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Aedes aegypti continuously exposed to *Bacillus thuringiensis* svar. *israelensis* does not exhibit changes in life traits but displays increased susceptibility for Zika virus

Karine da Silva Carvalho, Duschinka Ribeiro Duarte Guedes, Mônica Maria Crespo, Maria Alice Varjal de Melo-Santos and Maria Helena Neves Lobo Silva-Filha*

Abstract

Background: *Aedes aegypti* can transmit arboviruses worldwide, and *Bacillus thuringiensis* svar. *israelensis* (Bti)-based larvicides represent an effective tool for controlling this species. The safety of Bti and lack of resistance have been widely reported; however, little is known regarding the impact of the extensive use of these larvicides on the life traits of mosquitoes. Therefore, this study investigated biological parameters, including susceptibility to arbovirus, of an *Ae. aegypti* strain (RecBti) subjected to 29 generations of exposure to Bti compared with the RecL reference strain.

Methods: The biological parameters of individuals reared under controlled conditions were compared. Also, the viral susceptibility of females not exposed to Bti during their larval stage was analysed by oral infection and followed until 14 or 21 days post-infection (dpi).

Results: RecBti individuals did not display alterations in the traits that were assessed (fecundity, fertility, pupal weight, developmental time, emergence rate, sex ratio and haematophagic capacity) compared to RecL individuals. Females from both strains were susceptible to dengue serotype 2 (DENV-2) and Zika virus (ZIKV). However, RecBti females showed significantly higher rates of ZIKV infection compared with RecL females at 7 (90% versus 68%, Chi-square: $\chi^2 = 7.27$, $df = 1$, $P = 0.006$) and 14 dpi (100% versus 87%, Chi-square: $\chi^2 = 7.69$, $df = 1$, $P = 0.005$) and for dissemination at 7 dpi (83.3% versus 36%, Fisher's exact test: $P < 0.0001$, OR = 0.11, 95% CI 0.03–0.32). Quantification of DENV-2 and ZIKV viral particles produced statistically similar results for females from both strains.

Conclusions: Prolonged exposure of *Ae. aegypti* larvae to Bti did not alter most of the evaluated biological parameters, except that RecBti females exhibited a higher vector susceptibility for ZIKV. This finding is related to a background of Bti exposure for several generations but not to a previous exposure of the tested females during the larval stage. This study highlights mosquito responses that could be associated with the chronic exposure to Bti in addition to the primary larvicidal effect elicited by this control agent.

Keywords: Fitness, DENV, Artificial infection, Infection, Vector competence

Background

Infections caused by arbovirus dengue (DENV), chikungunya (CHIKV) and Zika (ZIKV) are global public health threats, and their transmission to humans relies primarily on *Aedes aegypti* and *Aedes albopictus* mosquitoes [1,

*Correspondence: mhelena.neves@fiocruz.br
Department of Entomology, Instituto Aggeu Magalhães-Fiocruz, Recife, Pernambuco, Brazil

2]. Brazil is among the most severely affected countries and is hyperendemic for all DENV, and the introduction of CHIKV and ZIKV has amplified the burden caused by arboviruses in this country [3]. The major approach for interrupting the transmission of these diseases relies on vector control, as effective and accessible vaccines and therapeutic drugs are not available to date [4]. Controlling *Aedes* spp. has been a challenge, since these species display outstanding survival strategies that enable them to occupy, spread and establish in the environment successfully [5–8]. Currently, emerging and conventional control methods can be integrated to fight *Aedes*, and the use of larvicides remains an important intervention that can be adopted in this context [9, 10]. These include environmentally safe products with specific modes of action, such as *Bacillus thuringiensis* svar. *israelensis* (Bti)-based larvicides [11]. The active principle of these larvicides is an insecticidal crystal produced by Bti that contains four major protoxins (Cry11Aa, Cry4Aa, Cry4Ba and Cyt1Aa) that kill mosquito larvae [11]. Bti crystals act by ingestion and, after they are solubilized in the midgut, protoxins are released and processed into active toxins that interact with midgut receptors. After the toxins specifically bind to receptors, they cause pore formation and are internalized in the cells, leading to osmotic lysis and larval death [12, 13]. Bti crystals display high larvicidal activity, but they have a selective spectrum, since they target only certain Diptera species, including those from the *Aedes* genus [11]. The three-domain Cry-type toxins from Bti (Cry11Aa, Cry4Ba, and Cry4Aa) can specifically bind to different membrane-bound receptors, such as cadherins, aminopeptidases and alkaline phosphatases that have been identified in *Ae. aegypti* larvae [12, 14, 15]. On the other hand, Cyt1Aa is a cytolytic toxin with the intrinsic ability to insert itself into the cell membrane and form pores [16, 17], and this toxin plays an important role in the mode of action of the Bti crystal. Cyt1Aa can act as a receptor for the other Cry toxins, binding and promoting their oligomerization, which enables them to bind to their midgut receptors with a higher affinity and form pores on the cell membrane [18–20]. The ability of Cyt1Aa to synergize with Cry toxins considerably reduces the selection of resistance caused by receptor alteration, which is the most common resistance mechanism reported for *Bacillus thuringiensis* toxins [21, 22].

Therefore, Bti is one of the most effective recommended larvicides for controlling *Aedes* spp. worldwide in view of its complex and safe mode of action, having been used over the last 4 decades [5, 7, 23–26]. Due to safety issues, only a few larvicides, including Bti larvicides, remain authorized for mosquito control according to the legislation of the European Union [27, 28]. In view of the increase in Bti utilization, the impact of the continuous exposure of mosquito populations needs to be assessed, particularly considering the scenario of endemic countries that conduct vector control actions throughout the year [29]. To date, most investigations have focused on resistance and environmental safety. These studies have indicated the absence of resistance to the Bti crystal [25, 26, 29–33] and showed its safety over almost those decades of use [34–36]. Notably, resistance to individual toxins has been reported using laboratory selection procedures but not resistance to the whole crystal, which is the active principle of Bti-based products [37–40].

Another important aspect, the influence of Bti exposure on the life traits of target insects, has only rarely been investigated. Most studies have investigated the biological cost associated with mosquitoes that are resistant to chemical insecticides [41–43] and the vector competence that could affect their capacity to become infected and transmit pathogens to humans [44–46]. Our hypothesis is that, although continuous exposure to Bti did not elicit the selection of resistance, other parameters of vector biology could be modified in response to this condition and should be investigated to understand the consequences of the chronic exposure to this larvicide. The major goal of this study was to investigate whether an *Ae. aegypti* strain continuously exposed to Bti for 29 generations displays changes in life traits, such as fecundity, fertility, pupal weight, developmental time, emergence rate, sex ratio, haematophagic capacity and vector susceptibility for DENV and ZIKV. This local strain, RecBti, was first established in our laboratory to evaluate the selection of Bti resistance after exposing larvae to Bti for 30 generations under controlled conditions [33]. No resistance to Bti was detected, and RecBti larvae were also susceptible to other insecticidal compounds, such as temephos and diflubenzuron. Similarly, the activity of the detoxifying enzymes involved in the metabolism of insecticidal compounds was unchanged. Taken together, these findings indicate that Bti use is compatible with other classes of insecticides with a low risk of resistance to Bti itself and of cross-resistance to other control agents [33].

Methods

Aedes aegypti strains

Two *Ae. aegypti* strains were used in this study, RecL and RecBti, which were maintained at the insectary of the Instituto Aggeu Magalhães (IAM)-FIOCRUZ at 26 ± 1 °C, 70% humidity and a 14 h:10 h light:dark photoperiod. Larvae were reared in dechlorinated tap water and fed cat food (Friskies®). Adults were fed a sucrose solution (10%), and females were also artificially fed defibrinated rabbit blood once per week. RecL and RecBti strains were founded using larvae from Recife city;

therefore, they share the same geographic origin. RecL is a local reference strain that has been maintained in the insectary without contact with any mosquito control agent since 1996 [47]. The test strain RecBti was established in 2011, and larvae from each generation have been continuously exposed to a Bti-based larvicide for more than 30 generations and selection procedures were described in our previous study [33]. The selection pressure imposed on the large samples of individuals from the RecBti strain, originated from Recife as the RecL strain, was strong and continuous using a commercial Bti-based product containing 37.4% Bti crystals/spores as active ingredient. Briefly, around 9,500 third-instar larvae from every generation were treated with Bti, and around 74% mortality was detected during the pre-imaginal phase. At least 2,000 adults that survived the Bti exposure per generation were used to compose the next parental generation. Bti exposure was carried during 30 generations and involved > 290,000 larvae, as fully described by Carvalho et al. [33]. Susceptibility bioassays performed using larvae from generations F_5 to F_{30} demonstrated they were susceptible to Bti, despite continuous exposure to this agent, as shown by resistance ratios (RR at LC_{50}): 1.6 (F_1), 2.8 (F_5), 2.3 (F_{10}), 1.5 (F_{15}), 1.1 (F_{20}), 0.9 (F_{25}) and 1.5 at F_{30} [33]. Individuals of the F_{30} generation were employed for this study and the last Bti exposure was performed on F_{29} third-instar larvae. Therefore, F_{30} larvae were not treated with Bti before adult emergence because the goal of this study was to record the chronic response of the colony and not the induced response to a recent Bti exposure during the immature stage of the individuals analysed.

