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7/22/2015

**Impact of insecticide resistance on reproductive fitness and larval performance
of the dengue vector *Aedes aegypti***

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Abstract

Impact of insecticide resistance on reproductive fitness and larval performance of the dengue vector *Aedes aegypti*

By Nicole Marie Dzuris

Background: Rising levels of insecticide resistance (IR) may compromise efforts made to control transmission of vector-borne diseases, including dengue, which causes significant morbidity and potential mortality worldwide. Heavy insecticide use affects the fitness of mosquito populations and ultimately vectorial capacity. IR needs to be monitored in order to maintain the integrity of vector control, which is currently our best defense against the dengue virus vector *Aedes aegypti*.

Methods: This study aimed to experimentally quantify and assess measures of adult fitness (fecundity, egg viability, and longevity) and larval performance (time to pupation and time to emergence) in three populations of *Ae. aegypti* with different IR profiles. Two populations originating from the Yucatan peninsula of Mexico and a control population (Rockefeller) were reared in the absence of further insecticide treatment. A separate group of adult mosquitoes from each population was exposed to the pyrethroid insecticide permethrin in bottle bioassays to evaluate their degree of phenotypic resistance. Real-time PCR was conducted to identify the presence of knockdown resistance (*kdr*) mutations (V1016I and F1534C) in mosquitoes used in both the fitness experiments and bottle bioassays. Biochemical assays were completed to characterize the activity of large enzyme families associated with insecticide detoxification.

Results: Fitness of the field populations was evaluated relative to the control population. Resistance level was positively correlated with time to pupation, time to emergence, and female longevity. Although egg viability was negatively correlated with resistance, the resistant population had higher average lifetime fecundity. Females homozygous resistant for the V1016I mutation, had the shortest longevity. Higher enzymatic activity was present in both field strains.

Discussion: Pyrethroid insecticides are especially useful as they are highly toxic to insects and relatively non-toxic to humans, therefore maintaining their efficacy and preventing the development of pyrethroid resistance is essential. This study describes the associations between resistance mechanisms and life-history traits in *Ae. aegypti* originating from the Yucatan peninsula of Mexico, an area of the world where DENV is hyperendemic. These findings can help provide a deeper understanding of how insecticide resistance may affect *Ae. aegypti* fitness, and impact the effectiveness of insecticide-based vector control strategies.

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Background

It is estimated that over 2.5 billion people live in areas considered at high-risk for dengue virus (DENV) transmission, and an estimated 390 million people are infected each year, leading to a substantial proportion of the morbidity and potential mortality in tropical countries [1,2]. Over the past few decades the global health burden of disease attributed to DENV has increased at a startling rate. Before 1970, only 9 countries had experienced severe DENV epidemics, but the disease is now endemic in 128 countries [3,4]. DENV has surpassed malaria and become the most rapidly expanding mosquito-borne infectious disease in the world [5]. Rapid urban growth, continual deforestation, increased worldwide travel, international trade, and ineffective vector control have fueled this process of DENV range expansion, which is expected to continue in the forthcoming years [6-8]. The direct and indirect costs of DENV are considerable and create an immense burden on the economies and health systems of developing tropical nations [9]. Worldwide, costs due to lost productivity, premature death, and health care utilization are estimated to be \$39.3 billion per year, with 25% attributed to the Americas [10]. DENV afflicts all ages and levels of society, but the burden is often highest among the pediatric population living in poorer communities and where conditions are supportive of DENV vectors, primarily of the *Aedes* genus of mosquitoes [11].

Dengue viruses are mosquito-borne, single positive-stranded RNA viruses of the Flaviviridae family [12]. Infections are caused by four antigenically distinct serotypes, DENV-1, DENV-2, DENV-3, and DENV-4 [13]. DENV infections in humans are often asymptomatic, but symptoms may appear 3-14 days after an infective bite [14]. Symptoms range from mild to severe, and often include fever, headache, pain behind the eyes, rash, and muscle and joint pain [14]. All serotypes have the ability to cause the most severe form of the disease called severe dengue. Recovery from infection of a single serotype is believed to provide lifelong immunity against that specific serotype, but also increases the risk of developing severe dengue upon secondary infection of another serotype (a process termed antibody-dependent enhancement) [3,15]. Severe dengue is most common in children and

characterized by high fever, abdominal pain, persistent vomiting, difficulty breathing, and loss of fluid due to capillary permeability which may cause hypovolemic shock [11,14].

There is no specific treatment for dengue fever other than supportive care; particularly in severe cases, intravenous rehydration can help reduce the fatality rate to less than 1% [5,11]. There are currently no licensed vaccines or drugs to guard against DENV, but vaccine trials are ongoing [5]. Sanofi Pasteur's CYD live attenuated tetravalent chimeric vaccine is the first to complete phase III trials [5]. So far the vaccine has been shown to be safe and have high protective efficacy against severe disease, but with differing levels of effectiveness against each of the four main serotypes [5]. Without licensed vaccines as a prevention measure, at-risk populations must rely on personal protective measures and vector control [2].

The main DENV vector, the highly anthropophilic mosquito *Aedes aegypti* mosquito, tends to live in tropical and subtropical urban areas in close contact with human dwellings. It is also known to be a competent vector of yellow fever and Chikungunya [16]. This species has a diurnal blood-feeding behavior, being most active in the early morning and late afternoon, and often bites humans in and around the home [2,17,18]. In a series of mark-release-capture studies conducted by Harrington *et al.* most *Ae. aegypti* remained in their release houses and the majority of those that left were captured within 100 meters of the release site, suggesting that they do not disperse far [19]. Unlike many other species, *Ae. aegypti* frequently take several human blood meals per gonotrophic cycle, resulting in fitness advantages for the mosquito, and an exponential increase in the potential transmission of DENV [20]. Even though human movement often complicates pathogen transmission, these close and frequent human-host interactions help explain why clusters of dengue patients are often from the same household and have a similar onset date of the illness [21].

Common vector control strategies include environmental management, individual protection, biological control, and chemical control [11]. These methods vary in cost, delivery, effectiveness, and some are preferred by public health programs, but all are potentially useful for customizing the needs of different communities [22,23]. Methods of

environmental management include interventions to reduce vector larval habitats, such as the installation of a reliable piped water supply, and the removal of non-essential containers from yards that collect water, such as buckets and tires [11,24]. To reduce human contact with adult mosquitoes, the installation of screens, notably long-lasting insecticide-treated house screens, on windows and other entry points into the home have been found to be effective [11,25]. Individual protection methods include wearing clothing that covers exposed areas of skin, treating clothing with insecticides such as permethrin, and the application of repellants containing DEET, IR3535, or Picaridin [11,26]. The use of well-maintained insecticide-treated bed nets (ITNs) can help control mosquito populations short-term and are especially effective in protecting infants and others who sleep during the day [11,27]. Biological control includes the use of organisms that reduce mosquito populations such as using *Bacillus thuringiensis israelensis* (Bti) as a microbial larvicide, introducing *Wolbachia* bacteria into mosquitoes to induce cytoplasmic incompatibility, the application of entomopathogenic fungi to kill adult mosquitoes, and the introduction of larvivorous fish and copepods into mosquito breeding sites [11,22]. Different methods of chemical control include insecticides and insect growth regulators applied via thermal fogging, indoor residual spray (IRS), ultra-light volume spray (ULV), attractive toxic sugar baits (ATSB), and insecticide treated materials (ITMs) [22].

There are four main classes of insecticides approved for public health use: carbamates, organochlorines, organophosphates, and pyrethroids [28,29]. Over the past two decades, pyrethroids have been increasingly used in place of organophosphates due to their superior toxicity to insects and relative non-toxicity to mammals [30,31]. Pyrethroids are commonly used for agricultural pest control and are the only class of insecticides that the World Health Organization (WHO) recommends using on ITMs [29].

Since 1950, vector control programs in Mexico have used various insecticides to control mosquitoes and reduce vector-borne disease transmission [32]. From 1950 to 1960, the organochlorine insecticide DDT was used largely for IRS and was even used in some locations until 1998 [32]. Throughout the 1980's and 1990's, organophosphate insecticides

principally malathion were widely used for ULV space spraying [32]. In 2000, Mexico switched over to pyrethroid insecticides with active ingredients such as deltamethrin and permethrin [32]. The widespread use of a relatively small number of insecticides over many generations, and in the case of *Ae. aegypti* possibly greater than 20 generations per year, has led to the development of insecticide resistance, potentially compromising the efforts to control these vectors and the pathogens they transmit [29,33]. Due to its extensive use, pyrethroid resistance has evolved in many locations worldwide [34].

There are two principal mechanisms of pyrethroid resistance: target site mutations and increased metabolic detoxification [35]. One of the key target sites for pyrethroid resistance is the voltage-gated sodium channel. In susceptible insects, pyrethroids bind to voltage-gated sodium channels and cause the insect to lose coordinated activity (or become ‘knocked down’) and die [36]. In resistant insects with knockdown resistance (*kdr*) mutations, conformational changes at the pyrethroid binding site result in reduced binding, and therefore inhibits the insecticide’s ability to knock down and kill the insect [35,37]. Having target site mutations at positions 1016 (V1016I) and/or 1534 (F1534C) of the voltage-gated sodium channel is strongly associated with the *kdr* phenotype [35,37]. Metabolic resistance results from increased activity in enzymes that metabolize the insecticide. Elevated activity of multi-function oxidases (including the cytochrome P450 monooxygenases) (MFO), glutathione-S-transferases (GST), and non-specific esterases (NSE) is strongly associated with pyrethroid resistance in *Ae. aegypti* [38,39].

Due to cross-resistance between chemicals with shared modes of action, it is possible that DDT resistant populations with *kdr* mutations could also be resistant to pyrethroids [37,40,41]. Among different pyrethroid insecticides the target sites involved in resistance are often shared, making regular surveillance of the development of pyrethroid resistance essential [38]. Permethrin has been one of the most widely utilized pyrethroid insecticides in the suppression of adult *Ae. aegypti* populations and it is believed that its intense use has contributed to the rapid rise of *kdr* [32,34].

The Ile1016 and Cys1534 mutations have become widely dispersed throughout Latin America and are rapidly increasing in areas with high pyrethroid use such as Mexico, Brazil and the Caribbean [32,42,43]. Between 1996 and 2009 there was a dramatic increase in the frequency of Ile1016 mutations in *Ae. aegypti* in several states in Mexico, thought to be caused by the heavy use of permethrin-based insecticides in space spraying in and around homes to control adult mosquitoes [32]. The city of Merida, capital of the Yucatan state of Mexico, had initial Ile1016 mutation frequencies of 0% in 1999, but by 2007, some areas around the city had frequencies as high as 54% [32]. High frequencies of the Cys1534 mutation were found in Grand Cayman in 2008 and were associated with both permethrin and DDT resistance [35].

