyg. 2007; 76: 111–117. PMID: 17255238

15. Guerra CA, Reiner RC, Perkins T, Lindsay SW, Midega JT, Brady OJ, et al. A global assembly of adult female mosquito mark-release-recapture data to inform the control of mosquito-borne pathogens. Parasit Vectors. 2014; 7: 276. https://doi.org/10.1186/1756-3305-7-276 PMID: 24946878

16. Ritchie SA, Montgomery LB, Hoffmann AA. Novel estimates of Aedes *aegypti* (Diptera: Culicidae) population size and adult survival based on *Wolbachia* releases. J Med Entomol. 2013; 50: 624–631. PMID: 23802459

17. R Core Team. R: A language and environment for statistical computing. Vienna, Austria; 2014. URL http://www.R-Proj.Org. 2015;

18. Lunn DJ, Thomas A, Best N, Spiegelhalter D. WinBUGS-a Bayesian modelling framework: concepts, structure, and extensibility. Stat Comput. 2000; 10: 325–337.

19. Su Y-S, Yajima M. R2jags: A package for running jags from R. R package version 003–08 URL HttpCRAN R-Proj Orgpackage R2jags. 2012;

20. Plummer M. JAGS: A program for analysis of Bayesian graphical models using Gibbs sampling. Proceedings of the 3rd international workshop on distributed statistical computing. Technische Universit at Wien; 2003. p. 125.

21. Gaskell TJ, George BJ. A Bayesian modification of the Lincoln Index. J Appl Ecol. 1972; 9: 377–384. https://doi.org/10.2307/2402438

22. Focks DA, Chadee DD. Pupal survey: an epidemiologically significant surveillance method for *Aedes aegypti*: an example using data from Trinidad. Am J Trop Med Hyg. 1997; 56: 159–167. PMID: 9080874

23. Reisen WK, Mahmood F, Azra K. *Anopheles culicifacies* giles: Adult ecological parameters measured in rural punjab province, pakistan using capture-mark-release-recapture and dissection methods, with comparative observations on *An. stephensi* liston and *An. subpictus* grassi. Res Popul Ecol. 1981; 23: 39–60. https://doi.org/10.1007/BF02514092

24. Coelho GE, Burattini MN, da Teixeira MG, Coutinho FAB, Massad E. Dynamics of the 2006/2007 dengue outbreak in Brazil. Mem Inst Oswaldo Cruz. 2008; 103: 535–539. PMID: 18949321

25. MacKenzie DI, editor. Occupancy estimation and modeling: inferring patterns and dynamics of species. Amsterdam ; Boston: Elsevier; 2006.

26. Royle JA, Karanth KU, Gopalaswamy AM, Kumar NS. Bayesian inference in camera trapping studies for a class of spatial capture-recapture models. Ecology. 2009; 90: 3233–3244. PMID: 19967878

27. Conway GR, Trpis M, McClelland GAH. Population parameters of the mosquito *Aedes aegypti* (L.) estimated by mark-release-recapture in a suburban habitat in Tanzania. J Anim Ecol. 1974; 43: 289. https://doi.org/10.2307/3366

28. Unruh TR, Chauvin RL. Elytral punctures: a rapid, reliable method for marking Colorado potato beetle. Can Entomol. 1993; 125: 55–63. https://doi.org/10.4039/Ent12555-1

29. Krebs CJ, Boonstra R, Gilbert S, Reid D, Kenney AJ, Hofer EJ. Density estimation for small mammals from livetrapping grids: rodents in northern Canada. J Mammal. 2011; 92: 974–981. https://doi.org/10.1644/10-MAMM-A-313.1

30. Smith DL, McKenzie FE. Statics and dynamics of malaria infection in *Anopheles* mosquitoes. Malar J. 2004; 3: 13. https://doi.org/10.1186/1475-2875-3-13 PMID: 15180900

31. Brady OJ, Godfray HCJ, Tatem AJ, Gething PW, Cohen JM, McKenzie FE, et al. Adult vector control, mosquito ecology and malaria transmission. Int Health. 2015; 7: 121–129. https://doi.org/10.1093/inthealth/ihv010 PMID: 25733562

32. Brady OJ, Godfray HCJ, Tatem AJ, Gething PW, Cohen JM, McKenzie FE, et al. Vectorial capacity and vector control: reconsidering sensitivity to parameters for malaria elimination. Trans R Soc Trop Med Hyg. 2016; 110: 107–117. https://doi.org/10.1093/trstmh/trv113 PMID: 26822603

Capítulo III:

A implementação da estratégia *Wolbachia* para controle de arboviroses no Brasil

Justificativa

No Brasil, o projeto “Eliminar a Dengue” apresenta algumas particularidades em relação aos países que já foram bem-sucedidos, em áreas-teste, com a invasão de *Ae. aegypti* com *Wolbachia*. Aqui, encontramos altos índices de infestação do vetor, além de ocorrer um uso intenso de inseticidas, que resultou em populações naturais de *Ae. aegypti* altamente resistentes, em especial aos adulticida piretroides (PI). Essas são questões potencialmente determinantes para o sucesso desta estratégia em campo. Neste contexto, acompanhar as primeiras liberações de *Ae. aegypti* com *Wolbachia* no Rio de Janeiro, considerando o impacto destas questões na invasão da bactéria, é uma oportunidade única para adquirir maior conhecimento sobre esta estratégia e auxiliar em liberações mais racionais de mosquitos com *Wolbachia* no país.

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RESEARCH ARTICLE

Using *Wolbachia* Releases to Estimate *Aedes aegypti* (Diptera: Culicidae) Population Size and Survival

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OPEN ACCESS

Citation: Garcia GdA, dos Santos LMB, Villela DAM, Maciel-de-Freitas R (2016) Using *Wolbachia* Releases to Estimate *Aedes aegypti* (Diptera: Culicidae) Population Size and Survival. PLoS ONE 11(8): e0160196. doi:10.1371/journal.pone.0160196

Editor: Immo A. Hansen, New Mexico State University, UNITED STATES

Received: April 25, 2016

Accepted: July 15, 2016

Published: August 1, 2016

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was partially supported by FAPERJ (E-26/110.168/2014), CNPq, the Brazilian Ministry of Health (DECIT/SVS) as well as a grant to Monash University from the Foundation for the National Institutes of Health through the Vector-Based Transmission of Control: Discovery Research (VCTR) program of the Grand Challenges in Global Health Initiatives of the Bill and Melinda Gates Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Mosquitoes carrying the endosymbiont bacterium *Wolbachia* have been deployed in field trials as a biological control intervention due to *Wolbachia* effects on reducing transmission of arboviruses. We performed mark, release and recapture (MRR) experiments using *Wolbachia* as an internal marker with daily collections with BG-Traps during the first two weeks of releases in Rio de Janeiro, Brazil. The MRR design allowed us to investigate two critical parameters that determine whether *Wolbachia* would successful invade a field population: the probability of daily survival (PDS) of *Wolbachia*-infected *Aedes aegypti* females, and the wild population density during releases. Released females had a PDS of 0.82 and 0.89 in the first and second weeks, respectively, immediately after releases, which is well within the range of previous estimates of survivorship of wild mosquitoes in Rio de Janeiro. Abundance estimation of wild population varied up to 10-fold higher depending on the estimation method used (634–3565 females on the average-difference model to 6365–16188 females according to Lincoln-Petersen). *Wolbachia*-released mosquitoes were lower than the density estimation of their wild counterparts, irrespectively of the model used. Individually screening mosquitoes for the presence of *Wolbachia* reduced uncertainty on abundance estimations due to fluctuation in capturing per week. A successful invasion into local population requires *Ae. aegypti* fitness is unaffected by *Wolbachia* presence, but also reliable estimates on the population size of wild mosquitoes.

Introduction

The distribution of diseases such as malaria and dengue frequently overlaps with tropical and subtropical zones in which primary vectors of these diseases are more abundant [1,2]. Considering dengue virus (DENV), about half of the world's population lives at risk of getting infected. In particular, Brazil registered more than one million cases annually in the last three years [3,4]. Since 2010 Brazil has all four DENV serotypes circulating in the country, promoting

Competing Interests: The authors have declared that no competing interests exist.

dengue outbreaks every 4–5 years, often due to the arrival of a new serotype in a susceptible human host population [5–7]. Apart from dengue, two other arboviruses were recently detected in Brazil: chikungunya (CHIKV) [8] and Zika (ZIKV) [9]. The potential association between ZIKV during pregnancy and microcephaly in newborn has raised additional concerns about vector control efforts to mitigate arboviruses transmission. Current evidence shows DENV, CHIKV and ZIKV to be overwhelmingly transmitted by *Aedes* mosquitoes, especially *Aedes aegypti* [10,11], but other mosquitoes in the case of ZIKV [12]. The main role of *Ae. aegypti* as vector of these three arboviruses is probably due to its close association with human dwellings, since females lay eggs in man-made containers, bite preferably human hosts and are more abundant in urbanized landscapes with low vegetation coverage [13–16].

Since there is no vaccine currently available, the best way to reduce arboviruses transmission still relies on vector control, which ultimately aims to maintain *Ae. aegypti* density below a theoretical threshold to avoid outbreaks [17]. Thus, estimations on mosquito population size, survivorship and spatial distribution in endemic areas becomes critical for improving practices in vector control, e.g., directing the intensification of mechanical and chemical control activities in the districts in which vector population is higher [6, 18, 19].

In Brazil, *Ae. aegypti* density is often estimated by indexes derived from infestation rates based on larval surveys, in which a sample of around 10% of houses are randomly selected and inspected 4–6 times yearly [20]. These indexes do not provide good estimators on adult mosquito abundance because container productivity and larval mortality are not taken into account [21,22]. Estimates on adult mosquito population density might be achieved through adult sampling using traps [23,24] and mark, release and recapture (MRR) experiments [25–27]. MRR-based estimation has been proposed as a more reliable approach to determine *Ae. aegypti* population size because it focuses on adult sampling, providing more robust estimates on the mosquito life cycle stage directly responsible for disease transmission [28,29].

One of the most promising strategies designed to reduce arboviruses transmission uses the intracellular endosymbiont, maternally inherited bacterium *Wolbachia pipientis*, which is naturally present in up to 65% of all insects [30–32]. This approach explores the fact that *Wolbachia* reduces transmission of key pathogens, including DENV and CHIKV viruses in the *Ae. aegypti* mosquitoes [33,34]. The *w*Mel strain causes a phenotypic effect called cytoplasmic incompatibility (CI), which is a reproductive incompatibility that prevents females without *Wolbachia* from producing viable offspring after mating with *Wolbachia*-infected males. By contrast, *Wolbachia*-infected females can successfully reproduce after mating with either *Wolbachia*-infected or wild male [35]. This reproductive advantage increases the frequency of *Wolbachia* infection in a given population with each subsequent generation. Thus, the control strategy is to release mosquitoes with this bacterium for 10 or more consecutive weeks [36]. The expected outcome is the replacement of wild, vector competent, mosquitoes with *Wolbachia*-infected mosquitoes, potentially ameliorating the burden of arboviruses.

In the first two weeks of releases, *Wolbachia* may be seen as an internal marker, allowing estimates on the probability of daily survival rates of released *Wolbachia*-infected *Ae. aegypti* females, but also the population size of wild mosquitoes during releases [37]. These estimates are critical for understanding invasion, because: (1) the daily survival rate of *Wolbachia*-released mosquitoes is a strong indicator whether *Ae. aegypti* females are fit to field conditions, (2) the displacement of wild vector populations by refractory *Wolbachia*-carrying mosquitoes have been shown to have a threshold conditions dictated by *Wolbachia* frequency in the total population [38]; and (3) to calculate the ideal minimum number of *Wolbachia*-carrying mosquitoes that have to be released per week to succeed invasion promoting low nuisance in local residents. Therefore, our main objective is to estimate the probability of daily survival rates of

Ae. aegypti infected with *Wolbachia* and population density of wild *Ae. aegypti* for better characterize *Wolbachia* invasion.

Materials and Methods

Study area

We conducted MRR studies in the district of Tubiacanga, Rio de Janeiro (area: 8.6 ha; 22° 47′08″S; 43°13′36″W). Such locality is an isolated middle-class suburban area located on the offshores of Guanabara Bay and distant 2.1Km for the closest community, which discourages mosquito migration. This residential area has paved streets, well-maintained sidewalks and low-moderate vegetation coverage, with around 2902 people living in 867 houses. Most houses have 2–3 bedrooms, large yards, regular water supply and garbage collection.

Mosquitoes release

The strain *w*MelBr was derived from the backcrossing of Australian *Ae. aegypti* females *w*Mel-infected with Brazilian males (collected from four districts in Rio de Janeiro) during nine generations [39]. Larvae were reared under optimal rearing conditions, in plastic trays in 3 liters of filtered and dechlorinated water (500 larvae per tray), and fed with 0.45g of Tetramin® Tropical Flakes Fish Food every day. Adult mosquitoes were maintained in a climate controlled insectary, at 27 ± 1°C and 65 ± 5% RH, with a 12:12 hour light:dark cycle, and received constant 10% sucrose solution up to the release day. Before every release, a sample of 100 mosquitoes was screened for *Wolbachia* to confirm the infection. Releases were conducted outdoors at 05:00AM, once a week, with 5–6 days-old adults at 1:1 sex ratio, with 50 mosquitoes released every four houses. A total of 2,350 *Wolbachia*-carrying females were released in field each week (2.71 per premise). Releases lasted 20 weeks, but our analyzes considered only the first two weeks, when captured mosquitoes positive for *Wolbachia* were those we released.

Mosquito collection

Mosquitoes were captured using 30 BG-Sentinel® Traps (BGS, BioGents, Regensburg, Germany) uniformly distributed across Tubiacanga. The kind of trap consists of a collapsible white bucket with white gauze covering its opening. In the middle of the gauze cover, a black tube through which a down flow is created by a fan that causes any mosquito surrounding the trap to be sucked into a catch bag. BGS-Trap uses local energy power and captures predominantly host-seeking *Ae. aegypti* females, seldom males [40,41]. After starting releases, traps were inspected daily for 14 days, and only female counts were included in the analyses.

Monitoring Wolbachia infection in field-collected mosquitoes

All mosquitoes collected in the BGS-Trap were brought to lab in order to proceed with identification using taxonomic keys [10]. Those classified as *Ae. aegypti* were screened for the presence of *Wolbachia*, following the approach taken in Dutra et al 2016 [42].

Estimating the daily survival of Wolbachia-carrying mosquitoes in field

The daily captures of mosquitoes allowed us to estimate the probability of daily survival (PDS) by using the exponential model [43]. Traditionally, the exponential model has been used to describe mortality patterns in MRR experiments with *Ae. aegypti* procedures [15, 28, 44]. This model assumes that marked, captured individual counts vary over time as an exponential decay with a constant rate, i.e., mosquito mortality is independent of age. This assumption is reasonable given typical mosquito life span.

Estimating the population size of wild mosquitoes using conventional MRR models

The wild population size of *Ae. aegypti* in Tubiacanga was estimated by conventional models such as the Lincoln-Petersen index, which can be defined as $N_L = \frac{M n}{m}$, where M is the number of released females; n is the total number of females captured; and m is the number of marked individuals captured. Moreover, we also used the Fisher-Ford model, which takes into account mortality rate, which was directly estimated in the same MRR experiment, given by the function $N_t = \frac{R_t M \phi^t}{r_t}$, where ϕ is the PDS and t is the number of days elapsed after releases [27].

Estimating the population size of wild mosquitoes using average-difference model

The population size of wild *Ae. aegypti* was estimated using an average-difference model based on *Wolbachia* releases on North Queensland, Australia. Population size was estimated by the increase of *Ae. aegypti* captures on BGS-Traps in the district in which *Wolbachia* was released in comparison with collections on a control site, in 2 weeks before and 2 weeks after releases [37]. We performed similar analysis in Rio, using the district of Jurujuba as a control site, where conditions for mosquito collections was similar to Tubiacanga. For comparing the control site with Tubiacanga we used the expanded data analysis to 4 weeks before and 4 weeks after releases.

Ethics Statement

Mosquito releases in Tubiacanga were authorized by CONEP (CAAE 02524513.0.1001.0008), the National Research Council. The release of mosquitoes does not involve directly endangered or protected species and, from our experience, it does not have any significant impact on endangered or protected species.

Results

BGS Trap Collections

Ae. aegypti density was weekly monitored in Tubiacanga (release area) and Jurujuba (control area) before and after releasing *Wolbachia*-carrying mosquitoes (Fig 1). After releases started, the number of captured females increased substantially. In the second week of releases, the number of *Ae. aegypti* females captured was twice the average capture number from the pre-release period.

Daily samples were taken in the first two weeks after starting releases in Tubiacanga (Table 1). The capture rates were 1.7% (n = 40) on week 1 and 2.5% (n = 59) on week 2. The number of wild females was higher than the *Wolbachia*-carrying mosquitoes in all recapture days. Days 7 and 14 were release days and were removed from the analysis to avoid biases because there were no reliable methods to disentangle those released from the ones released a week earlier. During the releases, males were captured in lower numbers than females in BGS trap: 93 males in week 1 (among them, 23 were *Wolbachia*-infected) and 94 in week 2 (23 *Wolbachia*-infected).

Estimation of daily survival of Wolbachia-carrying mosquitoes in field

Considering daily collections in the first two weeks of release, the estimation for the probability of daily survival (PDS) for *Wolbachia*-carrying mosquitoes in Tubiacanga was 0.82 in week 1, and 0.89 in week 2 (Table 2).

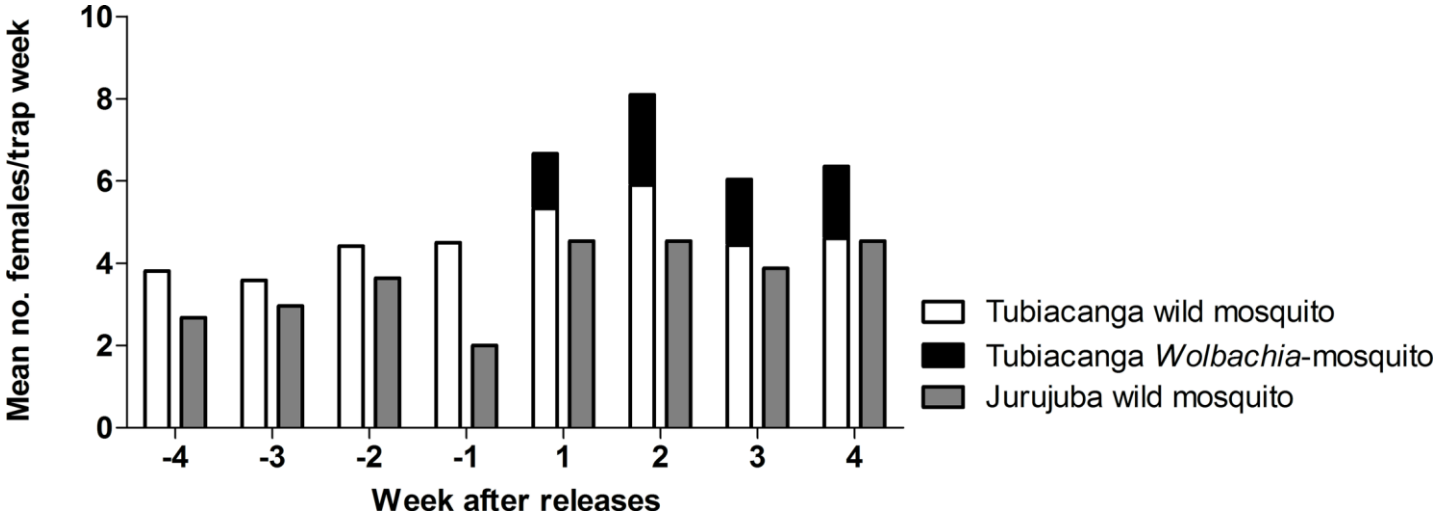

Fig 1. BG trap collections in field. Mean captures of female *Ae. aegypti* per BGS trap/week at the release site Tubiacanga and for the control area Jurujuba, for the periods of four weeks before and four weeks after the releases started. Mosquito captures increased after releases in Tubiacanga.

doi:10.1371/journal.pone.0160196.g001

Estimating the population size of wild mosquitoes based on Wolbachia releases in the field: average-difference model

Using average-difference model we estimate the population size based on changes on BGS capture, 2 weeks before and 2 weeks after the releases (Fig 1). In this method, we calculated the mean number of wild females before releases as 4.5 females/trap. A significant increase was observed after releases started, since this ratio augmented to 6.7 and 8.1 females/trap in weeks

Table 1. Daily capture rates after mosquito releases in Tubiacanga.

	Daily captures after release	
Week 1	*Wolbachia*-carrying mosquito	Wild mosquito
Day 1	12	42
Day 2	3	21
Day 3	8	16
Day 4	12	27
Day 5	3	28
Day 6	2	26
Total	40	160
Per BG trap (total/number of traps)	1.3	5.3
Capture rate (total/number of released)	1.7%	-
Week 2		
Day 8	12	47
Day 9	8	29
Day 10	17	35
Day 11	7	35
Day 12	10	33
Day 13	5	24
Total	59	203
Per BG trap (total/number of traps)	2.0	6.8
Capture rate (total/number of released)	2.5%	

doi:10.1371/journal.pone.0160196.t001

Table 2. Daily survival rate of *Wolbachia*-carrying females under field conditions.

		Wolbachia-carrying mosquito	Logarithm of counts		
Week 1	Day 1	12	2.56	Slope	-0.20
	Day 2	3	1.39	**exp(slope)**	**0.82**
	Day 3	8	2.20		
	Day 4	12	2.56		
	Day 5	3	1.39		
	Day 6	2	1.10		
Week 2	Day 8	12	2.56	Slope	-0.12
	Day 9	8	2.20	**exp(slope)**	**0.89**
	Day 10	17	2.89		
	Day 11	7	2.08		
	Day 12	10	2.40		
	Day 13	5	1.79		

doi:10.1371/journal.pone.0160196.t002

1 and 2, respectively (Table 3). The density of wild population was estimated at 70 and 55 females per trap (wk1 and wk2, respectably).

Estimating the population size of wild mosquitoes based on Wolbachia releases in the field: using MRR models

We used MRR models such as the Lincoln-Petersen and Fisher and Ford, to estimate the population size on a daily basis using *Wolbachia* as a marker (Table 4). The total number estimated by the average-difference method is shown for comparison purposes. The highest numbers were estimated from Lincoln-Petersen model and analysis using the average-difference model resulted in lowest abundance quantities.

Discussion

The endosymbiont bacterium *Wolbachia* has been deployed in field trials as a novel intervention aiming to reduce arboviruses transmission. *Wolbachia*-infected *Ae. aegypti* mosquitoes are

Table 3. Population size estimates using average-difference model.

Variable	Samples calculation (1/ 2 wk)
1) Mean no. of wild female *Ae. aegypti* per BGS trap, before release (95% CI) (-1/-2 wk)	4.5/4.5(1.3–7.5/ 2.7–6.3)
2) Mean no. of female *Ae. aegypti* per BGS trap after release of *Wolbachia*-infected mosquitoes (95% CI)	6.7/8.1(4.3–9.9/ 5.5–11.4)
3) Increase (mean ratio) in BGS collections of female due to released mosquitoes	2.2/3.6
4) Ratio of wild to released mosquitoes in BGS collections	2.0/1.3
5) Estimated no. of wild mosquitoes per trap = no. of released mosquitoes/trap x ratio of wild to released mosquitoes*	70/ 55
Estimated no. of wild mosquitoes per premise = no. of released mosquitoes/trap x ratio of wild to released mosquitoes x no. of traps / no. of premises	2.4/ 1.9
Estimated no. of wild mosquitoes per area (m^2) = no. of released mosquitoes/ trap x ratio of wild to released mosquitoes x no. of traps / total area	0.024/ 0.019

* The numbers of released mosquitoes/trap was estimated based on a mean of survival mosquitoes in field considering a daily survival of 0.8 (see more details on a S1 Table).

doi:10.1371/journal.pone.0160196.t003

Table 4. Wild population size (with confidence intervals) of *Ae. aegypti*.

		Fisher-Ford	Lincoln-Petersen	Average-difference
Week 1	Day 1	6,303	7,690	-
	Day 2	8,589	12,788	-
	Day 3	2,418	4,392	-
	Day 4	2,259	5,008	-
	Day 5	6,233	16,856	-
	Day 6	6,342	20,925	-
	Mean	5,357 (3,355–7,360)	11,276 (6,365–16,188)	2,100 (634–3,565)
	Per premise	6.2 (3.9–8.5)	13.0 (7.3–18.6)	2.4 (0.7–4.1)
Week 2	Day 8	7,641	8,585	-
	Day 9	6,140	7,750	-
	Day 10	3,279	4,650	-
	Day 11	6,567	10,463	-
	Day 12	4,015	7,186	-
	Day 13	4,818	9,688	-
	Mean	5,410 (4,086–6,735)	8,053 (6,406–9,700)	1,650 (987–2,313)
	Per premise	6.2 (4.7–7.8)	9.3 (7.3–11.2)	1.9 (1.1–2.7)

doi:10.1371/journal.pone.0160196.t004

established in areas of North Queensland, Australia and Vietnam and ongoing releases are taking place in Brazil, Colombia and Indonesia [36, 45, 46]. One of the milestones to obtain a successful invasion of *Wolbachia* into the local population is to release a sufficient number of mosquitoes which exceeds the threshold invasion [38]. Such threshold for invasion highlights the need for reliable estimates on the population size of wild mosquitoes in the target area. Additionally, invasion is dependent on releasing a mosquito population not only fit to survive in the natural environment but also with a strong cytoplasmic incompatibility, a crucial mechanism to facilitate *Wolbachia* spread [35]. Using an MRR approach in which mosquitoes are individually screened for *Wolbachia* presence, we are able to estimate significant aspects of vector biology by performing daily collections of *Wolbachia*-infected *Ae. aegypti* mosquitoes released once a week. Herein, we provide estimates of two critical parameters for invasion success: the population size of wild mosquitoes and the probability of daily survival of *Wolbachia*-carrying mosquitoes in the field.

Ritchie et al. (2013) [37] and Nguyen et al. (2015) [47] took advantage of the *Wolbachia* releases to estimate wild *Ae. aegypti* density. However, in their work, the bacteria was not used as an individual marker in the field. Using the average-difference model, they assumed the increase in the capture rates after releases was due to the addition of *Wolbachia*-infected *Ae. aegypti* through releases. Using data from the first *Wolbachia* release in Latin America, we proposed individual *Wolbachia* screening to use not only the average-difference model but also the Lincoln-Petersen and Fisher-Ford models, and thus compared population size estimates among methods. Different from our results, Ritchie et al. (2013) and Nguyen et al. (2015) showed that released mosquitoes were more abundant than the wild ones, suggesting low vector infestation in release areas. For example, in two sites from North Queensland, a ratio of 1:1.2–1.7 (wild:*w*Mel) was observed during the first two weeks of *w*Mel releases [37]. In Vietnan, a ratio of 1:2–4 was observed during the *w*MelPop releases. Our results in Rio de Janeiro/Brazil using the same average-difference model showed a ratio of 1: 0.5–0.7, which could hinder *Wolbachia* invasion due to high density of native mosquitoes. Thus, a successful invasion may require the period for *Wolbachia* releases to last longer or/and an increase in the number of released mosquitoes.

Our estimates of BGS trapping efficiency for female *Wolbachia*-carrying mosquito (ranging from 1.7 to 2.5%) were lower when compared to other MRR experiments in Rio de Janeiro. Usually the recapture rates ranged from 7 to 15% [15, 29, 48]. Ritchie et al 2013 [37] in North Queensland had sampling rates of 5 to 10% after releasing *Wolbachia*-carrying mosquitoes. Our lower recapture rate might be explained by the limited number of BGS traps installed, i.e., roughly, we had one BG every 30 houses. Probably, recapture rates increase if additional collecting methods such as backpack aspirator are used [15].

Mosquito population in Rio de Janeiro presents strong variation over short periods of time, eventually doubling its recapture rate on an interval of few weeks [49]. Under this scenario, estimates on *Ae. aegypti* population size using the average-difference model may have limited reliability because a fraction of the increase in collections after *Wolbachia* releases could be due to a natural fluctuation of mosquito population (see Fig 1). In this case, mosquito counts might not be accurate, because the excess numbers are considered to be all *Wolbachia*-mosquitoes.

We estimated the PDS of *Wolbachia*-infected mosquitoes under field conditions, which ranged from 0.82 to 0.89 in the first two weeks after releases in Tubiacanga. This is the first estimation of daily survival of *Wolbachia Ae. aegypti* mosquitoes in the field using methodology from MRR literature. Overall, it seems the *w*Mel strain did not affect significantly the daily survival of *Ae. aegypti* [37], since the PDS values observed are similar or even higher than PDS values found for wild mosquitoes in previous studies [15, 28, 50]. In particular, Maciel-de-Freitas et al. (2007) [15] found a PDS ranging from 0.71 to 0.75 in Tubiacanga during dry and wet seasons of 2007. Other studies in Rio de Janeiro raised the hypothesis that *Ae. aegypti* PDS might depend on the urban landscape. For instance, a PDS of ~0.93 was observed in a highly dense typical Brazilian slum, whereas in suburban districts, PDS ranged from 0.73 to 0.89 [48]. Finally, in a sparsely populated high-income neighborhood, we observed the lowest PDS of Rio, varying from 0.61 to 0.70 [15, 39, 48, 51]. Under such scenarios the effect of urban landscape on mosquito survival is potentially due to the availability of human hosts and breeding sites. Maciel-de-Freitas et al. (2007b) [52] observed a tendency towards higher *Ae. aegypti* survival in areas with a high human density. In crowded districts such as slums, mosquitoes would not present a long flight to find host or breeding sites, reducing the odds of mortality due to harsh environmental factors, insecticide use or even by the defensive host behavior [53].

Given the low capture rates in the field, the exponential model is used extensively for estimating the probability of daily survival. Analysis under the exponential model does not consider the number of individuals captured to be removed from environment due to daily collections, which might impact subsequent collections. A possible approach for estimation under such conditions is to use the method proposed by Buonaccorsi et al (2003) [54].

By individually screening *Ae. aegypti* females for *Wolbachia*, we were able to estimate the population size of wild mosquitoes using three different models. MRR models provided higher values of population size than the average-difference model. Probably, the difference is because the average-difference model misdetected some wild mosquitoes as the *Wolbachia* ones, contributing to underestimate the wild population size (calculated as 2,100 week 1 and 1,650 week 2). This occurs due to large oscillation in number of wild mosquitoes between weeks in Rio de Janeiro. Therefore, MRR models under individual screening by qPCR give us more accurate quantities because in this case, we know precisely which mosquitoes had *Wolbachia*. Due to high mortality rates, Lincoln-Petersen results possibly overestimated population size (estimated as 11,276 week 1 and 8,053 week 2), since the model does not consider mortality rate in released mosquitoes. Finally, the index from Fisher-Ford model seems to be a reliable estimator of population size (5,357 week 1 and 5,410 week 2), since the model considers mortality rate during the experiment studies.

Our MRR results suggest that fitness of *Wolbachia*-infected mosquitoes in the field is high enough to promote invasion. By screening mosquitoes individually and using this information to generate quantities to be applied to MRR models, we avoided inaccurate estimations due to fluctuation in the average number of mosquitoes per week. Such technique is deemed highly important to determining in future release sites the minimum number of released individuals, by taking into account the wild population size in order to achieve a sustainable *Wolbachia* invasion over time.

Supporting Information

S1 Table. Estimating the number of *Wolbachia*-carrying *Ae. aegytpi* females in field, based on a daily survival of 0.8.
(DOCX)

Acknowledgments

We are grateful to all members of the Mosquitos Vetores: Endossimbiontes e Interação Patógeno Vetor (MV–CPqRR/FIOCRUZ), especially Dr. Luciano A. Moreira and Dr. Fernando Braga Dias, and members of Laboratório de Transmissores de Hematozoários (LATHEMA–FIOCRUZ).

Author Contributions

Conceptualization: GAG DAMV RMF. Formal analysis: DAMV. Funding acquisition: RMF. Investigation: GAG LMBS RMF. Methodology: GAG LMBS DAMV. Resources: RMF. Software: DAMV. Writing - original draft: GAG DAMV RMF. Writing - review & editing: GAG LMBS DAMV RMF.

References

1. Schatzmayr HG. Dengue situation in Brazil by year 2000. Mem Inst Oswaldo Cruz. 2000; 95 Suppl 1:179–81. PMID: 11142711
2. Kraemer MU, Sinka ME, Duda KA, Mylne AQ, Shearer FM, Barker CM, et al. The global distribution of the arbovirus vectors *Aedes aegypti* and *Ae. albopictus*. Elife. 2015 Jun 30; 4:e08347. doi: 10.7554/eLife.08347 PMID: 26126267
3. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, et al. The global distribution and burden of dengue. Nature. 2013 Apr 25; 496(7446):504–7. doi: 10.1038/nature12060 PMID: 23563266
4. PAHO/WHO, 2016. Number of reported cases of dengue and severe dengue (SD) in Americas, by country. Avaiable from: http://www.paho.org/hq/index.php?option=com_topics&view=article&id=1&Itemid=40734
5. Focks DA, Brenner RJ, Hayes J, Daniels E. Transmission thresholds for dengue in terms of *Aedes aegypti* pupae per person with discussion of their utility in source reduction efforts. Am J Trop Med Hyg. 2000 Jan; 62(1):11–8. PMID: 10761719
6. Maciel-de-Freitas R, Valle D. Challenges encountered using standard vector control measures for dengue in Boa Vista, Brazil. Bull World Health Organ. 2014 Sep 1; 92(9):685–9. doi: 10.2471/BLT.13.119081 PMID: 25378760
7. Fares RC, Souza KP, Añez G, Rios M. Epidemiological scenario of dengue in Brazil. Biomed Res Int. 2015; 2015:321873. doi: 10.1155/2015/321873 PMID: 26413514
8. Azevedo RdS, Oliveira CS, Vasconcelos PF. Chikungunya risk for Brazil. Rev Saude Publica. 2015; 49:58. doi: 10.1590/S0034-8910.2015049006219 PMID: 26398876
9. Zanluca C, de Melo VC, Mosimann AL, Dos Santos GI, Dos Santos CN, Luz K. First report of autochthonous transmission of Zika virus in Brazil. Mem Inst Oswaldo Cruz. 2015 Jun; 110(4):569–72. doi: 10.1590/0074-02760150192 PMID: 26061233
10. Consoli RAGB, Lourenço-de-Oliveira R. Principais Mosquitos de Importância Sanitária no Brasil. 1rst ed. Rio de Janeiro: Fiocruz; 1994.

11. Chouin-Carneiro T, Vega-Rua A, Vazeille M, Yebakima A, Girod R, Goindin D, et al. Differential Susceptibilities of *Aedes aegypti* and *Aedes albopictus* from the Americas to Zika Virus. PLoS Negl Trop Dis. 2016 Mar 3; 10(3):e0004543. doi: 10.1371/journal.pntd.0004543 PMID: 26938868
12. Ayres CF. Identification of Zika virus vectors and implications for control. Lancet Infect Dis. 2016 Mar 16(3):278–9. doi: 10.1016/S1473-3099(16)00073-6 PMID: 26852727
13. Edman JD, Strickman D, Kittayapong P, Scott TW. Female *Aedes aegypti* (Diptera: Culicidae) in Thailand rarely feed on sugar. J Med Entomol. 1992 Nov 29(6):1035–8. PMID: 1460619
14. Braks MA, Lourenço-De-Oliveira R, Juliano SA, Lounibos LP. Convergent habitat segregation of *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) in southeastern Brazil and Florida. J Med Entomol. 2003 Nov; 40(6):785–94. PMID: 14765654
15. Maciel-de-Freitas R, Codeço CT, Lourenço-de-Oliveira R. Daily survival rates and dispersal of *Aedes aegypti* females in Rio de Janeiro, Brazil. Am J Trop Med Hyg. 2007a Apr; 76(4):659–65.
16. Castro M, Sánchez L, Pérez D, Sebrango C, Shkedy Z, Van der Stuyft P. The relationship between economic status, knowledge on dengue, risk perceptions and practices. PLoS One. 2013 Dec 12; 8(12): e81875. doi: 10.1371/journal.pone.0081875 PMID: 24349145
17. Cattand P, Desjeux P, Guzmán MG, Jannin J, Kroeger A, Medici A, et al. Tropical diseases lacking adequate control measures: dengue, leishmaniasis, and african trypanosomiasis. In: Jamison DT, Breman JG, Measham AR, Alleyne G, Claeson M, editors. Disease Control Priorities in Developing Countries. 2nd edition. Washington (DC): World Bank, Chapter 23; 2006.
18. Olanratmanee P, Kittayapong P, Chansang C, Hoffmann AA, Weeks AR, Endersby NM. Population genetic structure of *Aedes* (Stegomyia) *aegypti* (L.) at a micro-spatial scale in Thailand: implications for a dengue suppression strategy. PLOS Negl Trop Dis. 2013; 7(1):e1913. doi: 10.1371/journal.pntd.0001913 PMID: 23326609
19. Perkins TA, Scott TW, Le Menach A, Smith DL. Heterogeneity, mixing, and the spatial scales of mosquito-borne pathogen transmission. PLoS Comput Biol. 2013 9(12):e1003327. doi: 10.1371/journal.pcbi.1003327 PMID: 24348223
20. Coelho GE, Teixeira Mda G, Coutinho FA, Massad E. Dynamics of the 2006/2007 dengue outbreak in Brazil. Mem Inst Oswaldo Cruz. 2008 Sep; 103(6):535–9. PMID: 18949321
21. Focks DA, Chadee DD. Pupal survey: an epidemiologically significant surveillance method for *Aedes aegypti*: an example using data from Trinidad. Am J Trop Med Hyg. 1997 Feb; 56(2):159–67. PMID: 9080874
22. Morrison AC, Zielinski-Gutierrez E, Scott TW, Rosenberg R. Defining challenges and proposing solutions for control of the virus vector *Aedes aegypti* PLoS Med. 2008 Mar 18; 5(3):e68. doi: 10.1371/journal.pmed.0050068 PMID: 18351798
23. Johnson PH, Spitzauer V, Ritchie SA. Field sampling rate of BG-sentinel traps for *Aedes aegypti* (Diptera: Culicidae) in suburban Cairns, Australia. J Med Entomol. 2012 Jan 49(1): 29–34. PMID: 22308768
24. Codeço CT, Lima AW, Lima JB, Maciel-de-Freitas R, Honório NA, Galardo AK, et al. Surveillance of *Aedes aegypti*: comparison of house index with four alternative traps. PLoS Negl Trop Dis. 2015 Feb 10; 9(2):e0003475. doi: 10.1371/journal.pntd.0003475 PMID: 25668559
25. Sheppard PM, Macdonald WW, Tonn RJ. A new method of measuring the relative prevalence of *Aedes aegypti*. Bull World Health Organ. 1969; 40(3):467–8. PMID: 5306629
26. Trpis M, Hausermann W, Craig GB. Estimates of population size, dispersal, and longevity of domestic *Aedes aegypti aegypti* (Diptera: Culicidae) by mark-release-recapture in the village of Shauri Moyo in eastern Kenya. J. Med. Entomol. 1995 Jan; 32(1):27–33. PMID: 7869339
27. Silver JB. Mosquito ecology: field sampling methods. 3rd ed. New York: Springer; 2008.
28. Muir LE, Kay BH. *Aedes aegypti* survival and dispersal estimated by mark-release-recapture in northern Australia. Am J Trop Med Hyg. 1998 Mar; 58(3):277–82. PMID: 9546403
29. Villela DA, Figueiredo F, Garcia GA, Maciel-de-Freitas R, Struchiner CJ. A bayesian hierarchical model for estimation of abundance and spatial density of *Aedes aegypti*. PLoS One. 2015 Apr 23; 10(4): e0123794. doi: 10.1371/journal.pone.0123794 PMID: 25906323
30. Hilgenboecker K, Hammerstein P, Schlattmann P, Telschow A, Werren JH. How many species are infected with *Wolbachia*? A statistical analysis of current data FEMS Microbiol Lett. 2008 Apr; 281 (2):215–20. doi: 10.1111/j.1574-6968.2008.01110.x Epub 2008 Feb 28. PMID: 18312577
31. Zug R, Hammerstein P. Still a host of hosts for *Wolbachia*: analysis of recent data suggests that 40% of terrestrial arthropod species are infected. PLoS One. 2012; 7(6):e38544. doi: 10.1371/journal.pone.0038544 PMID: 22685581

32. de Oliveira CD, Gonçalves DS, Baton LA, Shimabukuro PH, Carvalho FD, Moreira LA. Broader prevalence of *Wolbachia* in insects including potential human disease vectors. 2015 Jun; 105(3):305–15. doi: 10.1017/S0007485315000085 PMID: 25772521

33. Moreira LA, Ormaetxe II, Jeffery JA, Lu G, Pyke AT, Hedges LM, et al. A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, chikungunya, and plasmodium. Cell. 2009 Dec 24; 139(7):1268–78. doi: 10.1016/j.cell.2009.11.042 PMID: 20064373

34. Ye YH, Carrasco AM, Frentiu FD, Chenoweth SF, Beebe NW, vanden Hurk AF, et al. *Wolbachia* Reduces the Transmission Potential of Dengue-Infected *Aedes aegypti*. PLoS Negl Trop Dis. 2015 Jun 26; 9(6):e0003894. doi: 10.1371/journal.pntd.0003894 PMID: 26115104

35. Werren JH, Baldo L, Clark ME. *Wolbachia*: master manipulators of invertebrate biology. Nat Rev Microbiol. 2008 Oct; 6(10):741–51. doi: 10.1038/nrmicro1969 Review PMID: 18794912

36. Hoffmann AA, Montgomery BL, Popovici J, Iturbe-Ormaetxe I, Johnson PH, Muzzi F, et al. Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission. Nature. 2011 Aug 24; 476(7361):454–7. doi: 10.1038/nature10356 PMID: 21866160

37. Ritchie SA, Montgomery BL, Hoffmann AA. Novel estimates of *Aedes aegypti* (Diptera: Culicidae) population size and adult survival based on *Wolbachia* releases.J Med Entomol. 2013 May; 50(3):624–31. PMID: 23802459

38. Turelli M, Hoffmann AA. Rapid spread of an inherited incompatibility factor in California *Drosophila*. Nature. 1991 Oct 3; 353(6343):440–2. PMID: 1896086

39. Dutra HL, Dos Santos LM, Caragata EP, Silva JB, Villela DA, Maciel-de-Freitas R, et al. From lab to field: the influence of urban landscapes on the invasive potential of *Wolbachia* in brazilian *Aedes aegypti* Mosquitoes. PLoS Negl Trop Dis. 2015 Apr 23; 9(4):e0003689. doi: 10.1371/journal.pntd.0003689 PMID: 25905888

40. Krockel U, Rose A, Eiras AE, Geier M. New tools for surveillance of adult yellow fever mosquitoes: comparison of trap catches with human landing rates in an urban environment. J Am Mosq Control Assoc. 2006 Jun; 22(2):229–38. PMID: 17019768

41. Maciel-de-Freitas R, Eiras AE, Lourenço-de-Oliveira R. Field evaluation of effectiveness of the BG-Sentinel, a new trap for capturing adult *Aedes aegypti* (Diptera: Culicidae). Mem Inst Oswaldo Cruz. 2006b May; 101(3):321–5.