Mosquito maintenance for biological assessment

To assess such biological parameters as fecundity, fertility, pupal weight, developmental time, emergence rate, sex ratio and haematophagic capacity, individuals from both strains were kept in the insectary under controlled rearing parameters, as described in this section. First, filter papers containing stored eggs were subject to induced eclosion by setting them in a recipient with grass infusion (6 g/l) for 24 h. Samples of 100 first-instar larvae, collected within 24 h after eclosion, were transferred to plastic recipients (20 cm length × 16 cm width × 8 cm deep, 3-l capacity) with 1 l tap water and fed 350 mg of cat food (Friskas®) provided on days 0 (100 mg), 4 (150 mg) and 5 (100 mg) during the assays. After emergence, samples of 20 females and 20 males (1:1 ratio) were set in a plastic container (12 cm height × 10 cm diameter, 2.3-l capacity) and fed sucrose solution (10%) *ad libitum*. Five days post-emergence, a single meal of defibrinated rabbit blood was offered to females using an artificial feeding system for 1 h at 37 °C. This system consisted of a blood sample (8 ml) set in a Petri dish (6 cm height × 1.5 cm diameter) covered by a double layer of Parafilm® membrane, placed on each mosquito cage, and kept at 37 °C using heat packs. Immediately after the blood meal, only engorged females, selected by visual abdomen inspection, were transferred to cages and fed sucrose solution (10%) *ad libitum*. Three days after the blood meal, two recipients each with water and two sections of filter paper (7 cm length × 5 cm width) were placed in cages as a substrate for female oviposition for two days. After that step, filter papers containing eggs were collected and allowed to dry at insectary room temperature to complete embryonic development. After four days of storage, eggs were counted, subjected to eclosion and reared, as described above.

Assessment of life traits

The life traits of RecBti (F_{30}) and RecL individuals reared according to the procedures described in the previous section were compared. For each parameter analysed, three replicates per colony were tested, and three independent experiments were carried out. Fecundity was determined as the total number of eggs recorded on the filter papers available for the oviposition of a group of 20 engorged females. Fertility was recorded as the total number of first-instar larvae that hatched from a sample of 2,551 eggs that were subjected to the protocol of eclosion for 24 h. Pupal weight was determined by measuring pools of 25 living pupae of each sex. For this purpose, pupae were collected until 14 h after the moult, water was drained, and samples were weighed and immediately placed back in their rearing containers, where they were kept until emergence. Adults obtained from these samples were used to evaluate adult rate, immature developmental time, sex ratio and haematophagic capacity, as described below. The adult rate was determined from a sample of 900 first-instar larvae that reached emergence within 15 days. The sex ratio among these males and females was also recorded. The haematophagic capacity was determined by the percentage of engorged females after the artificial blood meal from the pools of 20 females per cage.

Viral strains and sample preparation for vector susceptibility assays

The susceptibility of RecBti females to dengue serotype 2 (DENV-2) and Zika (ZIKV) viruses was investigated and compared to RecL females based on three independent artificial blood-feeding assays. The viral stocks used in this study were generously provided by the Laboratory of Virology and Experimental Therapy from IAM-Fiocruz. DENV-2 strain 3808/BR-PE and ZIKV strain 243/BR-PE were isolated from patients during dengue [48] and Zika [49] outbreaks in Recife in 1995 and 2015, respectively.

These strains are able to infect and disseminate in RecL females as previously described [50, 51]. Viral stocks were then produced in C6/36 cells after five passages for DENV-2 and in VERO cells for ZIKV after four passages, and both were stored at −80 °C until use. Prior to artificial blood-feeding experiments, DENV-2 viral stock was grown in C6/36 cells at a multiplicity of infection of 0.1 for 5–6 days, and ZIKV viral stock was grown in VERO cells at a multiplicity of infection of 0.5 for 4–5 days, when the cytopathic effects were visualized, which allowed the use of those samples for the infection assays [50, 51]. Noninfected cell cultures were maintained under the same conditions. The mean virus titration for the three assays based on the Tissue culture infection dose at 50% ($TCID_{50}$) for DENV-2 was 4.19×10^5 $TCID_{50}$/ml while the determination by assay plaque for ZIKV was 1.37×10^6 plaque-forming units, (PFU)/ml.

Oral artificial infection blood-feeding procedure

Each experiment was carried out using nulliparous 7- to 10-day-old females per strain starved for 24 h. A sample between 110 and 130 females from each strain was set in a cage (12 cm height × 10 cm diameter, 2.3-l capacity) and fed defibrinated rabbit blood containing cultures infected with DENV-2 or ZIKV. Another sample of 30 females per strain, set in another cage, was fed defibrinated rabbit blood containing uninfected cell cultures and used as the untreated control group. To prepare the blood meal, cell culture samples were subjected to a single cycle of freezing and thawing to lyse the cells releasing viral particles, allowing the use of comparable and reproducible titles [52]. To this end, cell culture flasks were kept at −80 °C for only 11 min, thawed in running water and then mixed with defibrinated rabbit blood in a 1:1 ratio. These samples were immediately used for feeding all experimental groups simultaneously, and the virus suspensions were also quantified, as described in the previous section. After feeding, only engorged females, selected by visual abdomen inspection, were transferred to another cage. These females were maintained under standard conditions for 21 days for DENV-2 and 14 days for ZIKV experiments, as previously described [50, 51].

RNA extraction and virus detection

RNA was extracted from each head and body part from individual females per experimental point ($n = 20$ females/point), and each sample was analysed in duplicate [50, 51]. For DENV-2 infection assays, females were collected just after feeding (0) and at 7, 14 and 21 day(s) post-infection (dpi) [51]. For ZIKV assays, females were collected at 0, 3, 7 and 14 dpi [50, 53]. Females fed uninfected blood were collected just after the feeding procedure ($n = 5$). Females were collected and killed in 70% alcohol at 2 °C for 2 s. Samples were washed twice in ultrapure water at 2 °C. Next, the body and head (with attached salivary glands) from each female were carefully dissected on a cold plate (2 °C). The dissected parts were immediately placed in separate DNase/RNase-free microtubes at 2 °C with 300 µl of mosquito diluent containing 0.1 M phosphate-buffered saline-PBS (Na_2HPO_4 7.7 mM, K_2HPO_4 1.1 mM, KCl 2.7 mM, NaCl 137 mM, pH 7.4) and supplemented with 10% foetal bovine serum (FBS) and 1% Fungizone® (Gibco #15290-018) [54]. Samples were stored at −80 °C until RNA extraction. Total RNA extraction of the tissue homogenate (100 µl) was performed using Trizol® following the manufacturer's protocol (Invitrogen #15596-026) with modifications as described in Guedes et al. [50]. Samples were further treated with Turbo DNase® (Ambion #AM2239) to prevent DNA contamination and stored at −80 °C. Virus detection and quantification in the samples were performed by quantitative real-time polymerase chain reaction (RT-qPCR) using an ABI Prism 7500 SDS Real-Time system® (Applied BioSystems). RT-qPCR assays for DENV-2 detection were performed using a SYBR Green RT-PCR Kit (QIAGEN #204245) according to previous protocols. The reaction contained the RNA sample (5 µl with an average concentration of 180 ng/µl), SYBR Green Master® mix 1X (10 µl), primers (0.2 µM) based on Kong et al. [55] (Additional file 1: Table S1), reverse transcriptase (0.2 µl) and ultrapure water for a 20-µl final volume. Reaction cycling conditions were as follows: 50 °C for 30 min; 95 °C for 15 min to activate Taq; 40 cycles of 94 °C for 15 s, 58 °C for 30 s and 72 °C for 30 s [51]. For ZIKV, those assays were performed using a QuantiNova Probe RT-PCR kit (QIAGEN #208352), and the reaction mix contained the following: the RNA sample (5 µl with an average concentration of 180 ng/µl), QuantiNova Probe RT-PCR Master Mix (2X, 20 µl), QuantiNova Probe RT Mix (0.2 µl), ROX Reference Dye (0.1 µl) and primers [56] (100 µM) (Additional file 1: Table S1) in a 20-µl final volume. RT-qPCR cycling included a single cycle of reverse transcription for 15 min at 45 °C followed by 5 min at 95 °C and then 45 cycles of 5 s at 95 °C and 45 s at 60 °C [50]. The viral RNA quantification of samples used for the assays was determined by absolute quantification using cycle threshold (Ct) values from a standard curve of known concentrations using a tenfold dilution (10^{-2} to 10^{-6}) of purified viral transcripts included in each PCR plate [55]. Positive control samples were RNA extracted from infected culture supernatant, and negative control samples were RNA extracted from uninfected culture supernatant and samples of RT-qPCR reaction mix without RNA. Samples with a melting curve for the specificity of the amplified products (~78.6 °C) were considered positive for DENV-2 RNA. Positive

samples for ZIKV RNA were those with a Ct value ≤ 38.5. The amount of DENV-2 and ZIKV viral RNA in each sample was calculated based on the Ct values from the standard curve of the viral RNA included in each PCR plate.