The constant selection pressure experienced by insects in the presence of insecticide results in a rapid increase of resistant genotypes. Most mutations encoding insecticide resistant phenotypes are expected to induce a fitness cost compared to the wild type in the absence of insecticide [29]. Here, fitness is defined as “the ability of organisms to survive and reproduce in the environment in which they find themselves [44].” Fitness costs are thought to be the result of pleiotropic effects of the resistant genes, and the reallocation of energy and resources needed to produce detoxifying enzymes [45,46]. Key physiological and reproductive life-history traits may be affected resulting in decreased adult longevity [47], larval performance [48], and fecundity [48].

These variables all have the potential to affect the efficiency of vector-borne disease transmission, or vectorial capacity. Theoretical models predict that if insecticide resistance decreases the vectorial capacity of a vector, pathogen transmission may decrease below the level experienced in the absence of resistance, and vice versa [49]. Slower development increases the chances of larvae predation, parasitism, and breeding site destruction, ultimately decreasing vectorial capacity [48,50]. Sodium channels are important in relaying olfactory cues to a mosquito’s nervous system, therefore the *kdr* mutation may cause *Ae. aegypti* to be less effective in sensing lactic acid, a chemical which helps them locate hosts [49,51,52]. This could potentially lead to a lower rate of bloodfeeding and less disease transmission (an

assumption not tested empirically). On the other hand, a study by Brito *et al.*, found that the amount of blood ingested by *Ae. aegypti* was independent of the *kdr* mutation, but that those with the mutation had a lower rate of insemination and therefore laid less eggs [48].

The same study by Brito *et al.* compared life-history parameters of the susceptible *Ae. aegypti* Rockefeller strain to a highly pyrethroid-resistant strain. Larvae with the *kdr* mutation developed slower, and fewer adult females laid eggs and also produced a smaller amount of eggs [48]. Adult longevity and egg viability (hatching) were not found to be significantly different [48]. Combined rearing of the two strains resulted in a decreased frequency of the mutant allele, suggesting that *kdr* mutations incur a fitness cost, and will be outbred in the absence of insecticide pressure [48]. In *Cx. pipiens* insecticide resistance has been associated with reduced longevity in a lab setting [47]. Longevity is expected to be especially reduced in insects with metabolic resistance, except in the case of those with increased GST activity, which has been shown to increase lifespan in some insects including *Drosophila melanogaster* [53].

The fitness costs related to permethrin resistance caused by *kdr* mutations are not well documented. There is currently a knowledge gap regarding the population level implications of insecticide resistance and its resulting fitness costs. Conducting routine surveillance of when, where, and which insecticides are being used, in addition to identifying and quantifying the mechanisms that lead to permethrin resistance and any associated fitness costs, will enable a better understanding of how resistance can be managed at a population level, as well as how resistance relates to the risk of pathogen transmission [48,54,55]. Such information would allow for the evidence-based selection of insecticides and aid in preserving the cost-effectiveness of vector control interventions, which are our main defense against DENV [56-58].

The dengue control campaign in Merida, Mexico invests heavily in insecticide applications to control the spread of DENV. Current vector control methods include strategies such as ULV spraying outdoors with the organophosphate insecticide chlorpyrifos, indoor space spraying with pyrethroids and carbamates to control the adult mosquito

population, and the application of the organophosphate insecticide temephos to control breeding sites [59]. Even with relatively consistent vector control, multiple DENV serotypes are hyperendemic to the area [60].

The aims of this study were:

Using a control and two field populations of *Ae. aegypti* from the Yucatan State of Mexico, with different natural exposures to insecticides:

Aim 1. Experimentally quantify and assess measures of *Ae. aegypti* adult fitness (fecundity, egg viability and female longevity) and larval performance (time to pupation, time to emergence and longevity) in the absence of further insecticide treatment.

Aim 2. Evaluate the degree of phenotypic resistance to permethrin using bottle bioassays.

Aim 3. Identify the independent and combined effects of various insecticide resistance mechanisms on key mosquito life history traits. Specifically, conduct molecular assays to identify the presence of the Ile1016 and Cys1534 *kdr* mutations and conduct biochemical assays to characterize the activity of enzymes associated with insecticide detoxification.

Methods

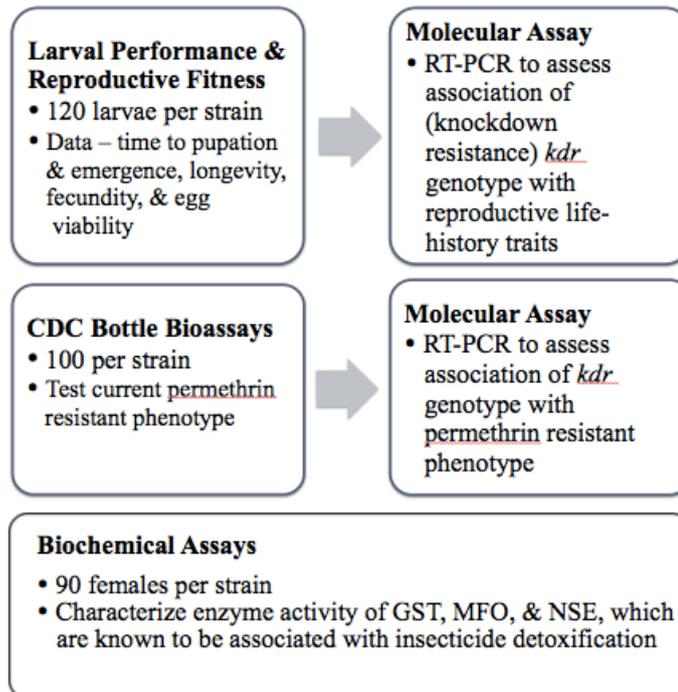
Experimental Design

This experimental study was designed to assess the impact of permethrin resistance on *Aedes aegypti* reproductive fitness and larval performance. Three strains of *Ae. aegypti* with differing levels of permethrin resistance were used for all experiments: Hunucma (F1; the offspring of mated field-collected material), Merida (F14), and the Rockefeller insecticide-susceptible reference strain. From a previous study, *Ae. aegypti* from the town of Hunucma (located in the Yucatan peninsula of Mexico) are known to have comparatively low levels of resistance to permethrin [61]. The Merida strain used in the experiments was originally established from *Ae. aegypti* collected in the city of Merida in the Yucatan peninsula of Mexico and has been reared in the CDC laboratories since 2011. Upon initial establishment, the strain was highly resistant to permethrin, but resistance has been declining over time. The susceptible Rockefeller strain, thought to be established in Havana, Cuba in 1881, has never been exposed to insecticides and was used as the permethrin-susceptible control in all experiments [62].

The experiments had been originally designed to compare 3 strains of *Ae. aegypti* expressing different levels of permethrin resistance: San Lorenzo (high resistance), Hunucma (low resistance), and Rockefeller (no resistance). Unfortunately, the eggs from San Lorenzo were damaged due to improper storage and were therefore unusable. The Merida strain was used as an alternative, since it originated from the same geographical area and had previously exhibited high levels of permethrin resistance.

Several laboratory methods were used to identify the independent and combined effects of different insecticide resistance mechanisms on key mosquito life history traits. Fitness experiments were done to experimentally quantify and assess measures of larval performance and reproductive fitness in the 3 strains of *Ae. aegypti* in the absence of insecticides. CDC bottle bioassays evaluated the degree of phenotypic resistance to permethrin [63]. Biochemical assays were conducted to characterize the activity of enzymes associated with insecticide detoxification. Molecular assays were conducted on female *Ae.*

aegypti from the fitness experiments and CDC bottle bioassays, to identify the presence of the *kdr* mutations, Ile1016 and/or Cys1534. All experiments took place at the Centers for Disease Control and Prevention (CDC) in Atlanta, GA.



Mosquito Collections and Rearing

The city of Merida has a subtropical climate, with mean monthly maximum temperatures ranging from 29°C in December to 34°C in July and a rainy season from May to October. Mosquito abundance and the number of DENV cases generally peak from July to October [64]. For this study, *Ae. aegypti* eggs originating from the city of Merida and the neighboring town of Hunucma (32km west of Merida) were used.

Ae. aegypti eggs were collected from the town of Hunucma during the summer of 2014, and from Merida in 2011 using the ovitrap design and collection methods described by Lenhart *et al.* [65]. Traps were checked weekly, and the eggs were collected, dried, and reared at the Universidad Autonoma de Yucatan, Unidad Colaborativa para Bioensayos Entomologicos (UCBE) to determine species. The mosquitoes were then allowed to mate, and the resulting eggs (F1) were dried and sent to the CDC insectary laboratories in Atlanta, GA.

Mosquitoes were reared in walk-in climate controlled chambers set at 29°C, 80% relative humidity, with a 16-hour light: 8-hour dark photoperiod. Eggs were hatched in trays (23x34cm) with 1 inch of de-chlorinated water, and emerging larvae separated into 4 trays containing 30 larvae each (to quantify larval development and performance). Simultaneously, additional trays were used to rear mosquitoes for CDC bottle bioassays and biochemical assays. Each tray of larvae was fed 65mg of ground koi food (Foster and Smith Aquatics Koi Food) every other day, coinciding with water changes. Pupae were then transferred into water-filled plastic cups placed inside mesh cages, where they later emerged and were fed a 10% sugar water solution using a soaked cotton ball, resting on the outer mesh of the cage.

Fitness experiments: Larval Performance and Reproductive Fitness

Larval performance was assessed by measuring time to pupation, time to emergence, and adult longevity. Time to pupation was considered the number of days it took each larva to pupate, and T_0 began when the eggs were placed in water. Each day, those that pupated were separated into a cage and remained together until after the first blood feeding. Time to emergence was considered the number of days it took pupae to emerge as adults, and T_0 began at pupation. Since pupae were maintained in groups until after the first blood feeding, information on each group's time to emergence was recorded rather than for each individual mosquito. Had they been separated earlier, mating would not have occurred. Longevity was calculated as the number of days the mosquito was known to be alive, and T_0 corresponded to the day at which 50% of adults had emerged. Adults were continuously provided the 10% sugar water solution throughout the experiment.