42. Dutra HL, Rocha MN, Dias FB, Mansur SB, Caragata EP, Moreira LA. *Wolbachia* blocks currently circulating Zika virus isolates in brazilian *Aedes aegypti* mosquitoes. Cell Host Microbe. 2016 Jun 8; 19 (6):771–4. doi: 10.1016/j.chom.2016.04.021 PMID: 27156023

43. Gillies MT. Studies on the dispersion and survival of *Anopheles gambiae* Giles in East Africa, by means of marking and releasing experiments. Bull Entomol Res. 1961 52: 99–127.

44. Harrington LC, Françoisevermeylen, Jones JJ, Kitthawee S, Sithiprasasna R, Edman JD, et al. Age-dependent survival of the dengue vector *Aedes aegypti* (Diptera: Culicidae) demonstrated by simultaneous release-recapture of different age cohorts. J Med Entomol. 2008 Mar; 45(2):307–13. PMID: 18402147

45. Hoffmann AA, Iturbe-Ormaetxe I, Callahan AG, Phillips BL, Billington K, Axford JK, et al. Stability of the *w*Mel *Wolbachia* Infection following Invasion into *Aedes aegypti* Populations. PLoS Negl Trop Dis. 2014 Sep 11; 8(9):e3115. doi: 10.1371/journal.pntd.0003115 eCollection 2014 Sep PMID: 25211492

46. Frentiu FD, Zakir T, Walker T, Popovici J, Pyke AT, van den Hurk A, et al. Limited dengue virus replication in field-collected *Aedes aegypti* mosquitoes infected with *Wolbachia*. PLoS Negl Trop Dis. 2014 Feb 20; 8(2):e2688. doi: 10.1371/journal.pntd.0002688 eCollection 2014 PMID: 24587459

47. Nguyen TH, Nguyen HL, Nguyen TY, Vu SN, Tran ND, Le TN, et al. Field evaluation of the establishment potential of *w*MelPop *Wolbachia* in Australia and Vietnam for dengue control. Parasit Vectors. 2015 Oct 28; 8:563. doi: 10.1186/s13071-015-1174-x PMID: 26510523

48. David MR, Lourenço-de-Oliveira R, Maciel-de-Freitas R. Container productivity, daily survival rates and dispersal of *Aedes aegypti* mosquitoes in a high income dengue epidemic neighbourhood of Rio de Janeiro: presumed influence of differential urban structure on mosquito biology. Mem Inst Oswaldo Cruz. 2009 Sep; 104(6):927–32. PMID: 19876569

49. Maciel-de-Freitas R, Lourenço-de Oliveira R 2011. Does targeting key-containers effectively reduce *Aedes aegypti*. Trop Med Int Health. 2011 Aug; 16(8):965–73. doi: 10.1111/j.1365-3156.2011.02797.x Epub 2011 May 23. PMID: 21605290

50. Harrington LC, Buonaccorsi JP, Edman JD, Costero A, Kittayapong P, Clark GG, et al. Analysis of survival of young and old *Aedes aegypti* (Diptera: Culicidac) from Puerto Rico and Thailand. J Med Entomol. 2001 Jul; 38(4):537–47. PMID: 11476334

51. Maciel-de Freitas R, Eiras AE, Lourenço-de Oliveira R. Calculating the survival rate and estimated population density of gravid *Aedes aegypti* (Diptera: Culicidae) in Rio de Janeiro, Brazil. Cad Saude Publica. 2008 Dec; 24(12):2747–54. PMID: 19082265

52. Maciel-de-Freitas R, Marques WA, Peres RC, Cunha SP, de Oliveira RL. Variation in *Aedes aegypti* (Diptera: Culicidae) container productivity in a slum and a suburban district of Rio de Janeiro during dry and wet seasons. Mem Inst Oswaldo Cruz. 2007b Jun; 102(4):489–96.

53. Clements AN, Paterson GD. The analysis of mortality and survival rates in wild populations of mosquitoes. J Appl Ecol. 1981 18:373–399.

54. Buonaccorsi JP, Harrington LC, Edman JD (2003) Estimation and comparison of mosquito survival rates with release-recapture-removal data. J Med Entomol, 40(1), 6–17. PMID: 12597647

Artigo 7

(Em preparação)

The riddle solved on a local grocery store: the release of *Aedes aegypti* as resistant to pyrethroids as the wild population is essential for *Wolbachia* invasion

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INTRODUCTION

Mosquito-borne diseases heavily impact human health around the world, especially considering the burden of arboviruses in the last decades. Dengue virus (DENV) is a flavivirus distributed mainly in tropical and subtropical areas, with around 400 million new infections yearly (Bhatt et al. 2013). Brazil is the country with the higher number of DENV cases, with more than one million cases per year in the last three years (PAHO 2016). Chikungunya virus (CHIKV) took a dramatic turn in the early 2000`s when a new epidemic strain emerged from an enzoonotic lineage (Weaver and Forrester 2015). In 2014, Brazil registered the first autochthonous CHIKV infections, from the Asian genotype in Oiapoque (Amapá State) but also from the East Central South Africa (ECSA) genotype in Feira de Santana-Bahia State (Nunes et al. 2015). The ECSA genotype increased its distribution range and is currently disseminated in larger areas of the country (Souza et al. 2017). The world also recently witnessed the rapid spread of Zika Virus (ZIKV) within the span of one year. In 2014 ZIKV was introduced in Brazil from the Pacific Islands and one year later an increase of microcephaly and neurological damage in newborn babies forced the World Health Organization (WHO) to declare a Public Health Emergency of International Concern (Peterson and Powers 2016, Barreto et al. 2016).

The three aforementioned arboviruses have at least one major similarity: they are overwhelmingly transmitted by *Aedes aegypti*. So far, vector control is still the most recommended action to mitigate disease transmission. Existing control programs are mostly reliant on the use of insecticides and larvae breeding source reduction. However, due to insecticide resistance in natural *Ae. aegypti* populations and the unfeasibility of extending breeding sites elimination over large cities, the effectiveness of traditional control methods has been discussed (Barreto et al. 2011, Marcombe et al. 2011, Maciel-de-Freitas et al. 2014a, Maciel-de-Freitas and Valle 2014b). Therefore, the development of new strategies to join traditional vector control methods must be encouraged (Yakob and Walker 2016).

One of these innovative approaches consist of deploying *Wolbachia pipientis* to block arboviruses transmission in endemic urban settlements (Hoffman et al. 2011, Walker et al. 2011). *Wolbachia* is an obligate endosymbiotic maternally transmitted bacterium that is

estimated to naturally infect around 60% of all arthropod species (Werren and Windsor 2000). The bacterium role as a natural control agent was reinforced after discovering of its blocking ability towards arboviruses transmission in *Ae. aegypti* mosquitoes (Moreira et al. 2009, Aliota et al. 2016, Dutra et al. 2016). *Wolbachia* is able to manipulate host behavior and produce a phenotype known as cytoplasmic incompatibility that together with high maternal transmission provides *Wolbachia*'s drive mechanism and thus facilitates its spread into a field population. Ultimately, the main objective of deploying *Wolbachia* is to replace a vector population with high vector competence for arboviruses to mosquitoes that block virus transmission.

Successful *Wolbachia* releases using *w*Mel strain have been conducted in Northern Queensland, Australia and Tri Nguyen Island, Vietnam (Hoffman et al. 2011, Walker et al. 2011, Nguyen et al. 2015). Considering a nation-wide dissemination of insecticide resistance to pyrethroids and organophosphates (Montella et al. 2007, Linss et al. 2014) and promising results on pilot releases, the Brazilian Ministry of Health and the Eliminate Dengue Program pursued *Wolbachia* insertion into a high dengue endemic district of Rio de Janeiro. After releasing a total of 180,000 mosquitoes carrying the *w*MelBr strain during 20 consecutive weeks, *Wolbachia* was not able to spread into Tubiacanga *Ae. aegypti* population. Herein, we report what jeopardized *Wolbachia* invasion, the steps required to produce a fit strain and the results of two successive releases at Rio de Janeiro.

MATERIALS AND METHODS

Study sites. Mosquitoes infected with *Wolbachia* were released in two isolated communities: Tubiacanga (22°47'06"S; 43°13'32"W) and Jurujuba (22 22°55'37"S; 43°07'11"W). Tubiacanga is a lower middle class community located on a lowland coastal area on the shores of Guanabara Bay, with around 3,000 residents in 690 houses. Jurujuba is located uphill on the shores of Guanabara Bay at Niteroi Municipality. It is a working-class community with 400 premises and around 1200 inhabitants, narrow paved streets, regular piped water distribution and garbage collection (Maciel-de-Freitas et al. 2007, Dutra et al.

2015). Weekly climatic data (mean, minimum and maximum temperature, accumulated rainfall) was collected on a meteorological station located 6Km from Tubiacanga (Figure S1).

Ethical considerations. *Wolbachia* deployment was authorized by the National Research Ethics Committee (CONEP, CAAE 02524513.0.1001.0008), and further regulatory approval was obtained by three Government agencies: IBAMA (Ministry of Environment), Anvisa (Ministry of Health) and MAPA (Ministry of Agriculture). According to ethical and regulatory approval, door-to-door activity and formal signed consent for releases of at least 70% of the sampled households (around 30% of households) was required. Questionnaires were responded by adults above 21 years old. One strong request of CONEP was not to change any practices performed by the community or the municipality vector control.

Backcrossing of *w*MelBr-infected *Ae. aegypti*. wMel-infected *Ae. aegypti* were imported from Australia (IBAMA license 11BR005873/DF) to produce a *Wolbachia*-infected line with a Brazilian genetic background, named *w*MelBr (details on Dutra et al. 2015). An inbreed laboratory colony was maintained before the obtaining the regulatory approvals for mosquito releases. Subsequently, every five generations, we added 10% of wild males per cage from a Rio de Janeiro population mix to refresh the genetic pool of that colony.

Community engagement (CE) and Communication (Comms) strategy for *w*MelBr release. Comms and CE activities were conducted in integration with the entomology team. CE initiated with the identification of local stakeholders, often households belonging to residents' association, local markets, churches or healthcare units. Representatives from the community formed the Community Reference Groups (CRG), meeting monthly with people from Eliminate Dengue project to share advances and concerns regarding public health issues, especially those related to vector-borne diseases. The main objective of community

engagement process was to promote awareness in the community before releases and provide substantial background information to households before the participation on the survey to authorize wMelBr mosquito release. The releases had a massive participation of local householders opening release cups with mosquitoes and received great media coverage, with national live broadcasting from Tubiacanga.

***w*MelBr colony quality assurance and mass rearing.** We randomly selected 100 3rd -4th instar larvae per generation to estimate the frequency of *Wolbachia* in the colony, with a threshold of at least 98% positivity to support releases. Larvae were reared with 0.45g of Tetramin Flakes® (Tetra GmbH, Herrenteich, Germany) every two days in a 3L plastic tray at 28° C. Under this condition, we had an average of >96% of pupae on day seven, with a standard deviation inferior to 5% (data not shown). Regulatory approval was obtained on May/2014, 12 months after the colony was closed. Releases started four months after regulatory approval, i.e., 17 generations after the lab colony has been closed.

Quality control of *w*MelBr released mosquitoes. Every week, ten of the release cups (50 mosquitoes per cup) were bring back to the lab to assess whether released mosquitoes were fit. We assessed male and female wing size, adult mortality and sex ratio. Wing length was defined as the distance from the axillary incision to the apical margin excluding the fringe (Harbach and Knight 1980). Mortality and sex ratio was monitored visually as soon as release cups returned to the insectary, i.e., when mosquitoes had roughly one week old.

***w*MelBr release.** In the first release of Tubiacanga, 50 mosquitoes with 4-6 days old were released early morning (05:00 AM) in front of every four houses. *w*MelBr deployment started on September 2014 and lasted 20 consecutive weeks, i.e., up to January 2015. The total number of released mosquitoes increased over time, starting with 7500 on week 1 and ending with 15000 on week 20.

***Wolbachia* detection.** Adult mosquitoes were individually screened through a multiplex qRT-PCR. After DNA extraction (0.1 M NaCl; 10 mM Tris Base; 1 mM EDTA; pH 8.2) supplemented with 9 µg of Proteinase K (Qiagen), *Wolbachia* was detected for WD0513 gene. We also analyzed a ribosomal gene of *Ae. aegypti* (Fergurson et al., 2008). The amplification was carried out on ViiA-7 Machine (Thermo Scientific) using Taqman Universal PCR Master Mix following manufacter's instructions.

Mitochondrial DNA. In order to test whether released mosquitoes had any leakage on *Wolbachia* maternal transmission, we conducted mitochondrial DNA analysis in *Ae. aegypti* field-caught females. Any wMelBr negative mosquito with mitochondrial DNA from Australian populations would point to an imperfect maternal transmission, which could jeopardize *Wolbachia* deployment. Two plates, consisting of 84 mosquitoes each were screened for *Wolbachia* infection, representing two time points: weeks 12 and 14.

***w*MelRio backcrossing.** Whenever the need of releasing a mosquito as resistant to pyrethroids as field populations became imperative, we started a new backcrossing using 500 females from *w*MelBr colony and 500 males from Urca, an urban district from Rio with high kdr frequency (Ademir Martins, unpublished data), every generation. During backcrossing, we had a total of six populations, each one representing one step of the backcrossing to produce *w*MelRio (*w*MelBr, Field (Urca), *w*MelF1CP, *w*MelB1, *w*MelB2, and *w*MelRio itself). We added a seventh population, *w*MelBrTet, which was treated with tetracycline to remove *Wolbachia* and maintain mosquito background) (Figure S2). Therefore, the frequency of *kdr* alleles increased over time. Every generation, 100 mosquitoes were randomly sampled for *Wolbachia* and *kdr* screening.

Monitoring kdr frequency during and after releases. Brazilian populations may present the susceptible wild-type (Na_vS) and two *kdr* alleles: substitution restricted to the 1534 position (Na_VR1) or concurrent substitutions in both 1534 and 1016 sites (Na_VR2)

(Linss et al. 2014), with Na_VR2 homozygous lineage exhibiting severe fitness cost (Brito et al. 2013). We estimated the frequency of *kdr* alleles (Val1016Ile and Phe1534Cys) as a proxy of pyrethroid resistance in Tubiacanga and released populations on the same DNA samples employed for *Wolbachia* identification. For that, on weeks 1, 8, 16, 20 (during *w*MelBr release), 26, 30, 34 (between releases), 51, 58, 66, 70 (during *w*MelRio release) and 76, 80 and 84 (after *w*MelRio release) we randomly selected at least 60 field-caught *Ae. aegypti* females per week, among which 30 were positive for *w*MelBr and other 30 negative, i.e., wild-type females (according to qPCR screening). Both 1016 (Val^+ and Ile^{kdr}) and 1534 (Phe^+ and Cys^{kdr}) sites of the *Ae. aegypti* Na_V were genotyped with a customized TaqMan genotyping assay (ThermoFischer Scientific) independently for each site. Primers, probes and reaction are described elsewhere (Brito et al. 2013). Allelic and genotypic frequencies considered 1016 and 1534 sites in a single locus, constituted by the alleles: Na_VS (1016 Val^+ + 1534 Phe^+), Na_VR1 (1016 Val^+ + 1534 Cys^{kdr}) and Na_VR2 (1016 Ile^{kdr} + 1534 Cys^{kdr}) (Linss et al 2014).

Insecticide resistance bioassays of field and released populations. We performed bioassays on *w*MelBr, *w*MelRio, and field populations (Tubiacanga, Jurujuba and Urca) to determine their insecticide resistance profile. Rockefeller lineage was used as a control parameter of insecticide susceptibility (Kuno 2010). Dose-response bioassays were performed with third instar larvae to the organophosphate temephos (Pestanal®, Sigma-Aldrich), and adults to the pyrethroid deltamethrin and the organophosphate malathion using 3 to 5 days-old females with WHO test tubes (WHO 1981, WHO 1998, Brito et al 2013; Bellinato et al 2016). Lethal concentrations were calculated with Probit analysis [software Polo-PC, LeOra Software, Berkeley, CA] (Raymond et al. 1985). Resistance Ratios (RR_{50} and RR_{90}) were obtained by the quotient of the concentrations or time values of the populations with the ones of the reference strain Rockefeller (WHO 1981, WHO 1998).

Estimating fitness costs of pyrethroid resistance on laboratory colony. The frequencies of *kdr* allele were obtained from a sample of 100 mosquitoes of F1 and F18 generations from the *w*MelBr backcrossing. Wild males were added every five generations to refresh colony genetics and consequently added resistance alleles. We estimated the fitness

cost due to insecticide resistance by applying a Monte-Carlo procedure over a two-allele model. The frequency of the resistance allele, observed in the first generation, is equal to the frequency of homozygous (simulated sample) plus half of the frequency of heterozygous individuals. Fitness cost applies for the genotype of homozygous resistant alleles (R1R1 and R2R2, similarly), whereas heterozygous individuals have a smaller cost by a multiplicative factor of 0.9. Successive population crossings are simulated for 17 generations assuming the frequency samples and fitness cost samples. In generations F10 and F15 we also simulate crossings with an added male population of resistant homozygous individuals by a ratio of one male to 10 females. After running 100,000 simulations, we obtain a subset whose results for a sample of frequencies in F18 is equal to the observed frequency for F18 generation plus/minus 1%.

Community engagement and Communication strategy for *w*MelRio release. The need to perform a second round of releases in Tubiacanga brought a big challenge for both the Community Engagement and Communication teams. We first focused on our most strong partners: the residents' association and CRG. We explained the reasons for *Wolbachia* not invading, listened to community expectations and clearly explained the timeline for the second release. This group adopted the intensification of door-to-door activities, meetings with householders and strengthened the collaboration with the primary school as the most relevant strategies to maintain a high community support. The Comms team produced flyers to support CE activities, banners for public areas with e-mail address and a telephone number to increase the feedback from community and schedule door-to-door visits whenever required. Local, national and international media filmed in Tubiacanga with volunteered residents. The second release was done with a great involvement of students from the local school and residents. *w*MelRio release in Jurujuba followed the same CE and Comms strategy.

Fitness assays. Mosquito life-history traits of backcrossing populations were measured to test whether the addition of alleles to promote pyrethroids resistance would affect the fitness of a mosquito already infected with *Wolbachia* (Figure S2).

Development time. Eggs of the seven tested populations were hatched and 3 groups of 100 first instar larvae per population were transferred to plastic trays (33 X 24 X 8 cm) that were monitored daily for 14 days to access the number of pupae per day. Survival analysis to censor the time larvae became pupae as the censoring event was performed among populations.

Adult survival. Sixty females per population were selected and isolated in labeled cylindrical plastic vials (6.5 cm height, 2.5 cm diameter) as Maciel-de-Freitas et al. (2011). Survival was checked daily and dead mosquitoes were stored in -80°C freezer for *Wolbachia* and kdr detection. *Ae. aegypti* longevity was normally distributed (Shapiro-Wilk W= 0.9942, P = 0.113). The effect of Wolbachia infection and density, frequency of kdr mutation and its interaction on mean *Ae. aegypti* longevity was analyzed by a three-way ANOVA. A Log-rank test followed by Bonferroni correction compared the survival distribution of *Ae. aegypti* females. We estimated by Cox proportional risk the hazard ratio of *Wolbachia* density and kdr frequency on *Ae. aegypti* survival.

Oviposition success, female fecundity and egg hatch. Females were fed once a week and eggs were counted 3-4 days later. Fecundity was analyzed by considering the first five clutches of eggs laid, since only a small number of females laid eggs beyond the fifth clutch, precluding adequate numbers for analysis. We analyzed the likelihood that a mosquito laid at least one egg (at a given clutch) with a logistic analysis that included Wolbachia presence and density, frequency of *kdr* mutation and clutch-number (i.e., age), backwards-eliminating the insignificant interactions. Second, we analyzed the number of eggs of the successful mosquitoes with a MANOVA repeated measures analysis. We included clutch-number as the repeat and estimated the effects of *Wolbachia* infection, *Wolbachia* density and frequency of kdr mutation.

Eggs laid on filter papers were kept for four days at room temperature to complete embryo development. Eggs were counted and the batch was hatched on plastic cups with Tetramim as food resource. Larvae were counted and divided by the number of eggs to access mosquito fertility. Egg hatch rate was monitored during the four first oviposition cycles.

Maternal transmission. All adult females and a sub-sample of up to 20 larvae per female was screened for *Wolbachia* on weeks 1 and 4. The main hypothesis was to test whether there was any failure on maternal transmission and if positive, to test if it was correlated with adding kdr during backcrossing.

Statistical analysis was conducted on R version 3.3.2 and JMP 13.

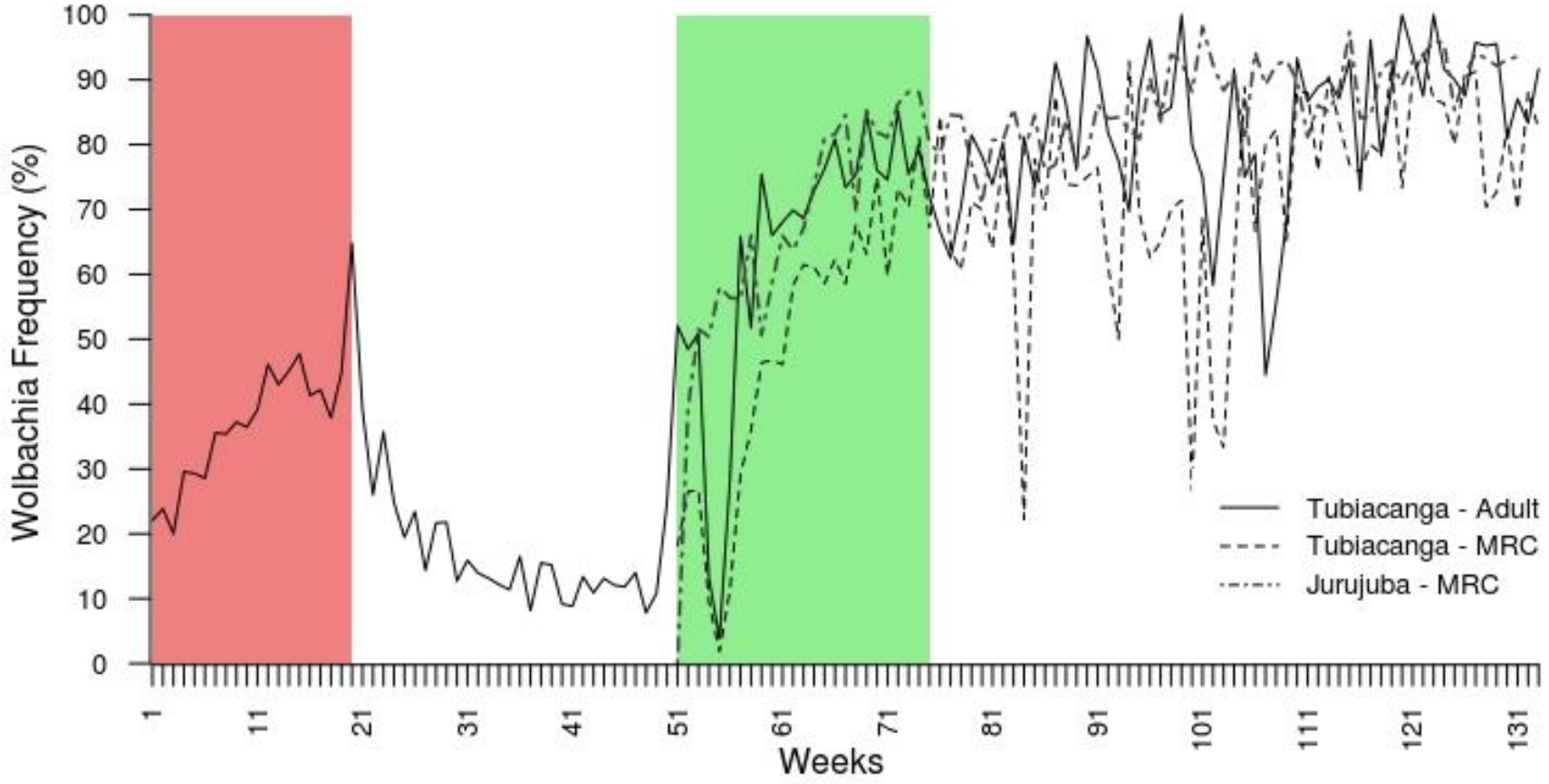
Genetic similarity among release and field strains. To assess the level of genetic similarity between released material and wild mosquitoes, we used double-digest RAD-sequencing, as previously described (Rašić et al. 2014). We analyzed 25 mosquitoes from each sample: the released colonies (*w*MelBr and *w*MelRio), field-caught individuals from Tubiacanga and Gordonvale (Queensland, Australia, as a proxy for the original *w*Mel transfected colony). The 100 bp PE reads from the Illumina HiSeq platform were deposited to NCBI (SRA PRJNA273913, PRJNA330553). The raw sequences were processed through a previously published pipeline (Rašić et al. 2014), retaining the high-quality reads (Phred >20), trimmed to the length of 90 bp, and uniquely aligned to the *Ae. aegypti* genome sequence (AaegL1) using Bowtie v0.12.7 (Langmead et al. 2009). SNPs and genotypes were called in the program Stacks v1.36 (Catchen et al. 2013), and further filtering of markers was done in the program VCFTools v0.1.12b (Danecek et al. 2011). Genome-wide similarity among samples was analysed using the Discriminant Analysis of Principal components (DAPC, Jombart et al. 2010) in the R package adegenet (Jombart and Ahmed 2011). The index of genetic structuring (F_{ST}) was calculated in VCFTools using the Weir and Cockerham's AMOVA-based method (Weir and Cockerham 1984).

RESULTS

Community approval. In June/2014, a house-to-house survey was conducted, by an external team, in 268 (38.8%) houses in Tubiacanga. Houses were randomly selected and presented an uniform distribution across Tubiacanga. A total of 235 (87.7%) householders declared support to mosquito releases, 20 (7.4%) opposed releases and 13 (4.85%) not answered this specific question. A new survey was required after a new release was confirmed. The approval for *w*MelRio releases was superior to 90% in Tubiacanga and Jurujuba (even with the need to do a second release in Tubiacanga), showing a long-term and strong community support for *Wolbachia* deployment.

***w*MelBr deployment in Tubiacanga.** The frequency of *w*MelBr in field-caught mosquitoes presented a constant but smooth increase from week 1-6. Surprisingly, the frequency plateaued between weeks 7-19 despite a total of 15,000 mosquitoes that were released per week. On week 20, the last week in which releases were conducted, we observed a peak of *w*MelBr frequency that reached 65% (Figure 1). However, *w*MelBr frequency dropped dramatically as soon releases stopped. For instance, *Wolbachia* frequency was around 20% only five weeks after releases ended (Figure 1). Interestingly, *w*MelBr not collapsed, but rather remained stable over a 10-20% frequency until the week 50, when new releases (with *w*MelRio) were conducted.

Figure 1: The frequency of *w*MelBr and *w*MelRio strains during *Wolbachia* deployment in Tubiacanga and Jurujuba. Red area represents the *w*MelBr releases and Green area the *w*MelRio releases.



As soon we observed that *Wolbachia* frequency plateaued between weeks 7-19, we conducted some investigations to understand the reasons to explain why *w*MelBr frequency remained constant over time despite strong CI, complete maternal transmission and deployment of thousands of mosquitoes per week. For that, we started by doubling the numbers of mosquitoes released per week: from 7,500 on weeks 1-7 to 15,000 on weeks 13-20. However, *w*MelBr frequency were still around 40%.

Quality control of *w*MelBr released mosquitoes.

Wing size. The wing size of released mosquitoes presented lower variation around the mean than observed in field mosquitoes (Figure S3-A). Released males were significantly bigger than those collected in the field in 8 out of 20 weeks. Released females were bigger than wild ones in 15 out of 20 weeks (Figure S3-B).

Adult mortality. The frequency of adult mosquito mortality during the first 15 weeks was remarkably low, often around 5% (Figure S3-C). There was no evidence of sex-biased mortality during the 20 weeks of mosquito releases.

Sex ratio. The sex ratio of released mosquitoes was in overall biased towards females (Figure S3-D). During five weeks only, we released more males than females. The averaged sex ratio during the 20 weeks was 1.21:1 (F:M).

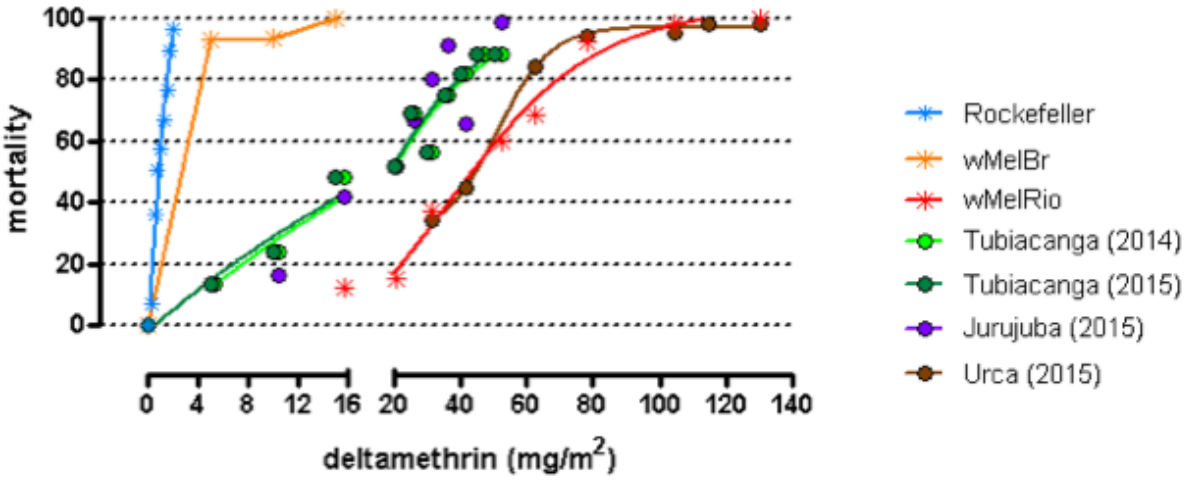
Mitochondrial DNA (mtDNA) during *w*MelBr release. One plate selected on week 12 presented an infection rate of 53.6% (45/84), and had 66 sequences validated to build the phylogeny tree. Another plate containing mosquitoes collected on week 14 had an infection rate of 44% (37/84), and 35 sequences were validated. Phylogeny trees were built using wild *Ae. aegypti* mosquitoes collected from Australia, Colombia and Indonesia by the Eliminate Dengue Program.

Considering the mtDNA sequences from all samples evaluated from weeks 12 and 14, all the *Wolbachia* positive samples grouped with *w*Mel control sequences. Furthermore, all the *Ae. aegypti-Wolbachia* negative mtDNA samples grouped with Colombian and Australian wild type control sequences. Therefore, based on the results of this analysis, there was no indication of maternal leakage on parental transmission.

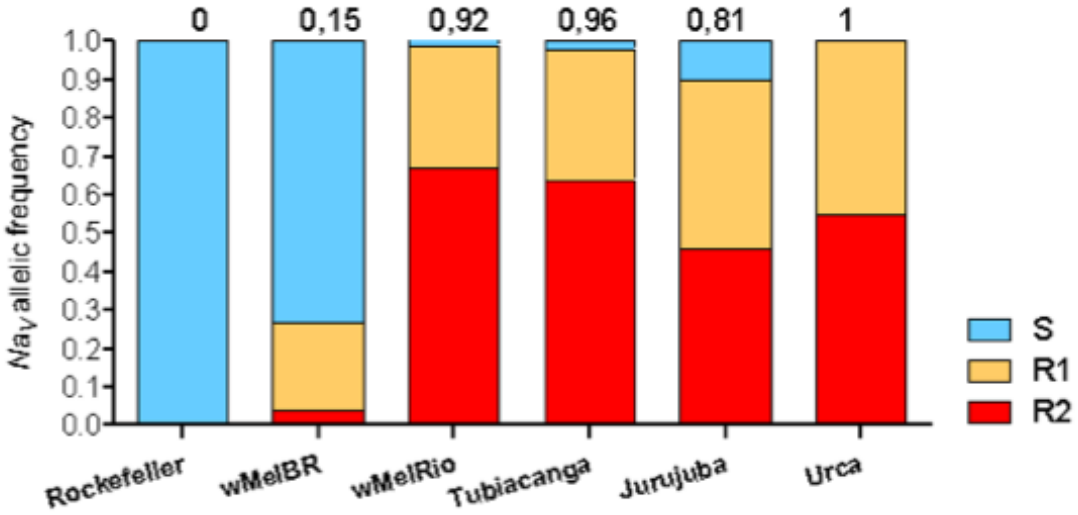
Bioassays with field populations and *Wolbachia* strains. The bioassays revealed a significant difference on the insecticide resistance status of *w*MelBr and *w*MelRio strains, especially to the pyrethroid deltamethrin and the organophosphate temephos. Considering adults, both strains were susceptible to the recently implemented malathion, but only wMelRio strain could be considered as resistant to deltamethrin (pyrethroids) as the three tested wild populations (Table S1, Figure 2A). On the other hand, individuals from the *w*MelBr population showed a similar trend to the susceptible strain Rockefeller: all individuals exposed to the same concentrations used to evaluate the deltamethrin resistance ratio of the field populations died (Table S1, Figure 2A). The allelic frequency of *kdr* mutations of all tested populations reinforces the phenotypic/genotypic response of pyrethroid susceptibility of *w*MelBr and resistance of *w*MelRio, as observed in field *Ae. aegypti* populations (Figure 2B). On the larval assays, all tested populations were susceptible to the newly adopted diflubenzuron. However, both *w*MelBr and *w*MelRio strains were diagnosed as resistant to temephos as the field populations (RR_{95}> 3,0) (Table S2, Figure S4).

Figure 2: Pyrethroid resistance of two *Wolbachia*-infected strains of *Aedes aegypti* and three field populations (Tubiacanga, Jurujuba and Urca) compared with the susceptible strain Rockefeller. A) Mortality profile of *Ae. aegypti* adult females exposed to the pyrethroid deltamethrin. B) Allelic frequency of populations testes; numbers above bars indicate the sum of resistance genotypes to pyrethroids.

(A)



(B)



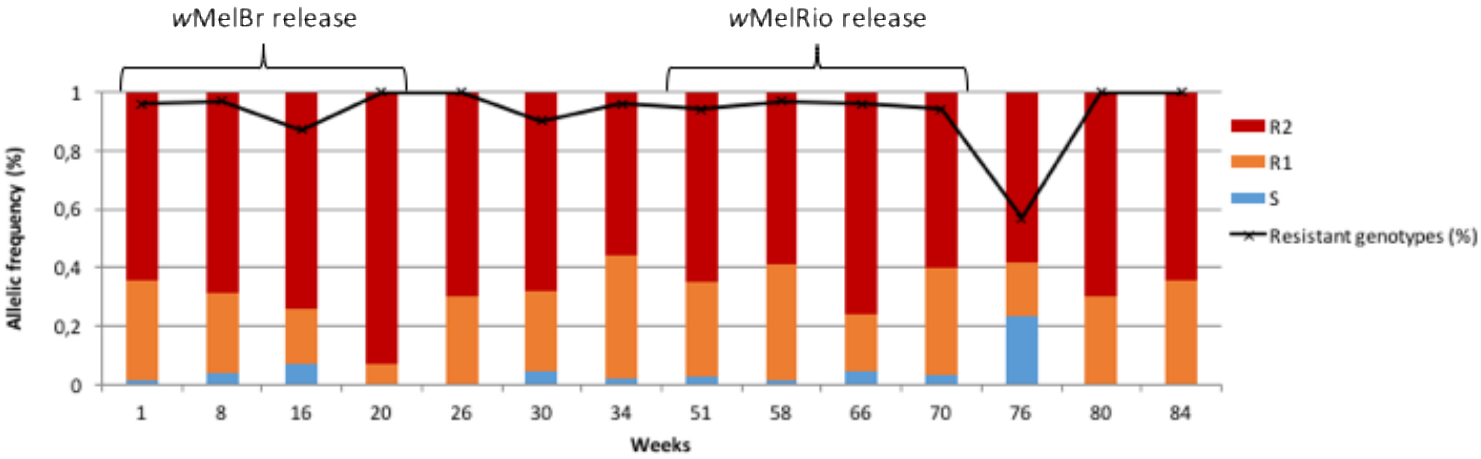
Therefore, *w*MelBr mosquitoes presented resistance levels like those of field populations if we consider temephos, diflubenzuron and malathion. But *w*MelBr was highly susceptible to deltamethrin. Instead, comparable levels of insecticide resistance/susceptibility were found between *w*MelRio strain and the three field populations tested, assuring we released mosquitoes as resistant as field to the chemical compounds used in the last two decades in Brazil (Table S1, S2).