Statistical analysis

Life traits from the two strains were statistically analysed by Student's t-test, with $P<0.05$ being considered significant. For virus susceptibility assays, two parameters were analysed based on the positive samples for the presence of virus RNA. The infection rate (IR) was calculated by the number of positive body samples divided by the total number of mosquitoes analysed. The disseminated infection rate (DIR) is the proportion of infected head samples considering the total number of infected body samples [57]. For statistical analysis, the Chi-square and Fisher's test were used to evaluate the IR and DIR between the two strains tested. To compare the number of RNA viral copies, the Kruskal-Wallis and Mann-Whitney tests were used, considering a *P*-value < 0.05 to be significant. The percentage of females blood-fed and non-fed of cells with uninfected or infected cultures were statistically analysed by Student's t-test, with $P<0.05$ being considered to be significant.

Results

Life traits

To investigate whether the continuous exposure of *Ae. aegypti* larvae to Bti larvicide for 29 generations affects their biological performance, seven life traits from RecBti individuals were assessed and compared to those of the RecL reference strain (Table 1). The fecundity of RecBti females, based on > 8,988 eggs from a pool of 20 females, showed a similar average of 93.2 eggs/female compared with 86.5 eggs/female from RecL females ($t_{(4)}=2.77$, $P=0.432$). A mean of 90.6% of eggs produced by RecBti females was viable, which was statistically similar to those of RecL females and indicated successful insemination. The pupal weight of a pool of 25 RecBti specimens weighed 69.1 mg and 122.2 mg on average for male and female pools, respectively, and similar measurements were found for RecL pupal pools. Considering the time of development of first-instar larvae to the adult phase, we found that emergence took an average of 10.4 and 11.1 days for RecBti and RecL individuals, respectively, which were statistically similar ($t_{(4)}=2.77$, $P=0.093$). From a sample of individuals analysed, the adult rate was notably high (96–97%); therefore, most first-instar larvae from both strains achieved emergence. The sex ratio recorded using samples of > 800 adults per strain showed that an equal proportion of males and females (1.1: 0.9) was produced by both strains. The haematophagic capacity of RecBti females was lower (80%) than that of RecL (89.2%) females, but this reduction was not significantly different ($t_{(4)}=2.77$, $P=0.170$). The rates of blood-fed females from both mosquito strains shown in the next sections are in keeping with these findings, except for females blood-fed and non-fed from assays DENV-2 with cell culture uninfected ($t_{(4)}=2.77$, $P=0.033$) (Additional file 2: Table S2). The parameters analysed in this section indicate that RecBti individuals exhibited characteristics similar to those of individuals from a reference colony.

Mosquito susceptibility to DENV-2

To investigate whether exposure to Bti would have an impact on the susceptibility of the RecBti strain to DENV-2, blood-fed females were subjected to viral detection in their bodies (IR) and heads (DIR) by RT-qPCR. Overall, a mean of 83.5% blood-fed females was

Table 1 Life traits of *Aedes aegypti* from RecBti and RecL strains

Parameters	RecBti		RecL		Student's t-test[a]
	n	Mean ± SD	*n*	Mean ± SD	
Fecundity (mean no. eggs/female)	8988[b]	93.2 ± 3.3	8463[b]	86.5 ± 7.6	$t_{(4)}=2.77$, $P=0.43$
Fertility (% first-instar larvae)	2551	90.6 ± 2.8	2526	89.9 ± 3.7	$t_{(4)}=2.77$, $P=0.79$
Pupal weight (mg), per pool of 25 males	16	69.1 ± 3.4	16	67.3 ± 3.9	$t_{(4)}=2.77$, $P=0.15$
Pupal weight (mg), per pool of 25 females	13	122.2 ± 7.4	12	121 ± 4.5	$t_{(4)}=2.77$, $P=0.61$
Development time (days)[c]	864	10.4 ± 0.5	873	11.1 ± 1.1	$t_{(4)}=2.77$, $P=0.09$
Adult rate (%)	900	96 ± 2.2	900	97 ± 2.1	$t_{(4)}=2.77$, $P=0.77$
Sex ratio (male:female)	864	1.1:0.9 ± 0.1	873	1.1:0.9	$t_{(4)}=2.77$, $P=0.74$
Haematophagic rate (% blood-fed)	120	80 ± 12.6	120	89.2 ± 8.0	$t_{(4)}=2.77$, $P=0.17$

[a] For $P \geq 0.05$, the mean of each parameter was not significantly different according to Student's t-test

[b] Number of 8988 and 8463 eggs laid by 96 and 108 blood-fed females, respectively

[c] Period from first-instar larvae to emergence

recorded, considering both strains and blood conditions analysed (Additional file 2: Table S2). The mortality among the blood-fed females recorded from the experimental groups during all assays was < 4% (Additional file 2: Table S2). In females fed uninfected blood DENV-2 was not detected. In females fed infected blood and collected immediately after the oral infection, between 80 and 100% were positive for DENV-2 for both strains, proving that females ingested the infected blood (Additional file 3: Table S3). At the other time points, 180 RNA samples from females of each strain derived from three independent assays were analysed to assess the presence of virus (Additional file 3: Table S3). The statistical analysis of data from those three assays performed (Additional file 3: Table S3) showed no differences; next, they were combined to represent the infection and dissemination profiles of these strains (Fig. 1, Additional file 4: Table S4). DENV-2 was found in body samples from both strains at each time point assessed (7, 14 and 21 dpi), with infection rates that were 35, 36.7 and 53.3% for RecBti against 36.7, 40 and 45.0% for RecL samples, which did not differ significantly (Chi-square: $\chi^2=0$, $df=1$, $P=1.000$, $\chi^2=0.03$, $df=1$, $P=0.851$ and $\chi^2=0.53$, $df=1$, $P=0.465$, respectively) (Fig. 1a, Additional file 4: Table S4). DENV-2 was also detected in head samples from females whose body samples were positive from both strains and at all time points analysed, demonstrating virus dissemination in that tissue. The dissemination increased over time, rising from 27% to > 70% positive samples, for both strains (Fig. 1b). The dissemination in RecBti after 7 dpi (41%) and 14 dpi (82.6%) was higher than that recorded for RecL, which was 27.3% and 66.7%, respectively; however, these values were statistically similar (Fisher's exact test: $P=0.511$, OR = 0.62, 95% CI 0.13–2.92; $P=0.317$, OR = 0.43, 95% CI 0.07–1.96, respectively) (Fig. 1b, Additional file 4: Table S4).

Viral quantification of DENV-2 in females samples varied from 3.6×10^8 to 2.7×10^{13} $\log_{10}$ RNA copies. The quantification of viral copies in the body (Fig. 2a) and head (Fig. 2b) was similar between RecBti and RecL females, which showed variability within samples among time points. DENV-2 copies increased particularly in head samples from 7 to 14 dpi for both strains (Fig. 2b). Nevertheless, no significant differences regarding the quantification of viral copies between strains were observed at any time point. Datasets from these assays showed that females of both strains were susceptible to DENV-2, and although an increased dissemination in RecBti females was recorded at two time points, these results were statistically similar.

Mosquito susceptibility to ZIKV

For ZIKV infection assays, a mean of 89.5% engorged females from both strains and conditions was recorded (Additional file 2: Table S2). Samples from females engorged with uninfected blood were negative for the presence of ZIKV, as expected. The mortality in all experimental groups was negligible (Additional file 2: Table S2). The presence of ZIKV RNA copies assessed immediately after oral infection was 100% in samples of both strains (Additional file 3: Table S3). Data from the three ZIKV assays performed (Additional file 3: Table S3) were combined for this analysis (Fig. 3, Additional file 4: Table S4). The data showed that both strains were more

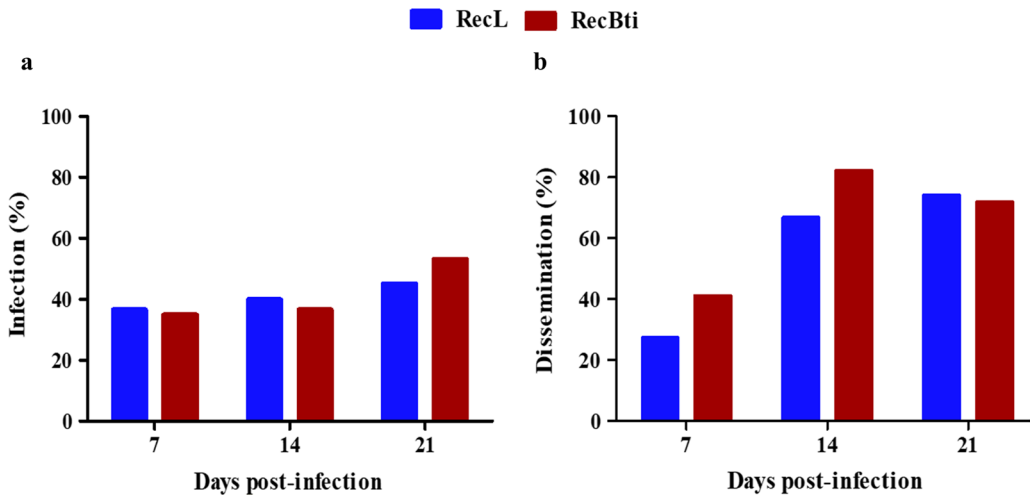

Fig. 1 Detection of DENV-2 RNA in *Aedes aegypti* females from RecBti and RecL strains. **a** Infection rate. **b** Dissemination rate. Each experimental point was based on a sample of 60 individuals from three independent assays. Each column represents positives samples in absolute numbers. According to Chi-square and Fisher's exact tests, the percentage between mosquito strains is not significantly different for $P \geq 0.05$