All female mosquitoes were bloodfed on an anesthetized rabbit 2 to 5 days after emerging, in order to assess reproductive fitness. The Hunucma mosquitoes, being an F1 generation, were not accustomed to feeding on rabbits or being in a lab setting, therefore they were given up to 7 opportunities to bloodfeed. The Merida mosquitoes were also bloodfed up to 7 times, but due to lab constraints after 2 bloodfeedings with the live rabbit, subsequent

feedings took place via the Hemotek Membrane Feeding System using expired human blood from a bloodbank. The Rockefeller mosquitoes were bloodfed once on a live rabbit.

During the initial bloodfeeding, males were present in the cages and all mosquitoes were left overnight to mate. The following day, bloodfed females were removed from the cages and placed in small, individual cages containing a small water cup lined with seed germination paper as an oviposition substrate. After 3 nights, the germination papers were removed from the cages, and the eggs were dried and counted to determine fecundity. Egg batches from each individual female were then placed into separate pans containing 1 inch of de-chlorinated water and the hatched larvae were counted to determine egg viability.

CDC Bottle Bioassay

Female *Ae. aegypti* that were 2-5 days old and not bloodfed were tested for permethrin resistance using the CDC bottle bioassay protocol [66]. The 3 strains were evaluated using the diagnostic dose and diagnostic time previously established for susceptible *Ae. aegypti* strains by the CDC [66]. Five 250 ml Wheaton bottles were used; 4 bottles were coated internally each with 15µg of permethrin dissolved in 1ml of acetone, and 1 control bottle was coated internally with 1ml of acetone. For each strain, 15-25 *Ae. aegypti* were introduced into each bottle and the number of mosquitoes knocked down was recorded at 0, 15, and 30 minutes. To be considered susceptible to the insecticide at the diagnostic time of 30 minutes, the mosquito had to be knocked down (unable to stand). All mosquitoes used in the bottle bioassay experiment were separated and categorized as susceptible or resistant and stored at -20°C for future molecular testing.

Biochemical Assays

Within 3 days of emergence, 90 non-blood-fed females from each strain were killed by freezing at -20°C and stored until biochemical assays were conducted. The activity of non-specific esterases (NSE)[67], mixed function oxidases (MFO)[68], glutathione-S-transferases (GST)[69], and acetylcholinesterase (AChE)[70] were determined according to the

methodologies described by Brogdon *et al.* [67-70]. Each individual whole mosquito was homogenized in 100 μ l of buffer (0.1M KPO₄, pH 7.2), and then diluted to 1ml with the addition of 900 μ l of buffer. In order to run multiple tests, the mosquito homogenate was further diluted with additional buffer, resulting in 2 - 1ml tubes, and all incubation times were also doubled. Each 96-well microplate allowed for 30 mosquitoes to be analyzed in triplicate. A variation coefficient of the triplicate means was calculated to identify possible manual errors made in the laboratory. Replicates with a variation coefficient of 0.15 or higher were omitted. The samples were analyzed using the Spectra Max M5e micro-plate reader and Soft Max Pro software. The Rockefeller strain was used as a susceptible reference population for all biochemical analyses.

For the NSE assay, the β -naphthyl acetate solution was prepared by dissolving 56mg of β -naphthyl acetate in 20ml of acetone and then mixed with 80ml of KPO₄ buffer. Each of 30 wells included 100 μ l of mosquito homogenate, 100 μ l of the β -naphthyl acetate solution, and 100 μ l of Fast Blue. The Fast Blue solution was made immediately before use by dissolving 100mg of Fast Blue in 100mL of dH₂O. The positive control consisted of 100 μ l of the β -naphthyl acetate solution, 100 μ l of Fast Blue, and 100 μ l of a β -naphthol solution, made with 25mg of β -naphthol dissolved in 5ml of acetone and 45ml of KPO₄ added. The negative control consisted of 300 μ l of KPO₄ buffer. The microplates were incubated for 4 minutes and then read at 540nm.

For the MFO assay, the TMBZ solution was made by dissolving 50mg of 3,3',5,5'-tetramethyl-benzidine dichloride in 25ml of methanol and then mixed with 75ml of 0.25M sodium acetate buffer, pH 5. Each of 30 wells included 100 μ l of mosquito homogenate, 200 μ l of TMBZ, and 25 μ l of 3% hydrogen peroxide. The positive control consisted of 200 μ l of TMBZ, 25 μ l of 3% hydrogen peroxide, and 100 μ l of cytochrome-C, made with 10mg of cytochrome-C (from bovine heart) dissolved in 100ml 0.25M sodium acetate buffer. The negative control consisted of 300 μ l of KPO₄ buffer. The microplates were incubated for 10 minutes and then read at 620nm.

For the GST assay, the reduced glutathione solution was made by mixing 61mg of reduced glutathione with 100ml of KPO_4 buffer. The cDNB solution was made by dissolving 20mg of 1-chloro-2,4'-dinitrobenzene in 10ml of acetone and then mixed with 90ml of KPO_4 buffer. Each of 30 wells included 100 μ l of mosquito homogenate, 100 μ l of the reduced glutathione solution, and 100 μ l of the cDNB solution. The microplates were read immediately (T_0) and 10 minutes later (T_{10}) at 340nm. T_0 was subtracted from T_{10} and these values were used for analyses.

For the AchE assay, the ATCH solution was made by dissolving 75mg of acetylthiocholine iodide in 10ml of acetone and 90ml of KPO_4 buffer. The DTNB solution was made by mixing 13mg of dithio-bis-2-nitrobenzoic acid with 100ml of KPO_4 buffer. The insensitive-ATCH solution differed from ATCH in that it also included 21mg of propoxur dissolved in acetone. One plate was read using the ATCH solution while the other used the insensitive-ATCH solution. Each of 30 wells included 100 μ l of mosquito homogenate, 100 μ l of ATCH (or insensitive-ATCH), and 100 μ l of DTNB solution. The negative control consisted of 300 μ l of KPO_4 buffer. The microplates were read immediately (T_0) and 20 minutes later (T_{20}) at 414nm. T_0 was subtracted from T_{20} and these values were used for statistical analyses.

Molecular Assays

To assess the associations of *kdr* genotype with permethrin resistant phenotype and *kdr* genotype with life history traits, all non-Rockefeller females used in the CDC bottle bioassay (n=93 Hunucma and n=96 Merida), and fitness studies (n=33 Hunucma and n=48 Merida) were genotyped for the Ile1016 and Cys1534 *kdr* alleles. DNA was extracted from each whole mosquito in a solution of 45 μ l of dH₂O and 5 μ l of Promega Taq DNA Polymerase10X Buffer with MgCl₂ (Madison, WI) in a 96-well PCR plate. Samples were incubated at 95°C in a Bio-Rad iCycler thermal cycler for 15 minutes, and stored at -4°C when not in use.

Real-Time Polymerase Chain Reaction (RT-PCR). When performing the PCR assays, positive controls were DNA previously obtained from *Ae. aegypti* reflecting all 3

possible genotypes for each mutation (homozygous susceptible, homozygous resistant, and heterozygous). The PCR reaction master mix for the Ile1016 allele consisted of: 6µl of dH₂O, 10µl of iQ™ SYBR® Green Supermix (BIO-RAD), 1µl (10 pmol) each of Val1016f, Ile1016f and Ile1016r primers, and 1µl of DNA (Table 1) [37]. Based on availability, SYBR for the Cys1534 allele was changed midway through the experiment from BIO-RAD to Quanta. The PCR reaction master mix using BIO-RAD SYBR for the Cys1534 allele consisted of: 7.67µl dH₂O, 10µl of iQ™ SYBR® Green Supermix (BIO-RAD), 1µl each of Phe1534+f, Phe1534+r, and 0.33µl of Cys1534+f primers, and 1µl of DNA [71]. The PCR reaction master mix using Quanta SYBR for the Cys1534 allele consisted of: 7.15µl of dH₂O, 9µl of Quanta BioSciences, Inc.™ PerfeCTa SYBR® Green SuperMix 0.6µl each of Phe1534+f, Phe1534+r, and 0.65µl of Cys1534+f primers, and 2µl of DNA [71].

Reaction cycling conditions for detection of the Ile1016 allele were: 95°C for 3 minutes, 40 cycles of 95°C for 10 seconds, 60°C for 10 seconds, 72°C for 30 seconds, and then a hold of 95°C for 10 seconds. The melting curve was then calculated by heating the plate from 65°C to 95°C in 0.2°C increments every 10 seconds. Reaction cycling conditions for detection of the Cys1534 allele using BIO-RAD SYBR were: 95°C for 3 minutes, 40 cycles of 95°C for 10 seconds, 57°C for 10 seconds, 72°C for 30 seconds, and then a hold of 95°C for 10 seconds. The melting curve was then calculated by heating the plate from 65°C to 95°C in 0.5°C increments every 5 seconds. Reaction cycling conditions for detection of the Cys1534 allele using Quanta SYBR were: 95°C for 3 minutes, 37 cycles of 95°C for 10 seconds, 57°C for 30 seconds, 72°C for 30 seconds, and then a hold of 95°C for 10 seconds. The melting curve was then calculated by heating the plate from 65°C to 95°C in 0.5°C increments every 5 seconds. The samples were analyzed using the BIO-RAD CFX96™ Real-Time System and C1000™ Thermal Cycler, and BIO-RAD CFX Manager™ software.

For the V1016I mutation, there are 3 potential melting peak patterns. A peak at 79°C corresponds to isoleucine (homozygous resistant), and a peak at 86°C corresponds to valine (homozygous susceptible). Mosquitoes with peaks at both 79°C and 86°C are considered heterozygotes (Figure 1). For the F1534C mutation there are 3 potential melting peak

patterns for both reactions using Quanta and BIO-RAD SYBR. For BIO-RAD SYBR a peak at 85°C corresponds to cysteine (homozygous resistant), and a peak at 80°C corresponds to phenylalanine (homozygous susceptible). Mosquitoes with peaks at both 80°C and 85°C are considered heterozygotes (Figure 2). For Quanta SYBR a peak at 82°C corresponds to cysteine (homozygous resistant), and a peak at 78°C corresponds to phenylalanine (homozygous susceptible). Mosquitoes with peaks at both 78°C and 82°C are considered heterozygotes (Figure 3).

Data Analysis

Time to pupation, time to emergence, lifetime fecundity and egg viability analyses by strain were calculated using one-way ANOVA tests. To compare adult mosquito longevity by strain, sex, and V1016I mutation Kaplan-Meier survival curves were constructed. For survival curve analyses, individuals that escaped or were accidentally killed were censored at time of escape or accidental death. To assess the association between the F1534C and V1016I mutations, and time to pupation, lifetime fecundity, egg viability, one-way ANOVA tests were conducted.