Frequency of *kdr* alleles in Tubiacanga. Among the wild types, the genotypes for resistance (R1R1, R1R2 and R2R2) were above 80% during the 84 weeks, i.e., during *w*MelBr and *w*MelRio releases (excepting week 76), evidencing that *Ae. aegypti* from Tubiacanga was highly resistant to pyrethroids (Figure 3A). However, field-collected *w*MelBr mosquitoes remained with high frequency of the Na_VS allele, ranging from 60% to 65% up to week 20, i.e. during *w*MelBr releases. Besides, the genotypes for resistance did not exceed 20% in the same period (Figure 3B). Remarkably, a shift in the *kdr* allelic frequency of *Wolbachia*-infected mosquitoes was observed after wMelBr releases stopped, as evidenced by a continuous increase in the frequency of Na_VR1 and Na_VR2 in weeks 26, 30 and further stabilization up to week 84 (Figure 3B). Starting on week 30, the *kdr* allelic frequency was similar between *Ae. aegypti* with and without *Wolbachia*. It is of note that the allele Na_VR2 of wild mosquitoes significantly increased during

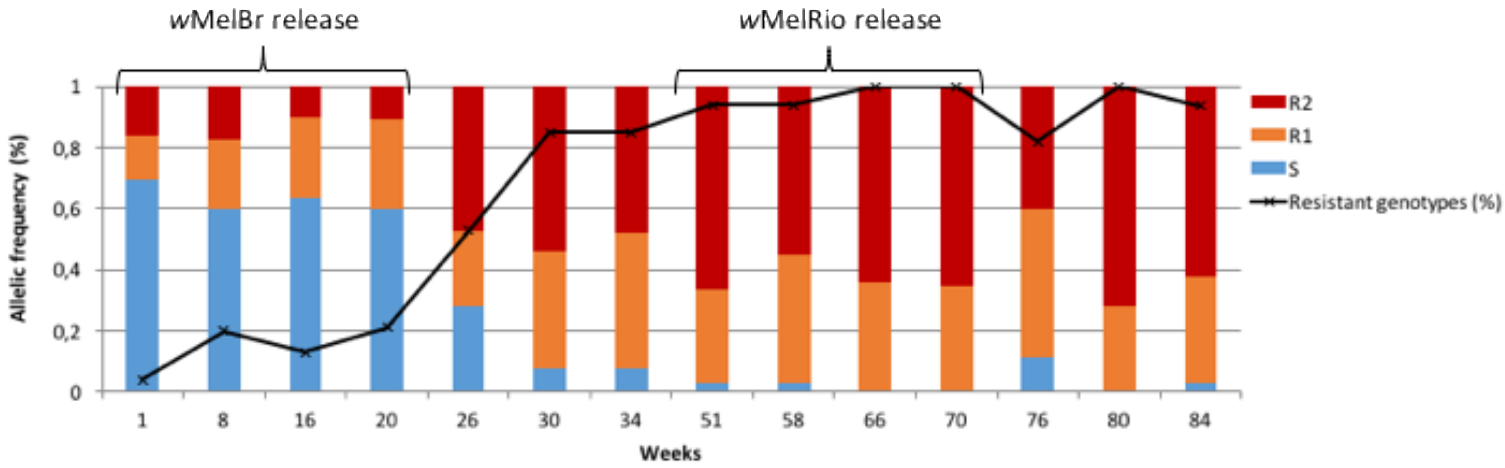
*w*MelBr releases, returning to the same level when wMelBr releases stopped, suggesting a strong selection for pyrethroids in Tubiacanga (Figure 3A).

Figure 3: Frequency of kdr alleles along *Wolbachia* releases in Tubiacanga, Brazil. At least 60 mosquitoes were analyzed per time point. (A) Wild mosquitoes, uninfected with *Wolbachia*, (B) Field-caught female *Ae. aegypti* infected with *Wolbachia*.

(A)



(B)

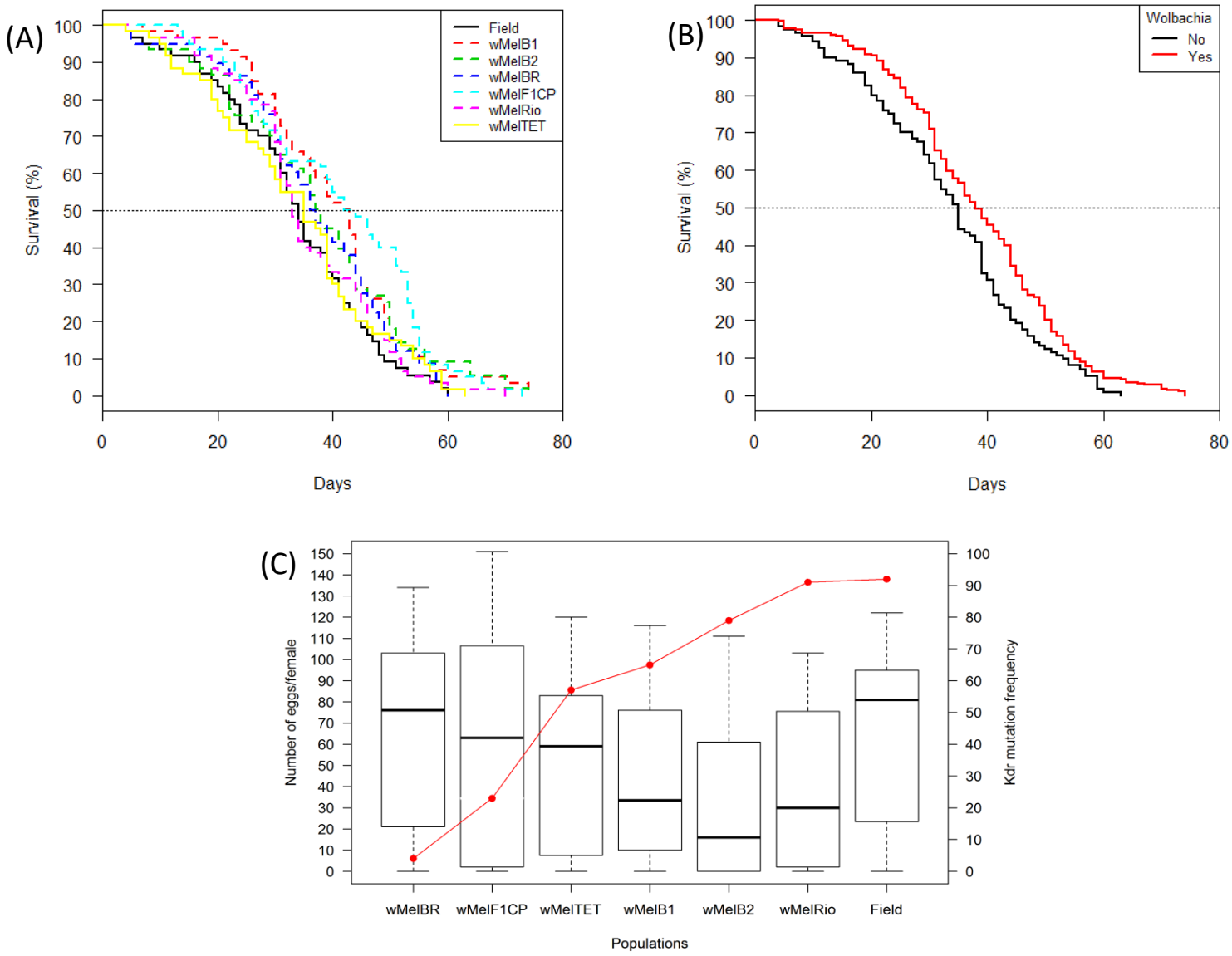


Insecticide resistance cost in *w*MelBr. The frequencies of the R2 kdr alleles obtained from samples in the F1 and F18 generations were 60% and 3%, respectively. We generated uniform samples (N=100,000) for frequencies of homozygous genotype of resistant allele (R2R2) within the interval 50-60% and samples of heterozygous frequencies were obtained to have a general frequency of resistant allele at 60% for F1 generation. Estimates produced a fitness cost in F18 generation at frequency of 3% plus/minus 1% generated a subset of 6490 values. This subset indicates a mean fitness cost of 34% (95% CI: 31-38%).

Fitness assays of *w*MelRio strain.

Survival. Survival of *w*MelRio, our population of interest, was not statistically different from any of the others (Figure 4A). *Ae. aegypti* survivorship was strongly influenced by *Wolbachia* density (F=9.458, d.f.=1, p=0.002), with mosquitoes with higher bacteria density presenting longer lifespan. Remarkably, the frequency of kdr mutation and *Wolbachia* infection, as well as the interactions among tested variables were not informative on mosquito mortality (Table S3). Survival of *w*MelRio, *w*MelBr and Field was statistically similar, suggesting that adding insecticide resistant alleles did not affect mosquito survival. Intriguingly, *Wolbachia* infected mosquitoes presented a survivorship 38% higher when compared with their uninfected counterparts (Log-rank: $\chi^2 = 8.9$, d.f. = 1, P = 0.0029) (Hazard ratio: z = -2.942, $\Psi = 1.38$, p = 0.003) (Figure 4B).

Figure 4. Survival curves of *Aedes aegypti* females from: (A) Seven populations from the backcrossing to produce an insecticide resistant line, (B) Populations infected and uninfected with *Wolbachia*, (C) Correlation between the number of eggs laid per *Aedes aegypti* female from the seven populations tested and the frequency of kdr mutation, represented by the red line.

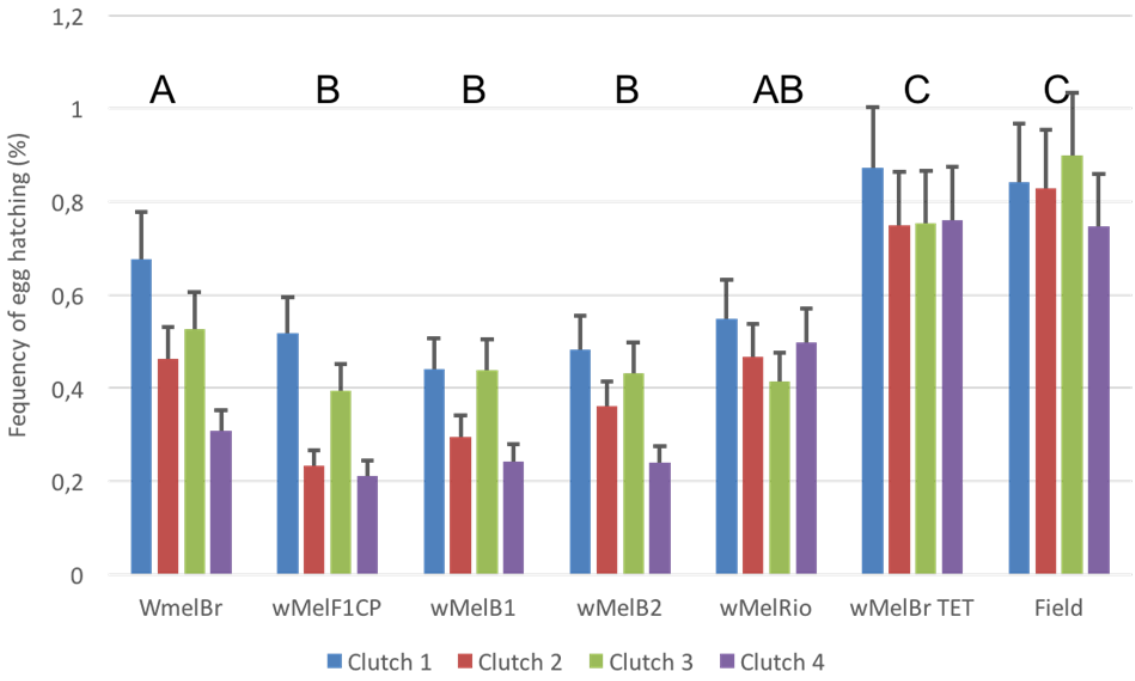


Oviposition success. The proportion of *Ae. aegypti* females laying at least one egg varied among populations, ranging from 67.7% (B2) to 86.3% (Field). Egg laying success was strongly affected by mosquito age (χ^2 = 58.01, d.f. = 4, P < 0.001). Female oviposition success was strongly affected by *Wolbachia* infection, with uninfected mosquitoes more often laying at least one egg. Oviposition success dropped more rapidly with age in mosquitoes from the populations with high kdr mutation frequency (Table S4).

Fecundity. We observed a slight effect of age on fecundity, with females laying less eggs when older in all populations tested. Eggs laid per successful females dropped from 50.5 eggs in the first clutch to 29.8 eggs in the fourth clutch. The number of eggs laid by females with *Wolbachia* dropped more rapidly than it did in uninfected populations, but bacteria density did not influence clutch size. Furthermore, populations with higher frequency of kdr mutation had a more dramatic decrease in fecundity over time (Table S5, Figure 4C).

Egg hatch. A great variation on the egg hatching was observed amongst mosquito populations, ranging from 21.1 (4th clutch of *w*MelF1CP) to 89.9% (3rd clutch of Field pop). *Wolbachia* infection was determinant to regulate egg hatching over populations, since higher egg hatching was observed in the absence of the *Wolbachia* (Figure 5).

Figure 5: Mean and standard deviation of *Aedes aegypti* egg hatching during the first four clutches of the seven populations from the backcrossing. Different letters indicate significant differences in egg hatch.



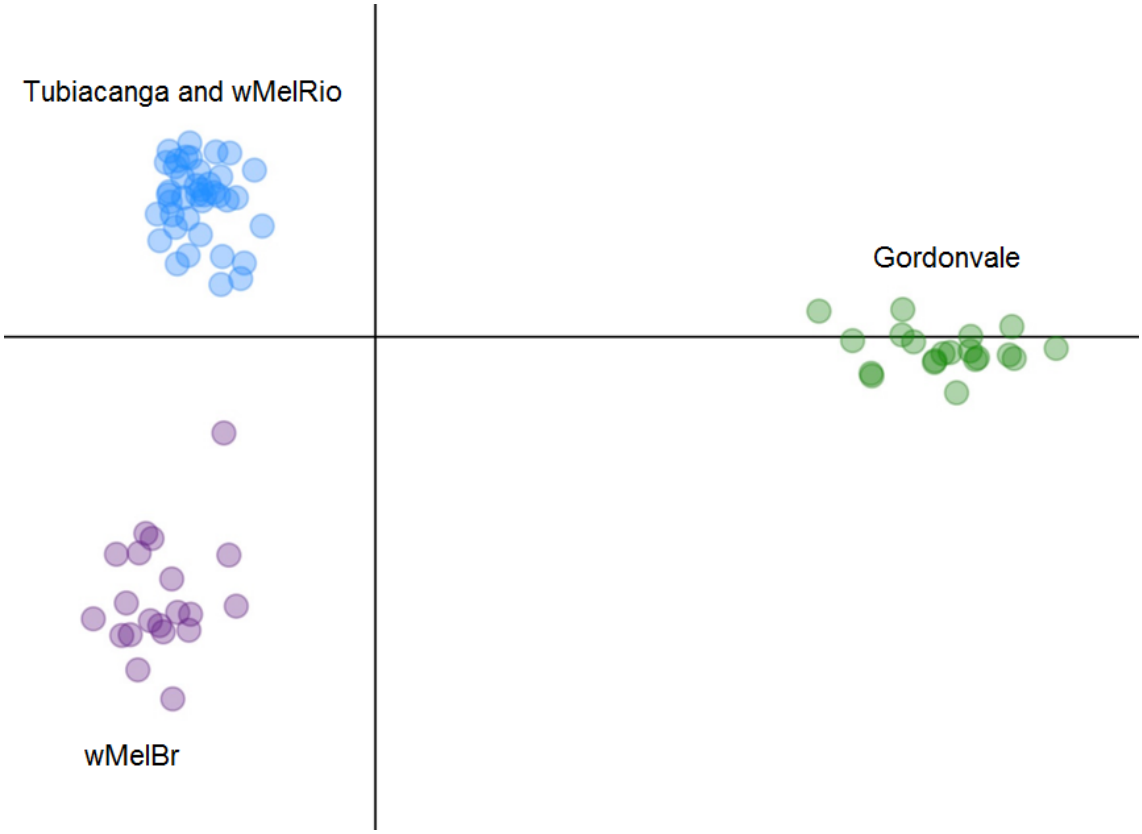
Maternal transmission. From the 249 tested mosquitoes, one female from F1CP population was not infected with *Wolbachia*, producing an overall 99.6% rate of

infection. From the 1932 individually screened larvae, 1898 of them (98.2%) were infected. The uninfected female of F1CP was responsible for producing 23 (67.6%) from the uninfected larvae. Therefore, if excluded from analysis, *Wolbachia* maternal transmission would be superior to 99.4% across the backcrossing to produce *w*MelRio (Figure S5).

Genetic similarities among release and field populations. After removing loci that were absent in more than 25% of individuals from each of the four samples, with minor allele frequency of 5% in at least one sample, and having high log-likelihood of heterozygous genotype calls (score $\leq$ -10), we retained 5,118 SNPs for downstream analysis. We also removed individuals that had more than 30% of missing data across all loci, retaining 20 individuals in each of the four samples: *w*MelRio, *w*MelBr, wild (Tubiacanga, Brazil), wildAu (Gordonvale, Australia). The average depth (SE) per locus per individual was 13.5 (5.1) for *w*MelRio, 17.6 (6.4) for *w*MelBr, 10.9 (2.3) for wild, and 8.4 (2.1) for wildAu.

Analysis of genetic variation and structuring revealed that the backcrossing strategy achieved a desirable genetic composition of the *w*MelRio release material. Namely, *w*MelRio and Tubiacanga were indistinguishable in the analysis of genetic structuring. DAPC showed that these two samples represent one genetic cluster clearly differentiated from the *w*MelBr strain and the wild Australian sample (wildAu) (Figure 6). Genome-wide F_{ST} (Weir and Cockerham 1984) between *w*MelRio and wild (Tubiacanga) was only 0.02, and this was significantly lower than the F_{ST} values between *w*MelRio and the *w*MelBr colony (0.06) or the wild Australian sample (0.114).

Figure 6: Genetic similarities and differences among *w*MelBr, *w*MelRio, Tubiacanga (Rio de Janeiro, Brazil) and Gordonvale (Cairns, Australia) *Aedes aegypti* mosquitoes.



***w*MelRio release in Tubiacanga and Jurujuba.** *w*MelRio releases in Tubiacanga started concomitantly with a 10-20% residual frequency of *w*MelBr. Release period lasted 24 weeks, and *Wolbachia* frequency reached 80% on week 18th after releases started. Releases in Jurujuba lasted 22 weeks, and *Wolbachia* reached an 80% frequency on week 9th (Figure 1). Remarkably, *w*MelRio invasion pattern in Tubiacanga was equally successful if mosquitoes were released as adults or through MRCs (Figure 1). One year after releases ended, the frequency of *Wolbachia* remained as 85-95% in both sites.

Keeping insecticide resistance in colony during *w*MelRio releases. The frequencies of Na_vR1 and Na_vR2 alleles in *w*MelRio strain were quite similar than field population during the six months (or 10 mosquito generations) in which releases were conducted in Tubiacanga. The frequency of NavR1 allele was above 30%, meanwhile for Na_vR2 it was above 50%. The Na_vS were rare, the frequencies were consistently lower than 2%. The resistant genotypes (R1R1, R1R2 and R2R2) were above 90% during all

releases. The same results were found for field samples during week 34 and 51 (Figure S6).

For the closed colony, that was maintained inbreeded for nine generation in lab, results showed a slightly tendency to the increase of Na_vR1 allele frequencies (about 60%) and decrease of Na_vR2 frequencies (about 40%) over time, when compared with *w*MelRio. Despite of that, the resistant genotypes continued in high numbers, above 90%-100%.

DISCUSSION

The endosymbiont *Wolbachia* has been deployed in endemic areas of several countries as a tool to reduce dengue transmission since mosquitoes with this bacterium have significant reduction on their vector competence to arboviruses (Moreira et al. 2009, Aliota et al. 2016, Dutra et al. 2016). Invasion is achieved after weekly releases of mosquitoes on the selected areas, which together with cytoplasmic incompatibility, pushes *Wolbachia* frequency upward. Herein, we detailed *Wolbachia* invasion in Rio de Janeiro and present consistent data on how the insecticide resistance statuses of released mosquitoes and native wild populations might jeopardized future *Wolbachia* release in endemic regions around the world.

Previous releases in places such as North Queensland, Tri Nguyen island and Yogyakarta showed a relatively constant and fast increase of *Wolbachia* frequency over weeks (Eliminate Dengue Program, unpublished data). For instance, an invasion of 98% was observed on Yorkeys Knob after 12 release weeks (Hoffmann et al. 2011). However, in Rio, invasion was plateaued at ~40-45% after the same period. Certainly, one of the major forces that may constrain *Wolbachia* invasion is the wild mosquito population size (Hoffmann et al. 2014, Garcia et al. 2016). Tubiacanga has an average of mosquito/trap/day superior than several sites of North Queensland, but slightly inferior to the Tri Nguyen island (Hoffmann et al. 2011, Ritchie et al. 2013, Hoffmann et al. 2015, personal communication Scott O'Neill). It is noteworthy that in the first weeks of releases in Rio, the ratio of wild:*w*MelBr was 1:0.5-0.7, while at least in North Queensland and Tri Nguyen Island, a ratio of 1:1.2-1.7 and 1:2-4 was observed, respectively (In Vietnam,

for *w*MelPop) (Ritchie et al. 2013, Hoffmann et al. 2015, Garcia et al. 2016). Therefore, we hypothesized increasing mosquito releases would overcome the *w*MelBr frequency plateau. From week 13 we doubled the number of mosquitoes released, but plateau remained unaltered, weakening relatedness with release numbers.

The second hypothesis was based on whether released mosquitoes were fit enough to survive in the field. We measured mosquito wing length, adult survivorship on the first week, and sex ratio as proxy of fitness. In 15 out of 20 weeks, we released females significantly bigger than wild ones, suggesting these females would not experience evident fitness loss due to poor rearing conditions (which was reinforced by the low mortality of adults on release cups). Finally, we checked if there were any deviation in sex ratio of released mosquitoes, e.g. release a cohort biased toward males would likely delay *Wolbachia* spread because it would rely more at cytoplasmic incompatibility to succeed. Nevertheless, the sex ratio was slightly biased for females. Given the evidence pointing that small wing size, low survival and male-biased sex ratio were responsible for the plateauing sounded discouraging.

The effects of temperature on the interaction between *Wolbachia* and its host has been documented for several systems (Hoffmann et al. 1990, Reynolds et al. 2002, Mouton et al. 2006, Mouton et al. 2007, Jia et al. 2009). Regarding *Ae. aegypti* mosquitoes, larval exposure to daily fluctuating temperatures of 30-40 °C during early development reduced *Wolbachia* levels in emerging females (Ulrich et al. 2016). When maintained at 26-37 °C (12:12 light:dark photoperiod, with 12 hours at each temperature), mosquitoes infected with *w*Mel strain presented a reduction in the occurrence of cytoplasmic incompatibility (Ross et al. 2016). During *Wolbachia* deployment in Rio, Tubiacanga experienced harsh climatic conditions, with an unexpected drought summer and a constant increase in temperature starting on week 12 (Figure S1). Nevertheless, despite extreme temperatures, there was no signal of incomplete maternal transmission of *Wolbachia* under climatic conditions faced in Tubiacanga.

Empirical data allowed us to refuse the effect of (a) releasing insufficient numbers of *Wolbachia*-infected mosquitoes, (b) their reduced fitness (measured by evaluating mosquito size, survival and sex ratio on released cohorts), and (c) incomplete maternal transmission to explain the plateau and the rapid decrease on *w*MelBr after the first release ended. Then, unexpectedly, field entomology team received an unexpected clue. Releases

were done early in the morning, at 05:00AM, every Thursday. As soon release was accomplished, field entomology team used to eat breakfast at the local grocery store. Spontaneously, the owner mentioned he wished releases never ended, because he was selling insecticide spray cans as never before. This awkward confession gave us an eureka moment and we started investigating a fourth hypothesis to explain the plateau: lack of resistance alleles in the released mosquito, but potentially present in wild population.

The first action was to evaluate the dynamics of resistance genes introgression during backcrossing of local males and *w*Mel-infected *Ae. aegypti* females. As *kdr* alleles were strictly correlated to pyrethroid resistance in *Ae. aegypti* Brazilian populations, they would serve as a good molecular marker for our assumptions. During backcrossing, the frequency of *kdr* alleles increased every generation up to reach an allelic frequency of around 80% (60% Na_VR2 and 20% Na_VR1). Therefore, a genetic background propitious for resistance introgression was expected, but the Na_VS allele was still present (Figure S7). The *knockdown* resistance is a recessive trait and the *kdr* alleles provide a fitness cost in the absence of the selective pressure of insecticide usage (Brito et al 2013). Thus, it is likely that the rapid decrease of "resistant alleles" in the colony was a function of their high fitness cost. Resistance to insecticides promote a fitness cost due to an energetic trade-off: insecticide resistance would deplete the energetic storage in mosquitoes, reducing the energy available for investment on other biological functions, creating trade-offs between insecticide resistance and key life history traits (Stearns 1989). Fitness cost due to insecticide resistance is well explored for the three most important mosquito genera: *Aedes*, *Anopheles* and *Culex*. There are several reports showing fitness cost on a range of relevant traits such as modification of wing shape on *Ae. aegypti* resistant to pyrethroids (Jaramillo et al. 2014), reduction in male mating competitiveness in *An. gambiae* and *Cx. pipiens* (Berticat et al. 2002, Platt et al. 2015). Overall, intense fitness cost is easier to distinguish when is related to the overproduction of molecules providing resistance, which is the main mechanism vectors evolve to become resistant to organophosphates (Hardstone et al. 2010, Rivero et al. 2011). There are some evidences pointing that *Ae. aegypti* mosquitoes have a significant fitness cost on several life history traits due to pyrethroid resistance, at least under laboratory conditions (Martins et al. 2012, Belinato et al. 2012). However, there are still few evidences pointing fitness cost under field conditions and how resistance genotypes fluctuate accordingly to insecticide

use. Most importantly, the need to investigate local insecticide resistance profile before *Wolbachia* deployment was evidenced for the first time.

The frequency of *kdr* alleles in *Wolbachia* positive mosquitoes captured during the first 20 weeks of releases remained unaltered. We started to see changes on *kdr* frequency on week 26. At this time, no released mosquito was likely to remain alive, i.e., this frequency was based on the offspring of *w*MelBr released mosquitoes. It is likely that the frequency of kdr alleles on field-caught insects during releases probably remained unaltered because *Ae. aegypti* captured the same mosquitoes we released. During the release period, driven by the high selection pressure of household pyrethroid spraying, a small proportion of *w*Mel-infected mosquitoes would be able to survive and reproduce due to their low *kdr* frequency. Therefore, the high mortality of released mosquitoes due to domestic use of pyrethroids kept the number of mosquitoes infected with *Wolbachia* below the threshold to promote bacterium invasion (Reference). Remarkably, *w*MelBr frequency remained 10-15% during nine months, when the release of *w*MelRio started in Tubiacanga (Garcia et al. 2017). Additionally, the hypothesis of overuse of pyrethroids spraying by local householders is reinforced by observing the fluctuation of *kdr* frequencies in wild mosquitoes (Figure 3-A). The frequency of Na_vR2, the genotype that confers high resistance and fitness cost to mosquitoes, increased over time up to the moment when releases stopped. After week 20, the pattern of genotype frequencies shifted and on week 34 it was similar to the one observed before *Wolbachia* deployment in Tubiacanga.

The main reason for failure in *w*MelBr invasion in Tubiacanga is strongly related with releasing mosquitoes susceptible to pyrethroids in a site where wild population is highly resistant to this class of insecticides. Adding 10% wild males every five generations was not enough to refresh the genetic pool of lab colony and, most importantly, made released mosquitoes less resistant as those in the field. Resistance is nation-wide spread and reversion of resistance in field populations may last thousands of mosquito generations (Linss et al. 2014, Schechtman & Souza 2015). Therefore, to accomplish success in *Wolbachia* deployment, countries will have to consider generating mosquitoes as resistant as the local wild population. Consequently, analyzing the insecticide resistance profiles of both the wild population and *Wolbachia* mosquitoes becomes a key factor to be monitored. We solved the problem by releasing “field-like” mosquitoes, named *w*MelRio. These mosquitoes had *Wolbachia* prevalence above 99.5%

and pyrethroid resistance as the Tubiacanga population. By that, we achieved invasion in Tubiacanga and Jurujuba, releasing adults or eggs (Figures 1 and 7). Therefore, as soon *Wolbachia* is inserted and released on a fit mosquito with a genetic like that of wild populations, the mode of release do not seem to be a critical issue to determine success. On the other hand, the use of MRCs might be helpful to stimulate local households to be engaged with the ongoing releases.

Mosquitoes with the *w*MelRio strain presented higher survival than their uninfected counterparts, showing *Wolbachia* may be advantageous to boost some aspects of *Ae. aegypti* fitness. The *w*MelRio backcross produced a mosquito with a similar genetic with field population, while the *w*MelBr revealed to be have more differences beyond the lack of insecticide resistance. In fact, *w*MelRio mosquitoes were resistant to pyrethroids and the organophosphate temephos, and susceptible to malathion and diflubenzuron, the chemical compounds currently used by Brazilian Ministry of Health. Therefore, *w*MelRio releases did not add pyrethroid resistance alleles into natural wild populations. Besides, Brazilian *Ae. aegypti* field populations have notoriously high resistance ratios to pyrethroids and temephos, highlighting the need of releasing a mosquito as resistance to field population to achieve invasion. We also provided additional information on how to sustain insecticide resistance in laboratory colonies to provide fit material for ongoing releases.

Figure S1. Ombrothermic curve of Tubiacanga during the 20 weeks of the first *Wolbachia* release in Rio de Janeiro. Bars represent weekly rainfall, solid line the mean temperature, the dotted line represents the average maximum temperature and dashed line represent the peak of temperature measured at 5 Km from Tubiacanga.

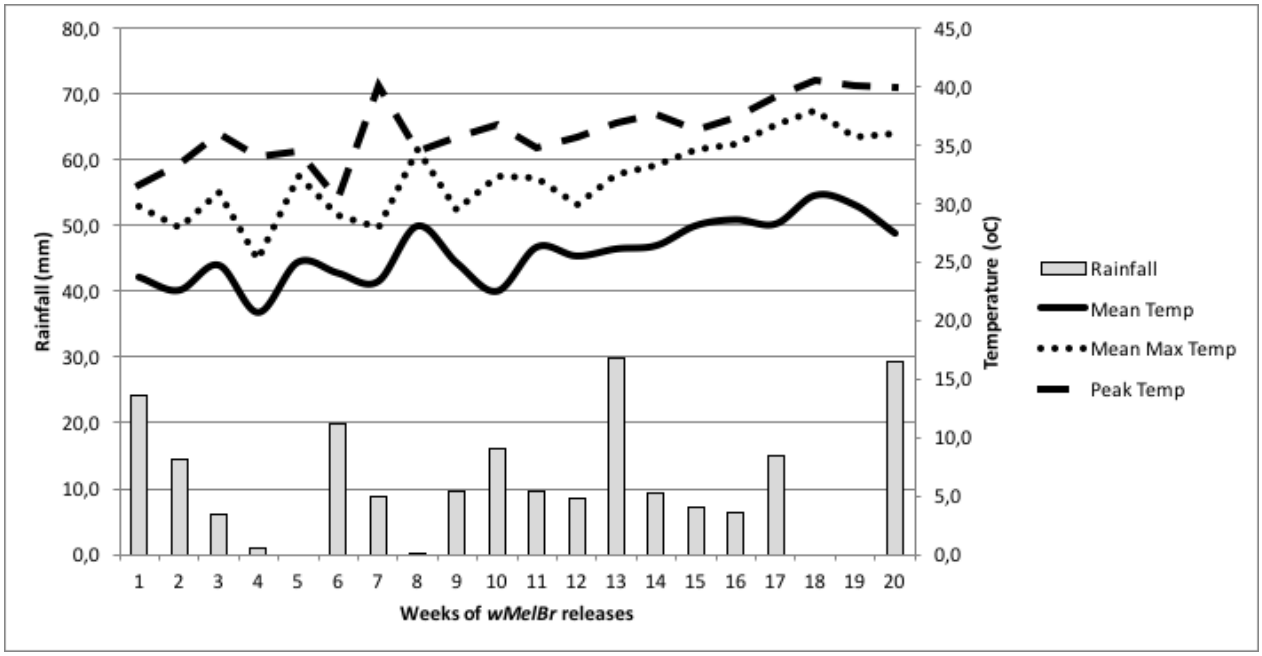
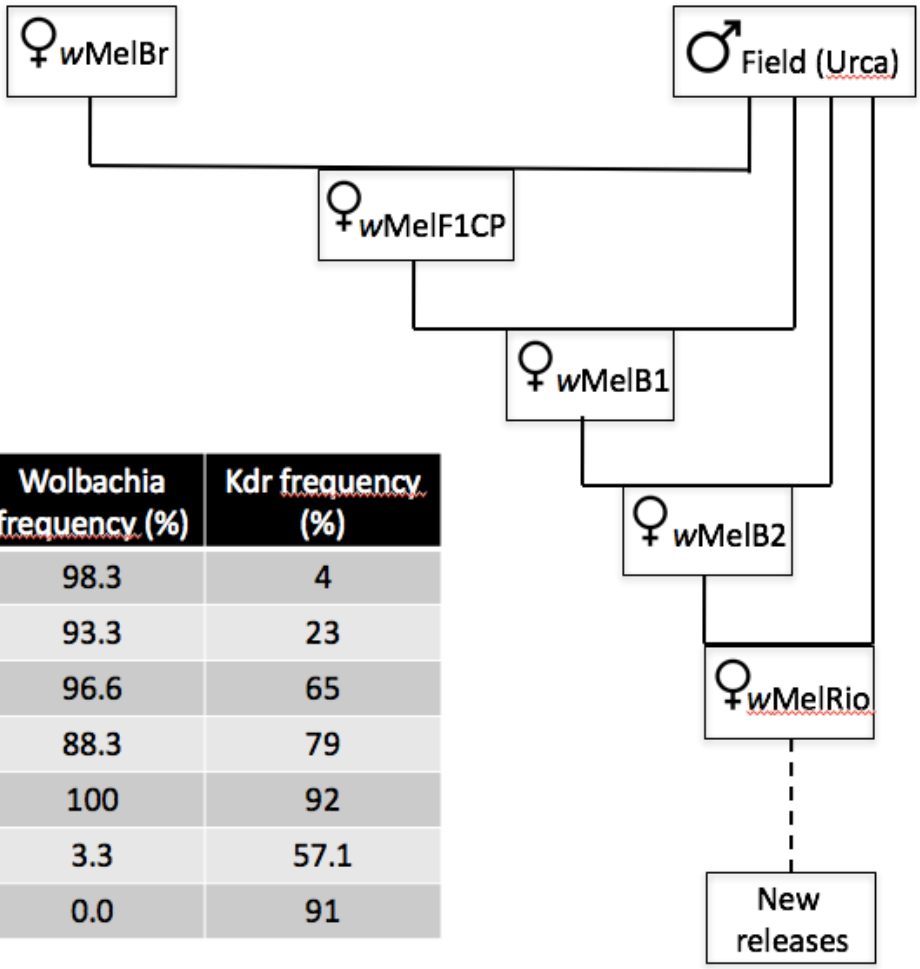


Figure S2: Schematic view of the backcrossing designed to produce an *Aedes aegypti* population resistant to pyrethroids for new releases in areas where wild mosquito population is highly resistant to insecticides.



	Wolbachia frequency (%)	Kdr frequency (%)
*w*MelBr	98.3	4
*w*MelF1CP	93.3	23
*w*MelB1	96.6	65
*w*MelB2	88.3	79
*w*MelRio	100	92
*w*MelBrTET	3.3	57.1
Field	0.0	91

Figure S3: Quality control of released *w*MelBr mosquitoes. (A) Wing size length of *Aedes aegypti* males (A) and females (B) released in the 20 weeks of *Wolbachia* deployment in Tubiacanga. Each week had 30 individuals randomly selected. The asterisk shows significance when releases mosquitoes had wing length significant bigger to wild ones. (C) Mean and confidence interval of the percentage of dead *Aedes aegypti* mosquitoes after release cups went to the field and back to the insectary. (D) Mean and confidence interval of sex ratio (female:male) of released mosquitoes. Points above the dotted line indicate sex ratio biased towards females. The red dotted line indicates the average sex ratio during releases.

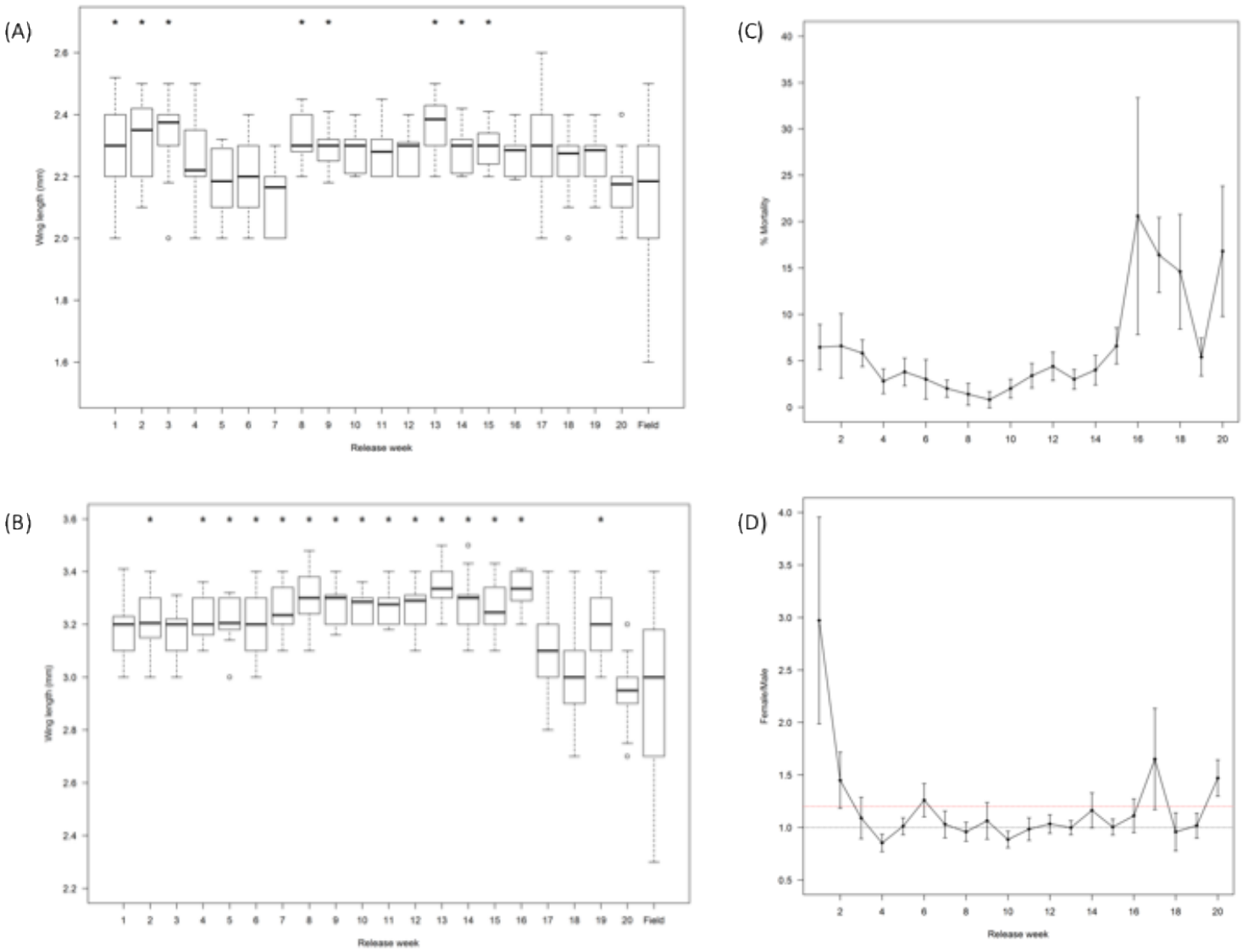


Figure S4: Dose x mortality profile of *Aedes aegypti* adult females exposed to a gradient of concentrations for the larvicide Temephos, being the mortality scored 24h later.