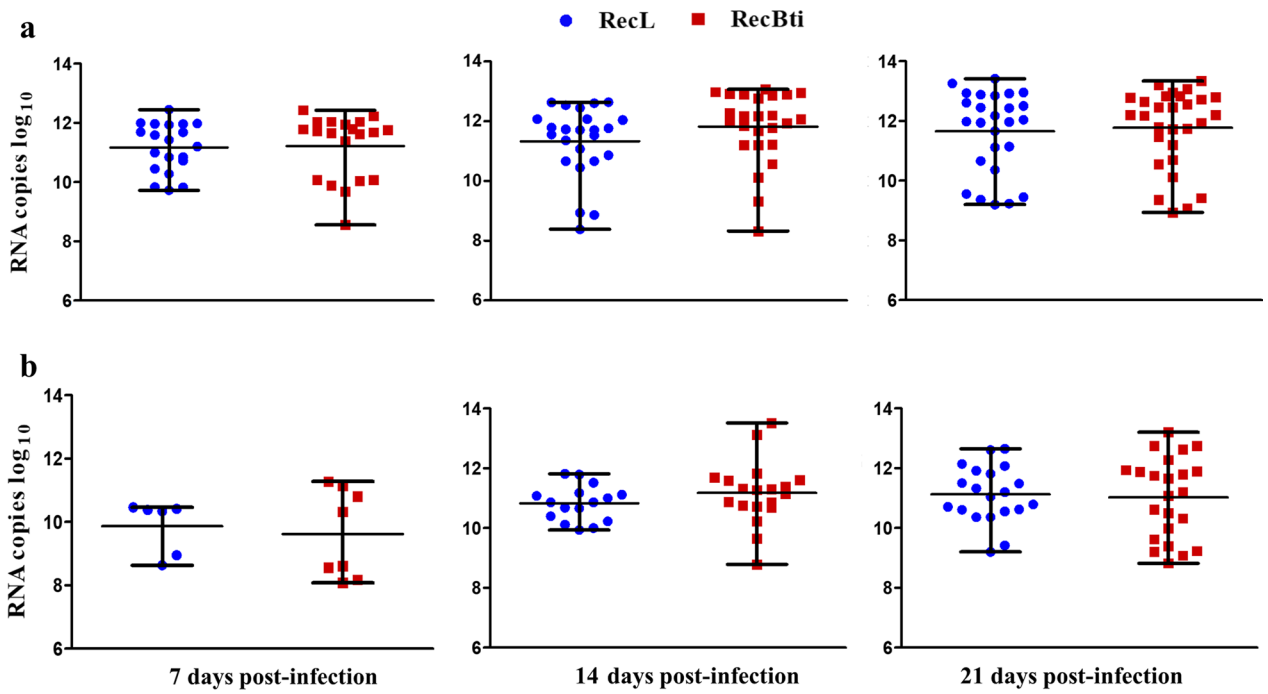

Fig. 2 Quantitation of DENV-2 RNA in *Aedes aegypti* females from RecBti and RecL strains. **a** Viral RNA in body samples. **b** Viral RNA in head samples. Experimental points represent positive individuals from three independent assays. According Kruskal-Wallis H-test, the percentage between mosquito strains is not significantly different for $P > 0.05$

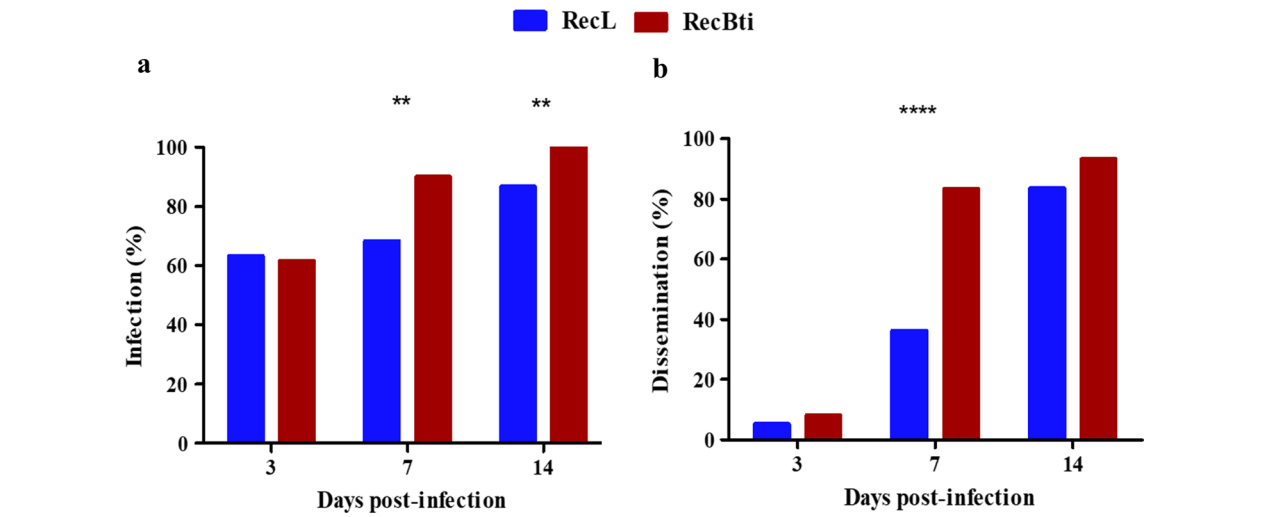

Fig. 3 Detection of ZIKV RNA in *Aedes aegypti* females from RecBti and RecL strains. **a** Infection rate. **b** Dissemination rate. Each experimental point was based on a sample of 60 individuals analysed from three independent assays. Each column represents positive samples in absolute numbers. Asterisks indicate significant differences between values (**$P \leq 0.006$; ****$P < 0.0001$), according Chi-square and Fisher's exact tests

susceptible to infection using ZIKV than the DENV-2 samples tested in this study, since approximately 60% of body samples were infected at 3 dpi. The comparison of ZIKV infection between the *Ae. aegypti* strains did not show a significant difference at this time point (Fig. 3a). Infection rates after 7 and 14 dpi reached 90–100% for RecBti and were significantly higher (Chi-square: $\chi^2 = 7.27$, $df = 1$, $P = 0.006$ and $\chi^2 = 7.69$, $df = 1$, $P = 0.005$,

respectively) than the 68.3% and 86.7% detected for RecL (Fig. 3a, Additional file 4: Table S4). ZIKV dissemination to head samples was more pronounced at 7 and 14 dpi (Fig. 3b). An increasing dissemination pattern throughout the time points was observed for RecBti (8.1–93.3%) and RecL (5.3–86.3%) samples, which was statistically higher for RecBti at 7 dpi (Fisher's exact test: $P<0.0001$, OR = 0.11, 95% CI 0.03–0.32) (Fig. 3b, Additional file 4: Table S4).

The quantification of ZIKV RNA determined in head and body samples from females that were found to be positive for the virus showed a number of RNA copies ranging from 1.8×10^{7} to 3.5×10^{14} $\log_{10}$ (Fig. 4). The number of RNA copies in the bodies of RecL and RecBti increased gradually over time, but no significant difference between strains was detected (Fig. 4a). In head samples, RNA copies increased primarily between 7 and 14 dpi and remained similar between the RecBti and RecL strains (Fig. 4b). Overall, RecBti females exhibited higher infection and dissemination rates for ZIKV than did RecL females, although the viral quantification in the positive samples from both strains was similar.

Discussion

The prolonged exposure of *Ae. aegypti* larvae to Bti over 29 generations had no impact on the life traits analysed from immature and adult phases, and this finding is compatible with the susceptibility status of this strain to Bti [33]. It is worth noting that RecBti strain was subjected to strong and continuous exposure of all larvae from each generation to the Bti crystals [33]. Therefore, the lack of resistance was not due to a low selection pressure. Biological costs have often been associated with significant levels of resistance to chemical insecticides due to the requirement of allocating energetic resources to ensure this phenotype [58–60]. However, the reduction in insect fitness associated with resistance or exposure to bacterial-based larvicides warrants further study, since contrasting results have been reported for *Culex quinquefasciatus* strains with a high resistance ratio (RR_{50} > 5,000-fold) to *Lysinibacillus sphaericus*-based larvicides, which exhibit only discrete alterations or no biological costs [61, 62]. An *Ae. aegypti* strain exposed to Bti for 22 generations without resistance to the Bti crystal but with resistance to individual Bti toxins (Cry4Aa $RR_{50}=35$, Cry4Ba $RR_{50}=11$) showed a reduction in fertility rates, larval viability and increased larval development time, while adult size, sex ratio, hatching time, longevity and survival were not changed [63]. Similarly,