Using the bottle bioassay data, time-mortality plots were constructed and Hunucma and Merida populations were compared with the permethrin-susceptible Rockefeller strain. To correct for any mortality induced by mosquito handling, Abbott's formula was used [63]:

$$\text{Corrected Mortality} = \frac{(\text{mortality in test bottles (\%)} - \text{mortality in control bottle (\%)})}{(100\% - \text{mortality in control bottle (\%)})} \times 100$$

When assessing the susceptibility of a mosquito strain or population, 98-100% mortality, is considered susceptible, 90-97% mortality suggests possible or developing resistance that should be confirmed, and <90% mortality in a sample size of >100 mosquitoes strongly suggests resistance [72].

Allele frequencies for the Ile1016 and Cys1534 mutations were calculated using the following equation:

$$\frac{n \text{ heterozygotes} + 2(n \text{ homozygotes})}{2(\text{total } n \text{ mosquitoes analyzed})}$$

Fisher's exact tests were performed to test the association between genotype and phenotypic resistance or susceptibility.

In order to determine statistically significant differences in the enzyme activity levels of the three populations, the enzymatic profiles of Merida and Hunucma were compared to those obtained for the susceptible Rockefeller strain by Kruskal-Wallis and Wilcoxon's non-parametric tests [38]. Statistical and survival analyses were carried out using SAS 9.4 (Cary, NC) and boxplots were created using R Studio. Results were considered statistically significant at $P < 0.05$.

Results

Fitness: Larval Performance and Reproductive Fitness

Of the 120 larvae successfully reared for each strain, 100% (n=120) of both Hunucma and Merida, and 99.2% (n=119) of Rockefeller mosquitoes pupated, and 97.5% (n=117) of Hunucma, 100% (n=120) of Merida, and 96.7% (n=116) of Rockefeller mosquitoes emerged (Table 2). Among the 353 mosquitoes that emerged, 16 either escaped or were accidentally killed and therefore were not included in reproductive fitness, or molecular assay analyses, but were included in time to pupation and time to emergence analyses. For survival curves analyses, these individuals were censored at time of escape or accidental death.

The mean time to pupation for Hunucma was the longest at 8.1 days, followed by 7.2 days for Merida and 7.0 days for Rockefeller (Figure 4a). The values for time to pupation were significantly different when comparing the 3 strains (one-way ANOVA: $F = 89.42$, $DF = 2$, $P < 0.0001$). The mean times for emergence followed the same pattern; 10.3 days for Hunucma, 9.7 days for Merida and 9.0 days for Rockefeller (Figure 4b). The values for time to emergence were significantly different when comparing the 3 strains (one-way ANOVA: $F = 99.59$, $DF = 2$, $P < 0.0001$). All 3 mosquito populations had a higher proportion of males than females; Hunucma had the largest proportion of males to females (2:1) (Table 2).

The mean adult longevity of all *Ae .aegypti* reared for fitness experiments was 40.4 days (SD 16.9). When separated by sex, males and females had mean longevities of 38.5 days (SD 14.0) and 42.9 days (SD 19.9), respectively. The Hunucma strain adults lived the longest overall with a mean longevity of 47.2 days (SD 17.5), followed by the Merida strain with a mean longevity of 46.7 days (SD 12.6), and the Rockefeller strain with a mean longevity of 27.3 days (SD 12.1) (Table 3). Hunucma females also lived the longest with a mean longevity of 64.5 days (SD 12.7), followed by Merida and Rockefeller females with mean longevities of 54.3 days (SD 8.4) and 24.0 days (SD 7.9), respectively. Analysis of the survival curves indicated a statistically significant difference between the longevities of the females of each strain (Kaplan-Meier Survival Analysis: $\chi^2=188.016$, $DF = 2$, log rank $P <$

0.0001). However, the difference between the longevities of the males of each strain was not statistically significant (Figure 5).

Even though Hunucma and Merida populations were given the chance to bloodfeed 7 times each, egg yields were low. The Rockefeller strain was not included in fecundity and egg viability analyses since it was bloodfed only once. During their lifetime, 87.9% (n=29/33) of the Hunucma females and 93.8% (n=45/48) of Merida females laid eggs at least once. Hunucma and Merida females laid an average total of 109.6 eggs (SD 72.4) and 87.7 eggs (SD 58.0), respectively. The values for fecundity were not statistically significant when comparing the 2 strains (one-way ANOVA: $F = 2.65$, $DF = 1$, $P = 0.1077$). Of all eggs laid, an average of 7.0% of Hunucma eggs and 20.5% of Merida eggs hatched into viable larvae. The values for egg viability were statistically significant when comparing the 2 strains (one-way ANOVA: $F = 17.51$, $DF = 1$, $P = 0.0001$).

Kdr Genotyping of Mosquitoes from Fitness Experiments

DNA was extracted and RT-PCR was completed for the 81 female Hunucma and Merida *Ae. aegypti* that were used in the fitness experiments. Of the Hunucma mosquitoes genotyped for the V1601I mutation (n=33), 39.4% (n=13) were wild-type (susceptible) homozygotes (SS), 48.5% (n=16) were heterozygotes (SR), and 12.1% (n=4) were homozygous resistant (RR). Of the Merida mosquitoes genotyped for the V1601I mutation (n=48), 87.5% (n=42) were wild-type (susceptible) homozygotes (SS), 6.3% (n=3) were heterozygotes (SR), none (n=0) were homozygous resistant (RR), and 6.3% (n=3) were unable to be determined. Of the Hunucma mosquitoes genotyped for the F1534C mutation (n=33), 39.4% (n=13) were SS, 48.5% (n=16) were SR, and 12.1% (n=4) were RR. Of the Merida mosquitoes genotyped for the F1534C mutation (n=48), 56.3% (n=27) were SS, 37.5% (n=18) were SR, and 6.3% (n=3) were RR.

Time to pupation was significantly associated with the V1016I mutation (one-way ANOVA: $F = 5.87$, $DF = 2$, $P = 0.0043$), but the F1534C mutation was not. Data were not available to test the association between time to emergence and the *kdr* mutations.

Independently, neither the F1534C, nor the V1016I mutation were significantly associated with fecundity (Figure 6a, b). The V1016I mutation was significantly associated with egg viability (one-way ANOVA: $F = 3.19$, $DF = 2$, $P < 0.0504$), whereas the F1534C was not (Figure 7a, b). Analysis of the survival curves indicated a statistically significant difference between mosquito longevity and V1016I haplotype (Kaplan-Meier Survival Analysis: $\chi^2=6.6399$, $DF = 2$, log rank $P = 0.0362$), but not for the F1534C mutation (Figure 8).

CDC Bottle Bioassays

A total of 3 CDC bottle bioassays were completed in which 289 female *Ae. aegypti* were tested for resistance to permethrin. Resistance to permethrin was detected in the Hunucma strain (84.2% mortality). Slight evidence of reduced susceptibility was detected in the Merida strain (97.4% mortality), and the Rockefeller strain showed complete susceptibility (100% mortality) (Figure 9).

Kdr Genotyping of CDC Bottle Bioassay Mosquitoes

DNA was extracted and PCR was completed for the 189 female Hunucma and Merida *Ae. aegypti* that were tested for permethrin resistance in the CDC bottle bioassays. Of the Hunucma mosquitoes genotyped for the V1016I mutation (n=93), 53.8% (n=50) were SS, 34.4% (n=32) were SR, and 11.8% (n=11) RR. Of the Merida mosquitoes genotyped for the V1016I mutation (n=96), 88.5% (n=85) were SS, 11.5% (n=11) were SR, and none (n=0) were RR. Of the Hunucma mosquitoes genotyped for the F1534C mutation (n=93), 43.0% (n=40) were SS, 40.9% (n=38) were SR, and 16.1% (n=15) were RR. Of the Merida mosquitoes genotyped for the F1534C mutation (n=96), 43.8% (n=42) were SS, 41.7% (n=40) were SR, and 14.6% (n=14) were RR.

The Fisher exact test resulted in a statistically significant association between the 1016I genotype and permethrin phenotypic resistance in the Hunucma strain ($P = 0.0197$), but not the Merida strain (Table 4). There was not a statistically significant association between the 1534C genotype and permethrin phenotypic resistance in either the Hunucma or Merida

strain. There were statistically significant associations between phenotypic resistance and 1016 haplotype (one-way ANOVA: $F = 17.78$, $DF = 2$, $P < 0.0001$), and phenotypic resistance and 1534 haplotype (one-way ANOVA: $F = 4.39$, $DF = 2$, $P = 0.0140$).

Among mosquitoes resistant to permethrin (i.e., surviving the bioassays), 56.8% ($n = 25/44$) were positive for 1016I and 20.5% ($n = 9/44$) were homozygous resistant (RR). Among resistant mosquitoes, 65.9% ($n = 29/44$) were positive for 1534C and 29.5% were RR. There were 10 Hunucma mosquitoes homozygous resistant to both 1016I and 1534C, 90% ($n = 9/10$) of which were resistant to permethrin. Of the Hunucma strain, 100% ($n = 9/9$) of 1016I RR individuals and 69.2% ($n = 9/13$) 1534C RR individuals were double homozygotes. The Merida strain did not have any double RR females.

Combining the female *Ae. aegypti* used in both the bottle bioassay and fitness experiments, the 1061I allele appeared in the two strains with an overall frequency of 17.2%; Hunucma had a frequency of 31.0% and Merida had a frequency of 4.9%. The 1534C allele appeared in the two strains with an overall frequency of 34.1%; Hunucma had a frequency of 36.5% and Merida had a frequency of 31.9% (Table 5).

Biochemical Assays

The 3 large enzyme families associated with pyrethroid resistance, GST, MFO, and NSE, were found to have significantly different median absorbances when comparing the Hunucma, Merida, and Rockefeller populations (Kruskal-Wallis non-parametric tests; MFO: $\chi^2 = 128.8743$, $DF = 2$, $P < 0.0001$; NSE: $\chi^2 = 37.3314$, $DF = 2$, $P < 0.0001$; GST: $\chi^2 = 93.7232$, $DF = 2$, $P < 0.0001$) (Figure 10). When comparing the Hunucma strain to the Rockefeller strain the median absorbances for MFO, GST, and NSE were significantly higher (Kruskal-Wallis non-parametric tests; MFO: $\chi^2 = 108.1259$, $DF = 1$, $P < 0.0001$; GST: $\chi^2 = 60.0145$, $DF = 1$, $P < 0.0001$; NSE: $\chi^2 = 50.6541$, $DF = 1$, $P < 0.0001$). When comparing the Merida strain to the Rockefeller strain the median absorbances for MFO and GST were significantly higher (Kruskal-Wallis non-parametric tests; MFO: $\chi^2 = 7.6064$, $DF = 1$, $P = 0.0058$, GST: $\chi^2 = 72.9739$, $DF = 1$, $P < 0.0001$).