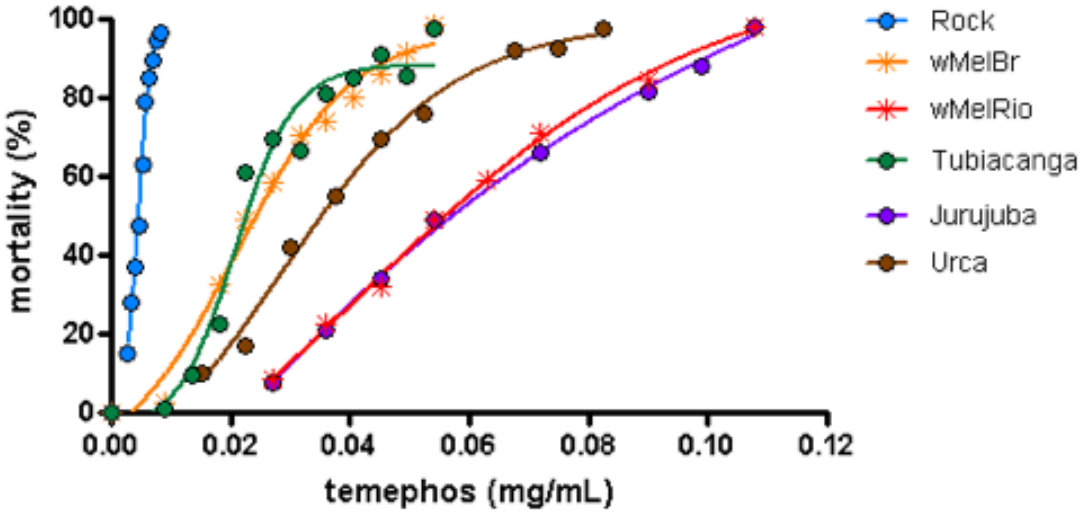


Figure S5: Frequency of *Wolbachia*-positive *Aedes aegypti* offspring during the first and fourth clutches of their relatives.

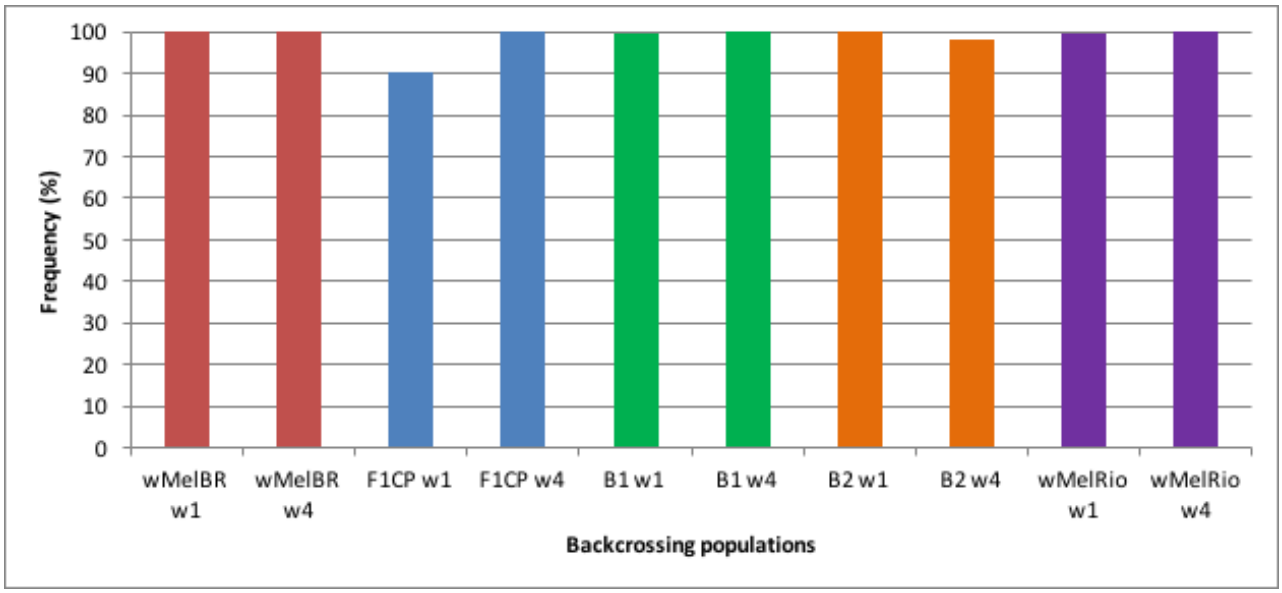


Figure S6: The frequencies of Na_vR1 and Na_vR2 alleles and genotypic frequency in *w*MelRio strain colony maintained under lab conditions during the second release in Tubiacanga. The two columns on the right represent the frequency of kdr alleles on the field population, week 34 as between *w*MelBr and *w*MelRio releases, and week 51 during *w*MelRio release.

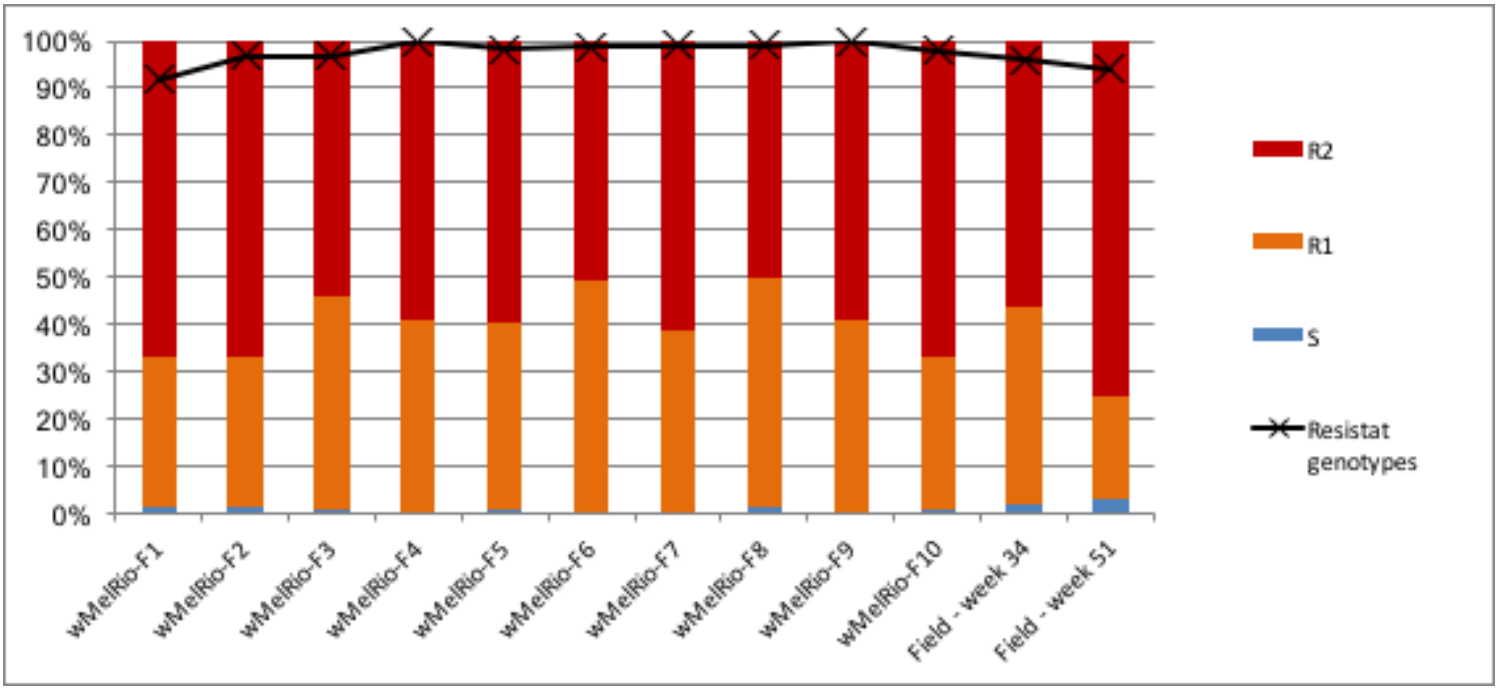


Figure S7: Allelic frequency of the susceptible wild-type (Na_vS), the *kdr* allele with a substitution restricted to the 1534 position (Na_v^{R1}) or concurrent substitutions in both 1534 and 1016 sites (Na_v^{R2}), during the backcrossing to produce the strain wMelBr (F1-CP to B4) and after backcrossing has finished (F5 to F18) .

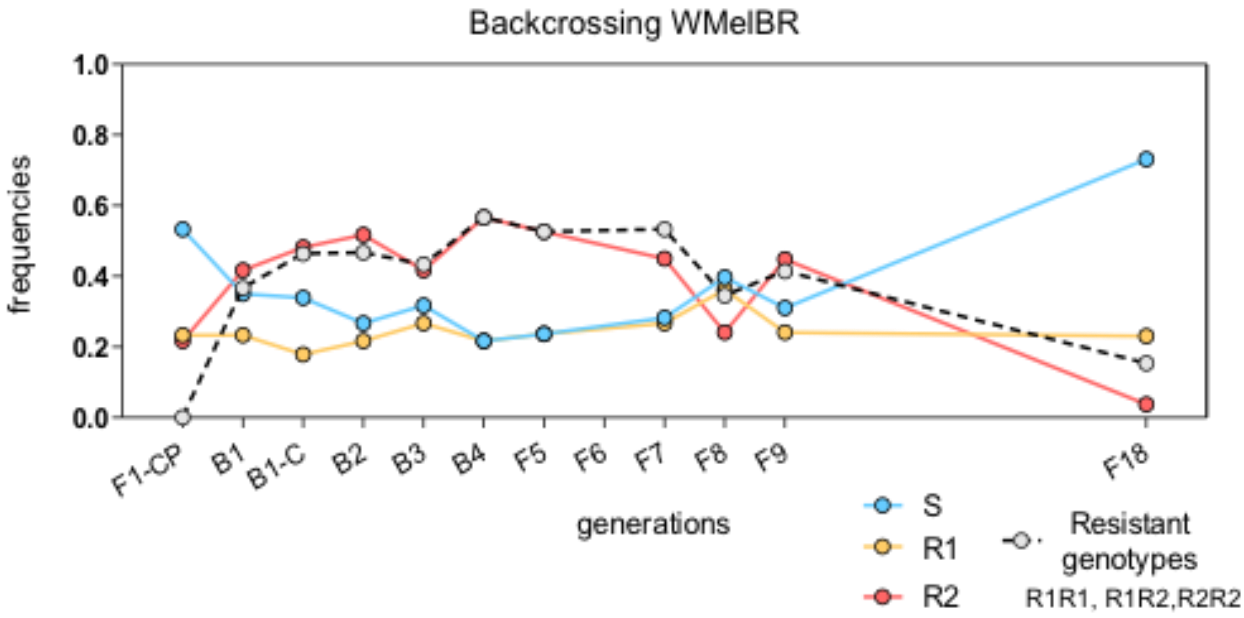


Table S1: Profile of *w*MelRio and three *Ae. aegypti* mosquitoes from local field populations (Tubiacanga, Jurujuba and Urca) exposed to two adulticides: (A) the organophosphate malathion (mg/m^2) and the pyrethroid deltamethrin (mg/m^2) up to 120 minutes. The dose used to evaluate the resistance ratio of mosquito populations to both insecticides killed all the individuals from the *w*MelBr and Rock populations.

(A) Malathion

Lineage/ populations	slope	LC_{50} (IC_{95})		RR_{50}	LC_{90} (IC_{95})		RR_{90}
Rockefeller	8.606	0.052	(0.026-0.105)	-	0.149	(0.066-0.337)	-
wMelRio	5.127	0.202	(0.171-0.239)	3,85	0.359	(0.279-0.463)	2,40
Jurujuba	6.320	0.197	(0.179-0.217)	3,75	0.314	(0.276-0.359)	2,10
Urca	6.322	0.198	(0.187-0.206)	3,76	0.315	(0.301-0.332)	2,10

(B) Deltamethrin

Lineage/ populations	slope	LC_{50} (IC_{95})		RR_{50}	LC_{90} (IC_{95})		RR_{90}
Rockefeller	4.953	1.051	(0.96-1.16)	-	1.915	(1.65-2.22)	-
wMelRio	3.591	38.842	(32.79-45.98)	37.0	88.310	(67.54-115.90)	46.2
Tubiacanga	2.048	17.931	(12.7-25.3)	17.1	62.602	(36.2-108.3)	32.8
Jurujuba	4.186	18.277	(16.35-20.35)	17.4	37.005	(32.55-42.32)	19.4
Urca	4.566	40.123	(35.57-45.05)	38.2	76.424	(67.32-87.28)	40.0

Table S2: Profile of *w*MelBr, *w*MelRio and three *Ae. aegypti* mosquitoes from local field populations (Tubiacanga, Jurujuba and Urca) exposed to two larvicides: (A) diflubenzuron (µg/L) and (B) the organophosphate temephos (mg/mL). Diflubenzuron is currently employed by Brazilian Ministry of Health.

(A) Diflubenzuron

Lineage/ populations	slope	LC_{50} (IC_{95})		RR_{50}	LC_{90} (IC_{95})		RR_{90}
Rockefeller	5.152	0.900	(0.415-1.951)	-	2.288	(1.001-5.229)	-
wMelBr	5.204	0.794	(0.243-2.594)	0.882	3.309	(0.543-20.167)	1.4
Tubiacanga	4.362	1.378	(0.682-2.787)	1.531	2.600	(1.520-4.449)	1.1

(B) Temephos

Lineage/ populations	slope	LC_{50} (IC_{95})		RR_{50}	LC_{90} (IC_{95})		RR_{90}
Rockefeller	5.962	0.0043	(0.0042-0.0044)	-	0.0071	(0.0068-0.0073)	
wMelRio	4.919	0.0541	(0.0510-0.0575)	12.61	0.0986	(0.0887-0.1103)	14.0
wMelBr	12.528	0.0239	(0.0157-0.0363)	5.62	0.0451	(0.0275-0.0738)	6.4
Tubiacanga	13.242	0.0255	(0.0176-0.0370)	6.05	0,0452	(0,0285-0,0716)	6.4
Jurujuba	4.760	0.0550	(0.0513-0.0590)	18.85	0.1022	(0.0914-0.1148)	14.5
Urca	4.303	0.0341	(0.0319-0.0363)	7.92	0.0676	(0.0617-0.0744)	9.6

Table S3: Analysis of variance of the influence of kdr frequency, Wolbachia presence and density on the survival of *Aedes aegypti* females.

Source	d.f.	Sum of squares	F	p
kdr	1	47.70	0.319	0.572
Wolbachia density	1	1413.75	9.458	**0.002**
Wolbachia infection	1	132.99	0.889	0.346
kdr AND Wolbachia infection	1	21.24	0.142	0.706
kdr AND Wolbachia density	1	4.42	0.029	0.863

Table S4: Logistic regression analysis of the influence of mosquito age, kdr frequency, Wolbachia presence and density on laying at least one egg during the first five clutches.

Source	d.f.	χ^2	*P-value*
Age	4	58.01	**<0.001**
Wolbachia infection	1	4.07	**0.043**
kdr	1	4.04	**0.044**
Wolbachia density	1	0.001	0.972
Wolbachia infection AND kdr	1	0.57	0.451
Wolbachia density AND kdr	1	0.46	0.497
Age AND Wolbachia infection	4	3.97	0.408
Age AND kdr	4	8.62	0.071
Age AND Wolbachia density	4	1.16	0.883

Table S5: Repeated measures analysis (with clutch size as the repeatedly measured variable) of the square-root of the number of eggs laid by successful *Aedes aegypti* females on the first five oviposition cycle.

Source	Numerator d.f.	Denominator d.f.	*F*	*P*
Clutch AND kdr	1	214	7.47	**<0.001**
Clutch AND Wolbachia density	1	214	0.39	0.818
Clutch AND Wolbachia infection	1	214	4.15	**0.003**

References

Aliota MT, Peinado SA, Velez ID, Osorio JE. The *w*Mel strain of *Wolbachia* reduces transmission of Zika virus by *Aedes aegypti*. Sci Rep. 2016; 6:28792.

Barreto ML, Teixeira MG, Bastos FI, Ximenes RA, Barata RB, Rodrigues LC. Successes and failures in the control of infectious diseases in Brazil: social and environmental context, policies, interventions, and research needs. Lancet. 2011; 377(9780):1877-89.

Barreto ML, Barral-Netto M, Stabeli R, Almeida-Filho N, Vasconcelos PF, Teixeira M, Buss P, Gadelha PE. Zika virus and microcephaly in Brazil: a scientific agenda. Lancet. 2016; 387(10022):919-21.

Belinato TA, Martins AJ, Valle D. Fitness evaluation of two Brazilian *Aedes aegypti* field populations with distinct levels of resistance to the organophosphate temephos. Mem Inst Oswaldo Cruz. 2012; 107(7):916-922.

Bellinato DF, Viana-Medeiros PF, Araújo SC, Martins AJ, Lima JBP, Valle D. Resistance status to the insecticides temephos, deltamethrin, and diflubenzuron in Brazilian *Aedes aegypti* populations. BioMed Research International. 2016; 8603263.

Berticat C, Boquien G, Raymond M, Chevillon C. Insecticide resistance genes induce a mating competition cost in *Culex pipiens* mosquitoes. Genet Res. 2002; 79(1):41-7.

Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, et al. The global distribution and burden of dengue. Nature. 2013; 496(7446): 504-7.

Brito L, Linss J, Lima-Camara T, Belinato T, Peixoto A, Lima J, et al. Assessing the effects of *Aedes aegypti* kdr mutations on pyrethroid resistance and its fitness cost. PLoS One. 2013; 8(4):e60878.

Catchen J, Hohenlohe PA, Bassham S, Amores A, Cresko WA. Stacks: an analysis tool set for population genomics. Mol Ecol. 2013; 22(11):3124-40.

Danecek P, Auton A, Abecasis G, Albers CA, Banks E, DePristo MA, et al; 1000 Genomes Project Analysis Group. The variant call format and VCF tools. Bioinformatics. 2011; 27(15):2156-8.

Dutra HLC, dos Santos LMB, Caragata EP, Silva JBL, Villela DAM, Maciel-de-Freitas R, et al. From Lab to Field: The influence of urban landscapes on the invasive potential of *Wolbachia* in Brazilian *Aedes aegypti* mosquitoes. PLoS Negl Trop Dis. 2015; 9(4):e0003689.

Dutra HLC, da Silva VL, Fernandes MR, Logullo C, et al. The influence of larval competition on Brazilian *Wolbachia*-infected *Aedes aegypti* mosquitoes. Parasit Vectors. 2016; 16;9(1):282.

Garcia GdA, dos Santos LMB, Villela DAM, Maciel-de-Freitas R. Using *Wolbachia* releases to estimate *Aedes aegypti* (Diptera: Culicidae) population size and survival. PLoS One. 2016; 11(8):e0160196.

Garcia GdA, Sylvestre G, Petersen MT, Aguiar R, Martins AJ, Lima JBP, et al. The riddle solved on a local grocery store: the release of *Aedes aegypti* as resistant to pyrethroid as the wild population is essential for *Wolbachia* releases. 2017. (Artigo em preparação)

Harbach RE & Knight K.L. Taxonomists' glossary of mosquito anatomy. Plexus Publ. Marlton New Jersey. 1980.

Hardstone MC, Huang X, Harrington LC, Scott JG. Differences in development, glycogen, and lipid content associated with cytochrome P450-mediated permethrin resistance in *Culex pipiens quinquefasciatus* (Diptera: Culicidae) J. Med. Entomol. 2010; 47:188-198.

Hoffmann AA, Iturbe-Ormaetxe I, Callahan AG, Phillips BL, Billington K, et al. Stability of the wMel *Wolbachia* Infection following Invasion into *Aedes aegypti* Populations. PLoS Negl Trop Dis. 2014; 8(9):e3115.

Hoffmann AA, Montgomery BL, Popovici J, Iturbe-Ormaetxe I, Johnson PH, et al. Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission. Nature. 2011; 476(7361):454-7.

Hoffmann AA, Turelli M, Harshman LG. Factors affecting the distribution of cytoplasmic incompatibility in *Drosophila simulans*. Genetics. 1990; 126:933-948.

Hoffmann AA, Ross PA, Rašić G. *Wolbachia* strains for disease control: ecological and evolutionary considerations. Evol Appl. 2015; 8:751-768.

Jaramillo-ON, Fonseca-González I, Chaverra-Rodríguez D. Geometric morphometrics of nine field isolates of *Aedes aegypti* with different resistance levels to lambda-cyhalothrin and relative fitness of one artificially selected for resistance. PLoS One. 2014; 9(5):e96379.

Jia FX, Yang MS, Yang WJ, Wang JJ. Influence of continuous high temperature conditions on *Wolbachia* infection frequency and the fitness of *Liposcelis tricolor* (Psocoptera: Liposcelididae) Environ. Entomol. 2009; 38:1365-1372.

Jombart T, Ahmed I. Adegenet 1.3-1: new tools for the analysis of genome-wide SNP data. Bioinformatics. 2011; 27(21):3070-1.

Jombart T, Devillard S, Balloux F. Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. BMC Genet. 2010; 11:94.

Kuno G. Early history of laboratory breeding of *Aedes aegypti* (Diptera: Culicidae) focusing on the origins and use of selected strains. J Med Entomol. 2010; 47(6):957-971.

Langmead B, Schatz MC, Lin J, Pop M, Salzberg SL. Searching for SNPs with cloud computing. Genome Biol. 2009; 10(11):R134.

Linss JGB, Brito LP, Azambuja GG, Araki AS, Bruno RV, et al. Distribution and dissemination of the Val1016Ile and Phe1534Cys Kdr mutations in *Aedes aegypti* Brazilian natural populations. Parasit Vectors. 2014; 7:5.

Maciel-de-Freitas R, Marques WA, Peres RC, Cunha SP, Lourenço-de-Oliveira R. Variation in *Aedes aegypti* (Diptera: Culicidae) container productivity in a slum and a suburban district in Rio de Janeiro during dry and wet seasons. Mem Inst Oswaldo Cruz. 2007; 102:489-496.

Maciel-de-Freitas R, Koella JC, Lourenço-de-Oliveira R. Lower survival rate, longevity and fecundity of Aedes aegypti (Diptera: Culicidae) females orally challenged with dengue virus serotype 2. Trans R Soc Trop Med Hyg. 2011; Aug;105(8):452-8.

Maciel-de-Freitas R, Avendanho FC, Santos R, Sylvestre G, Araújo SC, et al. Undesirable consequences of insecticide resistance following *Aedes aegypti* control activities due to a dengue outbreak. PloS One. 2014a; 9:e92424.

Maciel-de-Freitas R, Valle D. Challenges encountered using standard vector control measures for dengue in Boa Vista, Brazil. Bull World Health Organ. 2014b; 92(9): 685-9.

Marcombe S, Darriet F, Tolosa M, Agnew P, Duchon S, Etienne M, et al. Pyrethroid resistance reduces the efficacy of space sprays for dengue control on the island of Martinique (Caribbean). PLoS Negl Trop Dis. 2011; 5(6):e1202.

Martins AJ, Ribeiro CD, Bellinato DF, Peixoto AA, Valle D, Lima JB. Effect of insecticide resistance on development, longevity and reproduction of field or laboratory selected *Aedes aegypti* populations. PLoS One. 2012; 7(3):e31889.

Montella IR, Martins Jr AJ, Viana-Medeiros PF, Lima JBP, Braga IA, et al. Insecticide resistance mechanisms of Brazilian *Aedes aegypti* populations from 2001 to 2004. Am J Trop Med Hyg. 2007; 77:467-477.

Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, Lu G, Pyke AT, Hedges LM, et al. A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, Chikungunya, and *Plasmodium*. Cell. 2009;139(7):1268-78.

Mouton L, Henri H, Bouletreau M, Vavre F. Effect of temperature on *Wolbachia* density and impact on cytoplasmic incompatibility. Parasitol. 2006; 132(Pt1):49-56.

Mouton L, Henri H, Charif D, Boulétreau M, Vavre F. Interaction between host genotype and environmental conditions affects bacterial density in *Wolbachia* symbiosis. Biol Lett. 2007; 22;3(2):210-213.

Nguyen TH, Nguyen HL, Nguyen TY, Vu SN, Tran ND, Le TN, et al. Field evaluation of the establishment potential of wMelPop *Wolbachia* in Australia and Vietnam for dengue control. Parasit Vectors. 2015; 28; 8:563.

Nunes MRT, Faria NR, Vasconcelos JM, Golding N, Kraemer MUG, Oliveira LF, et al. Emergence and potential for spread of chikungunya virus in Brazil. BMC Med. 2015; 13:102.

PAHO/WHO. Number of reported cases of dengue and severe dengue (SD) in Americas, by country. 2016.

Petersen LR, Powers AM. Chikungunya: epidemiology. F1000Res; F1000 Faculty Rev. 2016; 5:82.

Platt N, Kwiatkowska RM, Irving H, Diabaté A, Dabire R, Wondji CS. Target-site resistance mutations (kdr and RDL), but not metabolic resistance, negatively impact male mating competiveness in the malaria vector *Anopheles gambiae*. Heredity (Edinb). 2015; 115(3):243-52.

Rašić G, Filipović I, Weeks AR, Hoffmann AA. Genome-wide SNPs lead to strong signals of geographic structure and relatedness patterns in the major arbovirus vector, *Aedes aegypti*. BMC Genomics. 2014; 15:275.

Raymond M. Presentation d'une programme d'analyse logprobit pour microordinateur. Cahiers Sér Ent Parasitol. 1985; 22:117-121.

Reynolds KT, Hoffmann AA. Male age, host effects and the weak expression or non-expression of cytoplasmic incompatibility in *Drosophila* strains infected by maternally transmitted *Wolbachia*. Genet Res. 2002; 80(2):79-87.

Ritchie SA, Montgomery BL, Hoffmann AA. Novel estimates of *Aedes aegypti* population size and adult survival based on *Wolbachia* releases. J Med Entomol. 2013; 50:624-631.

Rivero A, Magaud A, Nicot A, Vézilier J. Energetic cost of insecticide resistance in *Culex pipiens* mosquitoes. J. Med. Entomol. 2011; 48:694-700.

Ross PA, Endersby NM, Hoffmann AA. Costs of three *Wolbachia* infections on the survival of *Aedes aegypti* larvae under starvation conditions. PLoS Negl Trop Dis. 2016; 10(1):e0004320.

Schechtman H, Souza OM. Costly Inheritance and the Persistence of insecticide resistance in *Aedes aegypti* populations. PLoS One. 2015; 7:450.

Souza RL, Mugabe VA, Paploski IAD, Rodrigues MS, et al. Effect of an intervention in storm drains to prevent *Aedes aegypti* reproduction in Salvador, Brazil. Parasites & Vectors. 2017; 10:328.

Stearns SC. Trade-offs in life history evolution. Funct. Ecol. 1989; 3:259-268.

Ulrich JN, Beier JC, Devine GJ, Hugo LE. Heat sensitivity of *w*Mel *Wolbachia* during *Aedes aegypti* development. PLoS Negl Trop Dis. 2016; 10(7):e0004873.

Walker T, Johnson PH, Moreira LA, Iturbe-Ormaetxe I, Frentiu FD, McMeniman CJ, et al. The wMel *Wolbachia* strain blocks dengue and invades caged *Aedes aegypti* populations. Nature. 2011; 476(7361):450-3.

Weaver SC, Forrester NL. Chikungunya: evolutionary history and recent epidemic spread. Antiviral Res. 2015; 120:32-39.

Weir BS, Cockerham CC. Estimating F-statistics for the analysis of population structure. Evolution. 1984;38(6):1358-1370.

Werren JH, Windsor DM. *Wolbachia* infection frequencies in insects: Evidence of a global equilibrium? Proc R Soc Lond B Biol Sci. 2000; 267:1277-1285.

WHO. Instructions for determining the susceptibility or resistance of mosquito larvae to insecticides. Tech. Rep. WHO/VBC/81.807, World Health Organization, Geneva, Switzerland, 1981.

WHO. Test Procedures for Insecticide Resistance Monitoring in Malaria Vectors, Bio-Efficacy and Persistence of Insecticides on Treated Surfaces, WHO/CDS/CPC/MAL/98.12, World Health Organization, Geneva, Switzerland, 1998.

Yakob L, Walker T. Alternative vector control methods to manage the Zika virus outbreak: More haste, less speed - Authors' reply. Lancet Global Health. 2016; 4(6): e365-6.

Artigo 8

(Em preparação)

Aedes aegypti insecticide resistance elucidates the success (and failure) of *Wolbachia* population replacement

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Abstract

A novel strategy for controlling the spread of arboviral diseases, such as dengue, Zika and chikungunya, is to introduce *Aedes aegypti* populations with virus-suppressing *Wolbachia*. In general, *Wolbachia* transinfected into mosquitoes induces fitness costs through lower viability or fecundity. *Wolbachia* frequencies tend to increase only when frequency-dependent cytoplasmic incompatibility advantage exceeds frequency-independent costs. Those costs may be intrinsic to the *Wolbachia* or can be associated with the genetic background into which *Wolbachia* are introduced. In particular, we describe how insecticide resistance (or susceptibility) of transinfected mosquitoes can determine the success or failure of local *Wolbachia* introductions. Based on two Brazilian laboratory *Ae. aegypti* colonies with *Wolbachia*, *w*MelBr and *w*MelRio, susceptible and resistant to insecticide, respectively, the success of local introductions depends critically on the insecticide resistance/susceptibility of introduced *Wolbachia*-infected mosquitos and the level of selection for insecticide resistance induced by local pesticide use. Our theoretical results elucidate empirical examples, presented in accompanying papers, describing the failure and success of *Wolbachia*-infected *Ae. aegypti* introductions in an isolated population within one site in Rio de Janeiro, Brazil, highlighting some scenarios that may happen in *Wolbachia* releases areas around the world.

Introduction

The frequent reemergence of arboviral diseases and emergence of new arboviruses around the world is a constant and significant concern for public health authorities. High human mobility across countries, disorganized urban landscapes with poor sanitary conditions and climate change favor arthropod vector expansion (Cao-Lormeau and Musso 2014; Liang et al. 2015; Vasconcelos and Calisher 2016). Among the known arboviruses circulating in large geographical areas, dengue (DENV), chikungunya (CHIKV) and Zika (ZIKV) viruses have caused recent outbreaks in multiple countries, including Brazil (Mota et al 2016) .

These three arboviruses are overwhelmingly transmitted by *Aedes* mosquitoes, with *Ae. aegypti* as the principal vector (Lourenço-de-Oliveira et al. 2004; Vega-Rúa et al. 2015; Scully and Robinson 2016; Ferreira-de-Brito et al. 2016;). *Ae. aegypti* is closely associated with urban environments, blood feeding mainly on human hosts, laying eggs in manufactured containers around human dwellings and resting inside houses (Scott et al. 1993; Braks et al. 2003, Maciel-de-Freitas et al. 2007).

Since DENV, CHIK and ZIKV still do not have effective vaccines or specific antiviral drugs available for low income populations, the best strategy to reduce their incidence remains control practices targetting *Ae. aegypti* populations (Morrison et al. 2008, Liang et al. 2015). A promising strategy involves using the bacteria *Wolbachia*, an intracellular maternally transmitted endosymbiont that is present in around 60% of all known insects (Hilgenboecker et al. 2008; de Oliveira et al. 2015). This bacterium was transinfected into *Ae. aegypti* mosquitoes and has been treated as a biological agent able to block DENV, CHIKV (Moreira et al. 2009) and ZIKV (Dutra et al. 2016). Thus, *Wolbachia* deployment seeks to promote population replacement: an *Ae. aegypti*

population highly competent for arbovirus is replaced by a mosquito that blocks arboviruses transmission. Currently, *Wolbachia* has been established in more than 40 sites all over the world, including a variety of landscapes from Australia, Brazil, Colombia, Indonesia and Vietnam (Hoffmann et al. 2011, 2015, Eliminate Dengue Program).

To achieve a success invasion in wild population, *Wolbachia* produce a frequency-dependent advantage for infected females by inducing cytoplasmic incompatibility (CI), which kills the embryos produced by uninfected mothers mated to infected fathers (Werren et al. 2008). *Wolbachia* frequencies tend to increase only when on the frequency-dependent CI advantage exceed frequency-independent costs. Those costs may be intrinsic to the *Wolbachia*, such as reduction in fecundity (Turley et al. 2013; Hoffmann et al. 2014) and a decrease the likelihood of surviving under starvation (Ross et. al 2016), or can be associated with the genetic background into which *Wolbachia* are introduced, for example, insecticide resistance (or susceptibility). Resistance to insecticides also commonly has a fitness cost. Overexpression of a resistance-conferring gene may result in an energetic cost that involves resource and energy reallocation at the expense of metabolic and developmental processes. Additionally, mechanisms involving target-site modification may lead to a partial loss of function of a gene (Kliot and Ghanim 2012; Brito et al. 2013; Diniz et al. 2015).

Chemical control is the most common approach used to suppress *Ae. aegypti* populations in urban settlements in endemic areas, and can target both adult and larval stage of mosquito life cycle (Lima et al. 2015). Pyrethroids (PY) and organophosphate (OP) insecticides have been widely used over the past 50 years, the former for adults and the latter for larvae and adults (Baldacchino et al. 2014; Bellinato et al. 2016; Smith et al. 2016). Many studies have shown low insecticide efficiency due to resistance of wild *Ae. aegypti* populations (Marcombe et al. 2011; Maciel-de-Freitas et al. 2014, Bellinato et al.

2016; Plernsub et al. 2016). Intense selection pressure due to insecticide usage increases the frequency of resistance genes. Thus, mosquito populations acquire the ability to survive to doses that would be lethal to the most susceptible individuals of the same species (Beaty and Marquardt 1996). However, these changes are generally advantageous only in the presence of the insecticide due to the fitness costs of resistance (Kliot and Ghanim 2012; Brito et al. 2013; Diniz et al. 2015; Schechtman and Souza 2015). For example, mutations in the voltage sodium channel gene produces a phenotype known as knockdown resistance (*kdr*). These mutations give rise to PY resistance and has been related to fitness cost in the absence of that class of insecticide in many insects, including *Ae. aegypti* (Foster et al. 2003; Berticat et al. 2008; Brito et al. 2013).

In this context, during *Wolbachia* deployment aimed at replacement, mosquitoes are likely to be challenged by several factors that jeopardize bacterium invasion, such as high vector population density (Hancock et. al 2016; Garcia et al. 2016), high temperatures (Ulrich et al. 2016; Ross et al. 2016), insecticide resistance in wild population (Wuliandari et al. 2015) and an exacerbated superior fitness cost due to the presence of *Wolbachia* and alleles that confer resistance. Hoffmann and Turelli (2013) proposed an approach to facilitate *Wolbachia* invasion through insecticide-resistance selection. In this strategy, insecticide-resistant mosquitoes infected with *Wolbachia* are deployed into an area in which insecticide usage would suppress wild population, hence facilitating speed invasion. However, this strategy requires a susceptible wild population, and might have limited application if wild *Ae. aegypti* populations feature strong resistance, as is the case in Brazil (Linss et al. 2014).

In our work, we leveraged this model to analyze different scenarios of insecticide use and resistance, using as empirical examples of failure and success of *Wolbachia*-infected *Ae. aegypti* introductions in an isolated population within one site in Rio de

Janeiro, Brazil (Garcia et al. 2017), which enable us to understand how insecticides can undermine *Wolbachia* invasion in natural *Ae. aegypti* populations. First, we evaluate the fitness cost of a colony of *Ae. aegypti* infected with the *w*Mel strain maintained in laboratory for 18 generations (*w*MelBr), without insecticide pressure. Second, we studied the potential effects of the following features on *Wolbachia* invasion: (1) releasing *Wolbachia* in a mosquito with susceptible and resistant strains (*w*MelBr and *w*MelRio, respectively); (2) varying the insecticide use by local householders during the releases; (3) levels of insecticide resistance in *Ae. aegypti* wild populations; and (4) the fitness cost of *Wolbachia* infection and insecticide resistance. Thus, we identified scenarios in which the insecticide resistance of wild *Ae. aegypti* populations challenge successful *Wolbachia* invasion.

Models and Methods

1) General model

The model is based on previous studies that have shown a fitness cost associated with PY target-site resistance, with a focus on two-allele representation of knockdown resistance based on the Val1016Ile *kdr* mutation (Martins el al. 2012; Brito et al. 2013; Linss et al. 2014; Vera-Maloof et al. 2015). Individuals can be classified by their genotypes and *Wolbachia* infection state. Genotypes in a two-allele representation are given by RR, RS or SS for homozygous resistant, heterozygous and homozygous susceptible genotypes, respectively. Heterozygous individuals are susceptible. The *Wolbachia* infection state is either uninfected (U) or infected (I). Homozygous resistant mosquitoes have a relative viability given by a factor i to the viability of susceptible mosquitoes, hence a fitness cost given by $1 - i$.

Turelli and Hoffmann (2013) developed a model in which a *Wolbachia* fitness cost F_c would apply over successive generations. We introduce into such model a parameter to describe the fitness cost due to insecticide resistance. The model is constructed from components that evaluate frequencies of genotypes in successive generations and that consider varying intensities of insecticides application.

The first component evaluates frequencies $f(XX,WS)_t$ of XX newly entering individuals (zygotes) at generation t where XX={RR, RS, SS} and WS is the *Wolbachia* infection state, WS={U, I}. The frequency of *Wolbachia* over time t is described by p_t and the frequencies of R alleles in either *Wolbachia* mosquitoes or non-*Wolbachia* mosquitoes is given by $r_{I,t}$ and $r_{U,t}$, respectively.

These frequencies can be modeled by recursive equations such as

$$f(RR,U)_{t+1} = \frac{i\,(1-p_t)^2\, r_{U,t}^2}{\overline{w}}$$

$$f(RS,U)_{t+1} = (1-p_t)^2 2\, r_{U,t}\, \frac{1-r_{U,t}}{\overline{w}}$$

$$f(SS,U)_{t+1} = \frac{(1-p_t)^2\left(1-r_{U,t}\right)^2}{\overline{w}}$$

$$f(RR,I)_{t+1} = i\, F_c\, p_t\, r_{I,t}\, \frac{p_t\, r_{I,t} + (1-p_t) r_{U,t}}{\overline{w}}$$

$$f(RS,I)_{t+1} = F_c\, p_t\, \frac{2p_t\, r_{I,t}\left(1-r_{I,t}\right) + (1-p_t)\left(r_{I,t}\left(1-r_{U,t}\right) + r_{U,t}\left(1-r_{I,t}\right)\right)}{\overline{w}}$$

$$f(SS,I)_{t+1} = F_c\, p_t\, \frac{\left(1-r_{I,t}\right)\left(p_t\left(1-r_{I,t}\right) + (1-p_t)\left(1-r_{U,t}\right)\right)}{\overline{w}}$$

where $\overline{w}$ is given by:

$$\bar{w} = i\,(1-p_t)^2\,r_{U,t}^2 + (1-p_t)^2\,2\,r_{U,t}\left(1-r_{U,t}\right) + (1-p_t)^2\left(1-r_{U,t}\right)^2 + i\,F\,p_t\,r_{I,t}(p_t\,r_{I,t} + (1-p_t)r_{U,t}) + F\,p_t\,(\,p_t\,2\,r_{I,t}\,(1-r_{I,t}) + (1-p_t)(r_{I,t}\left(1-r_{U,t}\right) + r_{U,t}\left(1-r_{I,t}\right))) + F\,p_t\,(1-r_{I,t})(p_t\left(1-r_{I,t}\right) + (1-p_t)\left(1-r_{U,t}\right))$$

The frequencies of *Wolbachia* and the R allele in adults will be impacted by the use of insecticides. We assume that a fraction 1-*s* survives to mate and generate new individuals. This follows Equations 2.4 given by Turelli and Hoffmann (2013). In the field *s* reflects insecticide use intensity, whereas in the laboratory for rearing *Wolbachia* individuals no insecticide is used, hence *s*=0. This model is used to assess a best fit for parameter *i*, based on frequency changes of the R allele when laboratory *Wolbachia* mosquitoes are maintained as closed populations or crossed with with field males.