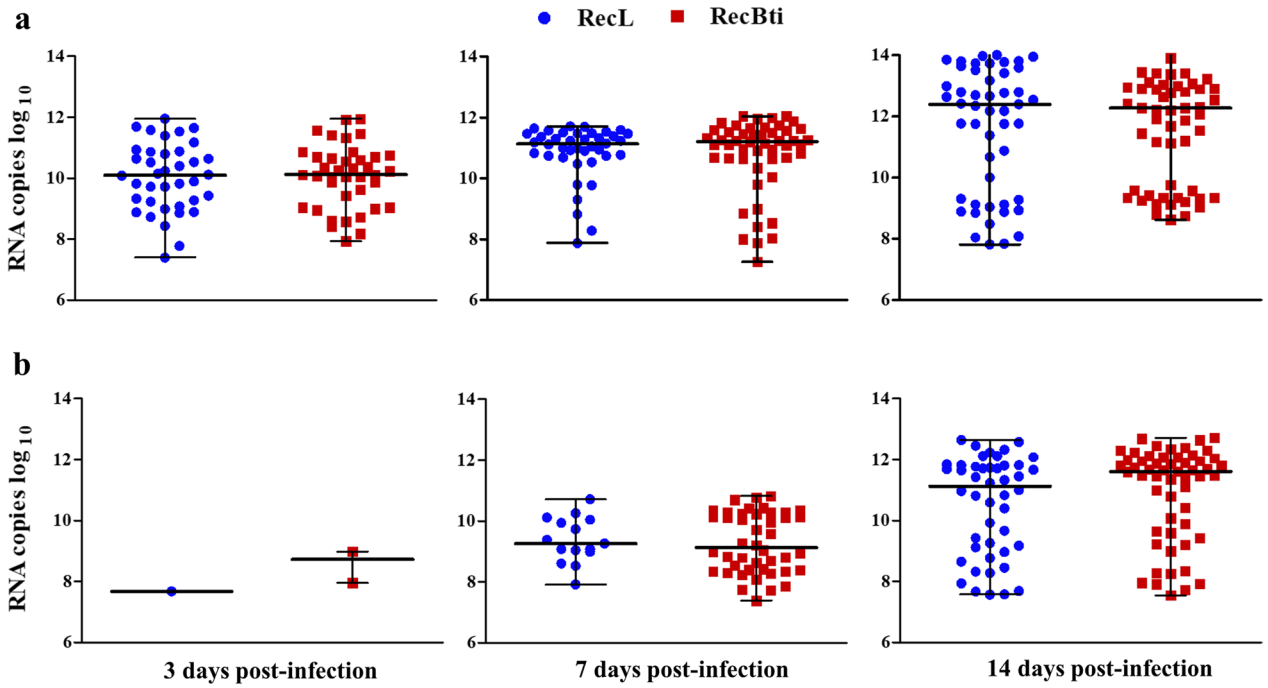

Fig. 4 Quantitation of ZIKV RNA in *Aedes aegypti* females from RecBti and RecL strains. **a** Viral RNA in body samples. **b** Viral RNA in head samples. Experimental points represent positive individuals from three independent assays. The percentage between mosquito strains is not significantly different, for $P>0.05$, according Kruskal-Wallis H-test

a *Culex pipiens* strain exposed to Bti for 20 generations and still susceptible to this agent ($RR_{50}=2.7$) showed a reduction in its fertility rate, while its longevity and time of blood-meal digestion were not altered [64]. Other studies that investigated the effects of sublethal doses of Bti on adult traits from susceptible strains of *Ae. aegypti* and *Anopheles coluzzii,* have noted both advantages and reductions in different biological parameters evaluated [65–67].

Differences recorded among those studies may be attributable to variations in the genetic background of mosquito species and viral strains, besides selection and rearing procedures. The assessment of insect fitness in response to exposure or resistance to *B. thuringiensis* toxins, in general, can be based on a large number of parameters [68]; in addition, variations in the adopted methodologies can lead to different results [43]. Attention should also be directed to certain specific biological mosquito features, such as protandry, that require suitable conditions for enabling the eclosion of larvae that will develop into males and females and their emergence [69]. Optimal conditions for immature development and adult maintenance are also critical for mosquito development. Therefore, under the controlled conditions of this study, *Ae. aegypti* from the RecBti strain did not exhibit changes in a number of major life traits analysed; however, other biological parameters might be affected, such as vector competence patterns.

Vector competence is the intrinsic ability of an insect to be infected with a pathogen and transmit it to another host. Few studies have investigated this feature in mosquito strains exposed to Bti, particularly after a long-term exposure period. In our study, *Ae. aegypti* RecBti females were determined to be susceptible to DENV-2 and ZIKV infection, as shown for the RecL reference strain previously assessed [50, 51]. Females from both strains were more susceptible to ZIKV than DENV, and this greater permissiveness of *Aedes* sp. to ZIKV agrees with the findings of other studies [50, 51, 70–72]. The comparison between these *Ae. aegypti* strains showed that RecBti females had significantly higher infection (at 7 and 14 dpi) and dissemination rates (at 7 dpi) for ZIKV than did the reference RecL strain at some time points. Notably, an increased dissemination in RecBti females was also observed for DENV-2, but this parameter was not statistically different that of from RecL females. Quantification of viral copies was similar for both arboviruses in the mosquito strains; therefore, the increased susceptibility to ZIKV was related to the greater infection and dissemination rates only [51, 53, 73, 74].

To the best of our knowledge, our study is the first to report the effect of chronic exposure of *Ae. aegypti* to Bti during their larval phase (29 preceding generations) on the susceptibility of females for ZIKV. Most studies have investigated the arbovirus susceptibility of females exposed to sublethal doses of Bti during the larval phase but without a history of previous Bti exposure. Moltini-Conclois et al. [75] assessed the susceptibility of *Ae. aegypti* to DENV-1 and CHIKV in females from a susceptible strain, a Cry4Aa-resistant strain ($RR=1018$) and a composite Bti-selected strain (susceptible to Bti with variable resistance from 5- to 14-fold to individual toxins). No alterations were observed for CHIKV, and enhanced susceptibility for DENV-1 was detected for Cry4A-resistant and Bti-selected females treated with sublethal Bti doses, similar to larvae. However, this effect was not observed in females that were not treated during their larval phase [75]. This finding suggests that increased DENV-1 susceptibility could be related to recent Bti larval exposure rather than to the previous status of Bti susceptibility of the strains tested. Another study showed no changes in DENV-1 susceptibility in *Ae. aegypti* females exposed as larvae to sublethal Bti doses [67]. Carlson et al. [76] investigated the carry-over effect of *Ae. aegypti* on susceptibility to ZIKV and DENV after larval and/or adult exposure to *Enterobacter ludwiggi* and to a *B. thuringiensis* (ATCC #35646) crystal-producing strain that was not specified as Bti. Significant alterations were seen in females exposed to *E. ludwiggi* during either larval (lower DENV-2 infection) or adult phases (higher ZIKV infection). Females originating from *B. thuringiensis*-treated larvae exhibited no alteration. Contrary to other published findings, our study showed an increased vector susceptibility to ZIKV not associated with an induced response related to the Bti exposure of tested individuals during their larval phase but rather a constitutive profile associated with the background of bacterial exposure during the 29 preceding generations.

The enhanced viral susceptibility of RecBti females might be further investigated, considering the role of the immune system and microbiota in modulating such responses, as reviewed by Caragata et al. [77], and few studies have investigated the role played by Bti in this process. An example was an *Ae. aegypti* strain exposed for 18 generations ($RR_{50}=2$) that exhibited reduced expression of genes from the Toll signalling pathway and genes coding antimicrobial peptides [78]. The Toll pathway is widely known in the defence of *Ae. aegypti* to DENV-2 [79] and ZIKV [80], as well as JAK/STAT [81], since they can activate effector molecules that limit viral spread. *Aedes aegypti* antimicrobial peptides, such as defensins and cecropins, with antiviral action have also been reported [82]. The microbiota in the mosquito larval environment and in the larval midgut [74, 83] can provoke changes in adult traits, including vector competence [74, 84]. Therefore, Bti, as a

bacterial pathogen present in the aquatic environment and ingested by mosquito larvae [11], might play an important role in these microbiota. Indeed, *Ae. aegypti* larvae from a laboratory-susceptible strain exposed to Bti for 25 h exhibited a reduction in the diversity of bacteria [85]. As previously described, the RecBti and RecL strains compared in our study derived from the same geographic area; they were maintained under identical and controlled conditions of insectary, except for the strong and continuous condition of Bti exposure imposed to the RecBti larvae. Although the reduced susceptibility to the arbovirus tested was found to be associated to this specific condition, those strains were not established in parallel; therefore, it is not possible to discard other factors that could have an influence on those findings.

It is important to note that the association of larval exposure to Bti and a higher susceptibility of *Ae. aegypti* females for the virus investigated in our study cannot be extrapolated to the epidemiological level. This finding is observed because the process of virus transmission to humans is complex and depends on multiple factors. Some of these properties are very powerful, such as mosquito density and longevity [86], features of viral strains and the susceptibility status of the human population, which might overcome the consequences of reduced or increased vector competence. Previous investigations showed that the status of mosquito susceptibility, per se, displays a wide range of variations among populations [53, 57, 87–89] in addition to other conditions that also modulate arbovirus transmission.

Conclusions

Our studies show that the RecBti *Ae. aegypti* strain exposed to Bti for 29 preceding generations exhibited no altered life traits, but females showed an increase in the susceptibility status to ZIKV, associated with a greater dissemination. This feature was not induced by larval exposure of tested females, as shown by other studies, but it was rather a response associated with long-term and continuous exposure to Bti. This work paves the way for further research investigating other mechanisms and phenotypes associated with chronic exposure to this control agent, which is of strategic importance for its rational utilization.