Discussion

The aim of this study was to better understand the independent and combined effects of various insecticide resistance mechanisms on key *Ae. aegypti* life-history traits. Molecular assays indicated the presence of Cys1534 and Ile1016 *kdr* alleles in Hunucma and Merida populations. Bottle bioassays confirmed a low level of permethrin resistance in Hunucma, and after 14 generations in the lab without insecticide exposure, the Merida strain's level of permethrin resistance was greatly diminished. Biochemical assays indicated higher enzymatic activity of GST and MFO in both Merida and Hunucma, and additionally NSE in Hunucma compared with Rockefeller, suggesting that these strains have higher levels of insecticide detoxification.

When we assessed fitness associations, it was also important to take into consideration how many generations each population had been reared in the lab. In this study the mosquito populations differed not only by insecticide exposure, but also by lab generation. This was the Hunucma strain's second generation in the lab, whereas the Merida and Rockefeller strains were accustomed to being reared in a lab setting for several years and therefore may have incurred a fitness advantage. Therefore, tests of association for variables of interest were completed for *kdr* mutations in addition to strain.

Time to pupation and time to emergence were significantly associated with both the Hunucma and Merida strains, and longest among the Hunucma strain, followed by Merida (Table 6). Time to pupation was also found to be statistically significantly associated with the V1016I mutation; homozygous resistant and heterozygote *Ae. aegypti* were associated with increased time to pupation, whereas homozygous susceptible tended to pupate earliest. The F1534C mutation was not associated with differing times to pupation (Table 7). There were significant differences between strain and longevity, and Ile1016 mutation presence and longevity. Interestingly, the Hunucma strain had the longest average life span, followed by the Merida strain. Since increased blood feeding has been associated with an increase in fitness [21], the Rockefeller strain was not an ideal comparison population as it was given

only one opportunity to bloodfeed, whereas the Hunucma and Merida strains were given 7 opportunities. Separating female longevity by V1016I haplotype showed that all homozygous resistant females survived until day 60 and then rapidly died off by day 67, whereas the heterozygote and homozygous susceptible females began dying at 25 days, and some lived as long as 84 days. The F1534C mutation was not statistically significantly associated with female longevity and therefore was not separated by haplotype. Both the Hunucma and Merida strains were statistically significantly associated with egg viability, but not with lifetime fecundity. Egg yields were poor for both the Hunucma and Merida strains, producing an average lifetime total of 109.6 and 84.2 eggs, respectively. Only 7.0% of Hunucma and 20.5% of Merida eggs hatched into viable larvae. The V1016I haplotypes were associated with egg viability; homozygous susceptible females had the highest hatching rate and homozygous resistant females had the lowest. Neither the F1534C nor the V1016I haplotype were statistically significantly associated with increased or decreased fecundity.

In addition to the statistically significant associations between the V1016I mutation and several life-history traits, the mutation was also associated with higher phenotypic resistance to permethrin. In agreement with a study by Plernsub *et al.*, the presence of the F1534C mutation in *Ae. aegypti* was not associated with a statistically significantly negative impact on life-history traits [73], but was associated with higher phenotypic resistance. Interestingly, the Hunucma and Merida *Ae. aegypti* used in the bottle bioassays had similar F1534C allele frequencies, 37.0% and 36.2% respectively, but mortality levels were 84.2% and 97.4% respectively. This suggests that the V1016I allele has stronger links to permethrin resistance, especially for those mosquitoes that are homozygous resistant at that locus [32]. The biochemical assays revealed significantly higher activity levels of GST and MFO in both the Hunucma and Merida populations and of NSE in Hunucma. Having higher levels of metabolic resistance suggests that energy is committed to the production of detoxifying enzymes therefore inducing fitness costs. Interestingly, longevity is expected to be especially lower in insects with metabolic resistance except in the case of those with higher GST activity, which was found to increase lifespan [53]. GST was most prevalent in Merida,

followed by Hunucma and may help explain their comparatively long lifespans [53].

Reductions in reproductive fitness are often explained by pleiotropic effects, which reduce the energy available for other functions [73,74]. There are, however, large variations in fitness costs between different insect species, genetic backgrounds, and mechanisms of resistance [73]. Consistent with several other studies, the average time to pupation in this study was longest among the strain with the highest level of resistance [48,75,76]. A study of developmental and reproductive fitness in the southern African malaria vector, *Anopheles funestus*, also found a significantly longer development time from first instar larva to pupa among mosquitoes highly resistant to permethrin compared to a fully susceptible strain [75]. Another study found that a pyrethroid resistant strain of *Ae. aegypti* took longer to pupate than did its non-resistant comparison strain, but the difference was not statistically significant [48].

The study by Brito *et al.* found that male longevity was shorter than female longevity, and mortality was lower among bloodfed females than females fed only sugar; however, there was no evidence that *kdr* mutations reduced longevity [48]. The study by Okoye *et al.* analyzed populations of highly resistant and susceptible *An. funestus* females and did not find a statistically significant difference in the length of female longevity between the two groups [75]. Similar to the current study, in a study by Plernsub *et al.* of *Ae. aegypti* in Thailand, it was found that at day 60 a higher proportion of females resistant to DDT and permethrin remained alive compared to females that were resistant only to DDT [73].

Interestingly, the Brito *et al.* found no statistically significant difference between the weight of resistant and susceptible females before and after blood feeding, but less resistant females laid eggs, suggesting that less resistant females were inseminated [48]. On the other hand, Plernsub *et al.* found that reduced egg viability could not be explained by decreased mating ability, since the insemination rates and quantity of spermatozoa were similar in both strains of *Ae. aegypti* [73]. They concluded that it was likely the more highly resistant strain consumed lower amounts of blood [43], or required a higher number of blood meals to lay eggs [73,75]. Hardstone *et al.* also reported that permethrin resistant *Culex quinquefasciatus*

females that were provided sugar survived longer than did susceptible ones [77]. In the present study, fecundity was not statistically significantly different between the Merida and Hunucma strains, but both fecundity and egg viability were poor overall.

This study had several limitations. The Merida eggs used in these experiments were not an ideal comparison for the Hunucma strain; whereas the original San Lorenzo eggs collected from the field for this experiment came from adults with a high level of insecticide resistance and would have provided a better contrast for the experiments. In addition, the Merida strain had adapted to rearing in insectary conditions over multiple generations, while the Hunucma strain had been recently collected from the field. Additionally, the labs were inaccessible during weekends and holidays; therefore, the longevity data were less detailed than they may have been otherwise. Longevity was recorded as the last day a mosquito was known to be alive; thus, some mosquitoes may have lived longer than recorded. Another limitation of this study was that the Rockefeller population was offered a blood meal only once, whereas the Hunucma and Merida populations which were offered one blood meal per week for 7 weeks, for approximately 6 minutes each. Scott *et al.* found that *Ae. aegypti* take about 0.63-0.73 bloodmeals per day [78], suggesting that in a non-lab habitat it is likely that they would have had more chances to blood feed, possibly resulting in higher fitness.

When in their natural habitat, more than 90% of female *Ae. aegypti*'s blood meals come from humans [79]. In this study most blood feedings were completed using an anaesthetized rabbit for convenience, and due to lab constraints some were fed on expired human blood via a Hemotek Membrane Feeding System. This system heats the blood and allows mosquitoes to take blood meals through a thin membrane. It is thought that mosquitoes see the expired blood as less attractive and this may have contributed to the poor feeding rate and low egg production [80]. Egg yields were unexpectedly low among all strains; therefore, it is suspected that having separated each female into its own small cage may have prevented the females from visual cues encouraging them to bloodfeed. Additionally, the Hunucma strain had only been reared in the lab for 2 generations, so it was less well-adapted to breeding in insectary conditions.

Despite these limitations, this study indicated that the V1016I mutation was statistically significantly associated with permethrin resistance, lower *Ae. aegypti* egg viability, longer time to pupation, and longevity. The F1534C mutation was not statistically significantly associated with any life-history traits, but was associated with permethrin resistance. Overall, the Hunucma and Merida strains were similar except that the Merida strain did not have elevated NSE activity, and associations were generally stronger in the Hunucma strain.

It would be useful to continue collecting these data over time in order to track the changes in fitness and mechanisms of insecticide resistance by generation, in both the absence and presence of insecticide pressure. Additionally, weighing the females before and after blood meals and determining the quantity of spermatozoa would help create a deeper understanding of what causes decreased fecundity and egg viability. Increased vector density, longevity, and biting frequency have the potential to increase the burden of vector-borne disease such as DENV [49]. Gaining a better understanding of female longevity is of particular interest, since a longer lifespan is the most important parameter associated with increased vectorial capacity [81]. A recent study found that target-site resistance mutations, particularly the *kdr* mutations, affect vector competence by increasing the probability of a *Plasmodium falciparum* infection and decreasing the intensity of infection in *Anopheles gambiae*, therefore leading to an increase in the transmission of malaria [82]. Understanding how insecticide resistance affects fitness, which in turn affects vectorial capacity of *Ae. aegypti* populations is necessary for effective vector control.

Recent studies from Mexico and Brazil reported a rapid dissemination of pyrethroid resistance and fixation of the V1016I mutation in *Ae. aegypti* populations [32,43]. Although an effective DENV vaccine may be on the horizon, vector control is still essential to controlling pathogen transmission [23,83]. A DENV vaccine would elevate herd immunity and vector control will lower the burden of infection [23]. Like with malaria and lymphatic filariasis, pathogen transmission is more rapidly and efficiently controlled when anti-parasitic drugs are combined with various forms of vector control [23]. Pyrethroid insecticides are

especially useful since they are highly toxic to insects and relatively non-toxic to humans, therefore maintaining their efficacy and preventing the development of pyrethroid resistance is essential. This study described the associations between resistance mechanisms and life-history traits in *Ae. aegypti* originating from the Yucatan peninsula of Mexico, an area of the world where DENV is hyperendemic. These findings may help provide a deeper understanding of how insecticide resistance may affect *Ae. aegypti* fitness, and as such, impact the effectiveness of insecticide-based vector control strategies.