2) Quantifying the fitness cost due to insecticide resistance (Laboratory conditions)

Since insecticides are not used during rearing of *Wolbachia* mosquito colonies, we model laboratory conditions by assuming *s*=0. Therefore, fitness costs due to *Wolbachia* presence and to insecticide resistance can be measured. Different sets of theoretical values can be obtained from the model using a fixed *Wolbachia* fitness cost and multiple costs due to insecticide resistance. We vary the insecticide resistance *Fc* by increments of 0.01 and obtain for each set of frequencies the sum of squared residuals considering the theoretical values and the frequencies observed in some of our lab generations (F5, F6, F7, F8, F9 and F18). The fitness cost due to the insecticide resistance is estimated from the relationship having lowest sum of squared residuals.

3) Parameters used in the *Wolbachia* invasion model

We analyze different scenarios considering initial levels of insecticide resistance among wild mosquitoes, as well as the insecticide application during releases. In order to define scenarios we also need the initial conditions on presence of *Wolbachia* in the field and levels of insecticide resistance. For all simulations we consider *Wolbachia* to be absent in the field prior to releases. We consider a frequency *rUinic* of the R allele in the local population prior to releasing *Wolbachia* mosquitoes. This parameter shows the level of insecticides resistance gene in *Ae. aegypti* wild population that receive *Wolbachia* releases. We use a value of 0.95 in our analyses, which considers the wild mosquitoes homozygous for resistance (RR).

Furthermore, our model considers that *Wolbachia* mosquitoes are released on a weekly basis during *nrel* weeks. In our analyses we considered *nrel* = 20 releases in all simulations based on *Wolbachia* release carried out in Rio de Janeiro, from Sept/2014 to Jan/2015 (Maciel-de-Freitas et al., 2017). Each release of *Wolbachia* mosquitoes requires a number of mosquitoes given by a *ratio r.rel of number of released individuals by the total number (released + local) per week.* This parameter considers the density of wild mosquitoes and the number of *Wolbachia* mosquitoes released per week. We use a value based on releases in Brazil of 0.10 (Garcia et al. 2017) of ratio and we generally consider a total number of 40 generations over a one year-period (Table 1). Furthermore, our analysis permits us to obtain the frequency of the resistance allele within the total population of *Wolbachia* mosquitoes in the field, including the released mosquitoes (which we set to last 20 weeks), plus the offspring born in field, over the 40 generations. Wild mosquitoes (without *Wolbachia*) were not taken into account due to lack of gene flow from *Wolbachia* mosquitoes to the wild ones, due to cytoplasmic incompatibility and complete maternal transmission (Hoffmann and Turelli 2013).

Parameters	Description	Values	References
I	Fitness of homozygous resistant mosquitoes (0.0 - 1.0)	0.75	Brito et al. 2013
H	Fitness of heterozygous mosquitoes (resistance nearly recessive)	0.8	Brito et al. 2013
Fc	Fitness of *Wolbachia*-carrying mosquitoes	0.8	Turley et al. 2013; Hoffmann et. al 2014; Ross et al. 2016; Garcia et al. 2017
rUinic	Local population has frequency of R (95%)	0.95	Linss et al. 2014; Bellinato et al. 2016
Nrel	Releases	20	Garcia et al. 2017
r.rel	Ratio of released individuals by the total number (released + local) per week	0.10	Garcia et al. 2017
totT	Total number of generations	40	-

Table 1. Fixed parameters used in simulations.

3.1) Construction of potential invasion scenarios (Table 2):

Our scenarios consider the intensity of insecticide used by local human population and the resistance of *Wolbachia* mosquitoes. We first consider that insecticide intensity *s* varies in the simulation scenario. We consider some scenarios with no application (*s* = 0.0), low use (s = 0.4), moderate use (s = 0.7), or high insecticide use (*s* = 0.9). We also define the frequencies *freq.rel* of genotypes (RR, RS, SS) in released *Wolbachia* mosquitoes. For the simulations done by releasing *Wobachia* susceptible mosquitoes the

frequency profile was *freq.rel* = (0.0, 0.0, 1.0). When releasing *Wolbachia* resistant mosquitoes, the *freq.rel* = (0.95, 0.0, 0.05), values based on wild resistant mosquitoes status observed in previous studies (Garcia et al. 2017).

	Insecticide intensity	Frequencies of genotyping (RR, RS, SS)
Scenario 1: Releasing susceptible *Wolbachia* mosquitoes x wild resistant mosquitoes	0.0 (no), 0.4 (little), 0.7 (moderate), 0.9 (high)	(0,0,1)
Scenario 2: Releasing resistant *Wolbachia* mosquitoes x wild resistant mosquitoes	0.0 (no), 0.4 (little), 0.7 (moderate), 0.9 (high)	(0.95,0,0.05)

Table 2. Variable parameters used in simulations.

4) Simulated scenarios

Scenario 1: Releasing susceptible mosquitoes with *Wolbachia* into an area with wild resistant mosquitoes, with no, little, moderate and high insecticide intensity (*s* = from 0.0 to 0.9).

Scenario 2: Releasing insecticide resistant *Wolbachia* mosquitoes into an area with wild resistant mosquitoes (same levels of insecticide resistant in both populations), with no, little, moderate and high insecticide intensity (*s* = from 0.0 to 0.9).

Results

1. Quantifying the fitness cost due to insecticide resistance

We analyze the frequencies of 1016Ile kdr mutation in the *w*Mel colony without insecticide pressure during 18 generations in one Brazilian *w*Mel colony (called wMelBr).

The frequency of the resistance gene decreased across generations (Fig 1). In fact, after 18 generations, the frequency of resistant genotypes dropped from 0.75 to 0. We estimate resistance fitness at a value of 0.75. This value was applied in the scenario analyzes for the *Wolbachia* invasion model (parameter *i*).

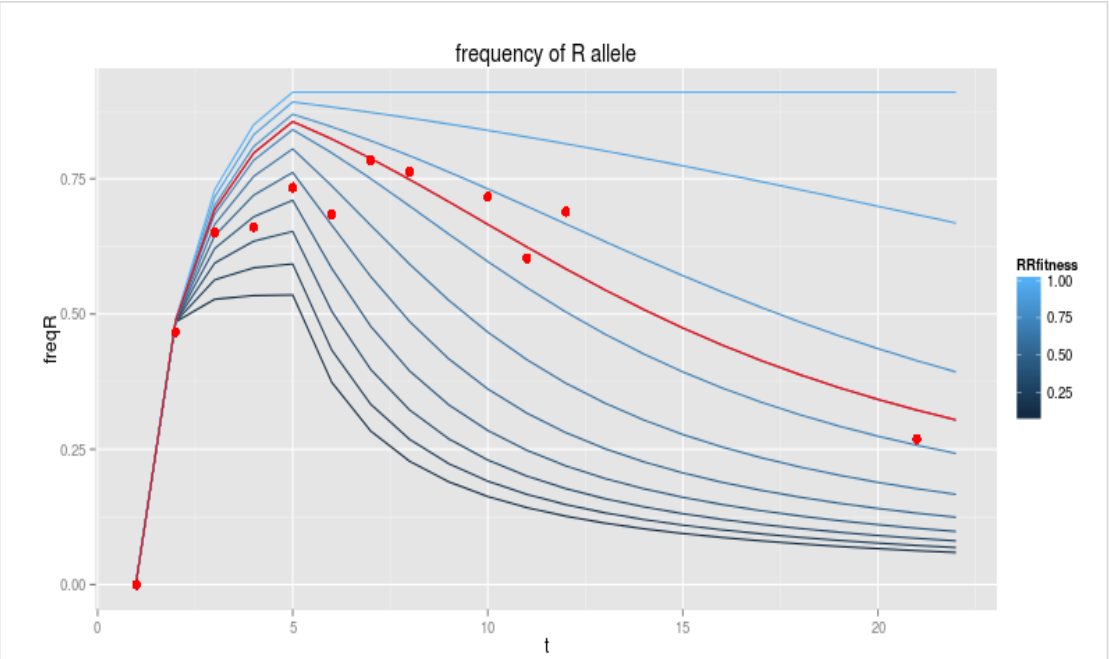

Fig 1. Observed and expected changes in the frequency of the resistance allele over time (laboratory generations) assuming different fitness costs due to insecticide resistance based on the frequency of the kdr mutation, 1016Ile, along 18 generations when maintained under laboratory conditions, i.e., without insecticide pressure. Dots show the observed values and various curves constructed using the model show the expected frequencies when varying fitness of homozygous mosquitoes (factor *i*) from 0.1 to 1.0. The best fit using the lowest sum of residuals is shown in red for a relative fitness factor *i* =0.75.

2. Simulation scenarios

We studied scenarios for *Wolbachia* releases considering that local wild *Ae. aegypti* mosquitoes are resistant to the insecticide. We proposed two different scenarios: deployment of *Wolbachia* infecting mosquitoes susceptible or resistant to insecticides.

Moreover, the intensity of insecticide application by local householders also was also allowed to vary in the simulations.

2.1) Scenario 1: Deployment of *Wolbachia* infecting a susceptible mosquito with wild mosquitoes resistant, with insecticide application ranging from 0.0 to 0.9.

Two outcomes of successful invasion of *Wolbachia* were observed by releasing susceptible mosquitoes: under a scenario of no insecticide use (s=0.0) and under a low application intensity of s=0.4 (Fig 2A, blue and yellow line). As expected, in the absence of insecticide *Wolbachia* invades faster. Additionally, the frequency of the R allele in the mosquitoes with *Wolbachia* (those released weekly plus the offspring born in field) increases due to introgression of the R allele in the first few generations. However, the frequency of R decreases rapidly and is effectively lost, due to the continuous introduction of susceptible alleles through *Wolbachia* releases and also due to fitness costs, resulting in a possible reversion of insecticide resistance status in the field after *Wolbachia* invasion (Fig 2B, blue line). However, even a occasional insecticide applications in the field is enough to select R alleles in *Wolbachia* mosquitoes (Fig 2B, yellow line).

Wolbachia is not able to invade in the two situations in which insecticide susceptible mosquitoes are released and local householders undertake moderate or high insecticide applications (Fig 2A, red and brown line). In these two scenarios, *Wolbachia* frequency did not increase above 25%, probably due to the selective suppression of released and susceptible individuals. The R alleles are rapidly selected in *Wolbachia* mosquitoes, despite the constant release of hundreds of *Ae. aegypti* on a weekly basis (Fig 2B, red and brown).

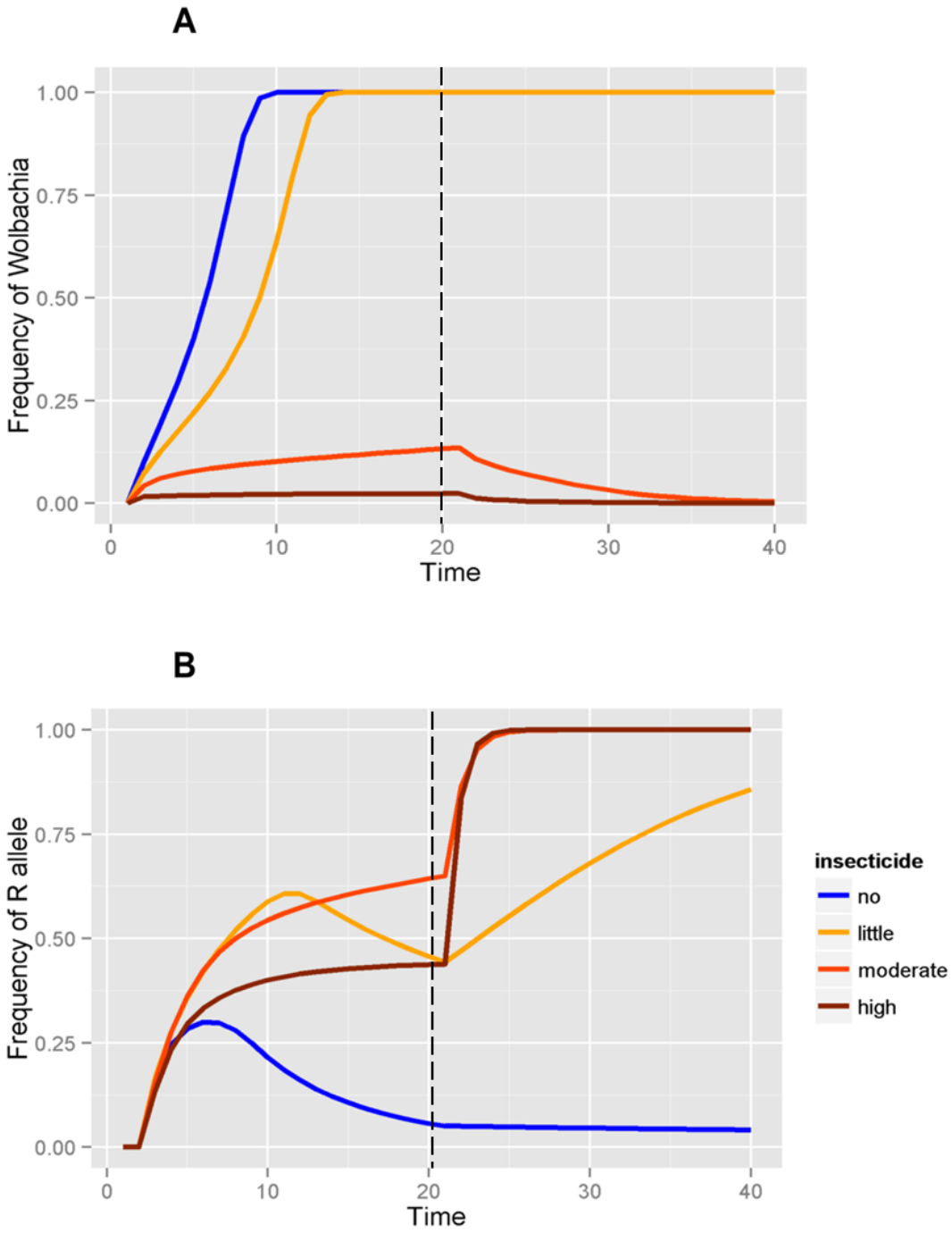

Fig 2. Releases of *Wolbachia* mosquitoes susceptible to insecticides. Frequency of (A) *Wolbachia* and (B) resistance alleles under different levels of insecticide use by local householders. Dashed line represents the end of releases.

2.2) Scenario 2: Deployment of *Wolbachia* infecting a resistant mosquito with wild mosquitoes resistant, with insecticide application ranging from 0.0 to 0.9

When releasing *Wolbachia* associated with mosquitoes resistant to insecticides, invasion always succeeds (Fig 3A). In this scenario the insecticide application intensity is allowed to vary from s = 0.0 to s=0.9 (Fig 3B, blue, yellow, red and brown line). Interestingly, the different levels of insecticide application did not alter the *Wolbachia* invasion profile. However, there is a minor tendency for faster *Wolbachia* invasion when insecticide intensity is low. In the absence of insecticide, the frequency of R allele decreases in the field (Fig 3B, line blue), as shown in scenario 1. However, in this case, it is slower due to fitness cost of insecticide resistance in the absence of insecticide, rather than the introduction of susceptible alleles by *Wolbachia* mosquitoes, as happened in scenario 1. However, if local householders use few, moderate or high insecticide application intensity, R alleles reach fixation in *Wolbachia* mosquitoes in field (Fig 3B, yellow, red and brown line).

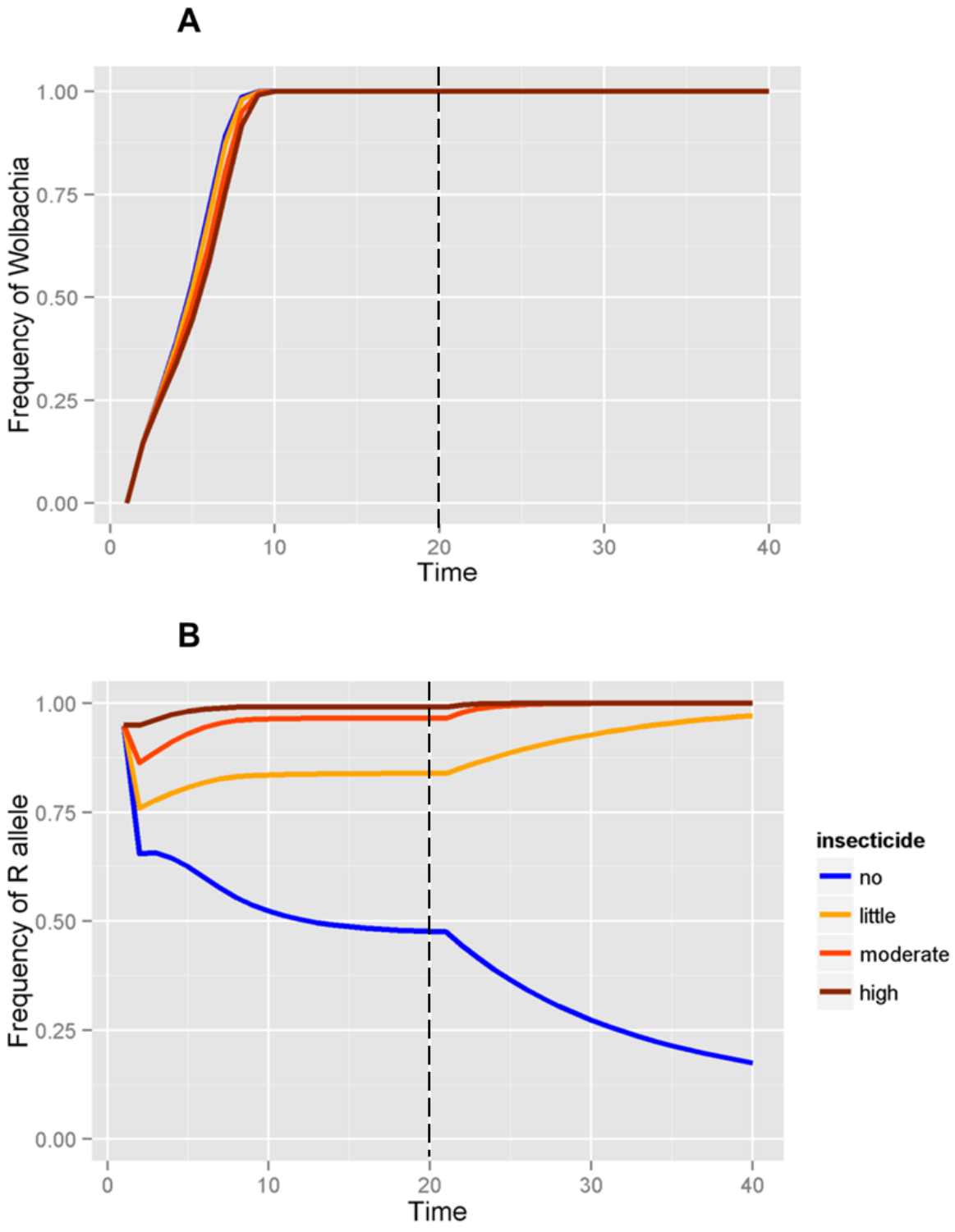

Fig 3. Releases of *Wolbachia* mosquitoes as resistant to insecticides as those in the wild population. Frequency of (A) *Wolbachia* and (B) resistance alleles in field considering different levels of insecticide use by local householders. Dashed line represents the end of releases.

Discussion

We investigated how *Wolbachia* invasion success is influenced by the presence of insecticide resistance alleles in the released and/or wild *Aedes aegypti* populations. Our model is based on the one proposed by by Hoffmann and Turelli (2013), but we take into account the fitness cost due to insecticide resistance in order to analyze different scenarios of insecticide use and resistance.

We estimated the fitness cost due to insecticide resistance at 0.25, based on empirical observations of *w*MelBr resistance allele loss over 18 generations. This finding points to an expected performance decrease in an insecticide-free environment. Since several vector control programs historically rely on chemicals, this would likely lead to distinct levels of resistance between wild and released populations (Garcia et al. 2017). Therefore, simulations of *Wolbachia* invasion must take into account the insecticide resistance status of both released and natural populations. Our results indicate that invasion of a susceptible strain is only possible if local householders use insecticides at low levels. By contrast, we show that invasion is possible if we release a population as resistant as the one in the field. Therefore, we set the conditions to determine how the frequency of insecticides application by local householders can affect *Wolbachia* invasion in field.

If no insecticide is used, *Wolbachia* invades faster and insecticide susceptibility status in field mosquitoes may increase rapidly, due to the introduction of S alleles by *Wolbachia* mosquitoes. We also demonstrated that although *Wolbachia* invades, R alleles are still selected even if local householders engage in a low level of insecticide applications. For moderate or high frequencies of insecticide application, *Wolbachia* released mosquitoes die quickly as wild-resistant mosquitoes are in advantage to survive

and reproduce. In these situations *Wolbachia* was not able to increase above 25% of its frequency in field, and after releases stopped, *Wolbachia* were almost lost from the field. This scenario is in agreement to explain the unsuccessful invasion of *w*Mel in one site of Rio, where a susceptible *Ae. aegypti* infected with *w*Mel (*w*MelBr) was released into a highly resistant field population. A high insecticide pressure by local householders culminated in the first failure of *w*Mel deployment in an *Ae. aegypti* wild population (Garcia et al. 2017).

When releasing a *Wolbachia* strain as resistant as the wild population, simulations indicate that *Wolbachia* is able to invade irrespective of the intensity of insecticide application, although a faster *Wolbachia* invasion happens in the absence of insecticide. We observe an expected decrease in the frequency of R alleles in the absence of insecticide, but this decrease is slower than the one when a susceptible strain introduces S alleles in field populations. This finding is in agreement with other studies that demonstrate faster insecticide resistance reversal when S alleles are at a higher frequency (Melo-Santos et al. 2010; Schechtman and Souza 2015). For the other three intensities of insecticide application by local householders (0.4, 0.7, 0.9), a selection of high frequencies of R alleles in field happens at a consistent use of insecticides, since both released and wild populations are resistant. Indeed, a second round of releases in the same site in Rio de Janeiro has recently resulted in successful invasion using a new strain with the same levels of insecticide resistance as the wild *Ae. aegypti* population (Garcia et al. 2017).

The use of insecticides, since the advent of DDT in 1940's (Naggash et al. 2016), still has an essential role in vector control. However, indiscriminate use of insecticides has selected resistant *Ae. aegypti* populations in many countries, mainly in tropical and subtropical areas where arboviruses´ outbreaks and chemical control are frequent

(Rodríguez et al. 2007; Saavedra-Rodriguez et al. 2007). Many *Ae. aegypti* populations were found resistant to pyrethroids (PY) in Brazil, Puerto Rico, Costa Rica and Mexico (Bellinato et al. 2016; Ponce-Garcia et al. 2016; Bisset et al. 2013; Linss et al. 2014; Vera-Maloof et al. 2015). PY is the most used insecticide class against adult mosquitoes, and has the unique characteristic of domestic use, including indoors, which favors an abusive use of this class (Bellinato et al. 2016). Insecticide resistance is also described in *Ae. aegypti* for the larvicide temephos, an organophosphate (OP) used in vector control routine in some countries (Mazzari 1995; Suarez et al. 1998; Rodríguez et al. 2002; Biber et al. 2006; Flores et al. 2006; Ocampo et al. 2011; Marcombe et al. 2011, 2012; Chediak et al. 2016;). In this context, the strategy of releasing *Wolbachia* mosquitoes for population replacement may face wild *Ae. aegypti* populations with various levels of insecticide resistance depending on insecticides types, and also different levels of insecticide use in different localities over countries.

The most studied insecticide resistance mechanisms in *Ae. aegypti* are metabolic resistance due to an increase in detoxifying enzymes activities and modifications in insecticides target sites (Hemingway et al. 2000). These mechanisms are not specific to a single insecticide type and can confer resistance to multiple chemical compounds. Modifications in target sites are often associated to PY resistance in *Ae. aegypti*, and can occur due to mutations in voltage-gated sodium channel gene (PY target site), known as knockdown resistance (*kdr*). Generally, only homozygous for kdr alleles are resistant, hence the kdr mutation has a recessive genetic trait (Huang et al. 2004; Brito et al. 2013). Occasionally, more than one mutation can happen in the same population, possibly increasing the resistance levels more than 300 fold (super kdr) (Soderlund 2008). In Brazil, *kdr* mutations are found in two loci of *Ae. aegypti* genome, Phe1534Cys and Val1016Ile, and double mutation (1534Cys+1016Ile) probably confers an even higher PY

resistance (Vera-Maloof et al. 2015). Importantly, 1016Ile mutation allele is often found in association with 1534Cys mutation in *Ae. aegypti* for Brazilian cities (Linss et al. 2014).

Insecticide resistance is frequently associated with a fitness cost. Larval development time and adult fecundity, longevity and locomotor activity are altered in resistant mosquitoes (Diniz et al. 2015; Brito et al. 2013; Schechtman and Souza 2015; Jaramillo et al. 2014; Martins et al. 2012). In our model, the fitness cost due to insecticide resistance is assessed by the decrease of kdr mutation in the strain *w*MelBr. This strain was backcrossed with Rio de Janeiro local populations (Dutra et al. 2015), thus having high levels of PI resistance and high frequencies of *kdr* mutations, for Val1016Ile and Phe1534Cys (Linss et al. 2014). After backcrossing stopped, *w*MelBr had a frequency of almost 70% of resistant genotypes and, after eighteen generations under laboratory conditions without insecticide pressure, resistant genotypes dropped to a frequency of 4%. The decrease in frequency of 1016Ile allele over the generations were used to model the fitness cost of the resistant allele, resulting in a fitness loss estimate of 0.25. Brito et al. (2013) also observed 1016Ile *kdr* frequency decreasing to less than 30%, after 15 generations of *Ae. aegypti* without *Wolbachia* in laboratory cages, when starting from frequencies of 70% and 50% of kdr allelic frequency. Our results show a relative fitness of 0.75 when compared to population without resistance alleles, which makes the resistant mosquitos in disadvantage in an environment without insecticide use.

We assume in the model that insecticide resistance is governed by a single diallelic locus, with alleles denoted R and S (Ranson et al. 2009; Hoffmann and Turelli 2013). We also considered fitness cost due to *Wolbachia* infection. *w*Mel infection in *Ae. aegypti* has small fitness cost, with slight alterations in larval competitions (Ross et al., 2016), fecundity (Turley et al. 2013, Hoffmann et al. 2014) and fertility (Garcia et al. 2017).

Thus, in our model both *Wolbachia* infection and insecticide resistance are considered to affect mosquito fitness. As a consequence, an increase in fitness cost of released mosquitoes could make them less competitive than their counterparts in field, increasing the likelihood that *Wolbachia* invasion would fail.

Insecticide resistance reversal was also demonstrated under laboratory conditions as introduction of S alleles alters resistance status over generations (Brito et al. 2013; Melo-Santos et al. 2010; Schechtman and Souza 2015). Melo-Santos et al. (2010), showed the interruption of insecticide use or its interruption combined with introduction of 30% of susceptible individuals in cages, decreased temephos resistance gradually to low levels. However, the introduction of 50% was enough to recover totally the susceptibility for this larvicide in only three generations. Additionally, such reversal is unlikely to happen naturally in Brazilian cities since in most of them frequency of susceptible field populations remains low. However, Melo-Santos et al. (2010) comment that the strategy of introduction of susceptible individuals in the field may be considered as an additional alternative for methods where occur releases of thousands of mosquitoes (such as transgenic mosquitoes or *Wolbachia* mosquitoes). Such approach requires close association with social scientists and a strong engagement effort to aware the households of a rational insecticide use.

We showed possibilities of *Wolbachia* releases scenarios in association with insecticide use and fitness costs due to insecticide resistance. Studying these scenarios is important to evaluate whether *Wolbachia* is able to invade in field at different levels of insecticide use and frequency of resistance alleles. We also showed a possible insecticide resistance reversal in the field when we take advantage of releasing susceptible mosquitoes. However, achieving insecticide reversal requires to stop or to reduce insecticide usage from local householders, which requires a significant effort from social

scientists to change community behavior and vector control good practices. Finally, if the insecticide application is intense in field, *Wolbachia* only invades if we use a *Wolbachia* strain as resistant as wild mosquitoes. These informations are important in decision processes regarding *Wolbachia* releases in the field.

References

Baldacchino F, Caputo B, Chandre F, Drago A, della Torre A, Montarsi F, et al. Control methods against invasive *Aedes* mosquitoes in Europe: a review. Pest Manag Sci. 2015 Nov;71(11):1471-85. doi: 10.1002/ps.4044. Epub 2015 Jun 26. Review.

Beauty BJ, Marquardt WC 1996. The biology of disease vectors. Colorado: University Press of Colorado, 632p.

Bellinato DF, Viana-Medeiros PF, Araújo SC, Martins AJ, Lima JB, Valle D. Resistance Status to the Insecticides Temephos, Deltamethrin, and Diflubenzuron in Brazilian *Aedes aegypti* Populations. Biomed Res Int. 2016;2016:8603263.

Berticat C, Bonnet J, Duchon S, Agnew P, Weill M, Corbel V. Costs and benefits of multiple resistance to insecticides for *Culex quinquefasciatus* mosquitoes. BMC Evol Biol. 2008 Apr 8;8:104.

Biber PA, Dueñas JR, Almeida FL, Gardenal CN, Almirón WR. Laboratory evaluation of susceptibility of natural subpopulations of *Aedes aegypti* larvae to temephos. J Am Mosq Control Assoc. 2006 Sep;22(3):408-11.

Bisset JA, Marín R, Rodríguez MM, Severson DW, Ricardo Y, French L, et al. Insecticide resistance in two *Aedes aegypti* (Diptera: Culicidae) strains from Costa Rica. J Med Entomol. 2013 Mar;50(2):352-61.

Braks MA, Honório NA, Lourenço-De-Oliveira R, Juliano SA, Lounibos LP. Convergent habitat segregation of *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) in southeastern Brazil and Florida. J Med Entomol. 2003 Nov;40(6):785-94.

Brito LP, Linss JG, Lima-Camara TN, Belinato TA, Peixoto AA, Lima JB, Valle D, Martins AJ. Assessing the effects of *Aedes aegypti* kdr mutations on pyrethroid resistance and its fitness cost. PLoS One. 2013 Apr 8;8(4):e60878.

Cao-Lormeau VM, Musso D. Emerging arboviruses in the Pacific. Lancet. 2014 Nov 1;384(9954):1571-2.

Chediak M, G Pimenta F Jr, Coelho GE, Braga IA, Lima JB, Cavalcante KR, et al. Spatial and temporal country-wide survey of temephos resistance in Brazilian populations of *Aedes aegypti*. Mem Inst Oswaldo Cruz. 2016 May;111(5):311-21.

de Oliveira CD, Gonçalves DS, Baton LA, Shimabukuro PH, Carvalho FD, Moreira LA. Broader prevalence of *Wolbachia* in insects including potential human disease vectors. Bull Entomol Res. 2015 Jun;105(3):305-15.

Diniz DF, de Melo-Santos MA, Santos EM, Beserra EB, Helvecio E, de Carvalho-Leandro D, et al. Fitness cost in field and laboratory *Aedes aegypti* populations associated with resistance to the insecticide temephos. Parasit Vectors. 2015 Dec 30;8:662.

Dutra HL, Dos Santos LM, Caragata EP, Silva JB, Villela DA, Maciel-de-Freitas R, Moreira LA. From lab to field: the influence of urban landscapes on the invasive potential of *Wolbachia* in Brazilian *Aedes aegypti* mosquitoes. PLoS Negl Trop Dis. 2015 Apr 23;9(4):e0003689.

Dutra HL, Rocha MN, Dias FB, Mansur SB, Caragata EP, Moreira LA. *Wolbachia* Blocks Currently Circulating Zika Virus Isolates in Brazilian *Aedes aegypti* Mosquitoes. Cell Host Microbe. 2016 Jun 8;19(6):771-4.

Eliminate Dengue Program. www.eliminatedengue.com

Ferreira-de-Brito A, Ribeiro IP, Miranda RM, Fernandes RS, Campos SS, Silva KA, et al. First detection of natural infection of *Aedes aegypti* with Zika virus in Brazil and throughout South America. Mem Inst Oswaldo Cruz. 2016 Oct;111(10):655-658.

Flores AE, Grajales JS, Salas IF, Garcia GP, Becerra MH, Lozano S, et al. Mechanisms of insecticide resistance in field populations of *Aedes aegypti* from Quintana Roo, Southern Mexico. J Am Mosq Control Assoc. 2006 Dec;22(4):672-7.

Foster SP, Young S, Williamson MS, Duce I, Denholm I, Devine GJ. Analogous pleiotropic effects of insecticide resistance genotypes in peach-potato aphids and houseflies. Heredity (Edinb). 2003 Aug;91(2):98-106.

Garcia GA, Sylvestre G, Petersen MT, Aguiar R, Martins AJ, Lima JBP, Lourenço-de-Oliveira R, et al. The riddle solved on a local grocery store: the release of *Aedes aegypti* as resistant to pyrethroids as the wild population is essential for *Wolbachia* invasion. Submitted to XXXXX. 2017.

Garcia Gde A, Dos Santos LM, Villela DA, Maciel-de-Freitas R. Using *Wolbachia* Releases to Estimate *Aedes aegypti* (Diptera: Culicidae) Population Size and Survival. PLoS One. 2016 Aug 1;11(8):e0160196.

Hancock PA, White VL, Ritchie SA, Hoffmann AA, Godfray HC. BMC Biol. Predicting *Wolbachia* invasion dynamics in *Aedes aegypti* populations using models of density-dependent demographic traits. 2016 Nov 8;14(1):96.

Hemingway J, Ranson H. Insecticide resistance in insect vectors of human disease. Annu Rev Entomol. 2000;45:371-91. Review.

Hilgenboecker K, Hammerstein P, Schlattmann P, Telschow A, Werren JH. How many species are infected with *Wolbachia*? A statistical analysis of current data. FEMS Microbiol Lett. 2008 Apr;281(2):215-20.

Hoffmann AA, Montgomery BL, Popovici J, Iturbe-Ormaetxe I, Johnson PH, Muzzi F, et al. Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission. Nature. 2011 Aug 24;476(7361):454-7.

Hoffmann AA, Turelli M. Facilitating *Wolbachia* introductions into mosquito populations through insecticide-resistance selection. Proc Biol Sci. 2013 Apr 10;280(1760):20130371.

Hoffmann AA, Iturbe-Ormaetxe I, Callahan AG, Phillips BL, Billington K, Axford JK, et al. Stability of the wMel *Wolbachia* Infection following invasion into *Aedes aegypti* populations. PLoS Negl Trop Dis. 2014 Sep 11;8(9):e3115.

Hoffmann AA, Ross PA, Rašić G. *Wolbachia* strains for disease control: ecological and evolutionary considerations. Evol Appl. 2015 Sep;8(8):751-68.

Huang J, Kristensen M, Qiao CL, Jespersen JB. Frequency of kdr gene in house fly field populations: correlation of pyrethroid resistance and kdr frequency. J Econ Entomol. 2004 Jun;97(3):1036-41.

Jaramillo-O N, Fonseca-González I, Chaverra-Rodríguez D. Geometric morphometrics of nine field isolates of *Aedes aegypti* with different resistance levels to lambda-cyhalothrin and relative fitness of one artificially selected for resistance. PLoS One. 2014 May 6;9(5):e96379.

Kliot A, Ghanim M. Fitness costs associated with insecticide resistance. Pest Manag Sci. 2012 Nov;68(11):1431-7. doi: 10.1002/ps.3395. Epub 2012 Sep 4. Review.

Liang G, Gao X, Gould EA. Factors responsible for the emergence of arboviruses; strategies, challenges and limitations for their control. Emerg Microbes Infect. 2015 Mar;4(3):e18.

Lima EP, Goulart MO, Rolim Neto ML. Meta-analysis of studies on chemical, physical and biological agents in the control of *Aedes aegypti*. BMC Public Health. 2015 Sep 4;15:858.

Linss JG, Brito LP, Garcia GA, Araki AS, Bruno RV, Lima JB, et al. Distribution and dissemination of the Val1016Ile and Phe1534Cys Kdr mutations in *Aedes aegypti* Brazilian natural populations. Parasit Vectors. 2014 Jan 15;7:25.

Lourenço-de-Oliveira R, Vazeille M, de Filippis AM, Failloux AB. *Aedes aegypti* in Brazil: genetically differentiated populations with high susceptibility to dengue and yellow fever viruses. Trans R Soc Trop Med Hyg. 2004 Jan;98(1):43-54.

Maciel-de-Freitas R, Marques WA, Peres RC, Cunha SP, de Oliveira RL. Variation in *Aedes aegypti* container productivity in a slum and a suburban district of Rio de Janeiro during dry and wet seasons. Mem Inst Oswaldo Cruz. 2007 Jun;102(4):489-96.

Maciel-de-Freitas R, Valle D. Challenges encountered using standard vector control measures for dengue in Boa Vista, Brazil. Bull World Health Organ. 2014 Sep 1;92(9):685-9.

Marcombe S, Darriet F, Tolosa M, Agnew P, Duchon S, Etienne M, et al. Pyrethroid resistance reduces the efficacy of space sprays for dengue control on the island of Martinique (Caribbean). PLoS Negl Trop Dis. 2011 Jun;5(6):e1202.

Marcombe S, Mathieu RB, Pocquet N, Riaz MA, Poupardin R, Sélior S, et al. Insecticide resistance in the dengue vector *Aedes aegypti* from Martinique: distribution, mechanisms and relations with environmental factors. PLoS One. 2012;7(2):e30989.

Martins AJ, Ribeiro CD, Bellinato DF, Peixoto AA, Valle D, Lima JB. Effect of insecticide resistance on development, longevity and reproduction of field or laboratory selected *Aedes aegypti* populations. PLoS One. 2012;7(3):e31889.

Mazzari MB, Georghiou GP. Characterization of resistance to organophosphate, carbamate, and pyrethroid insecticides in field populations of *Aedes aegypti* from Venezuela. J Am Mosq Control Assoc. 1995 Sep;11(3):315-22.

Melo-Santos MA, Varjal-Melo JJ, Araújo AP, Gomes TC, Paiva MH, Regis LN, et al. Resistance to the organophosphate temephos: mechanisms, evolution and reversion in an *Aedes aegypti* laboratory strain from Brazil. Acta Trop. 2010 Feb;113(2):180-9.

Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, Lu G, Pyke AT, Hedges LM, et al. A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, Chikungunya, and Plasmodium. Cell. 2009 Dec 24;139(7):1268-78.

Morrison AC, Zielinski-Gutierrez E, Scott TW, Rosenberg R. Defining challenges and proposing solutions for control of the virus vector *Aedes aegypti*. PLoS Med. 2008 Mar 18;5(3):e68.

Mota MT, Terzian AC, Silva ML, Estofolete C, Nogueira ML. Mosquito-transmitted viruses - the great Brazilian challenge. Braz J Microbiol. 2016 Dec;47 Suppl 1:38-50.

Naqqash MN, Gökçe A, Bakhsh A, Salim M. Insecticide resistance and its molecular basis in urban insect pests. Parasitol Res. 2016 Apr;115(4):1363-73.

Ocampo CB, Salazar-Terreros MJ, Mina NJ, McAllister J, Brogdon W. Insecticide resistance status of *Aedes aegypti* in 10 localities in Colombia. Acta Trop. 2011 Apr;118(1):37-44.

Plernsub S, Saingamsook J, Yanola J, Lumjuan N, Tippawangkosol P, Walton C, et al. Temporal frequency of knockdown resistance mutations, F1534C and V1016G, in *Aedes aegypti* in Chiang Mai city, Thailand and the impact of the mutations on the efficiency of thermal fogging spray with pyrethroids. Acta Trop. 2016 Oct;162:125-32.

Ponce-García G, Del Río-Galvan S, Barrera R, Saavedra-Rodriguez K, Villanueva-Segura K, Felix G, et al. Knockdown Resistance Mutations in *Aedes aegypti* From Puerto Rico. J Med Entomol. 2016 Nov;53(6):1410-1414.