Abbreviations

DENV: Dengue virus; CHIKV: Chikungunya virus; ZIKV: Zika virus; Bti: *Bacillus thuringiensis* svar. *israelensis*; RR: Resistance ratio; LC: Lethal concentration; DENV-2: Dengue virus serotype 2; RNA: Ribonucleic acid; Ct: Cycle threshold; dpi: Day(s) post-infection; $TCID_{50}$: Tissue culture infection dose at 50%; PFU: Plaque-forming unit; RT-qPCR: Real-time quantitative polymerase chain reaction; IR: Infection rate; DIR: Disseminated infection rate.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13071-021-04880-6.

Additional file 1: Table S1. Primers used for the detection and quantitation of DENV-2 and ZIKV.

Additional file 2: Table S2. Summary of the artificial blood-meal assays offered to *Aedes aegypti* females from RecBti and RecL strains using uninfected and infected DENV-2 and ZIKV samples.

Additional file 3: Table S3. Infection and dissemination rates of DENV-2 and ZIKV in *Aedes aegypti* females from RecBti and RecL strains. Data from three independent assays.

Additional file 4: Table S4. Infection and dissemination rates of DENV-2 and ZIKV in *Aedes aegypti* females from RecBti and RecL strains. Combined data from three independent assays (see Additional file 3: Table S3).

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Authors' contributions

MHNLSF and KdSC conceived the study; MHNLSF, MAVMS, DRDG, MMC, KdSC collaborated for the design of the experiments and analysed the experimental data; KdSC, DRDV, MMC performed the experiments; KdSC and MHNLSF wrote the manuscript. All authors read and approved the final version.

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Availability of data and materials

Data supporting the conclusions are included within the article and the additional files.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

1. Patterson J, Sammon M, Garg M. Dengue, zika and chikungunya: emerging arboviruses in the new world. West J Emerg Med. 2016;17(6):671–9. https://doi.org/10.5811/westjem.2016.9.30904.
2. Messina JP, Brady OJ, Golding N, Kraemer MUG, Wint GRW, Ray SE, et al. The current and future global distribution and population at risk of dengue. Nat Microbiol. 2019;4(9):1508–15. https://doi.org/10.1038/s41564-019-0476-8.

3. Paixao ES, Teixeira MG, Rodrigues LC. Zika, chikungunya and dengue: the causes and threats of new and re-emerging arboviral diseases. BMJ Glob Health. 2018;3(Suppl 1):e000530. https://doi.org/10.1136/bmjgh-2017-000530.
4. Achee NL, Grieco JP, Vatandoost H, Seixas G, Pinto J, Ching-Ng L, et al. Alternative strategies for mosquito-borne arbovirus control. PLoS Negl Trop Dis. 2019;13(1):e0006822. https://doi.org/10.1371/journal.pntd.0006822.
5. Regis LN, Acioli RV, Silveira JC Jr, Melo-Santos MA, Souza WV, Ribeiro CM, et al. Sustained reduction of the dengue vector population resulting from an integrated control strategy applied in two Brazilian cities. PLoS One. 2013;8(7):e67682. https://doi.org/10.1371/journal.pone.0067682.
6. Regis L, Souza WV, Furtado AF, Fonseca CD, Silveira JC Jr, Ribeiro PJ Jr, et al. An entomological surveillance system based on open spatial information for participative dengue control. An Acad Bras Cienc. 2009;81(4):655–62.
7. Flacio E, Engeler L, Tonolla M, Luthy P, Patocchi N. Strategies of a thirteen year surveillance programme on *Aedes albopictus* (Stegomyia albopicta) in southern Switzerland. Parasit Vectors. 2015;8:208. https://doi.org/10.1186/s13071-015-0793-6.
8. Barbosa RMR, Melo-Santos MAV, Silveira JC Jr, Silva-Filha M, Souza WV, Oliveira CMF, et al. Infestation of an endemic arbovirus area by sympatric populations of *Aedes aegypti* and *Aedes albopictus* in Brazil. Mem Inst Oswaldo Cruz. 2020;115:e190437. https://doi.org/10.1590/0074-02760190437.
9. Killeen GF, Tatarsky A, Diabate A, Chaccour CJ, Marshall JM, Okumu FO, et al. Developing an expanded vector control toolbox for malaria elimination. BMJ Glob Health. 2017;2(2):e000211. https://doi.org/10.1136/bmjgh-2016-000211.
10. Achee NL, Gould F, Perkins TA, Reiner RC Jr, Morrison AC, Ritchie SA, et al. A critical assessment of vector control for dengue prevention. PLoS Negl Trop Dis. 2015;9(5):e0003655. https://doi.org/10.1371/journal.pntd.0003655.
11. Lacey LA. *Bacillus thuringiensis* serovariety *israelensis* and *Bacillus sphaericus* for mosquito control. J Am Mosq Control Assoc. 2007;23(2 Suppl):133–63. https://doi.org/10.2987/8756-971X(2007)23[133:BTSIAB]2.0.CO;2.
12. Soberón M, Fernández LE, Pérez C, Gill SS, Bravo A. Mode of action of mosquitocidal *Bacillus thuringiensis* toxins. Toxicon. 2007;49(5):597–600. https://doi.org/10.1016/j.toxicon.2006.11.008.
13. Pardo-Lopez L, Soberon M, Bravo A. *Bacillus thuringiensis* insecticidal three-domain Cry toxins: mode of action, insect resistance and consequences for crop protection. FEMS Microbiol Rev. 2013;37(1):3–22. https://doi.org/10.1111/j.1574-6976.2012.00341.x.
14. Likitvivatanavong S, Chen J, Evans AM, Bravo A, Soberón M, Gill SS. Multiple receptors as targets of Cry toxins in mosquitoes. J Agricult Food Chem. 2011;59(7):2829–38. https://doi.org/10.1021/jf1036189.
15. Bravo A, Gill SS, Soberón M. Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. Toxicon. 2007;49(4):423–35. https://doi.org/10.1016/j.toxicon.2006.11.022.
16. Rodriguez-Almazan C, de Escudero RI, Canton PE, Munoz-Garay C, Perez C, Gill SS, et al. The amino- and carboxyl-terminal fragments of the *Bacillus thuringensis* Cyt1Aa toxin have differential roles in toxin oligomerization and pore formation. Biochemistry. 2011;50(3):388–96. https://doi.org/10.1021/bi101239r.
17. de Maagd RA, Bravo A, Berry C, Crickmore N, Schnepf HE. Structure, diversity, and evolution of protein toxins from spore-forming entomopathogenic bacteria. Ann Rev Genetics. 2003;37:409–33. https://doi.org/10.1146/annurev.genet.37.110801.143042.
18. Pérez C, Fernandez LE, Sun J, Folch JL, Gill SS, Soberón M, et al. *Bacillus thuringiensis* subsp. *israelensis* Cyt1Aa synergizes Cry11Aa toxin by functioning as a membrane-bound receptor. Proc Natl Acad Sci United States Am. 2005;102(51):18303–8. https://doi.org/10.1073/pnas.0505494102.
19. Elleuch J, Jaoua S, Darriet F, Chandre F, Tounsi S, Zghal RZ. Cry4Ba and Cyt1Aa proteins from *Bacillus thuringiensis israelensis*: interactions and toxicity mechanism against *Aedes aegypti*. Toxicon. 2015;104:83–90. https://doi.org/10.1016/j.toxicon.2015.07.337.
20. Cantón PE, Reyes EZ, de Escudero RI, Bravo A, Soberón M. Binding of *Bacillus thuringiensis* subsp. *israelensis* Cry4Ba to Cyt1Aa has an important role in synergism. Peptides. 2011;32(3):595–600. https://doi.org/10.1016/j.peptides.2010.06.005.
21. Tabashnik BE, Carriere Y. Global patterns of resistance to Bt crops highlighting pink bollworm in the United States, China, and India. J Econ Entomol. 2019;112(6):2513–23. https://doi.org/10.1093/jee/toz173.
22. de Bortoli CP, Jurat-Fuentes JL. Mechanisms of resistance to commercially relevant entomopathogenic bacteria. Curr Opin Insect Sci. 2019;33:56–62. https://doi.org/10.1016/j.cois.2019.03.007.
23. Williams GM, Faraji A, Unlu I, Healy SP, Farooq M, Gaugler R, et al. Area-wide ground applications of *Bacillus thuringiensis* var. *israelensis* for the control of *Aedes albopictus* in residential neighborhoods: from optimization to operation. PLoS One. 2014;9(10):e110035. https://doi.org/10.1371/journal.pone.0110035.
24. Setha T, Chantha N, Benjamin S, Socheat D. Bacterial larvicide, *Bacillus thuringiensis israelensis* strain am 65–52 water dispersible granule formulation impacts both dengue vector, *Aedes aegypti* (l.) population density and disease transmission in Cambodia. PLoS Negl Trop Dis. 2016;10(9):e0004973. https://doi.org/10.1371/journal.pntd.0004973.
25. Guidi V, Patocchi N, Luthy P, Tonolla M. Distribution of *Bacillus thuringiensis* subsp. *israelensis* in soil of a swiss wetland reserve after 22 years of mosquito control. Appl Environ Microbiol. 2011;77(11):3663–8. https://doi.org/10.1128/AEM.00132-11.
26. Becker N, Ludwig M, Su T. Lack of resistance in *Aedes vexans* field populations after 36 years of *Bacillus thuringiensis* subsp. *israelensis* applications in the upper Rhine Valley, Germany. J Am Mosq Control Assoc. 2018;34(2):154–7. https://doi.org/10.2987/17-6694.1.
27. Pocquet N, Darriet F, Zumbo B, Milesi P, Thiria J, Bernard V, et al. Insecticide resistance in disease vectors from Mayotte: an opportunity for integrated vector management. Parasit Vectors. 2014;7:299. https://doi.org/10.1186/1756-3305-7-299.
28. Bellini R, Zeller H, Van Bortel W. A review of the vector management methods to prevent and control outbreaks of West Nile virus infection and the challenge for Europe. Parasit Vectors. 2014;7:323. https://doi.org/10.1186/1756-3305-7-323.
29. Araujo AP, Diniz ADF, 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;69(1):297. https://doi.org/10.1186/1756-3305-6-297.
30. Suter T, Crespo MM, de Oliveira MF, de Oliveira TSA, de Melo-Santos MAV, de Oliveira CMF, et al. Insecticide susceptibility of *Aedes albopictus* and *Ae. aegypti* from Brazil and the Swiss-Italian border region. Parasit Vectors. 2017;10(1):431. https://doi.org/10.1186/s13071-017-2364-5.
31. Marcombe S, Darriet F, Agnew P, Etienne M, Yp-Tcha MM, Yebakima A, et al. Field efficacy of new larvicide products for control of multi-resistant *Aedes aegypti* populations in Martinique (French West Indies). Am J Trop Med Hyg. 2011;84(1):118–26. https://doi.org/10.4269/ajtmh.2011.10-0335.
32. Liu H, Cupp EW, Guo A, Liu N. Insecticide resistance in Alabama and Florida mosquito strains of *Aedes albopictus*. J Med Entomol. 2004;41(5):946–52.
33. Carvalho KDS, Crespo MM, Araujo AP, da Silva RS, de Melo-Santos MAV, de Oliveira CMF, et al. Long-term exposure of *Aedes aegypti* to *Bacillus thuringiensis* svar. *israelensis* did not involve altered susceptibility to this microbial larvicide or to other control agents. Parasit Vectors. 2018;11(1):673. https://doi.org/10.1186/s13071-018-3246-1.
34. Schneider S, Tajrin T, Lundstrom JO, Hendriksen NB, Melin P, Sundh I. Do multi-year applications of *Bacillus thuringiensis* subsp. *israelensis* for control of mosquito larvae affect the abundance of *B. cereus* group populations in riparian wetland soils? Microb Ecol. 2017;74(4):901–9. https://doi.org/10.1007/s00248-017-1004-0.
35. Lacey LA, Siegel JP. Safety and ecotoxicology of entomopathogenic bacteria. In: Charles JF, Nielsen-LeRoux C, Delecluse A, editors. Entomopathogenic bacteria: from laboratory to field application. Dordrecht, The Netherlands: Kluwer Academic Publishers; 2000. p. 253–73.
36. Derua YA, Kweka EJ, Kisinza WN, Githeko AK, Mosha FW. Bacterial larvicides used for malaria vector control in sub-Saharan Africa: review of their effectiveness and operational feasibility. Parasit Vectors. 2019;12(1):426. https://doi.org/10.1186/s13071-019-3683-5.
37. Tetreau G, Stalinski R, David JP, Despres L. Monitoring resistance to *Bacillus thuringiensis* subsp. *israelensis* in the field by performing bioassays with