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Table 1. Sequences of the primers used to make the RT-PCR reaction master mixes

Primer name	Primer sequence (5'-3')
Val1016f	GCGGGCGGCGGGGGCGGGGCCACAAATTGTTTCCCAC CCGCACCGG
Ile1016f	GCGGGCACAAATTGTTTCCCACCCGCACTGA
Ile1016r	TGATGAACCSGAATTGGACAAAAGC
Cys1534+f	GCGGGCAGGGCGGCGGGGGCGGGGCCTCTACTTTGTG TTCTTCATCATGTG
Phe1534+f	GCGGGCTCTACTTTGTGTTCTTCATCATATT
Phe1534+r	TCTGCTCGTTGAAGTTGTCGAT

Table 2. Pupation and emergence of *Ae. aegypti* mosquitoes by strain (expressed as percentage of initial 120 mosquitoes from each strain).

	Hunucma	Merida	Rockefeller
Pupated (%)	100	100	99.2
Emerged (%)	97.5	100	96.7
Female (%)	33.3	43.3	58.6

Table 3. Mean (\pm SD) adult longevity in days of *Ae. aegypti* mosquitoes by sex and population.

	Hunucma	Merida	Rockefeller
Females	64.5 (\pm 12.7)	54.3 (\pm 8.4)	24.0 (\pm 7.9)
Males	39.8 (\pm 13.6)	41.2 (\pm 12.2)	32.2 (\pm 15.2)
Both males and females	47.2 (\pm 17.5)	46.7 (\pm 12.6)	27.3 (\pm 12.1)

Table 4. Summary of data relating permethrin resistance phenotype to *kdr* genotype by population.

			V1016I					F1534C					Double Homozygotes	
Strain	Permethrin Phenotype	n	V/V	V/I	I/I	Freq. I	p*	F/F	F/C	C/C	Freq. C	p*	V/V & F/F	I/I & C/C
Merida	susceptible	74	64	10	0	0.067		32	32	10	0.351		32	0
	resistant	2	1	1	0	0.250		0	1	1	0.75		0	0
	Total	76	65	11	0	0.072	0.2702	32	33	11	0.362	0.5060	32	0
Hunucma	susceptible	31	22	8	1	0.161		17	13	1	0.242		17	1
	resistant	42	18	15	9	0.392		15	15	12	0.464		15	9
	Total	73	40	23	10	0.294	0.0197	32	28	13	0.370	0.1521	32	10

V/V and F/F are homozygous susceptible (SS); V/I and F/C are heterozygotes (SR); I/I and C/C are homozygous resistant (RR)

Table 5. Overall allele frequencies for the 1016I and 1534C mutations in Hunucma and Merida female *Ae. aegypti* from both the fitness and bottle bioassay experiments.

Strain	Allele	n	Frequency
Hunucma	I	63	0.310
	C	73	0.365
	Total Tested	126	
Merida	I	11	0.049
	C	54	0.319
	Total Tested	144	
Total	I	74	0.172
	C	127	0.341
	Total Tested	270	

Table 6. Associations of fitness, *kdr* genotype, phenotypic resistance, and enzyme activity by field strains in comparison with the susceptible Rockefeller lab strain.

	Hunucma	Merida
Life-history traits		
Time to pupation	*	*
Time to emergence	*	*
Female longevity	*	*
Fecundity [#]	-	-
Egg viability [#]	*	*
<i>kdr</i> mutation		
V1016I	*	*
F1534C	*	*
Enzyme activity		
glutathione- <i>S</i> -transferases (GST)	*	*
mixed function oxidases (MFO)	*	*
non-specific esterases (NSE)	*	-
Permethrin Resistance	Resistant	Suggestive of incipient resistance

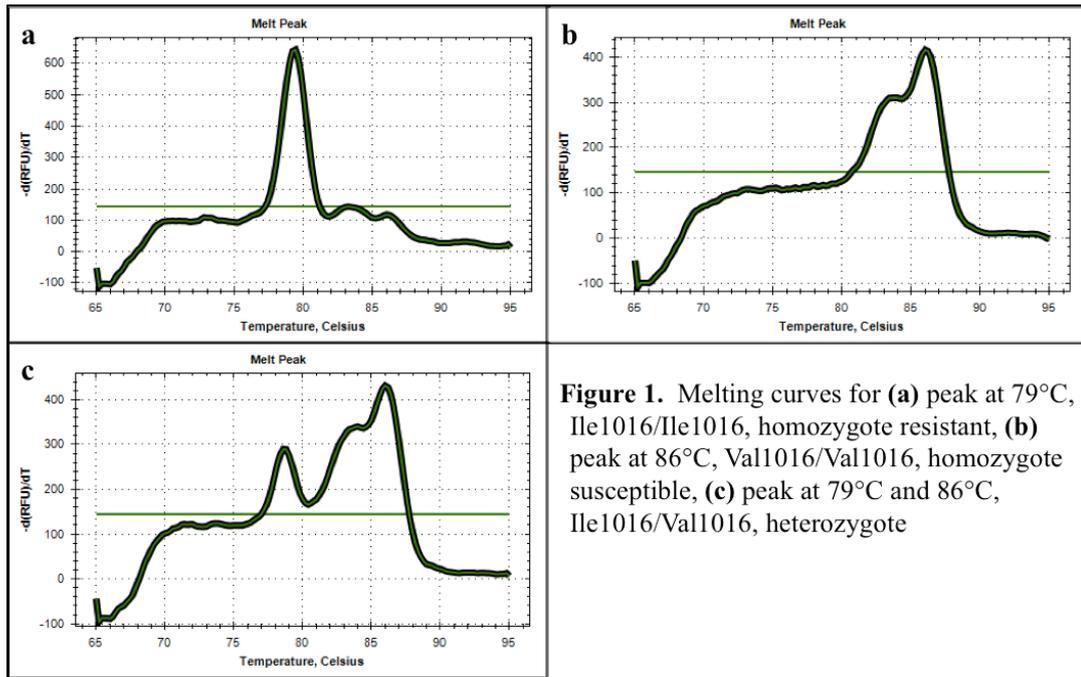
* Significant association ($P < 0.05$)

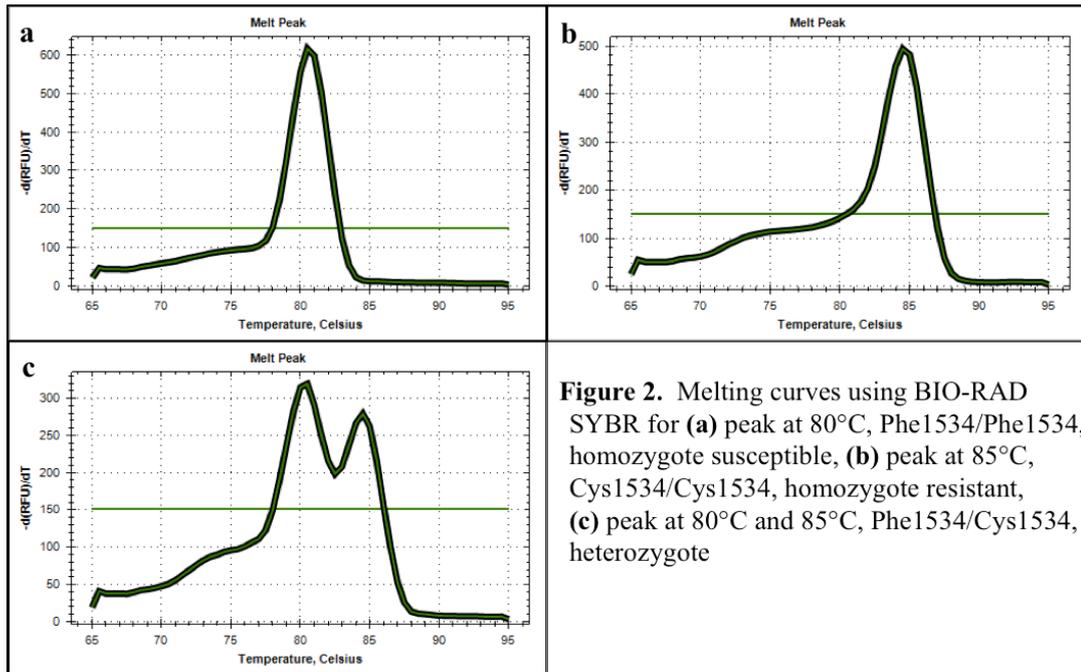
- Non-significant association ($P > 0.05$)

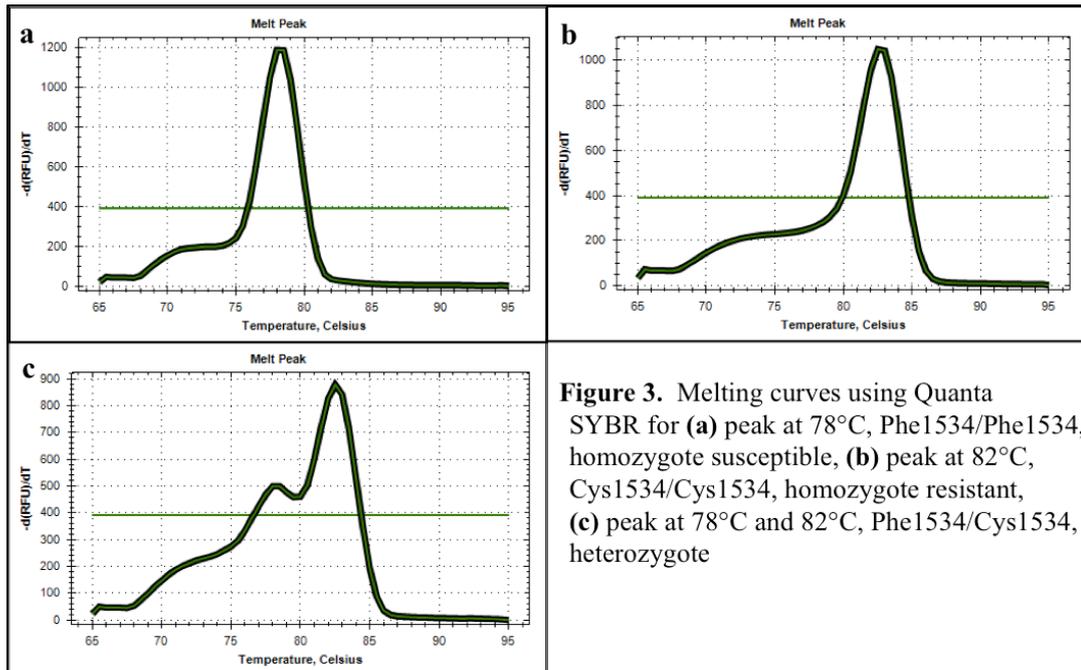
[#] Rockefeller was only bloodfed once, therefore egg viability and fecundity of Hunucma and Merida were compared to each other.