Ranson H, Burhani J, Lumjuan N, Black WCI. 2010. Insecticide resistance in dengue vectors. TropiKanet 1

Rodríguez MM, Bisset J, Ruiz M, Soca A. Cross-resistance to pyrethroid and organophosphorus insecticides induced by selection with temephos in *Aedes aegypti* from Cuba. J Med Entomol. 2002 Nov;39(6):882-8.

Rodríguez MM, Bisset JA, Fernández D. Levels of insecticide resistance and resistance mechanisms in *Aedes aegypti* from some Latin American countries. J Am Mosq Control Assoc. 2007 Dec;23(4):420-9.

Ross PA, Endersby NM, Hoffmann AA. Costs of Three *Wolbachia* Infections on the Survival of *Aedes aegypti* Larvae under Starvation Conditions. PLoS Negl Trop Dis. 2016 Jan 8;10(1):e0004320.

Saavedra-Rodriguez K, Urdaneta-Marquez L, Rajatileka S, Moulton M, Flores AE, Fernandez-Salas I, et al. A mutation in the voltage-gated sodium channel gene associated with pyrethroid resistance in Latin American *Aedes aegypti*. Insect Mol Biol. 2007 Dec;16(6):785-98.

Schechtman H, Souza MO. Costly Inheritance and the Persistence of Insecticide Resistance in *Aedes aegypti* Populations. PLoS One. 2015 May 1;10(5):e0123961.

Scott TW, Chow E, Strickman D, Kittayapong P, Wirtz RA, Lorenz LH, Edman JD. Blood-feeding patterns of *Aedes aegypti* collected in a rural Thai village. J Med Entomol. 1993 Sep;30(5):922-7.

Smith LB, Kasai S, Scott JG. Pyrethroid resistance in *Aedes aegypti* and *Aedes albopictus*: Important mosquito vectors of human diseases. Pestic Biochem Physiol. 2016 Oct;133:1-12.

Soderlund DM. Pyrethroids, knockdown resistance and sodium channels. Pest Manag Sci. 2008 Jun;64(6):610-6.

Suarez MF, Gonzales R, Morales CA. Temephos resistance to Aedes aegypti in Cali, Colombia. Am J Trop Med Hyg.1998.55: 257.

Turley AP, Zalucki MP, O'Neill SL, McGraw EA. Transinfected *Wolbachia* have minimal effects on male reproductive success in *Aedes aegypti*. Parasit Vectors. 2013 Feb 11;6:36.

Ulrich JN, Beier JC, Devine GJ, Hugo LE. Heat Sensitivity of wMel *Wolbachia* during *Aedes aegypti* Development. PLoS Negl Trop Dis. 2016 Jul 26;10(7):e0004873.

Vasconcelos PF, Calisher CH. Emergence of Human Arboviral Diseases in the Americas, 2000-2016. Vector Borne Zoonotic Dis. 2016 May;16(5):295-301.

Vega-Rúa A, Lourenço-de-Oliveira R, Mousson L, Vazeille M, Fuchs S, Yébakima A, et al. Chikungunya virus transmission potential by local *Aedes* mosquitoes in the Americas and Europe. PLoS Negl Trop Dis. 2015 May 20;9(5):e0003780.

Vera-Maloof FZ, Saavedra-Rodriguez K, Elizondo-Quiroga AE, Lozano-Fuentes S, Black Iv WC. Coevolution of the Ile1,016 and Cys1,534 Mutations in the Voltage Gated Sodium Channel Gene of *Aedes aegypti* in Mexico. PLoS Negl Trop Dis. 2015 Dec 11;9(12):e0004263.

Werren JH, Baldo L, Clark ME. *Wolbachia*: master manipulators of invertebrate biology. Nat Rev Microbiol. 2008 Oct;6(10):741-51.

Wuliandari JR, Lee SF, White VL, Tantowijoyo W, Hoffmann AA, Endersby-Harshman NM. Association between Three Mutations, F1565C, V1023G and S996P, in the Voltage-Sensitive Sodium Channel Gene and Knockdown Resistance in *Aedes aegypti* from Yogyakarta, Indonesia. Insects. 2015 Jul 23;6(3):658-85.

5. Discussão

Em função da ascensão de diferentes arboviroses no Brasil, e no mundo, torna-se imprescindível a busca por novas ferramentas de controle destas doenças. Neste contexto, diferentes abordagens têm sido testadas em áreas endêmicas ao redor do mundo para aumentar a eficiência do controle quando somado aos métodos tradicionais. Atualmente, por exemplo, encontra-se em andamento liberações em campo de mosquitos com *Wolbachia*, uma bactéria que bloqueia infecções por patógenos transmitidos por *Ae. aegypti*, como os vírus dengue, chikungunya e Zika (www.eliminatedengue.com). Testes em campo em áreas da Austrália, Indonésia e Vietnã confirmaram a invasão de *Ae. aegypti* infectados com a cepa *w*Mel sobre a população selvagem (Hoffmann et al. 2011; Frentiu et al. 2014). No Brasil, as liberações de *Ae. aegypti* com *Wolbachia* foram iniciadas pelo Rio de Janeiro, em setembro de 2014. Entretanto, diferentemente do observado em outros países que obtiveram rápida invasão desta bactéria, a primeira liberação de *Wolbachia* na América Latina não logrou êxito. Acreditamos que essa falha seja consequência de algumas particularidades de populações nativas de *Ae. aegypti* do Rio, tais como: (i) elevada resistência a inseticidas, em especial os da classe dos piretroides (PI), e (ii) alta densidade populacional. Nessa tese exploramos como estes dois fatores impactaram na implementação da estratégia *Wolbachia* em uma pequena região do Rio de Janeiro, e mostramos que esses efeitos podem se apresentar em diferentes cidades e regiões do país.

Contextualizando um pouco esta técnica, um dos primeiros, e principais, quesitos para obter uma invasão bem sucedida da *Wolbachia* em uma população selvagem de *Ae. aegypti*, é a liberação de um número suficiente de mosquitos em campo. Ou seja, é necessário ter uma quantidade de insetos com a bactéria que exceda o limiar de invasão

(Turelli e Hoffmann 1991; Turelli e Barton 2017). Para tal, semanalmente, são liberados cerca de 15.000 mosquitos (razão sexual de 1:1) nas áreas-teste, durante 20-30 semanas consecutivas. Os insetos liberados possuem uma marcação interna, a presença da *Wolbachia*, que pode ser detectada por PCR. Assim sendo, foi possível tirar proveito de liberações de *Ae. aegypti* com *Wolbachia* para se estimar a densidade da população nativa e, assim, ajustar o número de mosquitos a serem soltos ao longo das semanas subsequentes de acordo com o perfil de invasão (Garcia et al. 2016 – artigo 6). Ademais, outro ponto de extrema relevância que deve ser levado em conta para o sucesso da *Wolbachia*, é a liberação de mosquitos que sejam aptos a sobreviver no ambiente natural. Os mosquitos com a bactéria devem ser bons competidores frente aos nativos, tanto as fêmeas (em relação à fecundidade e sobrevivência diária, por exemplo), quanto os machos (os responsáveis pela efetivação da ocorrência da incompatibilidade citoplasmática nas fêmeas inseminadas, um mecanismo crucial que facilita a disseminação da *Wolbachia*) (Werren et al. 2008; Nguyen et al. 2015; Jiggins 2017; Garcia et al. 2017b – artigo 7). Em síntese, para os mosquitos com *Wolbachia* substituírem a população selvagem, esses dois fatores devem atuar em conjunto: liberações de um número suficiente de mosquitos e boa aptidão (*fitness*) em campo por parte dos insetos liberados.

Liberações de *Ae. aegypti* com *Wolbachia*, cepa *w*Mel, realizadas em áreas do Norte de Queensland (Austrália), Ilha Tri Nguyen (Vietnã) e Yogyakarta (Indonésia) apresentaram um aumento rápido e constante na frequência da bactéria em campo (Eliminate Degue, dados não publicados). Em Yorkeys Knob, uma área na Austrália, após 12 semanas de liberações a invasão da *Wolbachia* alcançou uma frequência de 98% (Hoffmann et al. 2011). Em setembro de 2014, o Brasil iniciou liberações em uma área-teste no Rio de Janeiro (bairro de Tubiacanga/Ilha do Governador), liberando-se, pela primeira vez, *Ae. aegypti* com *Wolbachia*, uma linhagem brasileira chamada de *w*MelBr.

Todavia, foi observado durante as liberações que a invasão da *Wolbachia* se manteve em um nível de 40-45% entre as semanas 7-19, finalizando na última semana de liberações (semana 20) em 60%. Esse resultado, teoricamente, caracterizaria uma invasão de sucesso, pois a frequência excedeu o limiar de invasão de 20-30% (Turelli e Barton 2017). Entretanto, após somente quatro semanas do fim das liberações, a frequência da *Wolbachia* caiu drasticamente para cerca de 10%, e assim permaneceu. Notadamente, pela primeira vez na literatura, a cepa *w*Mel não obteve êxito em invadir a população selvagem de *Ae. aegypti*.

Certamente, como comentado anteriormente, um dos principais fatores que impacta na invasão da *Wolbachia* é a densidade populacional dos mosquitos selvagens (Hoffmann et al. 2014). Por isso, nossa primeira hipótese para explicar a falha da invasão da *w*MelBr foi uma liberação de um número insuficiente de mosquitos com a bactéria, devido a uma elevada abundância de mosquitos nativos. A fim de estudar mais sobre esse aspecto, nas duas primeiras semanas de solturas no Rio, realizamos experimentos MSR utilizando a *Wolbachia* como um marcador em campo (Garcia et al. 2016 – artigo 6).

Nesta tese, primeiramente, investigamos as vantagens de se utilizar diferentes modelos matemáticos para MSR, desde os "clássicos" até os mais complexos, como os Bayesianos hierárquicos, que geram estimativas do tamanho da população, juntamente com intervalos de confiança (Villela et al. 2015 – artigo 4; Villela et al. 2017 – artigo 5). Em experimentos de MSR, o uso de mosquitos é particularmente desafiador. Fatores como seu pequeno tamanho corporal, dificuldade de múltiplas recapturas do mesmo indivíduo e baixa longevidade do mosquito tornam difícil a obtenção de dados robustos e expressivos, comumente encontrados para outros animais (Service 1993; Cianci et al. 2013; Villela et al. 2017 – artigo 5). Os modelos matemáticos "clássicos" mais empregados em MSR, que estimam o tamanho populacional, incluem o índice de Lincoln

e Fisher-Ford (Service 1993; Constantini et al. 1996; Maciel-de-Freitas et al. 2008; Cianci et al. 2013). Destes, o índice de Lincoln na maioria das vezes é o método de escolha, devido a sua simplicidade. No entanto, esta simplicidade comumente gera estimativas frágeis e, portanto, de baixa serventia.

Em nosso primeiro estudo, fêmeas de *Ae. aegypti* foram marcadas com pó fluorescente e liberadas no bairro Z-10, Ilha do Governador/RJ, e recapturadas diariamente com a armadilha BG-Sentinela. Estimativas de sobrevivência e tamanho populacional foram realizadas utilizando-se o modelo Fisher-Ford e o modelo hierárquico Bayesiano. Este último foi desenvolvido pelo nosso grupo e permite avaliar concomitantemente a distribuição espacial do vetor (Villela et al. 2015 – artigo 4). Dois experimentos de MSR foram feitos, um no verão e outro no inverno, e nossos resultados indicaram que a distribuição espacial dos mosquitos é agrupada durante o inverno (0,99 mosquitos/casa) e mais homogênea durante o verão, no período de alta abundância de mosquitos (5,2 mosquitos/casa). Em relação à probabilidade de sobrevivência diária (PDS) de fêmeas, observamos valores similares nas duas estações (0,71 - 0,80), apesar da diferença de temperatura. Isso indica uma estimativa média de vida de 7,5 a 9 dias (adicionando os quatro dias no laboratório), valores similares aos apresentados previamente na literatura (Maciel-de-Freitas et al. 2007b; Villela et al. 2015 – artigo 4).

Em nosso segundo trabalho, apresentamos um conjunto de modelos bayesianos para serem usados em experimentos de MSR de mosquitos (Villela et al. 2017 – artigo 5). Análises por esses modelos resultam em uma distribuição posterior dos parâmetros, ou seja, significa que podemos obter um intervalo de credibilidade para os vários parâmetros avaliados em um estudo de MSR. Usando dados de campo e simulações, avaliamos fatores importantes dos modelos, como eficiência de captura, taxas de sobrevivência dos mosquitos, remoção de indivíduos devido à captura, taxa de

recrutamento por coleta de pupas, abundância dos mosquitos, entre outros. Em nossas análises, apresentamos versões Bayesianas correspondentes aos estimadores de Lincoln e Fisher-Ford, e ainda avaliamos a precisão das estimativas em múltiplas simulações, variando os valores de alguns parâmetros. Observamos que esses métodos podem suportar baixas eficiências de captura, como geralmente relatadas na literatura (de 2% a 10%) (Guerra et al. 2014), mas que a sua precisão depende do número de mosquitos liberados, sua abundância e sobrevivência. Mostramos também que a coleta e contagem de pupas permitem estimar as diferenças de sobrevivência entre mosquitos liberados e a população selvagem, não marcada. Além disso, a contagem de pupas por pesquisas de campo foi usada como ferramenta para obter a taxa de recrutamento da população selvagem, uma abordagem pouco explorada em experimentos de MSR (Fock e Chadee 1997). Estas estimativas são particularmente interessantes, pois coleta de imaturos em criadouros é uma das atividades mais comuns feita no controle vetorial.

É importante destacar que experimentos de MSR, e seus modelos, podem exercer um papel fundamental durante as liberações de *Ae. aegypti* com *Wolbachia*, tornando-as um processo mais racional. Uma vez que a bactéria é um marcador interno dos mosquitos liberados, podemos tirar proveito das liberações para estimar a densidade da população nativa e, assim, ajustar o número de mosquitos soltos ao longo das semanas subsequentes, de acordo com o perfil de invasão em cada área (Garcia et al. 2016 – artigo 6). Em caso de invasão lenta, aumenta-se o número de indivíduos liberados. Em caso de invasão rápida, poderia até se interromperem as solturas de novos mosquitos e reduzirem-se as picadas nos moradores. Ademais, o custo adicional para realizar análises de MSR seria praticamente nulo nos casos onde já se monitora a frequência da *Wolbachia* por análise molecular, identificando-se presença/ausência da bactéria em mosquitos capturados em campo.

No contexto, realizamos experimentos de MSR nas duas primeiras semanas de liberações, utilizando a própria bactéria como marcador em campo. Com esta abordagem, investigamos a primeira hipótese para explicar a falha da invasão de *Ae. aegytpi* com *Wolbachia* (*w*MelBr) no Rio: a liberação de um número insuficiente de mosquitos. Para tal, calculamos a densidade populacional de mosquitos nativos durante a liberação de *w*MelBr, aplicando diferentes modelos matemáticos (Garcia et al. 2016 – artigo 6). Nossos resultados apresentaram estimativas que variaram cerca de 10 vezes, dependendo do modelo usado. Por exemplo, pelo modelo de diferença de média (baseado em Ritchie et al. 2013), foram calculadas 634 - 3.565 fêmeas e, no Lincoln, 6365-16.188 fêmeas em campo. Mas, independente do modelo utilizado, os mosquitos liberados estavam em um número menor que os selvagens, em todos os momentos do estudo (Garcia et al. 2016 – artigo 6). Assim sendo, Tubiacanga apresentou uma média de mosquitos nativos/armadilha/dia superior aos observados nas áreas da Austrália, onde a *Wolbachia* foi bem sucedida. Mas, níveis de infestação por *Ae. aegypti* até maiores foram observados na Ilha Tri Nguyen, onde a invasão se deu com sucesso (Hoffmann et al. 2011; Ritchie et al. 2013; Hoffmann et al. 2015; Scott O'Neil comunicação pessoal; Garcia et al. 2016 – artigo 6). Entretanto, deve-se destacar que, nas primeiras semanas de liberações no Rio, observamos uma razão de mosquitos selvagens: *w*Mel de 1: 0,5 – 0,7, enquanto nas áreas da Austrália e Vietnã essa razão era bem maior, de 1: 1,2 – 1,7 e 1: 2-4, respectivamente (Ritchie et al. 2013; Hoffman et al. 2015; Garcia et al. 2016 – artigo 6). Portanto, ocorreu liberação de um número muito maior de mosquitos com *Wolbachia* em relação à população selvagem local nas áreas da Austrália e do Vietnã, diferentemente do observado no Rio (Garcia et al. 2016 – artigo 6).

Diante destas constatações, aumentamos, subsequentemente, o número de mosquitos soltos em Tubiacanga. E, após a semana 13, dobrou-se a quantidade de

mosquitos soltos, como uma tentativa de reverter o platô (nível de 40-45%) que a invasão apresentava. No entanto, a manutenção do platô, mesmo após o aumento do número de mosquitos soltos, nos levou a descartar nossa primeira hipótese, ou seja, que teria havido liberação de um número insuficiente de mosquitos com *Wolbachia* em campo (Garcia et al. 2017b – artigo 7).

O teste de nossa segunda hipótese baseou-se em investigar se *Ae. aegytpi* com *Wolbachia* (*w*MelBr) liberados em campo estavam aptos o suficiente para sobreviver e competir com os nativos. Para tal, medimos o comprimento da asa, sobrevivência e razão sexual dos mosquitos liberados, como parâmetros para avaliar a aptidão física (*fitness*). Já é bem documentado na literatura que a oferta de alimento e/ou competição durante o desenvolvimento das larvas pode afetar significativamente vários aspectos da aptidão dos adultos, incluindo o tamanho corporal do mosquito, longevidade e a suscetibilidade às infecções por arbovírus (Nasci e Mitchell 1994; Tun-Lin et al. 2000; Alto et al. 2005). As fêmeas de *Ae. aegypti* com tamanho corporal pequeno tendem a se alimentar com mais frequência do que fêmeas grandes, pois emergem com reservas lipídicas insuficientes para desenvolverem seus ovários, e para completarem a oogênese, após um repasto sanguíneo (Macdonald 1956; Edman et al. 1992). Em 15 das 20 semanas, liberamos fêmeas significativamente maiores que as selvagens, sugerindo que essas fêmeas não teriam uma perda no *fitness* devido às condições de criação em laboratório. Testamos também a mortalidade dos adultos que foram liberados em campo. Para isso, observamos os copos de liberação do grupo controle. Após a soltura, eles retornavam ao laboratório com parte de mosquitos do mesmo lote solto. A mortalidade raramente alcançou mais de 5%, sugerindo baixa relevância neste aspecto. Finalmente, verificamos se houve algum desvio na razão sexual dos mosquitos liberados. Mas também não encontramos problemas com esse parâmetro. Portanto, ao analisarmos todas essas evidências levantadas, nossa

segunda hipótese sobre a baixa aptidão (*fitness*) dos mosquitos com *Wolbachia* liberados, também foi descartada (Garcia et al. 2017b – artigo 7).

O efeito da temperatura na interação entre a *Wolbachia* e seu hospedeiro já foi mostrado para diferentes sistemas (Hoffmann et al. 1990; Reynolds et al. 2002; Mouton et al. 2006, 2007; Jia et al. 2009). Por isso, nossa terceira hipótese estava relacionada com a perda da bactéria nos mosquitos de campo, em função da exposição a uma temperatura elevada. Em laboratório, a exposição diária de larvas de *Ae. aegypti*, no início de seu desenvolvimento, a temperaturas que variam de 30-40º C, reduz a densidade da bactéria nas fêmeas adultas que irão emergir (Ulrich et al. 2016). Quando mantidos a 26-37ºC (12 horas para cada temperatura/ dia), os mosquitos com *w*Mel apresentam uma menor eficiência na incompatibilidade citoplasmática (Ross et al. 2016). Assim sendo, durante a implementação da *Wolbachia* no Rio, Tubiacanga apresentou condições climáticas extremas, com um verão bastante seco, o que poderia ser um ambiente hostil para a bactéria. O retrocruzamento feito no Brasil para gerar a linhagem *w*MelBr foi iniciado com fêmeas australianas com *Wolbachia*. Assim, todas as gerações subsequentes apresentam o DNA mitocondrial (mtDNA) oriundo do *background* genético australiano (herdado da mãe, assim como a *Wolbachia*). Portanto, ao analisar o mtDNA de indivíduos capturados em campo, somos capazes de detectar transmissão materna imperfeita da *Wolbachia* (identificando indivíduos com mtDNA australiano e sem *Wolbachia*). Entretanto, apesar da temperatura elevada em Tubiacanga, não observarmos indícios de falha na transmissão materna da bactéria em campo (Garcia et al. 2017b – artigo 7).

Em resumo, após testar as diferentes hipóteses para explicar a falha da invasão da *Wolbachia* (*w*MelBr) no Rio, descartamos as seguintes possibilidades: (a) número insuficiente de mosquitos liberados, (b) baixo *fitness* dos mosquitos e (c) transmissão

materna incompleta. Nenhuma destas hipóteses foi capaz de explicar a ocorrência do platô, durante a invasão, e a rápida queda da *Wolbachia* quando as liberações terminaram.

As liberações dos mosquitos com *Wolbachia* eram feitas em Tubiacanga no período da manhã, às 5:00AM, toda quinta-feira. Após o término das liberações dos mosquitos, a equipe de entomologia costumava tomar café da manhã em uma mercearia local. Foi então que, espontaneamente, o dono da mercearia expressou seu desejo de que as liberações com *Wolbachia* nunca parassem, porque ele estava enriquecendo com a venda de inseticidas sprays (PI) para os moradores locais. Essa confissão nos deu um momento "eureka", e nós começamos a investigar uma quarta hipótese para a falha da invasão da *Wolbachia*: o uso de inseticidas e a falta de alelos de resistência na linhagem liberada de *Ae. aegytpi,* a *w*MelBr.

A primeira etapa desta investigação foi avaliar como sucedeu a dinâmica da introdução dos genes brasileiros, e os de resistência, durante o primeiro retrocruzamento em laboratório, realizado entre machos nativos do Rio com fêmeas australianas infectadas com *w*Mel. Este processo originou a linhagem *w*MelBr (*Ae. aegypti* com *Wolbachia* + genética brasileira), que foi a linhagem liberada em Tubiacanga. Uma vez que as mutações *knockdown resistance* (*kdr*) (mutações no canal de sódio, alvo dos PI) estão fortemente relacionados com a resistência a PI em populações de *Ae. aegypti* do Rio, e que os mosquitos australianos não possuem estas mutações (Endersby e Hoffmann 2011; Gabriela Garcia comunicação pessoal), elas serviram de marcadores moleculares em nossas análises.

Observamos, durante o retrocruzamento, que a frequência dos alelos *kdr* aumentou a cada geração, até alcançar 80% (60% Na_VR2 e 20% Na_VR1) no momento em que a colônia foi fechada (apenas endocruzamento), e o retrocruzamento cessou. Portanto,

esperava-se haver um perfil genético de resistência a inseticidas PI na linhagem *w*MelBr igual ao encontrado em populações de campo, que foram utilizadas no retrocruzamento. Entretanto, o alelo selvagem de susceptibilidade, o Na_VS, ainda estava presente (cerca de 20%) em *w*MelBr. Visto que a resistência por mutações *kdr* se expressa em caráter recessivo e que alelos *kdr* geram um custo no *fitness* dos mosquitos, os alelos de resistência decairam rapidamente após o término do retrocruzamento, dando lugar aos alelos de susceptibilidade (Brito et al. 2013). Assim, após 18 gerações, a linhagem *w*MelBr apresentou um perfil de susceptibilidade a inseticidas PI, que era comparável à linhagem de referência de susceptibilidade a inseticidas Rockefeller - "Rock" (Garcia et al. 2017b – artigo 7).

É provável que a rápida diminuição de alelos de resistência tenha ocorrido em função do alto custo no *fitness* causado pela resistência (Brito et al. 2013; Kliot e Ghanim 2014). Esta queda rápida na frequência de mutações *kdr* também foi observada numa linhagem de *Ae. aegypti* (sem *Wolbachia*) em apenas 15 gerações em laboratório (Brito et al. 2013). Neste estudo, introduziu-se a mutação Val1016Ile em *Ae. aegypti* no "background" genético da linhagem suscetível "Rock", o que possibilitou a detecção de diversas alterações nos parâmetros biológicos dos insetos resistentes. Outros trabalhos também exploram o fato de populações de *Ae. aegypti* resistentes apresentarem um custo no *fitness*, quando comparados às suscetíveis (Belinato et al. 2012; Martins et al. 2012; Diniz et al. 2015; David et al. 2017 – artigo 2). Assim, nesta tese, foi avaliado o *fitness* de quatro populações de *Ae. aegypti* brasileiras (Garcia et al. 2017a – artigo 1), correlacionando-o, ainda, com dados obtidos para resistência a inseticidas (Garcia et al. 2017a – artigo 1; David et al. 2017 – artigo 2). Observamos, de maneira geral, que as populações de campo aparentam ter uma desvantagem no *fitness* quando comparadas com a linhagem "Rock", como atraso no tempo de desenvolvimento e menor longevidade

(David et al. 2017 – artigo 2). Esses efeitos negativos podem resultar da realocação de recursos energéticos para a manutenção dos mecanismos de resistência (por exemplo, maior produção de enzimas detoxificantes), fenômeno conhecido como "trade-off" energético. Além disso, modificações estruturais em sítios-alvo podem caracterizar uma perda da função da molécula, fato que também pode impactar na viabilidade do inseto (Roush e Mckenzie 1987; Kliot e Ghanim 2014). Portanto, de maneira geral, a manutenção de linhagens resistentes a inseticidas em laboratório, e em ambiente sem exposição a inseticidas, pode resultar em quedas dos níveis de resistência ao longo das gerações. Isto sugere fortemente um custo no *fitness* em insetos resistentes e justifica os níveis de susceptibilidade a PI encontrados na linhagem de *Ae. aegypti* com *Wolbachia*, *w*MelBr, utilizada nas liberações do Rio (Brito et al. 2013; Garcia et al 2017b – artigo 7).

A partir do momento que tomamos conhecimento sobre o perfil de susceptibilidade a inseticidas apresentado pela *w*MelBr, que contrastava com os altos níveis das populações de campo do Rio, partimos para o monitoramento da resistência durante as liberações em Tubiacanga. Foram avaliados ambos os grupos, *Ae. aegypti* com e sem a bactéria, capturados em campo durante e após o término das semanas de liberações. A frequência de alelos *kdr* em mosquitos com *Wolbachia* capturados durante as 20 semanas consecutivas de liberações permaneceu inalterada, isto é, a grande maioria era Na_VS, genótipo suscetível. Entretanto, curiosamente, começamos a ver mudanças no perfil alélico para o *kdr* após a semana 26. Neste momento, 6 semanas após o término das liberações, nenhum mosquito com a bactéria em campo era proveniente das liberações, ou seja, essa frequência foi baseada nos mosquitos com *Wolbachia* que emergiram dos criadouros de Tubiacanga, exclusivamente. Considerando que liberamos mosquitos com genótipo suscetível, e que a frequência de alelos *kdr* durante os lançamentos permaneceu inalterada, acreditamos que este resultado foi baseado na alta captura dos próprios

mosquitos que liberamos. Esse resultado "mascarou" o resultado real de invasão da *Wolbachia*, que deve considerar apenas os mosquitos que emergem dos criadouros locais (Garcia et al. 2017b – artigo 7).

Durante o processo de invasão da *Wolbachia* em Tubiacanga, em função da baixa frequência de *kdr* e forte pressão de seleção pela utilização doméstica de PI, apenas uma pequena proporção de mosquitos infectados com *w*Mel foi capaz de sobreviver e se reproduzir. Portanto, a alta mortalidade de mosquitos liberados devido ao uso doméstico de PI manteve o número de mosquitos infectados com *Wolbachia* abaixo do limiar para promover uma invasão bem-sucedida. Deve-se destacar que a frequência de *w*MelBr manteve-se em 10-15% durante nove meses em campo, quando uma nova linhagem de *Ae. aegypti* com *Wolbachia* (*w*MelRio) foi liberada em Tubiacanga. Além disso, a hipótese de uso excessivo de PI por moradores locais é reforçada quando observamos a flutuação das frequências de *kdr* em mosquitos selvagens. A frequência de Na_VR2, o genótipo que confere alta resistência e custo de *fitness* aos mosquitos *Ae. aegypti*, aumentou ao longo do tempo até o momento em que as liberações pararam (Garcia et al. 2017b – artigo 7).

Assim sendo, concluímos que o principal motivo do fracasso da invasão de *Wolbachia* em Tubiacanga foi a liberação de mosquitos com genótipos suscetíveis a PI, em um local com forte pressão seletiva, onde a população selvagem é altamente resistente a esta classe de inseticidas. Além disso, a introdução de 10% de machos selvagens a cada cinco gerações nas gaiolas foi insuficiente para manter o perfil genético de campo na colônia de laboratório. E, o mais importante, a manutenção em laboratório por 18 gerações antes das liberações em campo tornou os mosquitos liberados menos resistentes que os nativos. Considerando que a resistência a inseticidas PI é disseminada em todo o país, para ter sucesso no Brasil, e em vários outros países, a implementação da *Wolbachia*

deve considerar liberar mosquitos tão resistentes quanto os da população selvagem local. Portanto, analisar o perfil da resistência a inseticidas na população selvagem, e dos mosquitos com a bactéria, é um fator chave que não deve ser ignorado antes de se iniciarem as liberações de *Wolbachia*.

Para resolver este problema, a solução mais racional foi realizar novamente um retrocruzamento com machos nativos do Rio, dessa vez analisando alelos *kdr*, genótipos para resistência a PI, a cada geração. Para isto, usamos novamente as mutações *kdr* como marcadores, já que *w*MelBr estava com níveis baixos das mutações e os mosquitos de campo apresentavam níveis altos (Linss et al. 2014 – artigo 3; Garcia et al. 2017a – artigo 1).

Durante o novo retrocruzamento, observamos que a frequência das mutações *kdr* aumentou a cada geração. Após quatro retrocruzamentos consecutivos, conseguimos uma linhagem com *Wolbachia* com frequências alélicas de *kdr* semelhantes aos mosquitos nativos do Rio, e, finalizamos o retrocruzamento. Esta nova linhagem foi chamada de *w*MelRio. Em seguida, realizamos bioensaios em laboratório para checar se a resistência a inseticidas estava de acordo com o esperado. Bioensaios com PI, comparando *w*MelRio com populações de campo do Rio de Janeiro, confirmaram os mesmos níveis de resistência para este composto. Além disso, *w*MelRio apresentou também os mesmo níveis de resistência para o larvicida temephos (OP). Entretanto, ainda permaneceu com o *status* de susceptibilidade ao larvicida diflubenzuron (IGR) e ao adulticida malathion (OP), exatamente como observamos nos mosquitos nativos do Rio (Garcia et al. 2017b – artigo 7).

Caso nossa hipótese estivesse correta, a liberação de uma nova linhagem contendo alelos de resistência deveria promover uma disseminação bem sucedida. Assim, a nova

linhagem de *Ae. aegypti*, *w*MelRio, era a nova esperança de uma liberação da *Wolbachia* no Rio de Janeiro. Mas, para isto, a linhagem deveria ser *fitness* suficiente para se estabelecer em campo (Garcia et al 2017b – artigo 7). Neste contexto, uma preocupação adicional foi levantada pelo fato de tanto a infecção por *Wolbachia* quanto os alelos *kdr* causarem um custo no *fitness* em *Ae. aegypti* (Brito et al. 2013; Turley et al. 2013; Belinato et al. 2012; Hoffmann et. al 2014; Ritchie et al. 2015; Dutra et al. 2016; Ross et al. 2016).

Para avaliar se a introdução de alelos *kdr* afetaria na aptidão do *Ae. aegypti* com *Wolbachia* e comprometeria a invasão em populações nativas do Rio, realizamos experimentos para medir parâmetros da história de vida dos mosquitos. Usamos nesses experimentos sete linhagens, duas sem *Wolbachia* (Urca/RJ-(F1)-campo e *w*MelTet, esta última foi curada da *Wolbachia* pelo tratamento com tetraciclina, e cinco linhagens com *Wolbachia* que variavam a frequência das mutações *kdr* (*w*MelBr, e mais cada geração do retrocruzamento, F1CP, B1, B2 e *w*MelRio). Corroborando estudos anteriores, observamos que a presença da *Wolbachia* afeta fortemente a fecundidade e o sucesso de oviposição das fêmeas infectadas (Turley et al. 2013; Hoffman et al. 2014; Garcia et al. 2017b – 2017). Houve ainda uma interação significativa da idade dos mosquitos e da frequência de alelos *kdr*, o sucesso de oviposição diminuiu mais rapidamente conforme o aumento da idade e dos alelos *kdr* (Garcia et al. 2017b – artigo 7). Em relação à taxa de transmissão materna da *Wolbachia*, detectamos imperfeições (valores menores que 100%), principalmente na primeira semana de vida das fêmeas (portanto, primeira oviposição). Entretanto, na quarta semana de vida, a taxa de transmissão materna mostrou-se mais fidedigna: observamos somente valores de 100%, ou bem próximos, corroborando resultados já disponíveis na literatura (Dutra et al. 2015). Em relação à longevidade dos mosquitos infectados com *Wolbachia*, embora existam relatos de uma

redução de até 10% em *Ae. aegytpi* com *w*Mel (Walker et al. 2011), nossos resultados mostraram uma longevidade até maior para as linhagens com *Wolbachia* (Garcia et al. 2017b – artigo 7). Por fim, de forma curiosa, a eclosão de ovos apresentou forte influência da *Wolbachia*: observamos valores bem baixos para todas as linhagens com a bactéria (uma média de 60%), enquanto nas linhagens sem *Wolbachia*, Urca/RJ e *w*MelTet, esses valores eram próximos de 90% (Garcia et al. 2017b – artigo 7).

É importante destacar que após o término do retrocruzamento era necessário traçar um plano para manter a resistência a PI em níveis altos na *w*MelRio. Baseado em nossa experiência anterior, se a nova linhagem fosse mantida em laboratório, sem contato com inseticidas, a tendência seria a perda dos alelos *kdr* ao longo das gerações. Para evitar isso, optamos por introduzir 50% de machos de campo, a cada duas gerações, em todas as gaiolas de criação de *w*MelRio (10 gaiolas com 500 fêmeas e 500 machos). Esta estratégia foi mantida por 10 gerações, durante todo período de liberação de *Wolbachia* em campo entre 2015/2016. Desta forma, nos certificamos que os mosquitos liberados estavam com uma genética similar a dos nativos, incluindo os níveis de resistência a inseticidas (Garcia et al. 2017b – artigo 7).

Após a análise de *fitness* e definição da estratégia para manter da resistência a PI em *w*MelRio, em laboratório, concluímos que a mesma estava apta para ser liberada no Rio de Janeiro. Assim, partimos para novas liberações em campo em setembro de 2015, desta vez usando a nova linhagem *w*MelRio, com os mesmos níveis de resistência a PI que os mosquitos nativos do Rio. As liberações ocorreram novamente por 20-30 semanas consecutivas em dois bairros: Tubiacanga (segunda fase de solturas) e Jurujuba (na cidade de Niterói). Após as liberações, a frequência da *Wolbachia* não caiu em campo (como observado na primeira liberação com *w*MelBr), ou seja, a invasão foi bem sucedida nas duas áreas. Até o presente momento (agosto de 2017) mais de 90% dos *Ae. aegypti* de

campo apresentam a bactéria, sem ser necessário realizarem-se novas liberações nestas localidades (Garcia et al 2017b – artigo 7). Esses resultados foram muito promissores e, atualmente, o projeto se encontra em expansão, com planos de liberação da *Wolbachia* em todos os bairros do Rio de Janeiro e Niterói (em um período de 2 anos), e, ainda, em outras regiões do Brasil, a partir de 2018.

Portanto, conclui-se que ao introduzir uma nova estratégia de controle de arboviroses no Brasil, como o uso da bactéria *Wolbachia*, a resistência a inseticidas é uma questão relevante que precisa ser ponderada (Garcia et al 2017a – artigo 1; Garcia et al. 2017b – artigo 7). Este mesmo fato, possivelmente, será enfrentado em outros países da América Latina e da Ásia, regiões onde são observados níveis altos de resistência a PI (Corbel et al. 2017). Entretanto, nossa realidade contrasta com a de outros países, como na Austrália, por exemplo, onde as populações de *Ae. aegypti* não apresentam essa característica (Endersby e Hoffmann 2013). Neste contexto, deve-se destacar a longa história de uso exacerbado de inseticidas no Brasil, fato que culminou no quadro atual de resistência a diversas classes de inseticidas em populações nativas de *Ae. aegypti* (Braga e Valle 2007b; Bellinato et al. 2016; Garcia et al 2017a – artigo 1).

A resistência aos inseticidas repercute diretamente na propagação de arbovírus em campo por estar relacionada à capacidade vetorial (CV) dos insetos, uma vez que impede que um grande número dos mosquitos expostos a um inseticida venha a óbito. Num cenário onde alelos de resistência estão amplamente disseminados, a aplicação do composto químico pouco diminui a densidade populacional do vetor, e, portanto, pouco se altera o risco de transmissão (McCarrol et al., 2000; Rivero et al., 2010). Assim sendo, podemos traçar um paralelo deste conceito com a falha da invasão da *w*MelBr, em Tubiacanga/RJ, na primeira liberação de *Wolbachia* no Brasil. Neste caso, os mosquitos liberados com a bactéria eram suscetíveis, e, consequentemente, morriam com o uso

doméstico exacerbado de PI. Enquanto isso, os mosquitos nativos do Rio, resistentes, permaneciam vivos neste ambiente com alta pressão de seleção, persistindo em campo em altas densidades. Portanto, este é um indício evidente de como a resistência a inseticidas pode impactar na transmissão de arbovírus, por manter a infestação de mosquitos em níveis elevados em campo, além de indicar claramente a perda na eficiência desta importante ferramenta (os inseticidas PI) para controle vetorial no Brasil.

Historicamente, a primeira suspeita de resistência a inseticidas em populações brasileiras de *Ae. aegypti* ocorreu com o larvicida temephos (OP), em 1998, após a intensificação de seu uso a partir de surtos de dengue em 1986 (Braga e Valle 2007b). Em seguida, sucedeu a detecção da resistência a PI logo após sua implementação no controle de adultos no país, em 2000 (da-Cunha et al. 2005; Montella et al. 2007). Desde então, estudos detectaram diferentes níveis de resistência a estes dois inseticidas, com valores altos de Razão de Resistência - RR (razão calculada por bioensaios, pela divisão da CL da população avaliada pela CL da cepa referência de susceptibilidade a inseticidas (Rock) (Bellinato et al. 2016). Em concordância, foram apontados como potenciais mecanismos de resistência o aumento da atividade de enzimas detoxificantes, como as GST e EST, além de mutações *kdr* (Montella et al. 2007; Martins et al. 2009a; Lima et al. 2011; Bellinato et al. 2016). Assim sendo, em nosso trabalho, traçamos o perfil da dinâmica da resistência a inseticidas em populações brasileiras do vetor, de diferentes regiões, o que nos permitiu uma melhor compreensão sobre o cenário do controle químico efetuado no país (Capítulo I, artigos 1, 2 e 3). Esta informação ainda é crucial para a expansão das liberações de *Ae. aegypti* com *Wolbachia* no Brasil, uma vez que a resistência se mostrou um importante fator na implementação desta estratégia.