each Cry toxin separately. Mem Inst Oswaldo Cruz. 2013;108(7):894–900. https://doi.org/10.1590/0074-0276130155.
38. Stalinski R, Laporte F, Tetreau G, Despres L. Receptors are affected by selection with each *Bacillus thuringiensis israelensis* Cry toxin but not with the full Bti mixture in *Aedes aegypti*. Infect Genet Evol. 2016;44:218–27. https://doi.org/10.1016/j.meegid.2016.07.009.
39. Paris M, Tetreau G, Laurent F, Lelu M, Després L, David JP. Persistence of *Bacillus thuringiensis israelensis* (Bti) in the environment induces resistance to multiple Bti toxins in mosquitoes. Pest Manag Sci. 2011;67(1):122–8. https://doi.org/10.1002/ps.2046.
40. Georghiou GP, Wirth MC. Influence of exposure to single versus multiple toxins of *Bacillus thuringiensis* subsp. *israelensis* on development of resistance in the mosquito *Culex quinquefasciatus* (Diptera: Culicidae). Appl Environ Microbiol. 1997;63(3):1095–101.
41. 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;8:662. https://doi.org/10.1186/s13071-015-1276-5.
42. David MR, Garcia GA, Valle D, Maciel-de-Freitas R. Insecticide resistance and fitness: the case of four *Aedes aegypti* populations from different Brazilian regions. Biomed Res Int. 2018;2018:6257860. https://doi.org/10.1155/2018/6257860.
43. 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):916–22. https://doi.org/10.1590/S0074-02762012000700013.
44. Rivero A, Vezilier J, Weill M, Read AF, Gandon S. Insecticide control of vector-borne diseases: when is insecticide resistance a problem? PLoS Pathog. 2010;6(8):1001000. https://doi.org/10.1371/journal.ppat.1001000.
45. Muturi EJ, Alto BW. Larval environmental temperature and insecticide exposure alter *Aedes aegypti* competence for arboviruses. Vector Borne Zoonotic Dis. 2011;11(8):1157–63. https://doi.org/10.1089/vbz.2010.0209.
46. McCarroll L, Hemingway J. Can insecticide resistance status affect parasite transmission in mosquitoes? Insect Biochem Mol Biol. 2002;32(10):1345–51. https://doi.org/10.1016/s0965-1748(02)00097-8.
47. Melo-Santos MA, Araújo AP, Rios EM, Regis L. Long lasting persistence of *Bacillus thuringiensis* serovar. *israelensis* larvicidal activity in *Aedes aegypti* (Diptera: Culicidae) breeding places is associated to bacteria recycling. Biol Control. 2009;49:186–91.
48. Cordeiro MT, Silva AM, Brito CA, Nascimento EJ, Magalhaes MC, Guimaraes GF, et al. Characterization of a dengue patient cohort in Recife. Brazil Am J Trop Med Hyg. 2007;77(6):1128–34.
49. Donald CL, Brennan B, Cumberworth SL, Rezelj VV, Clark JJ, Cordeiro MT, et al. Full genome sequence and sfRNA interferon antagonist activity of Zika virus from Recife, Brazil. PLoS Negl Trop Dis. 2016;10(10):e0005048. https://doi.org/10.1371/journal.pntd.0005048.
50. Guedes DR, Paiva MH, Donato MM, Barbosa PP, Krokovsky L, Rocha S, et al. Zika virus replication in the mosquito *Culex quinquefasciatus* in Brazil. Emerg Microbes Infect. 2017;6(8):e69. https://doi.org/10.1038/emi.2017.59.
51. Carvalho-Leandro D, Ayres CF, Guedes DR, Suesdek L, Melo-Santos MA, Oliveira CF, et al. Immune transcript variations among *Aedes aegypti* populations with distinct susceptibility to dengue virus serotype 2. Acta Trop. 2012;124(2):113–9. https://doi.org/10.1016/j.actatropica.2012.07.006.
52. Diallo M, Nabeth P, Ba K, Sall AA, Ba Y, Mondo M, et al. Mosquito vectors of the 1998–1999 outbreak of Rift Valley Fever and other arboviruses (Bagaza, Sanar, Wesselsbron and West Nile) in Mauritania and Senegal. Med Vet Entomol. 2005;19(2):119–26. https://doi.org/10.1111/j.0269-283X.2005.00564.x.
53. da Moura AJ, de Melo Santos MA, Oliveira CM, Guedes DR, de Carvalho-Leandro D, da Cruz Brito ML, et al. Vector competence of the *Aedes aegypti* population from Santiago Island, Cape Verde, to different serotypes of dengue virus. Parasit Vectors. 2015;8:114. https://doi.org/10.1186/s13071-015-0706-8.
54. Lambrechts L, Chevillon C, Albright RG, Thaisomboonsuk B, Richardson JH, Jarman RG, et al. Genetic specificity and potential for local adaptation between dengue viruses and mosquito vectors. BMC Evol Biol. 2009;9:160. https://doi.org/10.1186/1471-2148-9-160.
55. Kong YY, Thay CH, Tin TC, Devi S. Rapid detection, serotyping and quantitation of dengue viruses by TaqMan real-time one-step RT-PCR. J Virol Methods. 2006;138(1–2):123–30. https://doi.org/10.1016/j.jviromet.2006.08.003.
56. Lanciotti RS, Kosoy OL, Laven JJ, Velez JO, Lambert AJ, Johnson AJ, et al. Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. Emerg Infect Dis. 2008;14(8):1232–9. https://doi.org/10.3201/eid1408.080287.
57. Bennett KE, Olson KE, Munoz Mde L, Fernandez-Salas I, Farfan-Ale JA, Higgs S, et al. Variation in vector competence for dengue 2 virus among 24 collections of *Aedes aegypti* from Mexico and the United States. Am J Trop Med Hyg. 2002;67(1):85–92. https://doi.org/10.4269/ajtmh.2002.67.85.
58. Rivero A, Magaud A, Nicot A, Vezilier J. Energetic cost of insecticide resistance in *Culex pipiens* mosquitoes. J Med Entomol. 2011;48(3):694–700. https://doi.org/10.1603/me10121.
59. Rigby LM, Rasic G, Peatey CL, Hugo LE, Beebe NW, Devine GJ. Identifying the fitness costs of a pyrethroid-resistant genotype in the major arboviral vector *Aedes aegypti*. Parasit Vectors. 2020;13(1):358. https://doi.org/10.1186/s13071-020-04238-4.
60. Kliot A, Ghanim M. Fitness costs associated with insecticide resistance. Pest Manag Sci. 2012;68(11):1431–7. https://doi.org/10.1002/ps.3395.
61. Oliveira CMF, Silva-Filha MH, Nielsen-Leroux C, Pei G, Yuan Z, Regis L. Inheritance and mechanism of resistance to *Bacillus sphaericus* in *Culex quinquefasciatus* (Diptera: Culicidae) from China and Brazil. J Med Entomol. 2004;41(1):58–64.
62. Amorim LB, de Barros RA, Chalegre KD, de Oliveira CM, Regis LN, Silva-Filha MH. Stability of *Culex quinquefasciatus* resistance to *Bacillus sphaericus* evaluated by molecular tools. Insect Biochem Mol Biol. 2010;40(4):311–6. https://doi.org/10.1016/j.ibmb.2010.02.002.
63. Paris M, David JP, Despres L. Fitness costs of resistance to Bti toxins in the dengue vector *Aedes aegypti*. Ecotoxicology. 2011;20(6):1184–94. https://doi.org/10.1007/s10646-011-0663-8.
64. Saleh MS, El-Meniawi FA, Kelada NL, Zahran HM. Resistance development in mosquito larvae *Culex pipiens* to the bacterial agent *Bacillus thuringiensis* var. *israelensis*. J Appl Entomol. 2003;127:29–32.
65. Gowelo S, Chirombo J, Spitzen J, Koenraadt CJM, Mzilahowa T, van den Berg H, et al. Effects of larval exposure to sublethal doses of *Bacillus thuringiensis* var. *israelensis* on body size, oviposition and survival of adult *Anopheles coluzzii* mosquitoes. Parasit Vectors. 2020;13(1):259. https://doi.org/10.1186/s13071-020-04132-z.
66. Flores AE, Garcia PG, Badii MH, Tovar MLR, Salas IF. Effects of sublethal concentrations of Vectobac on biological parameters of *Aedes aegypti*. J Am Mosq Control Assoc. 2004;20:412–7.
67. Alto BW, Lord CC. Transstadial effects of Bti on traits of *Aedes aegypti* and infection with dengue virus. PLoS Negl Trop Dis. 2016;10(2):e0004370. https://doi.org/10.1371/journal.pntd.0004370.
68. Gassmann AJ, Carriere Y, Tabashnik BE. Fitness costs of insect resistance to *Bacillus thuringiensis*. Annu Rev Entomol. 2009;54:147–63. https://doi.org/10.1146/annurev.ento.54.110807.090518.
69. Bellini R, Puggioli A, Balestrino F, Carrieri M, Urbanelli S. Exploring protandry and pupal size selection for *Aedes albopictus* sex separation. Parasit Vectors. 2018;11(Suppl 2):650. https://doi.org/10.1186/s13071-018-3213-x.
70. Richards SL, White AV, Balanay JAG. Potential for sublethal insecticide exposure to impact vector competence of *Aedes albopictus* (Diptera: Culicidae) for dengue and Zika viruses. Res Rep Trop Med. 2017;8:53–7. https://doi.org/10.2147/RRTM.S133411.
71. Mourya DT, Gokhale MD, Majumdar TD, Yadav PD, Kumar V, Mavale MS. Experimental Zika virus infection in *Aedes aegypti*: susceptibility, transmission & co-infection with dengue & chikungunya viruses. Indian J Med Res. 2018;147(1):88–96. https://doi.org/10.4103/ijmr.IJMR_1142_17.
72. Chaves BA, Orfano AS, Nogueira PM, Rodrigues NB, Campolina TB, Nacif-Pimenta R, et al. Coinfection with Zika Virus (ZIKV) and dengue virus results in preferential ZIKV transmission by vector bite to vertebrate host. J Infect Dis. 2018;218(4):563–71. https://doi.org/10.1093/infdis/jiy196.
73. Seixas G, Jupille H, Yen PS, Viveiros B, Failloux AB, Sousa CA. Potential of *Aedes aegypti* populations in Madeira Island to transmit dengue and chikungunya viruses. Parasit Vectors. 2018;11(1):509. https://doi.org/10.1186/s13071-018-3081-4.
74. Dickson LB, Jiolle D, Minard G, Moltini-Conclois I, Volant S, Ghozlane A, et al. Carryover effects of larval exposure to different environmental