Table 7. Associations between fitness parameters and phenotypic resistance by *kdr* mutation allele, Hunucma and Merida *Ae. aegypti*

	V1016I	F1534C
Life-history traits		
Time to pupation	*	-
Female longevity	*	-
Fecundity	-	-
Egg viability	*	-
Phenotypic resistance	*	*
* Significant association ($P < 0.05$)		
- Non-significant association ($P > 0.05$)		
Rockefeller mosquitoes were not included		







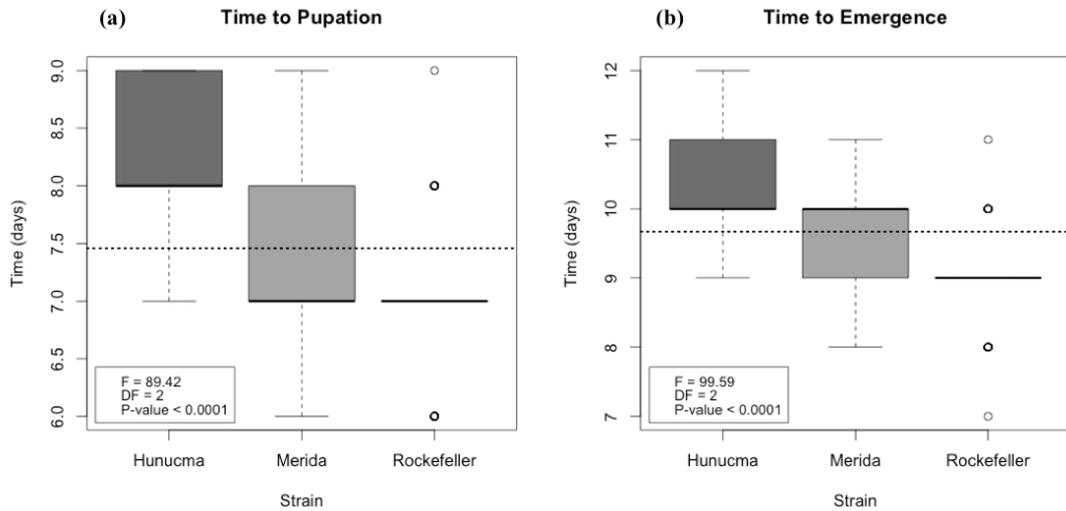


Figure 4. Box-plots of (a) time to pupation and (b) time to emergence in days of the 3 strains of *Ae. aegypti* mosquitos used for fitness experiments. The dotted line represents the mean time of all 3 strains; the black line represents the median; the box represents the 25th percentile (lower quartile) to the 75th percentile (upper quartile); the upper whisker represents 1.5(IQR) above 75th percentile; the lower whisker represents 1.5(IQR) below the 25th percentile; circles represent outliers.

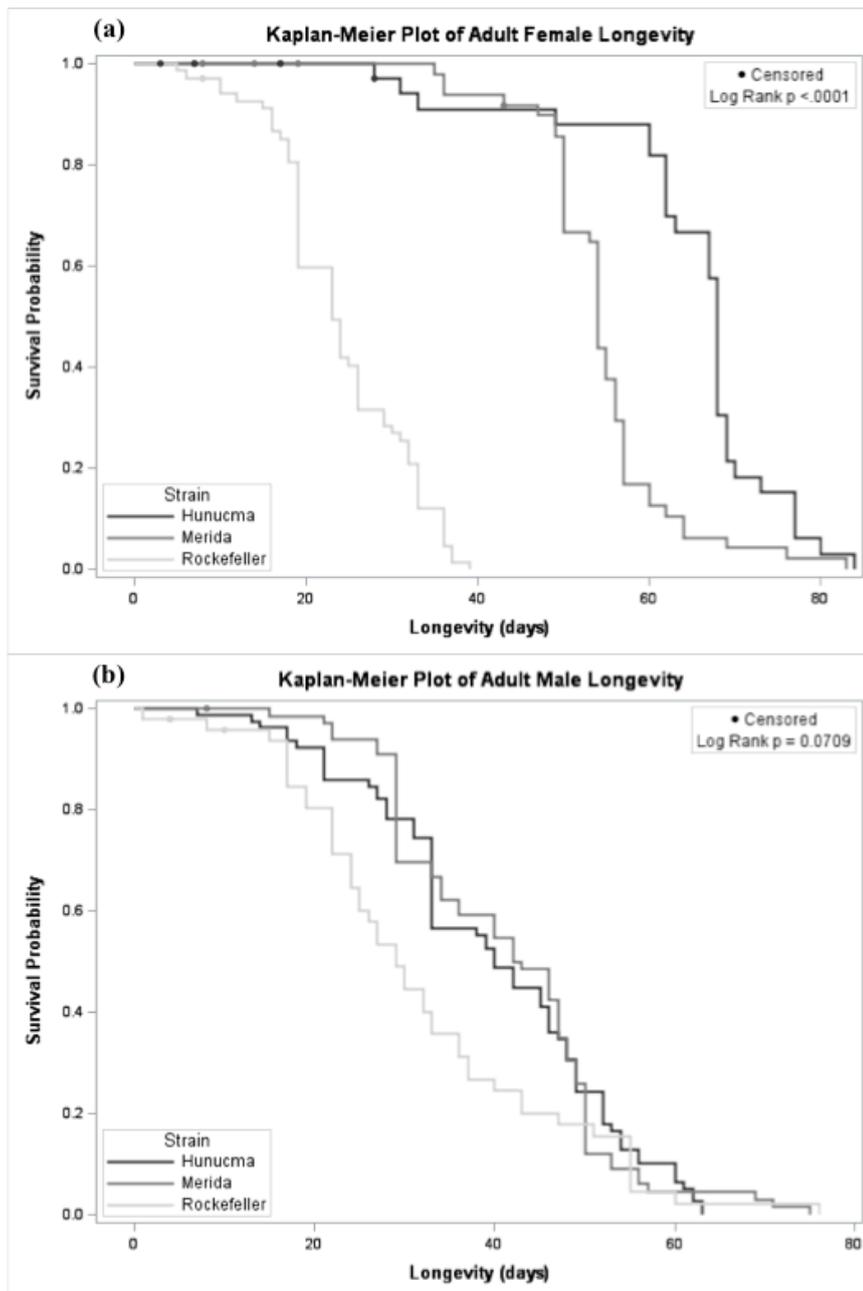


Figure 5. Kaplan-Meier Survival Analysis Curves of adult Hunucma, Merida, and Rockefeller *Ae. aegypti* mosquitoes reared for fitness experiments. **a:** female longevity; **b:** male longevity

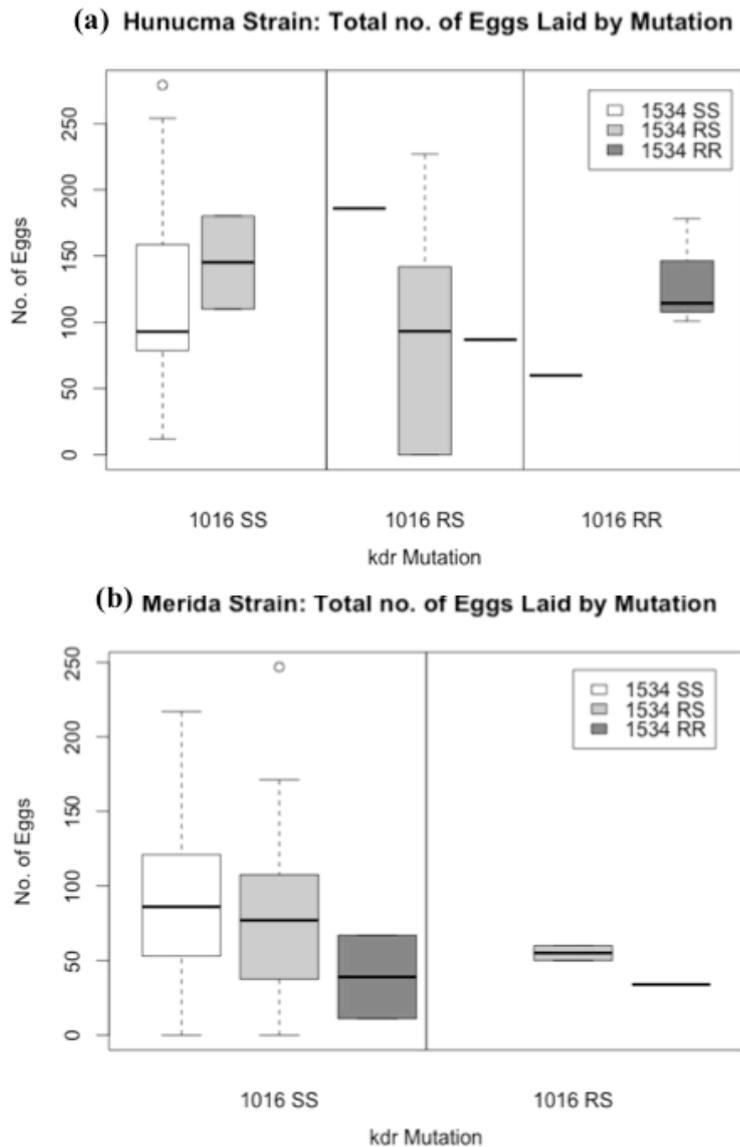


Figure 6. The total number of eggs laid by each female by *kdr* mutation **(a)** Hunucma and **(b)** Merida. V1016I, F534C; homozygous susceptible (SS); heterozygotes (SR); homozygous resistant (RR). A single line indicates there was only one female in this category; the black line represents the median; the box represents the 25th percentile (lower quartile) to the 75th percentile (upper quartile); the upper whisker represents 1.5(IQR) above 75th percentile; the lower whisker represents 1.5(IQR) below the 25th percentile; circles represent outliers.