O temephos (OP) foi o único larvicida utilizado em campo pelos serviços públicos de saúde, de 1967 até 2009 (Braga e Valle 2007b). Este panorama levou a uma forte

pressão de seleção, por um único composto, durante muitos anos no país. Consequentemente, diversas populações brasileiras de *Ae. aegypti* foram detectadas resistentes ao temephos, fato já bastante relatado na literatura (Lima et al. 2003; Lima et al. 2011; Chediak et al. 2016; Bellinato et al. 2016). Confirmamos, em nossos resultados, o *status* de resistência a este larvicida para as populações brasileiras (Garcia et al. 2017a – artigo 1). A resistência ao OP temephos também é observada em outros países América Latina, com relatos em populações de *Ae. aegypti* nativas da Colômbia, México, Cuba, Caribe e Argentina (Mazzari 1995; Suarez et al. 1998; Rodríguez et al. 2002; Biber et al. 2006; Flores et al. 2006; Ocampo et al. 2011; Marcombe et al. 2011, 2012; Goindin et al. 2017). Entretanto, apesar da resistência disseminada ao temephos em populações brasileiras de *Ae. aegypti*, destacamos uma tendência à diminuição dos níveis de resistência a este composto (Garcia et al 2017a – artigo 1). Esta queda pode ser justificada pelo custo no *fitness* causado pela resistência a inseticidas (Belinato et al. 2012; Diniz et al. 2015; David et al. 2017 – artigo 2;), em associação com a interrupção do uso do temephos em campo desde 2009. Porém, esta diminuição apresentou-se lenta e em nenhuma localidade estudada alcançou valores inferiores ao limiar definido pelo Ministério da Saúde (MS) (valor de corte: RR_{95} de 3,0), impossibilitando o retorno do uso de temephos em campo (Garcia et al. 2017a – artigo 1).

Em relação ao *status* da resistência de *Ae. aegypti* a outra classe de larvicidas recentemente introduzida no país, o diflubenzuron (um inibidor da síntese de quitina, *IGR*), as populações brasileiras apresentaram níveis compatíveis com situação de susceptibilidade (Martins et al. 2008; Fontoura et al. 2012; Bellinato et al. 2016; Garcia et al. 2017a – artigo 1). A recente introdução dos IGR em campo no país e seu mecanismo de ação distinto dos inseticidas neurotóxicos convencionais, certamente contribuem para os baixos índices de resistência encontrados até o presente momento. Fica ainda evidente

a necessidade de monitoramento contínuo da mortalidade de *Ae. aegypti* quando expostos ao diflubenzuron para se tentar retardar a perda deste composto pela seleção da resistência em populações naturais.

No que concerne ao uso de inseticidas para controle de adultos, os PI começaram a ser utilizados a partir do ano 2000-2001, depois da confirmação de disseminação da resistência aos OP em populações brasileiras (Funasa 1999). No entanto, amostras de campo coletadas pouco tempo depois, em 2002-2003, já exibiam indícios de resistência a PI (da-Cunha et al. 2005). Desde então, a resistência a PI foi detectada em diversas populações de *Ae. aegypti* do país (Luna et al. 2004; Macoris et al. 2007; Martins et al. 2009a; Lima et al. 2011; Bellinato et al. 2016). Ademais, todas as populações de campo estudadas em nosso trabalho apresentaram resistência a este composto. Especialmente, três delas (Santarém/PA, Campo Grand/MS e Duque de Caxias/RJ) exibiram níveis extremamente altos de resistência ao longo do nosso estudo, com valores da RR_{95} sempre acima de 25, e, por vezes, atingindo até valores mais altos, com RR_{95} entre 50- 100 (Garcia et al 2017a – artigo 1).

No Brasil, de acordo com o MS, diferentemente dos larvicidas (que são aplicados em ciclos periódicos, totalizando quatro a seis aplicações durante o ano), os adulticidas não são considerados ferramentas de prevenção de infestação. Tais produtos são utilizados apenas para bloqueio de surtos, ou em pontos estratégicos, como aeroportos e portos, ou outros potenciais pontos de entrada do vetor. Assim sendo, o MS recomenda que as aplicações de adulticidas, na forma de UBV, não devem ultrapassar cinco a sete vezes por ano, em épocas de epidemia e em situações e locais bastante específicos (MS 2009). Apesar disto, destaca-se em nosso trabalho, que os níveis mais altos de resistência a PI não apresentaram relação com as aplicações de adulticidas pelos gestores municipais de saúde, e sim, foi visto uma associação com os períodos de maior transmissão de

dengue. Assim, o uso doméstico exacerbado de inseticidas PI apresentou marcada sazonalidade, ocorrendo principalmente durante épocas de surtos e por vezes é acompanhado por elevação também sazonal dos níveis de resistência. Logo, a hipótese é que surtos de arboviroses provocam pânico na população, e há uma grande corrida para a aquisição de medidas individuais de proteção e de controle, resultando em grande aumento do uso doméstico de PI, disponíveis no mercado. Esta situação tem reflexo direto na elevação dos níveis de resistência encontrados durante os surtos epidêmicos (Garcia et al. 2017a – artigo 1). Estas constatações nos remetem novamente a falha da invasão de *Ae. aegypti* com *Wolbachia* (*w*MelBr) na primeira tentativa de estabelecimento da bactéria no Rio de Janeiro. Durante as liberações ocorreu um uso doméstico exacerbado de PI pelos moradores locais, resultando na morte dos mosquitos liberados que eram suscetíveis a estes compostos (Garcia et al. 2017b – artigo 7). Portanto, nesta tese, apontamos para diversos indícios que a aplicação doméstica de inseticidas tem causado uma forte pressão de seleção em campo. E, possivelmente, este tipo de uso tem colaborado intensamente para os altos níveis de resistência a PI encontrados em populações brasileiras de *Ae. aegypti*.

Tendo em vista que nos últimos anos tem-se usado excessivamente PI como adulticidas, buscamos investigar a distribuição e o envolvimento das mutações *kdr* na resistência a este composto em populações brasileiras de *Ae. aegypti* (Linss et al. 2014 – artigo 3). Neste trabalho, observamos a presença de duas substituições no Na_V de *Ae. aegypti*, a Val10116Ile, já detectada anteriormente no país (Martins et al. 2009a), e a Phe1534Cys, esta pela primeira vez descrita no Brasil (Linss et al. 2013 – artigo 3). Com estes resultados, descrevemos três diferentes alelos em *Ae. aegypti* no Brasil: o tipo selvagem, suscetível (chamado de Na_VS), e mais dois alelos *kdr*, um com a substituição restrita à posição 1534 (Na_VR1) e outro com substituições simultâneas, em ambos os sites

1016 e 1534 (Na_VR2). É importante ressaltar que também observamos um padrão claro de distribuição regional desses alelos. A região Nordeste é a que apresenta menores frequências de alelos *kdr*, já o Na_VR1 aparece presente em todas as localidades, enquanto o Na_VR2 foi mais frequente nas regiões Centro-Oeste e Sudeste (Linss et al. 2014 – artigo 3; Garcia et al. 2017a – artigo 1). Ou seja, independente dos mecanismos envolvidos, populações naturais brasileiras, em sua grande maioria, apresentam elevados níveis de resistência a PI. No contexto desta tese fica claro, portanto, como o conhecimento prévio do perfil de resistência e seus mecanismos podem auxiliar, por exemplo, na implementação da estratégia de controle mais promissora em uso atualmente (Linss et al. 2014 – artigo 3; Garcia et al. 2017a – artigo 1; Garcia et al. 2017b – artigo 7; Garcia et al. 2017c – artigo 8).

Ao percebermos a importância da contemplação da resistência e do uso de inseticidas nas liberações com *Wolbachia* no Brasil, buscamos investigar mais a fundo algumas hipóteses por modelagem matemática e simulações. Hoffmann e Turelli (2013), em um trabalho teórico de modelagem matemática, foram os primeiros a considerar a resistência a inseticidas nas liberações de *Ae. aegypti* com *Wolbachia*. Entretanto, os autores sugerem liberar mosquitos resistentes em uma área onde os mosquitos nativos são suscetíveis a inseticidas e, dessa forma, favorecer os mosquitos com a bactéria ao aplicar inseticidas durante as liberações. Os próprios autores comentam no trabalho a respeito dos problemas éticos em liberar mosquitos resistentes em áreas onde não existem problemas com resistência a inseticidas. Modificamos e adaptamos o modelo desenvolvido por Hoffmann e Turelli (2013) para estudar as estratégias de liberações vivenciadas em nosso país, utilizando nossos dados empíricos (Garcia et al. 2017c – artigo 8). Desta forma, avaliamos o efeito que a liberação de mosquitos resistentes e susceptíveis a PI exerceram na disseminação da *Wolbachia* em áreas do Rio de Janeiro (onde os

mosquitos nativos são resistentes a PI) e investigamos a frequência de alelos de susceptibilidade e resistência a inseticidas a partir de diferentes cenários entomológicos.

Primeiramente, nós estimamos o custo da resistência a inseticidas PI para considerar esse parâmetro nas modelagens de invasão da *Wolbachia*. Baseados nos dados empíricos da perda da resistência a PI em *w*MelBr, em 18 gerações mantida em laboratório (Garcia et al. 2017b – artigo 7), calculamos um valor de 25% de perda de *fitness* devido à resistência (Garcia et al. 2017c – artigo 8). Esse resultado aponta para uma baixa performance de mosquitos resistentes em um ambiente livre de inseticidas.

Em seguida, simulamos a viabilidade de buscar a invasão da *Wolbachia* com a liberação de mosquitos susceptíveis a PI, numa população de campo resistente. Desta maneira, obtivemos as condições necessárias de intensidade/ausência do uso de PI durante as liberações para disseminar a *Wolbachia* e também alelos de susceptibilidade a inseticidas e, ocasionalmente, mitigar ou até mesmo reverter o perfil de resistência a PI numa população natural de *Ae. aegypti* (após disseminação da bactéria). Este resultado corrobora outros estudos que demonstraram uma rápida reversão da resistência a inseticidas quando ocorre uma alta introdução de alelos de susceptibilidade (S) em ambiente sem inseticida (Melo-Santos et al. 2010; Brito et al. 2013; Schechtman e Souza 2015). Adicionalmente, observamos que a utilização de inseticidas em campo, durante as liberações de mosquitos com *Wolbachia*, pode trazer desvantagem para os mosquitos suscetíveis e impedir ou retardar a disseminação da *Wolbachia* em campo, como observado na primeira soltura de *Wolbachia* no Rio (usando *w*MelBr) (Garcia et al. 2017b,c –artigo 7 e 8).

Entretanto, mostramos, pela modelagem, que ao se utilizar uma linhagem com os mesmos níveis de resistência que os mosquitos nativos (como a *w*MelRio), não ocorrem

problemas com a utilização de inseticidas durante a soltura. A *Wolbachia* é capaz de invadir e se disseminar na população selvagem de *Ae. aegypti* sem complicações, assim como visto em campo nas segundas liberações bem sucedidas no Rio de Janeiro (Garcia et al. 2017b,c – artigo 7 e 8).

Portanto, nossas simulações mostraram possíveis cenários que as liberações de *Ae. aegypti* com *Wolbachia* podem enfrentar em campo, dando foco à resistência a inseticidas e o custo no *fitness* causado por ela. O estudo destes cenários é importante para avaliar quando a *Wolbachia* é capaz de invadir as populações de campo considerando diferentes níveis de uso de inseticidas e de frequência dos alelos de resistência. Nós também chamamos a atenção para a possibilidade de reversão da resistência a inseticidas quando ocorre a liberação de uma linhagem suscetível, em um ambiente com mosquitos resistentes. Entretanto, essa reversão só é alcançada se o uso de inseticidas for interrompido ou reduzido, o que envolveria uma forte campanha de cientistas sociais para mudar os hábitos do uso doméstico de inseticidas. Ainda, se a aplicação de inseticidas em campo for intensa e constante, a *Wolbachia* só invade se ocorrer a liberação de uma linhagem com os mesmos níveis de resistência que os mosquitos nativos.

Por fim, esta tese reúne importantes aportes ao conhecimento a respeito da nova estratégia que está sendo implementada no Brasil, o uso da *Wolbachia* em *Ae. aegypti* para controle de arboviroses. Neste trabalho, apresentamos dois fatores importantes que devem ser considerados ao liberar mosquitos com a bactéria em campo: a densidade populacional de mosquitos nativos e a resistência/uso de inseticidas. Relatamos, pela primeira vez na literatura, que a resistência/uso de inseticidas é um fator importante, capaz de levar a falha na implementação desta estratégia. Assim, esperamos com esta tese auxiliar futuras liberações de *Wolbachia* no Brasil e no mundo.

6. Conclusões

- Populações brasileiras de *Aedes aegypti* apresentam perfis e mecanismos de resistência variados, como esperado. Para o larvicida temephos (OP), identificamos tendência de queda da resistência após a interrupção do uso deste composto em campo. Já para os adulticidas (PI), foram encontrados níveis extremamente altos de resistência, com tendência de aumento durante o pico de casos de dengue, o que sugerimos ser reflexo do uso doméstico de inseticidas desta classe.

- Há forte correlação entre altos níveis de resistência a PI e a frequência de duas mutações *kdr* em *Ae. aegypti*: Na_VR1 (mutação Phe1534Cys, somente) e Na_VR2 (mutação no sítio Phe1534Cys + Val1016Ile). A observação espaço-temporal destes alelos mostra a rápida disseminação da resistência no país.

- Populações de *Ae. aegypti* resistentes a inseticidas parecem ter uma desvantagem na aptidão (*fitness*) quando comparadas à linhagem referência, Rockefeller, em especial um maior tempo de desenvolvimento larval e uma redução na longevidade na fase adulta.

- A complexidade incorporada pela inferência Bayesiana permite estimativas robustas de densidade populacional, sobrevivência diária e identificação de hot-spots de infestação pelo *Ae. aegypti*, juntamente com medidas de incerteza, diferentemente do observado para modelos "clássicos" de MSR.

- Usando dados de campo e simulações, mostramos que modelos bayesianos podem ser incorporados a atividades de controle, pois permitem estimar a taxa de

recrutamento através da coleta de pupas, assim como estimar a sobrevivência de mosquitos selvagens, não marcados, durante ensaio de MSR.

- Ao utilizar a própria *Wolbachia* como um marcador dos mosquitos liberados, estimativas de densidade populacional e sobrevivência permitem o ajuste do número de mosquitos soltos em liberações subsequentes, de acordo com o perfil de invasão da bactéria em cada área.

- A liberação de *Wolbachia* numa linhagem suscetível a PI (*w*MelBr), em área com alta pressão de inseticidas e população nativa resistente, resultou na falha desta estratégia. No entanto, a invasão da bactéria foi bem sucedida ao se liberar uma nova linhagem de *Ae. aegypti* com *Wolbachia* (*w*MelRio), com os mesmos níveis de resistência que os mosquitos nativos.

- A invasão da *Wolbachia* será bem-sucedida tão somente se: 1) mosquitos suscetíveis forem liberados em um ambiente sem uso de inseticidas (podendo inclusive reverter os níveis de resistência); e 2) liberando mosquitos resistentes em uma população resistente, mesmo com um elevado uso de inseticidas (caso de *w*MelRio).

- Considerando-se a vasta distribuição da resistência a inseticidas em *Ae. aegypti* provenientes de países da América Latina e da Ásia, ressalta-se que este aspecto deve ser considerado na expansão das liberações da *Wolbachia* pelo mundo.

7. Referências

Abad-Franch F, Zamora-Perea E, Luz SL. Mosquito-disseminated insecticide for citywide vector control and its potential to block arbovirus epidemics: entomological observations and modeling results from Amazonian Brazil. PLoS Med. 2017; 17;14(1):e1002213.

Ali S, Gugliemini O, Harber S, Harrison A, Houle L, Ivory J, et al. Environmental and social change drive the explosive emergence of Zika virus in the Americas. PLoS Negl Trop Dis. 2017; 9;11(2):e0005135.

Aliota MT, Walker EC, Uribe Yepes A, Velez ID, Christensen BM, Osorio JE. The *w*Mel strain of *Wolbachia* reduces transmission of chikungunya virus in *Aedes aegypti*. PLoS Negl Trop Dis. 2016; 28;10(4):e0004677.

Alout H, Weill M. Amino-acid substitutions in acetylcholinesterase 1 involved in insecticide resistance in mosquitoes. Chem Biol Interact. 2008; 25;175(1-3):138-141

Alphey L, Benedict M, Bellini R, Clark GG, Dame DA, Service MW, et al. Sterile-insect methods for control of mosquito-borne diseases: an analysis. Vector Borne Zoonotic Dis. 2010; 10(3):295-311.

Alto BW, Lounibos LP, Higgs S, Juliano SA. Larval competition differentially affects arbovirus infection in *Aedes* mosquitoes. Ecology. 2005; 86(12):3279-3288.

Angelini P, Macini P, Finarelli AC, Pol C, Venturelli C, Bellini R, et al. Chikungunya epidemic outbreak in Emilia-Romagna (Italy) during summer 2007. Parasitol. 2008; 50(1-2):97-98.

Araújo AP, Araujo Diniz DF, Helvecio E, de Barros RA, de Oliveira CM, Ayres CF, et al. The susceptibility of *Aedes aegypti* populations displaying temephos resistance to *Bacillus thuringiensis israelensis*: a basis for management. Parasit Vectors. 2013; 13;6(1):297.

Araújo HR, Carvalho DO, Ioshino RS, Costa-da-Silva AL, Capurro ML. *Aedes aegypti* control strategies in Brazil: incorporation of new technologies to overcome the persistence of dengue epidemics. Insects. 2015; 11;6(2):576-594.

Atyame CM, Pasteur N, Dumas E, Tortosa P, Tantely ML, Pocquet N, et al. Cytoplasmic incompatibility as a means of controlling *Culex pipiens quinquefasciatus* mosquito in the islands of the south-western Indian Ocean. PLoS Negl Trop Dis. 2011; 5(12):e1440.

Axford JK, Ross PA, Yeap HL, Callahan AG, Hoffmann AA. Fitness of *w*AlbB *Wolbachia* infection in *Aedes aegypti*: parameter estimates in an outcrossed background and potential for population invasion. Am J Trop Med Hyg. 2016; 94(3):507-516.

Azevedo Rdo S, Oliveira CS, Vasconcelos PF. Chikungunya risk for Brazil. Rev Saude Publica. 2015; 49:58.

Baldini F, Segata N, Pompon J, Marcenac P, Shaw WR, Dabiré RK, et al. Evidence of natural *Wolbachia* infections in field populations of *Anopheles gambiae*. Nat Commun. 2014; 6;5:3985.

Bandi C, Anderson TJ, Genchi C, Blaxter ML. Phylogeny of *Wolbachia* in filarial nematodes. Proc Biol Sci. 1998; 22;265(1413):2407-2413.

Bandi C, Trees AJ, Brattig NW. *Wolbachia* in filarial nematodes: evolutionary aspects and implications for the pathogenesis and treatment of filarial diseases. Vet Parasitol. 2001; 12;98(1-3):215-338.

Beauty BJ, Marquardt WC. The biology of disease vectors. University Press of Colorado, Colorado. 1996.

Belinato TA, Martins AJ, Valle D. Fitness evaluation of two Brazilian *Aedes aegypti* field populations with distinct levels of resistance to the organophosphate temephos. Mem Inst Oswaldo Cruz. 2012; 107(7):916-922.

Bellinato DF, Viana-Medeiros PF, Araújo SC, Martins AJ, Lima JB, Valle D. Resistance status to the insecticides temephos, deltamethrin, and diflubenzuron in Brazilian *Aedes aegypti* populations. Biomed Res Int. 2016; 2016:8603263.

Benedict MQ, Robinson AS. The first releases of transgenic mosquitoes: an argument for the sterile insect technique. Trends Parasitol. 2003; 19(8):349-355.

Berticat C, Bonnet J, Duchon S, Agnew P, Weill M, Corbel V. Costs and benefits of multiple resistance to insecticides for *Culex quinquefasciatus* mosquitoes. BMC Evol Biol. 2008; 8;8:104.

Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, et al. The global distribution and burden of dengue. Nature. 2013; 25;496(7446):504-507.

Biber PA, Duenas JR, Almeida FL, Gardenal CN, Almirón WR. Laboratory evaluation of susceptibility of natural subpopulations of *Aedes aegypti* larvae to temephos. J Am Mosq Control Assoc. 2006; 22:408–411.

Bosio CF, Fulton RE, Salasek ML, Beaty BJ, Black WC 4th. Quantitative trait loci that control vector competence for dengue-2 virus in the mosquito *Aedes aegypti*. Genetics. 2000; 156(2):687-698.

Braga IA, Lima JBP, Soares SS, Valle D. *Aedes aegypti* resistance to temephos during 2001 in several municipalities in the States of Rio de Janeiro, Sergipe and Alagoas, Brazil. Mem Inst Oswaldo Cruz. 2004; 99:199-203.

Braga IA, Valle D. *Aedes aegypti*: histórico do controle no Brasil. Epidemiol. Serv. Saúde. 2007a; 16(2):113-118.

Braga IA, Valle D. *Aedes aegypti*: inseticidas, mecanismos de ação e resistência. Epidemiol. Serv. Saúde. 2007b; 16(4):179-293.

Breeuwer JA, Jacobs G. *Wolbachia*: intracellular manipulators of mite reproduction. Exp Appl Acarol. 1996; 20(8):421-434.

Briegel H, Knüsel I, Timmermann SE. *Aedes aegypti*: size, reserves, survival, and flight potential. J Vector Ecol. 2001; 26:21-31.

Brito LP, Linss JG, Lima-Camara TN, Belinato TA, Peixoto AA, Lima JB, et al. Assessing the effects of *Aedes aegypti kdr* mutations on pyrethroid resistance and its fitness cost. PLoS One. 2013; 8;8(4):e60878.

Brogdon WG, McAllister JC. Insecticide resistance and vector control. Emerg Infect Dis. 1998; 4:605-613.

Buonaccorsi JP, Harrington LC, Edman JD. Estimation and comparison of mosquito survival rates with release-recapture-removal data. J Med Entomol. 2003; 40(1):6-17.

Busvine JR. Mechanism of resistance to insecticides in housefly. Nature. 1951; 168:193-195.

Campos GS, Bandeira AC, Sardi SI. Zika virus outbreak, Bahia, Brazil. Emerg Infect Dis. 2015; 21(10):1885-1886.

Caragata EP, Dutra HL, Moreira LA. Inhibition of Zika virus by *Wolbachia* in *Aedes aegypti*. Microb Cell. 2016; 27;3(7):293-295.

Carrière Y, Deland JP, Roff DA, Vincent C. Life-history costs associated with the evolution of insecticide resistance. Proc R Soc London B. 1994; 258(1351):35-40.

Carson R. Silent Spring. Hougthon Mifflin Company, New York. 1962.

Carvalho DO, Costa-da-Silva AL, Lees RS, Capurro ML. Two step male release strategy using transgenic mosquito lines to control transmission of vector-borne diseases. Acta Trop. 2014; 132Suppl:S170-177.

Carvalho DO, McKemey AR, Garziera L, Lacroix R, Donnelly CA, Alphey L, et al. Suppression of a field population of *Aedes aegypti* in Brazil by sustained release of transgenic male mosquitoes. PLoS Negl Trop Dis. 2015; 2;9(7):e0003864.

Casida JE. Pyrethrum flowers and pyrethroid insecticides. Environ Health Perspect. 1980; 34:189-202.

Casida JE, Quistad GB. Golden age of insecticide research: past, present, or future? Annu Rev Entomol. 1998; 43:1-16.

Cassanelli S, Reyes M, Rault M, Carlo Manicardi G, Sauphanor B. Acetylcholinesterase mutation in an insecticide-resistant population of the codling moth *Cydia pomonella*. Insect Biochem Mol Biol. 2006; 36(8):642-653.

Chan M, Johansson MA. The incubation periods of dengue viruses. PLoS One. 2012; 7(11):e50972.

Chandre F, Darriet F, Darder M, Cuany A, Doannio JM, Pasteur N, et al. Pyrethroid resistance in *Culex quinquefasciatus* from west Africa. Med Vet Entomol. 1998; 12(4):359-366.

Chavasse DC, Yap HH. Chemical methods for the control of vectors and pests of public health importance. WHO, Geneva. 1997.

Chediak M, G Pimenta F Jr, Coelho GE, Braga IA, Lima JB, Cavalcante KR, et al. Spatial and temporal country-wide survey of temephos resistance in Brazilian populations of *Aedes aegypti*. Mem Inst Oswaldo Cruz. 2016; 111(5):311-321.

Chen L, Zhu C, Zhang D. Naturally occurring incompatibilities between different *Culex pipiens pallens* populations as the basis of potential mosquito control measures. PLoS Negl Trop Dis. 2013; 7(1):e2030.

Chouin-Carneiro T, Vega-Rua A, Vazeille M, Yebakima A, Girod R, Goindin D, et al. Differential susceptibilities of *Aedes aegypti* and *Aedes albopictus* from the Americas to Zika virus. PLoS Negl Trop Dis. 2016; 3;10(3):e0004543.

Christophers SR. *Aedes aegypti* the yellow fever mosquito: its life history, bionomics and structure. Cambridge University Press, London. 1960.

Cianci D, Van den Broek J, Caputo B, Marini F, Della Torre A, Heesterbeek H, et al. Estimating mosquito population size from mark-release-recapture data. J Med Entomol. 2013; 50(3):533-542.

Coelho GE, Burattini MN, Teixeira Mda G, Coutinho FA, Massad E. Dynamics of the 2006/2007 dengue outbreak in Brazil. Mem Inst Oswaldo Cruz. 2008; 103(6):535-539.

Coffey LL, Forrester N, Tsetsarkin K, Vasilakis N, Weaver SC. Factors shaping the adaptive landscape for arboviruses: implications for the emergence of disease. Future Microbiol. 2013; 8(2):155-176.

Consoli RAGB, Lourenço-de-Oliveira R. Principais mosquitos de importância sanitária no Brasil. Editora Fiocruz, Rio de Janeiro. 1994.

Constantini C, Li S, Torre AD, Sagnon N, Coluzzi M, Taylor CE. Density, survival and dispersal of *Anopheles gambiae* complex mosquitoes in a West African Sudan savanna village. Med Vet Entomol. 1996; 10:203-219.

Coon KL, Brown MR, Strand MR. Mosquitoes host communities of bacteria that are essential for development but vary greatly between local habitats. Mol Ecol. 2016; 25(22):5806-5826.

Corbel V, Fonseca DM, Weetman D, Pinto J, Achee NL, Chandre F, et al. International workshop on insecticide resistance in vectors of arboviruses, December 2016, Rio de Janeiro, Brazil. Parasit Vectors. 2017 Jun 2;10(1):278.

Costa-da-Silva AL, Ioshino RS, Petersen V, Lima AF, Cunha MDP, Wiley MR, et al. First report of naturally infected *Aedes aegypti* with chikungunya virus genotype ECSA in the Americas. PLoS Negl Trop Dis. 2017; 14;11(6):e0005630.

Couto-Lima D, Madec Y, Bersot MI, Campos SS, Motta MA, Santos FBD, et al. Potential risk of re-emergence of urban transmission of Yellow Fever virus in Brazil facilitated by competent *Aedes* populations. Sci Rep. 2017; 7;7(1):4848.

Crotti E, Rizzi A, Chouaia B, Ricci I, Favia G, Alma A, et al. Acetic acid bacteria, newly emerging symbionts of insects. Appl Environ Microbiol. 2010; 76(21):6963-6970.

Crow JF. Genetics of insect resistance to chemicals. Annu Rev Entomol. 1957; 2:227-246.

D'Amato C, Torres JPM, Malm O. DDT (dicloro difenil tricloroetano): toxicidade e contaminação ambiental – uma revisão. Quim Nova. 2002; 25:995-1002.

da-Cunha MP, Lima JBP, Brogdon WG, Moya GE, Valle D. Monitoring of resistance to the pyrethroid cypermethrin in Brazilian *Aedes aegypti* populations collected between 2001 and 2003. Mem Inst Oswaldo Cruz. 2005; 100:441-444.

David MR, Lourenço-de-Oliveira R, Freitas RM. Container productivity, daily survival rates and dispersal of *Aedes aegypti* mosquitoes in a high income dengue epidemic neighbourhood of Rio de Janeiro: presumed influence of differential urban structure on mosquito biology. Mem Inst Oswaldo Cruz. 2009; 104(6):927-932.

David MR, Garcia GA, Valle D, Maciel-de-Freitas. Association of insecticide resistance with fitness traits in four field *Aedes aegypti* populations from Brazil. Submetido para PLoS Neglected and Tropical Diseases. 2017. **(Artigo - 2).**

Davies TG, Field LM, Usherwood PN, Williamson MS. DDT, pyrethrins, pyrethroids and insect sodium channels. IUBMB Life. 2007; 59(3):151-62.

de Oliveira CD, Moreira LA. Uso de *Wolbachia* no controle biológico. Tópicos Avançados de Entomologia Molecular, Belo Horizonte. 2012.

de Oliveira CD, Gonçalves DS, Baton LA, Shimabukuro PH, Carvalho FD, Moreira LA. Broader prevalence of *Wolbachia* in insects including potential human disease vectors. Bull Entomol Res. 2015; 105(3):305-315.

Devine GJ, Perea EZ, Killeen GF, Stancil JD, Clark SJ, Morrison AC. Using adult mosquitoes to transfer insecticides to *Aedes aegypti* larval habitats. Proc Natl Acad Sci U S A. 2009; 14;106(28):11530-11534.

Dick GW, Kitchen SF, Haddow AJ. Zika virus. I. Isolations and serological specificity. Trans R Soc Trop Med Hyg. 1952; 46(5):509-520.

Diniz DF, de Melo-Santos MA, Santos EM, Beserra EB, Helvecio E, de Carvalho-Leandro D, et al. Fitness cost in field and laboratory *Aedes aegypti* populations associated with resistance to the insecticide temephos. Parasit Vectors. 2015; 30;8:662.

Dorazio RM, Karanth KU. A hierarchical model for estimating the spatial distribution and abundance of animals detected by continuous-time recorders. PLoS One. 2017; 17;12(5):e0176966.

Dos Santos RL, Forattini OP, Burattini MN. *Anopheles albitarsis s.l.* (Diptera: Culicidae) survivorship and density in a rice irrigation area of the state of São Paulo, Brazil. J Med Entomol. 2004; 41(5):997-1000.

Dotson EM, Plikaytis B, Shinnick TM, Durvasula RV, Beard CB. Transformation of *Rhodococcus rhodnii*, a symbiont of the Chagas disease vector *Rhodnius prolixus*, with integrative elements of the L1 mycobacteriophage. Infect Genet Evol. 2003; 3(2):103-109.

Du Y, Nomura Y, Zhorov BS, Dong K. Sodium channel mutations and pyrethroid resistance in *Aedes aegypti*. Insects. 2016; 31;7(4).

Duffy MR, Chen TH, Hancock WT, Powers AM, Kool JL, Lanciotti RS, et al. Zika virus outbreak on Yap Island, Federated States of Micronesia. N Engl J Med. 2009; 11;360(24):2536-2543.

Dutra HL, Dos Santos LM, Caragata EP, Silva JB, Villela DA, Maciel-de-Freitas R, et al. From lab to field: the influence of urban landscapes on the invasive potential of *Wolbachia* in Brazilian *Aedes aegypti* mosquitoes. PLoS Negl Trop Dis. 2015; 23;9(4):e0003689.

Dutra HL, Rocha MN, Dias FB, Mansur SB, Caragata EP, Moreira LA. *Wolbachia* blocks currently circulating Zika virus isolates in Brazilian *Aedes aegypti* mosquitoes. Cell Host Microbe. 2016; 8;19(6):771-774.

Dye C. Vectorial capacity: must we measure all its components? Parasitol Today. 1986; 2:203-209.

Dye C. Epidemiological significance of vector-parasite interactions. Parasitol. 1990; 101:409-415.

Edman JD, Strickman D, Kittayapong P, Scott TW. Female *Aedes aegypti* in Thailand rarely feed on sugar. J Med Entomol. 1992; 29(6):1035-1038.

Endersby NM, Hoffmann AA. Effect of *Wolbachia* on insecticide susceptibility in lines of *Aedes aegypti*. Bull Entomol Res. 2013; 103(3):269-277.

Enserink M. Infectious diseases. Chikungunya: no longer a third world disease. Science. 2007; 21;318(5858):1860-1861.

Fares RC, Souza KP, Añez G, Rios M. Epidemiological scenario of dengue in Brazil. Biomed Res Int. 2015; 2015:321873.

Farnesi LC, Menna-Barreto RF, Martins AJ, Valle D, Rezende GL. Physical features and chitin content of eggs from the mosquito vectors *Aedes aegypti*, *Anopheles aquasalis* and *Culex quinquefasciatus*: Connection with distinct levels of resistance to desiccation. J Insect Physiol. 2015; 83:43-52.

Favia G, Ricci I, Marzorati M, Negri I, Alma A, Sacchi L, et al. Bacteria of the genus *Asaia*: a potential paratransgenic weapon against malaria. Adv Exp Med Biol. 2008; 627:49-59.

Faye O, Freire CC, Iamarino A, Faye O, de Oliveira JV, Diallo M, et al. Molecular evolution of Zika virus during its emergence in the 20(th) century. PLoS Negl Trop Dis. 2014; 9;8(1):e2636.

Ferguson NM, Kien DT, Clapham H, Aguas R, Trung VT, Chau TN, et al. Modeling the impact on virus transmission of *Wolbachia*-mediated blocking of dengue virus infection of *Aedes aegypti*. Sci Transl Med. 2015; 18;7(279):279ra37.

Ferrari JA. Insecticide resistance. The biology of disease vectors. University Press of Colorado, Colorado. 1996.

Ferreira C, Yang H, Esteva L. Assessing the suitability of sterile insect technique applied to *Aedes aegypti*. Journ Biolog Syst. 2008; 16(4):565–577.

Ferreira-de-Brito A, Ribeiro IP, Miranda RM, Fernandes RS, Campos SS, Silva KA, et al. First detection of natural infection of *Aedes aegypti* with Zika virus in Brazil and throughout South America. Mem Inst Oswaldo Cruz. 2016; 111(10):655-658.

ffrench-Constant RH, Pittendrigh B, Vaughan, Anthony N. Why there so few resistance-associated mutation target genes? Philos Trans R Soc Lond B Biol Sci. 1998; 353:1685-1693.

Figueiredo LT. The Brazilian flaviviruses. Microbes Infect. 2000;2(13):1643-1649.

Fine BC. Pattern of pyrethrin-resistance in houseflies. Nature. 1961; 191:884-88.

Fine P, Eames K, Heymann DL. "Herd immunity": a rough guide. Clin Infect Dis. 2011; 1;52(7):911-916.

Fiocruz. Recomendação técnica sobre a interrupção do uso de inseticidas piretroides no controle do *Aedes aegypti* no Brasil. NOTA TÉCNICA N.º 2/2011/IOC-FIOCRUZ/DIRETORIA. 2011.

Fisher RA, Ford EB. The spread of a gene in natural conditions in a colony of the moth *Panaxia dominula*. Heredity. 1947; 1:143–174;

Flores AE, Grajales JS, Salas IF, Garcia GP, Becerra MH, Lozano S, et al. Mechanisms of insecticide resistance in field populations of *Aedes aegypti* (L.) from Quintana Roo, Southern Mexico. J Am Mosq Control Assoc. 2006; 22: 672–677.

Focks DA, Chadee DD. Pupal survey: an epidemiologically significant surveillance method for *Aedes aegypti*: an example using data from Trinidad. Am J Trop Med Hyg 1997; 56:159-167.

Fontoura NG, Bellinato DF, Valle D, Lima JB. The efficacy of a chitin synthesis inhibitor against field populations of organophosphate-resistant *Aedes aegypti* in Brazil. Mem Inst Oswaldo Cruz. 2012; 107(3):387-395.

Forattini OP. Culicidologia médica: identificação, biologia, epidemiologia. Edusp, São Paulo. 2002.

Forattini OP. Entomologia Médica. Faculdade de Higiene e Saúde Pública, São Paulo. 1962.

Fournet F, Sannier C, Monteny N. Effects of two insect growth regulators on the susceptibility of *Aedes aegypti* (Diptera: Culicidae) to Molinema dessetae (Nematoda: Filarioidea). J Am Mosq Control. 1997; 13:40-42.

Franz AW, Sanchez-Vargas I, Adelman ZN, Blair CD, Beaty BJ, James AA, et al. Engineering RNA interference-based resistance to dengue virus type 2 in genetically modified *Aedes aegypti*. Proc Natl Acad Sci U S A. 2006; 14;103(11):4198-4203.

Frentiu FD, Zakir T, Walker T, Popovici J, Pyke AT, van den Hurk A, et al. Limited dengue virus replication in field-collected *Aedes aegypti* mosquitoes infected with *Wolbachia*. PLoS Negl Trop Dis. 2014; 20;8(2):e2688.

Funasa. Reunião técnica para discutir status de resistência de *Aedes aegypti* e definir estratégias a serem implantadas para monitoramento da resistência no Brasil. Fundação Nacional de Saúde, Ministério da Saúde, Brasília. 1999.

Garcia GA, David MR, Martins AJ, Maciel-de-Freitas, Linss JB, Araújo SC, et al. The impacto of insecticide applications on the dynamics of resistance: the case of four *Aedes aegypti* populations from diferente Brazilian regions. (Submetido para PLoS Neglected and Tropical Disease). 2017a. **(Artigo – 1)**

Garcia GA, Dos Santos LM, Villela DA, Maciel-de-Freitas R. Using *Wolbachia* releases to estimate *Aedes aegypti* population size and survival. PLoS One. 2016; 1;11(8):e0160196 (**Artigo - 6**).

Garcia GA, Sylvestre G, Petersen MT, Aguiar R, Martins AJ, Lima JBP, et al. The riddle solved on a local grocery store: the release of *Aedes aegypti* as resistant to pyrethroid as the wild population is essential for *Wolbachia* releases. 2017b. (Artigo em preparação) **(Artigo – 7)**

Garcia GA, Turelli M, Hoffmann AA, Maciel-de-Freitas R, Villela D. Insecticide resistance elucidates the sucess (and failure) of *Wolbachia* population replacement. 2017c. (**Artigo - 8**)

García GP, Flores AE, Fernández-Salas I, Saavedra-Rodríguez K, Reyes-Solis G, Lozano-Fuentes S, et al. Recent rapid rise of a permethrin knockdown resistance allele in *Aedes aegypti* in México. PLoS Negl Trop Dis. 2009; 13;3(10):e531.

Garret-Jones C. The human blood index of malaria vector in relation to epidemiological assessment. Bull World Health Organ. 1964; 30(2)241-261.

Georghiou GP. Principles of insecticide resistance management. Phytoprotection. 1994; 75:51-59.

Gillies MT. Studies on the dispersion and survival of *Anopheles gambiae* Giles in East Africa, by means of marking and releasing experiments. Bull Entomol Res. 1961; 52:99-127.

Gillies MT, Wilkes TJ. A study of the age composition of populations of *Anopheles gambiae* Giles and *An. funestus* Giles in northeastern Tanzania. Bull Entomol Res. 1965; 56:237-262.

Goindin D, Delannay C, Gelasse A, Ramdini C, Gaude T, Faucon F, et al. Levels of insecticide resistance to deltamethrin, malathion, and temephos, and associated mechanisms in *Aedes aegypti* mosquitoes from the Guadeloupe and Saint Martin islands (French West Indies). Infect Dis Poverty. 2017; 10;6(1):38.

Gotoh T, Noda H, Hong XY. *Wolbachia* distribution and cytoplasmic incompatibility based on a survey of 42 spider mite species (Acari: Tetranychidae) in Japan. Heredity. 2003; 91(3):208-216.

Graf JF. The role of insect growth regulators in arthropod control. Parasitol Today. 1993; 9:471-474.

Grange L, Simon-Loriere E, Sakuntabhai A, Gresh L, Paul R, Harris E. Epidemiological risk factors associated with high global frequency of inapparent dengue virus infections. Front Immunol. 2014; 11;5:280.

Grunnill M, Boots M. How important is vertical transmission of dengue viruses by mosquitoes? J Med Entomol. 2016; 53(1):1-19.

Gubler DJ, Kuno G. Dengue and dengue hemorrhagic fever. Wallingford, CAB International. 1997.

Gubler DJ. Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. Trends Microbiol. 2002; 10(2):100-103.

Guerra CA, Reiner RC Jr, Perkins TA, Lindsay SW, Midega JT, Brady OJ, et al. A global assembly of adult female mosquito mark-release-recapture data to inform the control of mosquito-borne pathogens. Parasit Vectors. 2014; 19;7:276.

Guimarães RM, Asmus CI, Meyer A. DDT reintroduction for malaria control: the cost-benefit debate for public health. Cad Saude Publica. 2007; 23(12):2835-2844.