bacteria drive adult trait variation in a mosquito vector. Sci Adv. 2017;3(8):e1700585. https://doi.org/10.1126/sciadv.1700585.
75. Moltini-Conclois I, Stalinski R, Tetreau G, Despres L, Lambrechts L. Larval exposure to the bacterial insecticide bti enhances dengue virus susceptibility of adult *Aedes aegypti* Mosquitoes. Insects. 2018. https://doi.org/10.3390/insects9040193.
76. Carlson JS, Short SM, Anglero-Rodriguez YI, Dimopoulos G. Larval exposure to bacteria modulates arbovirus infection and immune gene expression in adult *Aedes aegypti*. Dev Comp Immunol. 2020;104: 103540. https://doi.org/10.1016/j.dci.2019.103540.
77. Caragata EP, Tikhe CV, Dimopoulos G. Curious entanglements: interactions between mosquitoes, their microbiota, and arboviruses. Curr Opin Virol. 2019;37:26–36. https://doi.org/10.1016/j.coviro.2019.05.005.
78. Despres L, Stalinski R, Faucon F, Navratil V, Viari A, Paris M, et al. Chemical and biological insecticides select distinct gene expression patterns in *Aedes aegypti* mosquito. Biol Lett. 2014;10(12):20140716. https://doi.org/10.1098/rsbl.2014.0716.
79. Xi Z, Ramirez JL, Dimopoulos G. The *Aedes aegypti* toll pathway controls dengue virus infection. PLoS Pathog. 2008;4(7):1000098. https://doi.org/10.1371/journal.ppat.1000098.
80. Anglero-Rodriguez YI, MacLeod HJ, Kang S, Carlson JS, Jupatanakul N, Dimopoulos G. *Aedes aegypti* molecular responses to Zika Virus: modulation of infection by the toll and Jak/Stat immune pathways and virus host factors. Front Microbiol. 2017;8:2050. https://doi.org/10.3389/fmicb.2017.02050.
81. Souza-Neto JA, Sim S, Dimopoulos G. An evolutionary conserved function of the JAK-STAT pathway in anti-dengue defense. Proc Natl Acad Sci U S A. 2009;106(42):17841–6. https://doi.org/10.1073/pnas.0905006106.
82. Xiao X, Liu Y, Zhang X, Wang J, Li Z, Pang X, et al. Complement-related proteins control the flavivirus infection of *Aedes aegypti* by inducing antimicrobial peptides. PLoS Pathog. 2014;10(4):e1004027. https://doi.org/10.1371/journal.ppat.1004027.
83. Minard G, Mavingui P, Moro CV. Diversity and function of bacterial microbiota in the mosquito holobiont. Parasit Vectors. 2013;6:146. https://doi.org/10.1186/1756-3305-6-146.
84. Brown LD, Shapiro LLM, Thompson GA, Estevez-Lao TY, Hillyer JF. Transstadial immune activation in a mosquito: Adults that emerge from infected larvae have stronger antibacterial activity in their hemocoel yet increased susceptibility to malaria infection. Ecol Evol. 2019;9(10):6082–95. https://doi.org/10.1002/ece3.5192.
85. Tetreau G, Grizard S, Patil CD, Tran FH, Van Tran V, Stalinski R, et al. Bacterial microbiota of *Aedes aegypti* mosquito larvae is altered by intoxication with *Bacillus thuringiensis israelensis*. Parasit Vectors. 2018;11(1):121. https://doi.org/10.1186/s13071-018-2741-8.
86. Kramer LD, Ebel GD. Dynamics of flavivirus infection in mosquitoes. Adv Virus Res. 2003;60:187–232. https://doi.org/10.1016/s0065-3527(03)60006-0.
87. Serrato IM, Caicedo PA, Orobio Y, Lowenberger C, Ocampo CB. Vector competence and innate immune responses to dengue virus infection in selected laboratory and field-collected *Stegomyia aegypti* (= *Aedes aegypti*). Med Vet Entomol. 2017;31(3):312–9. https://doi.org/10.1111/mve.12237.
88. Lourenco-de-Oliveira R, Rua AV, Vezzani D, Willat G, Vazeille M, Mousson L, et al. *Aedes aegypti* from temperate regions of South America are highly competent to transmit dengue virus. BMC Infect Dis. 2013;13:610. https://doi.org/10.1186/1471-2334-13-610.
89. Goncalves CM, Melo FF, Bezerra JM, Chaves BA, Silva BM, Silva LD, et al. Distinct variation in vector competence among nine field populations of *Aedes aegypti* from a Brazilian dengue-endemic risk city. Parasit Vectors. 2014;7:320. https://doi.org/10.1186/1756-3305-7-320.

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