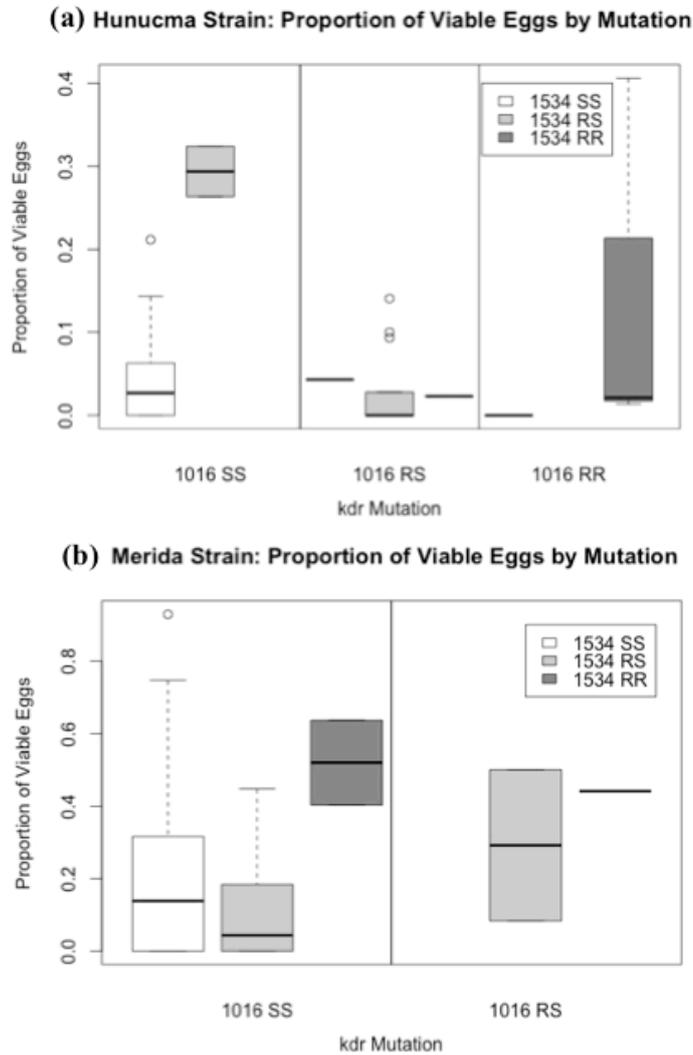


Figure 7. Proportion of viable eggs to total laid eggs, by *kdr* mutation **(a)** Hunucma and **(b)** Merida. V1016I, F534C; homozygous susceptible (SS); heterozygotes (SR); homozygous resistant (RR). A single line indicates there was only one female in this category; the black line represents the median; the box represents the 25th percentile (lower quartile) to the 75th percentile (upper quartile); the upper whisker represents 1.5(IQR) above 75th percentile; the lower whisker represents 1.5(IQR) below the 25th percentile; circles represent outliers.

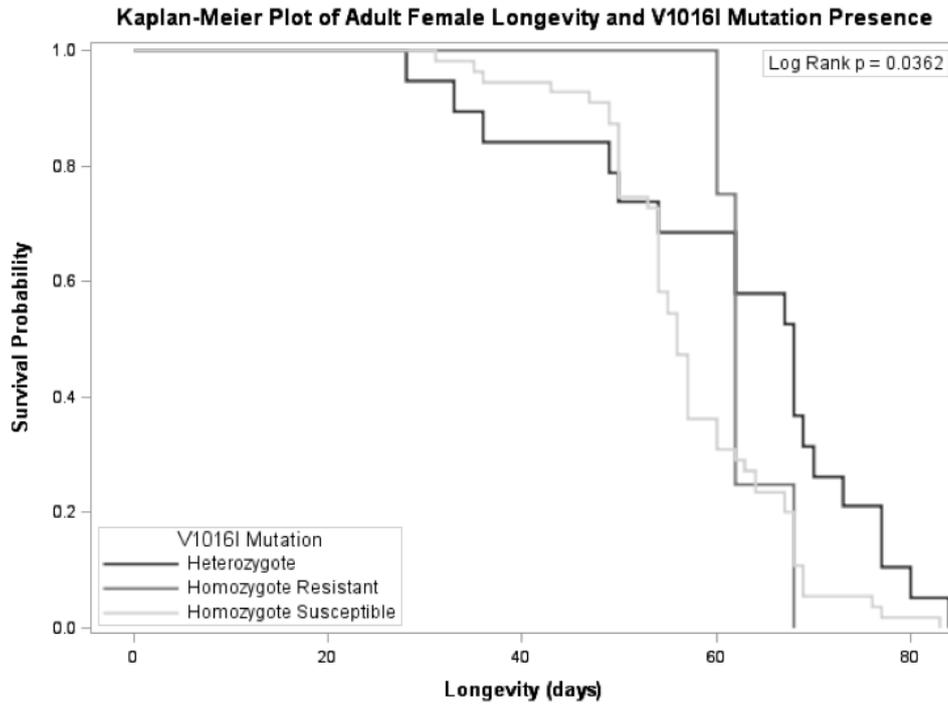


Figure 8. Kaplan-Meier Survival Analysis Curves of female longevity and V1016I allele among Hunucma, and Merida, and *Ae. aegypti* mosquitos reared for fitness experiments.

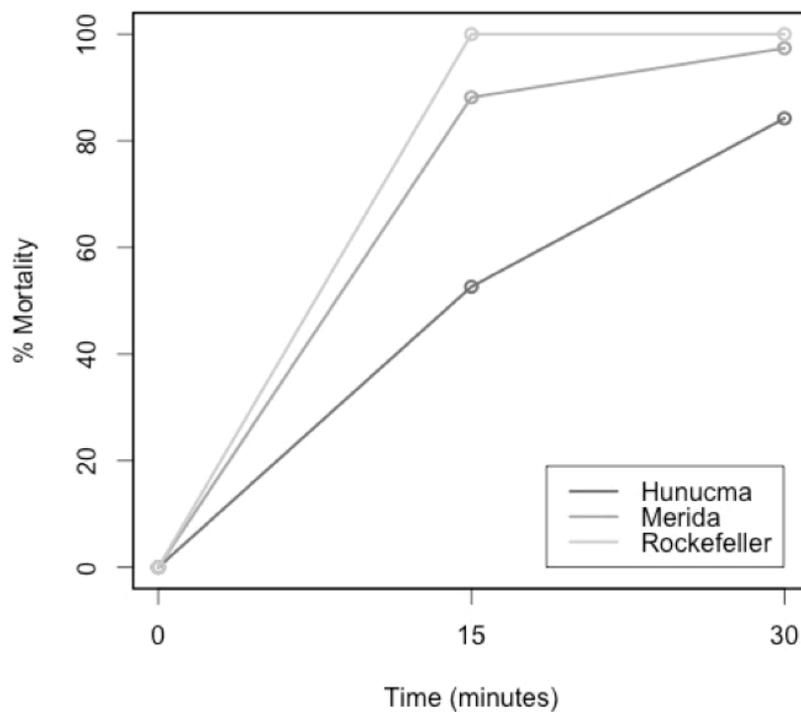


Figure 9. Knockdown times for female *Ae. aegypti* exposed to the diagnostic dose of permethrin (15 μ g) in CDC bottle bioassays for 30 minutes.

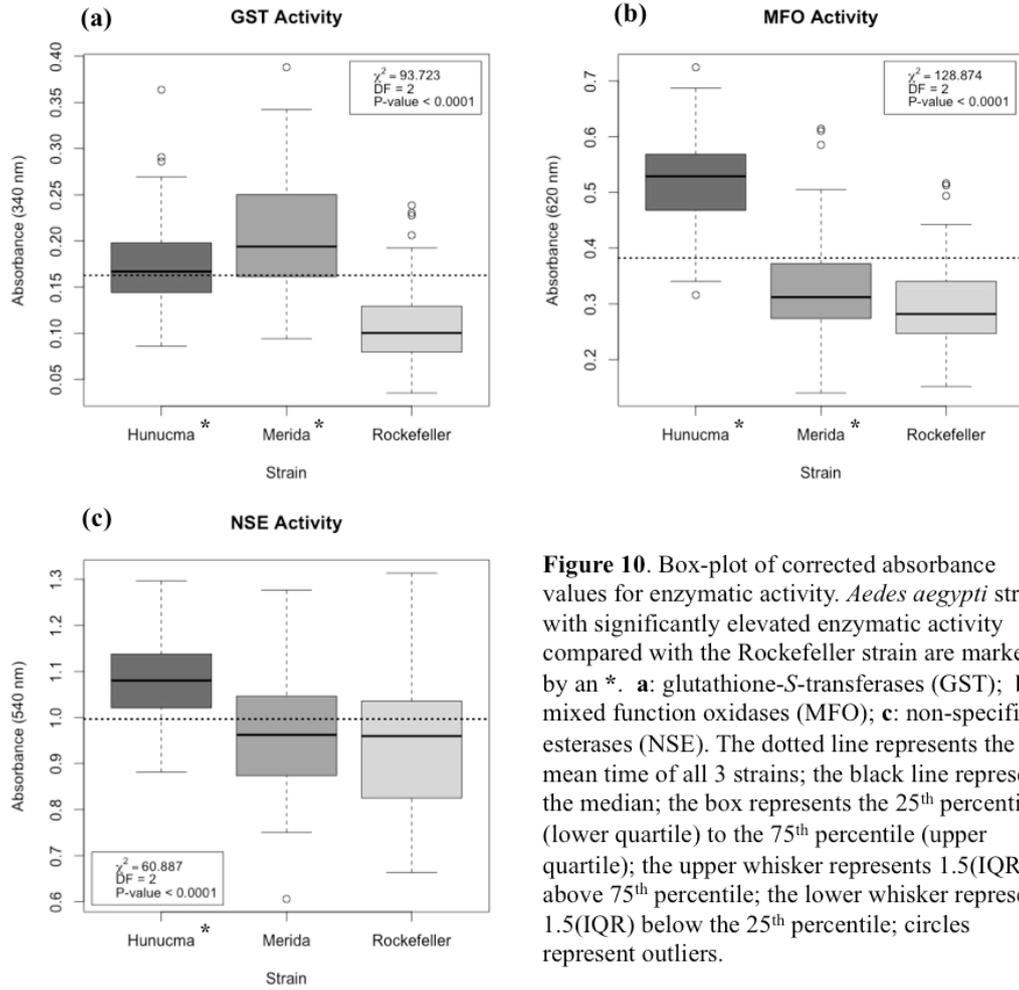


Figure 10. Box-plot of corrected absorbance values for enzymatic activity. *Aedes aegypti* strains with significantly elevated enzymatic activity compared with the Rockefeller strain are marked by an *. **a:** glutathione-S-transferases (GST); **b:** mixed function oxidases (MFO); **c:** non-specific esterases (NSE). The dotted line represents the mean time of all 3 strains; the black line represents the median; the box represents the 25th percentile (lower quartile) to the 75th percentile (upper quartile); the upper whisker represents 1.5(IQR) above 75th percentile; the lower whisker represents 1.5(IQR) below the 25th percentile; circles represent outliers.