Hancock PA, White VL, Ritchie SA, Hoffmann AA, Godfray HC. Predicting *Wolbachia* invasion dynamics in *Aedes aegypti* populations using models of density-dependent demographic traits. BMC Biol. 2016; 8;14(1):96.

Harrington LC, Vermeylen F, Jones JJ, Kithawee S, Sithiprasasna R, Edman JD, et al. Age-dependent survival of the dengue vector *Aedes aegypti* demonstrated by simultaneous release-recapture of different age cohorts. J Med Entomol. 2008; 45:307-313.

Hayes EB. Zika virus outside Africa. Emerg Infect Dis. 2009; 15(9):1347-1350.

Hedges LM, Brownlie JC, O'Neill SL, Johnson KN. *Wolbachia* and virus protection in insects. Science. 2008; 31;322(5902):702.

Hemingway J, Ranson H. Insecticide resistance in insect vectors of human disease. Annu Rev Entomol. 2000; 45:371-391.

Hemingway J, Hawkes NJ, McCarroll L, Ranson H. The molecular basis of insecticide resistance in mosquitoes. Insect Biochem Mol Biol. 2004; 34:653-665.

Hertig M. The rickettsia, *Wolbachia pipientis* (gen. et sp. n.) and associated inclusions of the mosquito, *Culex pipiens*. Parasitol. 1936; 28:453-486.

Hilgenboecker K, Hammerstein P, Schlattmann P, Telschow A, Werren JH. How many species are infected with *Wolbachia*? A statistical analysis of current data. FEMS Microbiol Lett. 2008; 281(2):215-220.

Hoffmann AA, Turelli M, Harshman LG. Factors affecting the distribution of cytoplasmic incompatibility in *Drosophila simulans*. Genetics. 1990; 126(4):933-948.

Hoffmann AA, Montgomery BL, Popovici J, Iturbe-Ormaetxe I, Johnson PH, Muzzi F, et al. Successful establishment of *Wolbachia* in *Aedes populations* to suppress dengue transmission. Nature. 2011; 24;476(7361):454-457.

Hoffmann AA, Turelli M. Facilitating *Wolbachia* introductions into mosquito populations through insecticide-resistance selection. Proc Biol Sci. 2013; 10;280(1760):20130371.

Hoffmann AA, Iturbe-Ormaetxe I, Callahan AG, Phillips BL, Billington K, Axford JK, et al. Stability of the *w*Mel *Wolbachia* Infection following invasion into *Aedes aegypti* populations. PLoS Negl Trop Dis. 2014; 11;8(9):e3115.

Hoffmann AA, Ross PA, Rašić G. *Wolbachia* strains for disease control: ecological and evolutionary considerations. Evol Appl. 2015; 8(8):751-768.

Honório NA, Lourenço-de-Oliveira. Frequência de larvas e pupas de *Aedes aegypti* e *Aedes albopictus* em armadilhas, Brasil. Rev Saúde Pública. 2001; 35(4):385-391.

Itoh T, Kawada H, Abe A, Eshita Y, Rongsriyam Y, Igarashi A. Utilization of bloodfed females of *Aedes aegypti* as a vehicle for the transfer of the insect growth regulator pyriproxyfen to larval habitats. J Am Mosq Control Assoc. 1994; 10(3):344-347.

Jeyaprakash A, Hoy MA. Long PCR improves *Wolbachia* DNA amplification: wsp sequences found in 76% of sixty-three arthropod species. Insect Mol Biol. 2000; 9(4):393-405.

Jia FX, Yang MS, Yang WJ, Wang JJ. Influence of continuous high temperature conditions on *Wolbachia* infection frequency and the fitness of *Liposcelis tricolor* (Psocoptera: Liposcelididae). Environ Entomol. 2009; 38(5):1365-1372.

Jiggins FM. The spread of *Wolbachia* through mosquito populations. PLoS Biol. 2017; 1;15(6):e2002780.

Joubert DA, Walker T, Carrington LB, De Bruyne JT, Kien DH, Hoang Nle T, et al. Establishment of a *Wolbachia* superinfection in *Aedes aegypti* mosquitoes as a potential approach for future resistance management. PLoS Pathog. 2016; 18;12(2):e1005434.

Jupatanakul N, Sim S, Angleró-Rodríguez YI, Souza-Neto J, Das S, Poti KE, et al. Engineered *Aedes aegypti* JAK/STAT pathway-mediated immunity to dengue virus. PLoS Negl Trop Dis. 2017; 12;11(1):e0005187.

Kambris Z, Cook PE, Phuc HK, Sinkins SP. Immune activation by life-shortening *Wolbachia* and reduced filarial competence in mosquitoes. Science. 2009; 2;326(5949):134-136.

Karabatsos N. International Catalogue of Arboviruses. Am Soc Trop Med Hyg, San Antonio. 1985.

Karunamoorthi K, Sabesan S. Insecticide resistance in insect vectors of disease with special reference to mosquitoes: a potential threat to global public health. Health Scope. 2012; 2(1):4-18.

Klempner MS, Unnasch TR, Hu LT. Taking a bite out of vector-transmited infectious diseases. N Engl J Med. 2007; 356:2567-2569.

Kliot A, Ghanim M. Fitness costs associated with insecticide resistance. Pest Manag Sci. 2012; 68(11):1431-1437.

Koyama J, Kakinohana H, Miyatake T. Eradication of the melon fly, Bactrocera cucurbitae, in Japan: importance of behavior, ecology, genetics, and evolution.Annu Rev Entomol. 2004;49:331-49.

Kraemer MU, Sinka ME, Duda KA, Mylne AQ, Shearer FM, Barker CM, et al. The global distribution of the arbovirus vectors *Aedes aegypti* and *Ae. albopictus*. Elife. 2015; 30;4:e08347.

Kuno G, Chang GJ. Biological transmission of arboviruses: reexamination of and new insights into components, mechanisms, and unique traits as well as their evolutionary trends. Clin Microbiol Rev. 2005; 18(4):608-637.

Kuno G. Review of the factors modulating dengue transmission. Epidemiol Rev. 1995;17(2):321-335.

Labbé P, Berthomieu A, Berticat C, Alout H, Raymond M, Lenormand T, Weill M. Independent duplications of the acetylcholinesterase gene conferring insecticide resistance in the mosquito *Culex pipiens*. Mol Biol Evol. 2007; 24(4):1056-1067.

Laven H. Eradication of *Culex pipiens* fatigans through cytoplasmic incompatibility. Nature. 1967; 28;216(5113):383-384.

Le Cren ED. A note on the history of mark-recapture population estimates. J Anim Ecol. 1965; 34:453–454.

Li X, Schuler MA, Berenbaum MR. Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. Annu Rev Entomol. 2007; 52:231-253.

Liang G, Gao X, Gould EA. Factors responsible for the emergence of arboviruses; strategies, challenges and limitations for their control. Emerg Microbes Infect. 2015; 4(3):e18.

Lima EP, Paiva MH, De Araújo AP, Da Silva EV, Da Silva UM, De Oliveira LN, et al. Insecticide resistance in *Aedes aegypti* populations from Ceará, Brazil. Parasit Vectors. 2011; 12:5-16.

Lima JBP, da-Cunha M, Silva-Jr RCS, Galardo AKR, Soares SS, Braga IA, et al. Resistance of *Aedes aegypti* to organophosphates in several municipalities in the States of Rio de Janeiro and Espírito Santo, Brazil. Am J Trop Med Hyg. 2003; 368: 329-333.

Lincoln FC. Calculating waterfowl abundance on the basis of banding returns. Department of Agriculture. 1930; 118:1-4.

Linss JG, Brito LP, Garcia GA, Araki AS, Bruno RV, Lima JB, et al. Distribution and dissemination of the Val1016Ile and Phe1534Cys *kdr* mutations in *Aedes aegypti* Brazilian natural populations. Parasit Vectors. 2014; 15;7:25. (**Artigo - 3**)

Lockwood JA, Sparks TC, Story RN. Evolution of insect resistance to insecticides: a reevaluation of the roles of physiology and behavior. Bull Entomol Soc Am. 1984; 30:41–51.

Lofgren CS, Dame DA, Breeland SG, Weidhaas DE, Jeffery G, Kaiser R, et al. Release of chemosterilized males for the control of *Anopheles albimanus* in El Salvador. 3. Field methods and population control. Am J Trop Med Hyg. 1974; 23(2):288-297.

Lourenço-de-Oliveira R, Vazeille M, de Filippis AM, Failloux AB. *Aedes aegypti* in Brazil: genetically differentiated populations with high susceptibility to dengue and yellow fever viruses. Trans R Soc Trop Med Hyg. 2004; 98(1):43-54.

Löwi I. Yellow fever in Rio de Janeiro and the Pasteur Institute mission (1901-1905): the transfer of science to the periphery. Medical History. 1990; 34:144-163.

Luna JE, Martins MF, Anjos AF, Kuwabara EF, Navarro-Silva MA. Susceptibility of *Aedes aegypti* to temephos and cypermethrin insecticides, Brazil. Rev Saude Publica. 2004; 38(6):842-843.

Luz PM, Codeço CT, Massad E, Struchiner CJ. Uncertainties regarding dengue modeling in Rio de Janeiro, Brazil. Mem Inst Oswaldo Cruz. 2003; 98(7):871-878.

Macdonald WW. *Aedes aegypti* in Malaya. II. Larval and adult biology. Ann Trop Med Parasitol. 1956; 50(4):399-414.

Maciel-de-Freitas R, Neto RB, Gonçalves JM, Codeço CT, Lourenço-de-Oliveira R. Movement of dengue vectors between the human modified environment and an urban forest in Rio de Janeiro. J Med Entomol. 2006; 43(6):1112-1120.

Maciel-de-Freitas R, Marques WA, Peres RC, Cunha SP, de Oliveira RL. Variation in *Aedes aegypti* container productivity in a slum and a suburban district of Rio de Janeiro during dry and wet seasons. Mem Inst Oswaldo Cruz. 2007**a**; 102(4):489-496.

Maciel-de-Freitas R, Codeço CT, Lourenço-de-Oliveira R. Daily survival rates and dispersal of *Aedes aegypti* females in Rio de Janeiro, Brazil. Am J Trop Med Hyg. 2007b; 76(4):659-665.

Maciel-de-Freitas R, Eiras AE, Lourenço-de-Oliveira R. Calculating the survival rate and estimated population density of gravid *Aedes aegypti* (Diptera, Culicidae) in Rio de Janeiro, Brazil. Cad Saude Publica. 2008; 24(12):2747-2754.

Maciel-de-Freitas R, Souza-Santos R, Codeço CT, Lourenço-de-Oliveira R. Influence of the spatial distribution of human hosts and large size containers on the dispersal of the mosquito *Aedes aegypti* within the first gonotrophic cycle. Med Vet Entomol. 2010; 24(1):74-82.

Macoris MLG, Andrighetti MT, Takaku L, Glasser CM, Garbeloto VC, Bracco JE. Resistance of *Aedes aegypti* from State of Sao Paulo, Brazil, to organophosphate insecticides. Mem Inst Oswaldo Cruz. 2003; 98:703-708.

Macoris MLG, Andrighetti MT, Otrera VC, Carvalho LR, Caldas Júnior AL, Brogdon WG. Association of insecticide use and alteration on *Aedes aegypti* susceptibility status. Mem Inst Oswaldo Cruz. 2007; 102: 895-900.

Macoris MLG, Andrighetti MT, Wanderley DM, Ribolla PE. Impact of insecticide resistance on the field control of *Aedes aegypti* in the State of São Paulo. Rev Soc Bras Med Trop. 2014; 47(5):573-578.

Mallet J. The evolution of insecticide resistance: Have the insects won? Trends Ecol Evol. 1989; 4(11):336-340.

Marcombe S, Darriet F, Tolosa M, Agnew P, Duchon S, Etienne M, et al. Pyrethroid resistance reduces the efficacy of space sprays for dengue control on the island of Martinique (Caribbean). PLoS Negl Trop Dis. 2011; 5: e1202.

Marcombe S, Mathieu RB, Pocquet N, Riaz MA, Poupardin R, Sélior S, et al. Insecticide resistance in the dengue vector *Aedes aegypti* from Martinique: distribution, mechanisms and relations with environmental factors. PLoS One. 2012; 7(2):e30989.

Marini F, Caputo B, Pombi M, Tarsitani G, della Torre A. Study of *Aedes albopictus* dispersal in Rome, Italy, using sticky traps in mark-release-recapture experiments. Med Vet Entomol. 2010; 24(4):361-368.

Martinez-Torres D, Chandre F, Williamson MS, Darriet F, Bergé JB, Devonshire AL, et al. Molecular characterization of pyrethroid knockdown resistance (*kdr*) in the major malaria vector *Anopheles gambiae s.s*. Insect Mol Biol. 1998; 7(2):179-184.

Martins AJ, Lima JB, Peixoto AA, Valle D. Frequency of Val1016Ile mutation in the voltage-gated sodium channel gene of *Aedes aegypti* Brazilian populations. Trop Med Int Health. 2009a; 14:1351-1355.

Martins AJ, Lins RM, Linss JG, Peixoto AA, Valle D. Voltage-gated sodium channel polymorphism and metabolic resistance in pyrethroid-resistant *Aedes aegypti* from Brazil. Am J Trop Med Hyg. 2009b; 81:108-115.

Martins AJ, Valle D. The pyrethroid knockdown resistance. Insecticides. Basic and Other Applications. Disponível em http://www.intechopen.com/books/insecticides-basic-and-other-applications. 2011.

Martins AJ, Ribeiro CD, Bellinato DF, Peixoto AA, Valle D, Lima JB. Effect of insecticide resistance on development, longevity and reproduction of field or laboratory selected *Aedes aegypti* populations. PLoS One. 2012; 7(3):e31889.

Martins AJ, Brito LP, Linss JG, Rivas GB, Machado R, Bruno RV, et al. Evidence for gene duplication in the voltage-gated sodium channel gene of *Aedes aegypti*. Evol Med Public Health. 2013; 2013(1):148-160.

Mayer SV, Tesh RB, Vasilakis N. The emergence of arthropod-borne viral diseases: A global prospective on dengue, chikungunya and Zika fevers. Acta Trop. 2017; 166:155-163.

Mazzari MB. Revisión del estado actual de la resistencia en *Aedes aegypti* a insecticidas utilizados en salud pública. Boletín de la dirección de malariología y saneamiento ambiental – Colombia. 1995; 35:90-95.

McCarroll L, Paton MG, Karunaratne SH, Jayasuryia HT, Kalpage KS, Hemingway J. Insecticides and mosquito-borne disease. Nature. 2000; 26;407(6807):961-962.

McMeniman CJ, Lane RV, Cass BN, Fong AW, Sidhu M, Wang YF, et al. Stable introduction of a life-shortening *Wolbachia* infection into the mosquito *Aedes aegypti*. Science. 2009; 2;323(5910):141-144.

Melo-Santos MA, Varjal-Melo JJ, Araújo AP, Gomes TC, Paiva MH, Regis LN, et al. Resistance to the organophosphate temephos: mechanisms, evolution and reversion in an *Aedes aegypti* laboratory strain from Brazil. Acta Trop. 2010; 113(2):180-189.

Metsky HC, Matranga CB, Wohl S, Schaffner SF, Freije CA, Winnicki SM, et al. Zika virus evolution and spread in the Americas. Nature. 2017; 15;546(7658):411-415.

Midega JT, Mbogo CM, Mwnambi H, Wilson MD, Ojwang G, Mwangangi JM, et al. Estimating dispersal and survival of *Anopheles gambiae* and *Anopheles funestus* along the Kenyan coast by using mark-release-recapture methods. J Med Entomol. 2007; 44(6):923-929.

Milani R. Comportamento mendeliano della resistenza alla azione del DDT e correlazione tra abbattimento e e mortalitá in *Musca domestica* L. Rev Parassit. 1954; 15: 513-542.

Milani R, Travaglino A. Information circular on insecticide resistance. Rev Parassit. 1957; 32:6.

MS - Ministério da Saúde. Diretrizes Nacionais para Prevenção e Controle de Epidemias de Dengue. Ministério da Saúde, Secretaria de Vigilância em Saúde, Departamento de Vigilância Epidemiológica. Brasília. 2009.

MS - Ministério da Saúde. Controle de Vetores. Portal da Saúde/SUS. Disponível em http://portalsaude.saude.gov.br. 2014a.

MS - Ministério da Saúde. Orientações técnica para utilização do larvicida pyriproxyfen (0,5G) no controle de *Aedes aegypti*. Disponível em http://portalarquivos.saude.gov.br/images/pdf/2014/maio/30/Instrucoes-para-uso-de-pyriproxifen-maio-2014.pdf. 2014b.

MS - Ministério da Saúde. Dengue: diagnóstico e manejo clínico: adulto e criança. Disponível em http://portalarquivos.saude.gov.br/images/pdf/2016/janeiro/14/dengue-manejo-adulto-crianca-5d.pdf. 5a Ed. Brasília. 2016a.

MS - Ministério da Saúde.. Protocolo de vigilância em resposta à ocorrência de microcefalia e/ou alterações do sistema nervoso central. Disponível em http://combateaedes.saude.gov.br/images/sala-de-situacao/Microcefalia-Protocolo-de-vigilancia-e-resposta-10mar2016-18h.pdf. Versão 2. 2016b.

MS - Ministério da Saúde. Boletim Epidemiológico. Disponível em http://combateaedes.saude.gov.br/images/pdf/2017-Dengue_Zika_Chikungunya-SE4.pdf. 2017.

Mittal PK. Biolarvicides in vector control: challenges and prospects. J Vect Born Dis. 2003; 40:20–32.

Montella IR, Martins AJ, Viana-Medeiros PF, Lima JB, Braga IA, Valle D. Insecticide resistance mechanisms of Brazilian *Aedes aegypti* populations from 2001 to 2004. Am J Trop Med Hyg. 2007; 77:467-477.

Montella IR, Schama R, Valle D. The classification of esterases: an important gene family involved in insecticide resistance - a review. Mem Inst Oswaldo Cruz. 2012; 107(4):437-49.

Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, Lu G, Pyke AT, Hedges LM, et al. A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, chikungunya, and *Plasmodium*. Cell. 2009; 24;139(7):1268-1278.

Morrison AC, Zielinski-Gutierrez E, Scott TW, Rosenberg R. Defining challenges and proposing solutions for control of the virus vector *Aedes aegypti*. PLoS Med. 2008; 18;5(3):e68.

Mouton L, Henri H, Bouletreau M, Vavre F. Effect of temperature on *Wolbachia* density and impact on cytoplasmic incompatibility. Parasitol. 2006; 132(Pt 1):49-56.

Mouton L, Henri H, Charif D, Boulétreau M, Vavre F. Interaction between host genotype and environmental conditions affects bacterial density in *Wolbachia* symbiosis. Biol Lett. 2007; 22;3(2):210-213.

Mustafa MS, Rasotgi V, Jain S, Gupta V. Discovery of fifth serotype of dengue virus (DENV-5): A new public health dilemma in dengue control. Med J Armed Forces India. 2015; 71(1):67-70.

Nasci RS, Mitchell CJ. Larval diet, adult size, and susceptibility of *Aedes aegypti* to infection with Ross River virus. J Med Entomol. 1994; 31(1):123-126.

Nguyen TH, Nguyen HL, Nguyen TY, Vu SN, Tran ND, Le TN,et al. Field evaluation of the establishment potential of *w*MelPop *Wolbachia* in Australia and Vietnam for dengue control. Parasit Vectors. 2015; 28;8:563.

Niebylski ML, Craig GB Jr. Dispersal and survival of *Aedes albopictus* at a scrap tire yard in Missouri. J Am Mosq Control Assoc. 1994; 10(3):339-343.

Nkya TE, Akhouayri I, Kisinza W, David JP. Impact of environment on mosquito response to pyrethroid insecticides: facts, evidences and prospects. Insect Biochem Mol Biol. 2013; 43(4):407-416.

Nogueira RM, Miagostovich MP, Schatzmayr HG, dos Santos FB, de Araújo ES, de Filippis AM, et al. Dengue in the State of Rio de Janeiro, Brazil, 1986-1998. Mem Inst Oswaldo Cruz. 1999; 94(3):297-304.

Nogueira RM, Miagostovich MP, de Filippis AM, Pereira MA, Schatzmayr HG. Dengue virus type 3 in Rio de Janeiro, Brazil. Mem Inst Oswaldo Cruz. 2001; 96(7):925-926.

Nogueira RM, Schatzmayr HG, de Filippis AM, dos Santos FB, da Cunha RV, Coelho JO, et al. Dengue virus type 3, Brazil, 2002. Emerg Infect Dis. 2005; 11(9):1376-1381.

Noor Afizah A, Roziah A, Nazni WA, Lee HL. Detection of *Wolbachia* from field collected *Aedes albopictus* Skuse in Malaysia. Indian J Med Res. 2015; 142(2):205-210.

Nunes MR, Faria NR, de Vasconcelos JM, Golding N, Kraemer MU, de Oliveira LF, et al. Emergence and potential for spread of chikungunya virus in Brazil. BMC Med. 2015; 30;13:102.

Ocampo CB, Salazar-Terreros MJ, Mina NJ, McAllister J, Brogdon W. Insecticide resistance status of *Aedes aegypti* in 10 localities in Colômbia. Acta Tropica. 2011; 118:37–44.

O'Reilly AO, Khambay BP, Williamson MS, Field LM, Wallace BA, Davies TG. Modelling insecticide-binding sites in the voltage-gated sodium channel. Biochem J. 2006; 396:255-263.

OMS - Organização Mundial da Saúde. Executive committee of the directing council the regional committee Pan American World Health. 120th Meeting CE120/21. Genebra, Suíça. 1997.

OMS - Organização Mundial da Saúde. Test procedures for insecticide resistance monitoring in malaria vectors; bio-efficacy and persistence of insecticides on treated surfaces. WHO/CDS/CPC/MAL/9812. Genebra, Suíça. 1998.

OMS - Organização Mundial da Saúde. Global insecticide use for vector-borne disease control. Department of control of neglected tropical diseases (NTD). WHO Pesticide Evaluation Scheme - 4th ed. 2009.

OMS - Organização Mundial da Saúde. The use of DDT in malaria vector control. Malaria WHO position statement. 2011.

OMS - Organização Mundial da Saúde. WHO recommended long-lasting insecticidal nets. Disponível em http://www.who.int/whopes/Long-lasting_insecticidal_nets_November_2015.pdf?ua=1. 2015.

OMS - Organização Mundial da Saúde. WHO recommended insecticides for space spraying against mosquitoes. Disponível em http://www.who.int/whopes/Space_Spray_products_February_2016.pdf?ua=1. 2016.

OMS - Organização Mundial da Saúde. Dengue and severe dengue. Disponível em http://www.who.int/mediacentre/factsheets/fs117/en/. Media Centre. 2017a.

OMS - Organização Mundial da Saúde. Chikungunya. Disponível em http://www.who.int/mediacentre/factsheets/fs327/en/. Media Center. 2017b.

Osanai CH, Travassos da Rosa AP, Tang AT, do Amaral RS, Passos AD, Tauil PL. Dengue outbreak in Boa Vista, Roraima. Preliminary report. Rev Inst Med Trop Sao Paulo. 1983; 25(1):53-54.

Patterson J, Sammon M, Garg M. Dengue, Zika and chikungunya: emerging arboviruses in the new world. West J Emerg Med. 2016; 17(6):671-679.

Penna MLF. Um desafio para a saúde pública brasileira: o controle do dengue. Cad Saúde Pública. 2003; 19:305-309.

Perry T, Batterham P, Daborn PJ. The biology of insecticidal activity and resistance. Insect Biochem Mol Biol. 2011; 41(7):411-422.

Powell JR, Tabachnick WJ. History of domestication and spread of *Aedes aegypti* - a review. Mem Inst Oswaldo Cruz. 2013; 108 Suppl 1:11-17.

Ranson H, Claudianos C, Ortelli F, Abgrall C, Hemingway J, Sharakhova MV, et al. Evolution of supergene families associated with insecticide resistance. Science. 2002; 4;298(5591):179-181.

Reigart J, Roberts J. Recognition and Management of Pesticide Poisonings. 5ª Ed. Environmental Protection Agency, Washington, DC. 1999.

Reisen WK, Baker RH, Sakai RK, Aziz-Javed A, Aslam Y, Siddiqui TF. Observations on mating behavior and survivorship of *Culex tritaeniorhynchus* Giles during late autumn. Southeast Asian J Trop Med Public Health. 1977; 8(4):537-545.

Reisen WK, Aslam Y, Siddiqui TF, Khan AQ. A mark-release-recapture experiment with *Culex tritaeniorhincus* Giles. Trans R Soc Trop Med Hyg. 1978; 72: 167-177.

Reisen WK, Mahmood F, Parveen T. *Anopheles culcifacies* Giles: a release recapture experiment with cohorts of known age with implications for malaria epidemiology and genetical control in Pakistan. Trans R Soc Trop Med Hyg. 1980; 74: 307-317.

Reynolds KT, Hoffmann AA. Male age, host effects and the weak expression or non-expression of cytoplasmic incompatibility in *Drosophila* strains infected by maternally transmitted *Wolbachia*. Genet Res. 2002; 80(2):79-87.

Ricci I, Cancrini G, Gabrielli S, D'Amelio S, Favi G. Searching for *Wolbachia* mosquitoes: large polymerase chain reaction survey and new identifications. J Med Entomol. 2002; 39(4):562-567.

Ridley M. Evolução. 3ª Ed. Artmed, Porto Alegre. 2006.

Ritchie SA, Montgomery BL, Hoffmann AA. Novel estimates of *Aedes aegypti* (Diptera: Culicidae) population size and adult survival based on *Wolbachia* releases. J Med Entomol. 2013; 50(3):624-631.

Ritchie SA, Townsend M, Paton CJ, Callahan AG, Hoffmann AA. Application of *w*MelPop *Wolbachia* strain to crash local populations of *Aedes aegypti*. PLoS Negl Trop Dis. 2015; 23;9(7):e0003930.

Rivero A, Vézilier J, Weill M, Read AF, Gandon S. Insecticide control of vector-borne diseases: when is insecticide resistance a problem? PLoS Pathog. 2010 Aug 5;6(8):e1001000.

Robinson MC. An epidemic of virus disease in Southern Province, Tanganyika Territory, in 1952-53. I Clinical features. Trans R Soc Trop Med Hyg. 1955; 49(1):28-32.

Rodriguez MH, Bown DN, Arredondo-Jimenez AD, Villareal C, Loyola EG, Frederickson DE. Gonotrophic cycle and survivorship of *Anopheles albimanus* in southern Mexico. J Med Entomol. 1992; 29:395-399.

Rose RI. Pesticides and public health: integrated methods of mosquito management. Emerg Infect Dis. 2001; 7:17-23.

Ross RW. The Newala epidemic. III. The virus: isolation, pathogenic properties and relationship to the epidemic. J Hyg (Lond). 1956; 54(2):177-191.

Ross PA, Endersby NM, Hoffmann AA. Costs of three *Wolbachia* infections on the survival of *Aedes aegypti* larvae under starvation conditions. PLoS Negl Trop Dis. 2016; 8;10(1):e0004320.

Roth A, Mercier A, Lepers C, Hoy D, Duituturaga S, Benyon E, et al. Concurrent outbreaks of dengue, chikungunya and Zika virus infections - an unprecedented epidemic wave of mosquito-borne viruses in the Pacific 2012-2014. Euro Surveill. 2014; 16;19(41).

Roush RT, McKenzie JA. Ecological genetics of insecticide and acaricide resistance. Annu Rev Entomol. 1987; 32:361-380.

Royle JA, Karanth KU, Gopalaswamy AM, Kumar NS. Bayesian inference in camera trapping studies for a class of spatial capture-recapture models. Ecology. 2009; 90(11):3233-3244.

Royle JA, Kéry M, Guélat J. Spatial capture-recapture models for search-encounter data. Methods Ecol Evol. 2011; 2: 602–611.

Royle JA, Dorazio RM. Parameter-expanded data augmentation for Bayesian analysis of capture-recapture models. J Ornithol. 2012; 152: 521–537.

Rozendaal JA.Vector control: methods for use by individuals and communities. Organização Mundial da Saúde, Genebra, Suiça. 1997.

Rudolph KE, Lessler J, Moloney RM, Kmush B, Cummings DA. Incubation periods of mosquito-borne viral infections: a systematic review. Am J Trop Med Hyg. 2014; 90(5):882-891.

Salazar MI, Richardson JH, Sánchez-Vargas I, Olson KE, Beaty BJ. Dengue virus type 2: replication and tropisms in orally infected *Aedes aegypti* mosquitoes. BMC Microbiol. 2007; 30;7:9.

San Martín JL, Brathwaite O, Zambrano B, Solórzano JO, Bouckenooghe A, Dayan GH, et al. The epidemiology of dengue in the americas over the last three decades: a worrisome reality. Am J Trop Med Hyg. 2010; 82(1):128-135.

Sarwar M, Salman M. Insecticides resistance in insect pests or vectors and development of novel strategies to combat its evolution. Intern Journ of Bioinfor Biomed Eng, 2015; 1(3):344-351.

Schatzmayr HG, Nogueira RM, Travassos da Rosa AP. An outbreak of dengue virus at Rio de Janeiro--1986. Mem Inst Oswaldo Cruz. 1986; 81(2):245-246.

Schechtman H, Souza MO. Costly inheritance and the persistence of insecticide resistance in *Aedes aegypti* populations. PLoS One. 2015; 1;10(5):e0123961.

Scott JG. Evolution of resistance to pyrethroid insecticides in *Musca Domestica*. Pest Manag Sci. 2016; 73(4):716-722.

Service MW. Mosquito ecology: field sampling methods. 2ª Ed. Elsevier Applied Science, London. 1993.

Sheppard PM, MacDonald WW, Tonn RJ, Grabs B. The dynamics of an adult population of *Aedes aegypti* in relation to dengue haemorrhagic fever in Bangkok. J Anim Ecol.1969; 38:661-702.

Simpson DI. Zika virus infection in man. Trans R Soc Trop Med Hyg. 1964; 58:335-338.

Sinkins SP, Curtis CF, O'Neill SL. The potential application of inherited symbiont systems to pest control. Oxford University Press, New York. 1997.

Smith LB, Kasai S, Scott JG. Pyrethroid resistance in *Aedes aegypti* and *Aedes albopictus*: Important mosquito vectors of human diseases. Pestic Biochem Physiol. 2016; 133:1-12.

Smith TJ, Lee SH, Ingles PJ, Knipple DC, Soderlund DM. The L1014F point mutation in the house fly Vssc1 sodium channel confers knockdown resistance to pyrethroids. Insect Biochem Mol Biol. 1997; 27(10):807-812.

Soderlund DM, Knipple DC. The molecular biology of knockdown resistance to pyrethroid insecticides. Insect Biochem Mol Biol. 2003; 33(6):563-577.

Soderlund DM. Pyrethroids, knockdown resistance and sodium channels. Pest Manag Sci. 2008; 64(6): 610-616.

Sougoufara S, Doucouré S, Backé Sembéne PM, Harry M, Sokhna C. Challenges for malaria vector control in sub-Saharan Africa: Resistance and behavioral adaptations in *Anopheles* populations. J Vector Borne Dis. 2017; 54(1):4-15.

Souza TM, Azeredo EL, Badolato-Corrêa J, Damasco PV, Santos C, Petitinga-Paiva F, et al. First Report of the East-Central South African Genotype of Chikungunya Virus in Rio de Janeiro, Brazil. PLoS Curr. 2017; 14;9.

Souza RP, Rocco IM, Maeda AY, Spenassatto C, Bisordi I, Suzuki A, et al. Dengue virus type 4 phylogenetics in Brazil 2011: looking beyond the veil. PLoS Negl Trop Dis. 2011; 5(12):e1439.

Stoddart JF. Comprehensive Organic Chemistry: The synthesis and reaction of organic compounds. 6ª Ed. Oxford. 1979.

Styer LM, Carey JR, Wang LM, Scott TW. Mosquitoes do senesce: departure from the paradigm of constant mortality. Am J Trop Med Hyg. 2007a; 76:111-117.

Styer LM, Minnick SL, Sun AK, Scott TW. Mortality and reproductive dynamics of *Aedes aegypti* fed human blood. Vector Borne Zoonotic Dis. 2007b; 7:86-98.

Suarez MF, Gonzales R, Morales CA. Temephos resistance to *Aedes aegypti* in Cali, Colombia. Am J Trop Med Hyg.1998; 55:257.

Suh E, Mercer DR, Dobson SL. Life-shortening *Wolbachia* infection reduces population growth of *Aedes aegypti*. Acta Trop. 2017; 172:232-239.

Sylvestre G, Gandini M, Maciel-de-Freitas R. Age-dependent effects of oral infection with dengue virus on *Aedes aegypti* feeding behavior, survival, oviposition success and fecundity. PLoS One. 2013; 8(3):e59933.

Teixeira MG, Barreto LM, Guerra Z. Epidemiologia e medidas de prevenção do dengue. Informe Epidemiológico do Sistema Único de Saúde. 1999; 8:5-33.

Teixeira L, Ferreira A, Ashburner M. The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. PLoS Biol. 2008; 23;6(12):e2.

Teixeira MG, Siqueira JB Jr, Ferreira GL, Bricks L, Joint G. Epidemiological trends of dengue disease in Brazil (2000-2010): a systematic literature search and analysis. PLoS Negl Trop Dis. 2013; 19;7(12):e2520.

Temporão JG, Penna GO, Carmo EH, Coelho GE, do Socorro Silva Azevedo R, Teixeira Nunes MR, et al. Dengue virus serotype 4, Roraima State, Brazil. Emerg Infect Dis. 2011; 17(5):938-940.

Touré YT, Dolo G, Petrarca V, Traoré SF, Bouaré M, Dao A, et al. Mark-release-recapture experiments with *Anopheles gambiae* s.l. in Banambani Village, Mali, to determine population size and structure. Med Vet Entomol. 1998; 12(1):74-83.

Tsuda Y, Komagata O, Kasai S, Hayashi T, Nihei N, Saito K, et al. A mark-release-recapture study on dispersal and flight distance of *Culex pipiens pallens* in an urban area of Japan. J Am Mosq Control Assoc. 2008 Sep;24(3):339-343.

Tun-Lin W, Kay BH, Barnes A. Understanding productivity, a key to *Aedes aegypti* surveillance. Am J Trop Med Hyg. 1995; 53(6):595-601.

Tun-Lin W, Burkot TR, Kay BH. Effects of temperature and larval diet on development rates and survival of the dengue vector *Aedes aegypti* in north Queensland, Australia. Med Vet Entomol. 2000; 14(1):31-37.

Turell MJ, Lundström JO. Effect of environmental temperature on the vector competence of *Aedes aegypti* and *Ae. taeniorhynchus* for Ockelbo virus. Am J Trop Med Hyg. 1990; 43(5):543-550.

Turelli M, Hoffmann AA. Rapid spread of an inherited incompatibility factor in California *Drosophila*. Nature. 1991; 3;353(6343):440-442.

Turelli M, Barton NH. Deploying dengue-suppressing *Wolbachia*: Robust models predict slow but effective spatial spread in *Aedes aegypti*. Theor Popul Biol. 2017; 115:45-60.

Turley AP, Zalucki MP, O'Neill SL, McGraw EA. Transinfected *Wolbachia* have minimal effects on male reproductive success in *Aedes aegypti*. Parasit Vectors. 2013; 11;6:36.

Ulrich JN, Beier JC, Devine GJ, Hugo LE. Heat sensitivity of *w*Mel *Wolbachia* during *Aedes aegypti* development. PLoS Negl Trop Dis. 2016; 26;10(7):e0004873.

van den Hurk AF, Hall-Mendelin S, Pyke AT, Frentiu FD, McElroy K, Day A, et al. Impact of *Wolbachia* on infection with chikungunya and yellow fever viruses in the mosquito vector *Aedes aegypti*. PLoS Negl Trop Dis. 2012; 6(11):e1892.

Vega-Rúa A, Lourenço-de-Oliveira R, Mousson L, Vazeille M, Fuchs S, Yébakima A, et al. Chikungunya virus transmission potential by local *Aedes* mosquitoes in the Americas and Europe. PLoS Negl Trop Dis. 2015; 20;9(5):e0003780.

Villela DA, Codeço CT, Figueiredo F, Garcia GA, Maciel-de-Freitas R, Struchiner CJ. A bayesian hierarchical model for estimation of abundance and spatial density of *Aedes aegypti*. PLoS One. 2015; 23;10(4):e0123794. (**Artigo - 4**)

Villela DAM, Garcia GA, Maciel-de-Freitas R. Novel inference models for estimation of abundance, survivorship and recruitment in mosquito populations using mark-release-recapture data. PLoS Negl Trop Dis. 2017; 26;11(6):e0005682. (**Artigo - 5)**

Walker T, Johnson PH, Moreira LA, Iturbe-Ormaetxe I, Frentiu FD, McMeniman CJ, et al. The *w*Mel *Wolbachia* strain blocks dengue and invades caged *Aedes aegypti* populations. Nature. 2011; 24;476(7361):450-453.

Ware GW. The Pesticide Book. Thomson Publications. 5ª Ed. Fresno, California. 2000.

Ware GW, Whitacre DM. The Pesticide Book. 6ª Ed. Meister Media Worldwide, Willoughby, Ohio. 2004.

Watts DM, Burke DS, Harrison BA, Whitmire RE, Nisalak A. Effect of temperature on the vector efficiency of *Aedes aegypti* for dengue 2 virus. Am J Trop Med Hyg. 1987; 36(1):143-152.

Weaver SC, Reisen WK. Present and future arboviral threats. Antiviral Res. 2010; 85(2):328-345.

Weaver SC, Forrester NL. Chikungunya: Evolutionary history and recent epidemic spread. Antiviral Res. 2015; 120:32-39.

Weill M, Malcolm C, Chandre F, Mogensen K, Berthomieu A, Marquine M, et al. The unique mutation in ace-1 giving high insecticide resistance is easily detectable in mosquitovectors. Insect Mol Biol. 2004; 13(1):1-7.

Werren JH, Zhang W, Guo LR. Evolution and phylogeny of *Wolbachia*: reproductive parasites of arthropods. Proc Biol Sci. 1995; 22;261(1360):55-63.

Werren JH. Biology of Wolbachia. Annu Rev Entomol. 1997;42:587-609.

Werren JH, Baldo L, Clark ME. *Wolbachia*: master manipulators of invertebrate biology. Nat Rev Microbiol. 2008; 6(10):741-751.

WHOPES - WHO Pesticide Evaluation Scheme. WHO specifications for pesticides used in public health. Disponível em http://www.who.int/whopes/quality/newspecif/en/. 2017.

Wilder-Smith A, Gubler DJ, Weaver SC, Monath TP, Heymann DL, Scott TW. Epidemic arboviral diseases: priorities for research and public health. Lancet Infect Dis. 2017; 17(3):e101-e106.

Wilson AJ, Morgan ER, Booth M, Norman R, Perkins SE, Hauffe HC, et al. What is a vector? Philos Trans R Soc Lond B Biol Sci. 2017; 5;372(1719).

Wing KD, Andaloro JT, McCann SF, Salgado VL. Indoxacarb and the sodium channel blocker insecticides: chemistry, physiology, and biology in insects. Compreh Mol Insect Science. Ed. Elsevier, New York. 2005.

Zabalou S, Riegler M, Theodorakopoulou M, Stauffer C, Savakis C, Bourtzis K. *Wolbachia*-induced cytoplasmic incompatibility as a means for insect pest population control. Proc Natl Acad Sci USA. 2004; 19;101(42):15042-15045.

Zammarchi L, Tappe D, Fortuna C, Remoli ME, Günther S, Venturi G, et al. Zika virus infection in a traveller returning to Europe from Brazil, March 2015. Euro Surveill. 2015; 11;20(23).

Zanluca C, Melo VC, Mosimann AL, Santos GI, Santos CN, Luz K. First report of autochthonous transmission of Zika virus in Brazil. Mem Inst Oswaldo Cruz. 2015; 110(4):569-572.

Zara AL, Santos SM, Fernandes-Oliveira ES, Carvalho RG, Coelho GE. *Aedes aegypti* control strategies: a review. Epidemiol Serv Saude. 2016; 25(2):391-404.