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Laboratory evaluation of the Ile1, 016 mutation-effect on several life-history parameters of *Aedes aegypti*

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Abstract
Pyrethroid insecticides were intensively applied in Mexico between 2000 and 2009. We evaluated the effect of the Ile1, 016 mutation on several life-history parameters of *Ae. aegypti* from Merida, Yucatan. *Aedes aegypti* colonies was established from larvae collected in houses between October and November 2015. Fifty adult females were analyzed for the frequency of the Ile1, 016 mutation at each collection site (n = 3). All biological parameters were evaluated by comparing the control group versus natural population of *Ae. aegypti*. The frequency of kdr homozygous (Val1, 016) was high in all sites studied, being 76% in Juan Pablo-II, 85% in Chuburna and 87% in Vergel-III. In *Ae. aegypti* with kdr homozygous were observed delay larval development, higher mortality in late stages, decrease in blood ingested and have a larger body sizes. Our results suggest that *Ae. aegypti* with kdr homozygous have a diminished reproductive fitness compared to susceptible group.

Keywords: *Aedes aegypti*, knockdown resistance, life-table, blood fed, morphometry

1. Introduction
Aedes (*Stegomyia*) *aegypti* (L.) is the primary vector for the arboviruses causing dengue, chikungunya and Zika in Mexico [1-3]. The co-circulation of these viruses represents a major public health challenge for the country. Effective methods to reduce transmission of these arboviruses has been through the control or eradication of *Ae. aegypti* using outdoor ultra-low volume application of insecticides coupled to indoor residual spray and more recently insecticide impregnated materials [4]. Since 1978, when dengue re-emerged in Mexico [5], the Ministry of Health has used a series of insecticides for vector. The adulticide malathion was used for ultra-low volume (ULV) space spraying from 1981 to 1999 of wide areas of *Ae. aegypti* distribution. In 2000, the vector control programs in Mexico switched to pyrethroids for adult mosquito control [6] which included synthetic pyrethroids such as permethrin and deltamethrin [7]. In addition, bed nets, curtains and other household items treated with pyrethroids for personal protection are seeing increased use [4]. The long-term application of insecticides has resulted in the intense selection pressure for resistance and emergence in *Ae. aegypti* [6]. Knockdown resistance' to pyrethroids (kdr) is frequently caused by nonsynonymous mutations in the voltage-gated sodium channel transmembrane protein (para) resulting in reduced pyrethroid binding [7].
The Ile1,016 mutation is known to be associated with resistance to type I and II pyrethroids (including permethrin and deltamethrin, respectively), while the Cys1534 allele is thought to be associated most strongly with resistance to type I pyrethroids [7-8]. In Mexico, the Ile1, 016 mutation in natural populations of *Ae. aegypti*, which is associated with very high knock-down resistance to the pyrethroid insecticide permethrin was detected between 1996-2009 [6, 7]. Recently, the Cys1, 534 mutation was found in the same mosquito collections that were previously analyzed for Ile1, 016 [8]. Knowledge of the resistance status and its underlying mechanisms are the first steps toward a more effective resistance management [7]. However, resistance has a significant adaptive value in the presence of insecticides [9]. Fitness costs have

been described as the adaptive consequences of resistance [10]; it has been observed that resistant and susceptible strains of *Ae. aegypti* frequently differ in fitness components, including longevity, fecundity and fertility [9-13]. Thus, since the advantage of fitness is not conclusive among resistant strains, a study in a well-defined geographical scale may provide evidence of fitness cost associated to site targeted mutation in natural populations.

Currently, there is limited knowledge of morphometry of *Ae. aegypti* associated with insecticide resistance [10, 14]. Specific phenotypes are selected under insecticide selection pressure [7]. This phenotypic variation can be scored by measurable changes in the insect morphology. By morphometric techniques it is possible to measure size, shape, and the relation between size and shape of insects [15]. This technique has been used in studies of differentiation of sympatric populations of *Anopheles* and *Culex* species [16] and can also be used to determine the morphological changes of mosquitoes associated to insecticide resistance.

The strong dependence on pyrethroids to control adult populations of *Ae. aegypti* in the State of Yucatan has led to the presence of the Ile1,016 and Cys1,534 mutations in these insects which confers knockdown resistance to permethrin and deltamethrin [6, 17]. However, the fitness cost associated to pyrethroid-resistance of *Ae aegypti* is unknown. In the present study, we evaluated the effect of the Ile1, 016 mutation on several life-history parameters of *Ae. aegypti*, in order to fill these gaps in our knowledge.

2. Materials and Methods

2.1 Rearing of *Ae. aegypti* laboratory colonies

Three *Ae. aegypti* colonies were used in this study and were established from larvae collected from buckets placed in houses in the city of Merida (population ~ 800,000) over a two-month period from October to November 2015. At each collection site, we collected immature larvae from at least 15 different buckets in each of three different neighborhoods located at least 10 km apart from each other (Figure 1). Emerged adults were identified to species and *Ae. aegypti* females were blood fed on mice to generate F1 eggs. Larvae development was maintained at 28±1 °C, 80% RH with a photoperiod of 12-h light-dark cycle. Merida city is located in the Yucatan State in southeastern Mexico. The area has a flat and low elevation (~0-250 m) with bedrock of limestone and a subtropical climate. In the last ten years (2006-2015) the city has experienced a mean annual temperature of 26.5 °C, with a maximum of 34.2 °C and minimum of 21.1 °C recorded. The mean annual rainfall was 871 mm, with peaks in June-September in the range of 1,161 mm to 1,792 mm (based on data from a weather station operated by Comision Nacional del Agua at the Merida airport, http://www.tutiempo.net/clima/Aerop Internacional_Merida _Yuc/766440.htm; last accessed December 2016). Vergel-III and Juan Pablo-II neighborhoods were selected in this study because they are neighborhoods with high dengue virus transmission [1] and the Ile1, 016 mutation has been reported in *Ae. aegypti* [6, 17]. Chuburna, a neighborhood with low dengue transmission where the *Ae. aegypti* Ile1, 016 mutation is unknown was included because we expected to find low frequency of this mutation. At the time of this study, local authorities used ultra-low volume (ULV) spraying with the organophosphate insecticides (chlorpyrifos and malathion) and indoor space spraying with pyrethroids (deltamethrin) and organophosphates (malathion) for adult *Ae. aegypti* control [18]. The study was conducted in the Arbovirus laboratory of the Universidad Autonoma de Yucatan between November 2015 and April 2016. The susceptible strain New Orleans donated by Colorado State University, was used as a control.

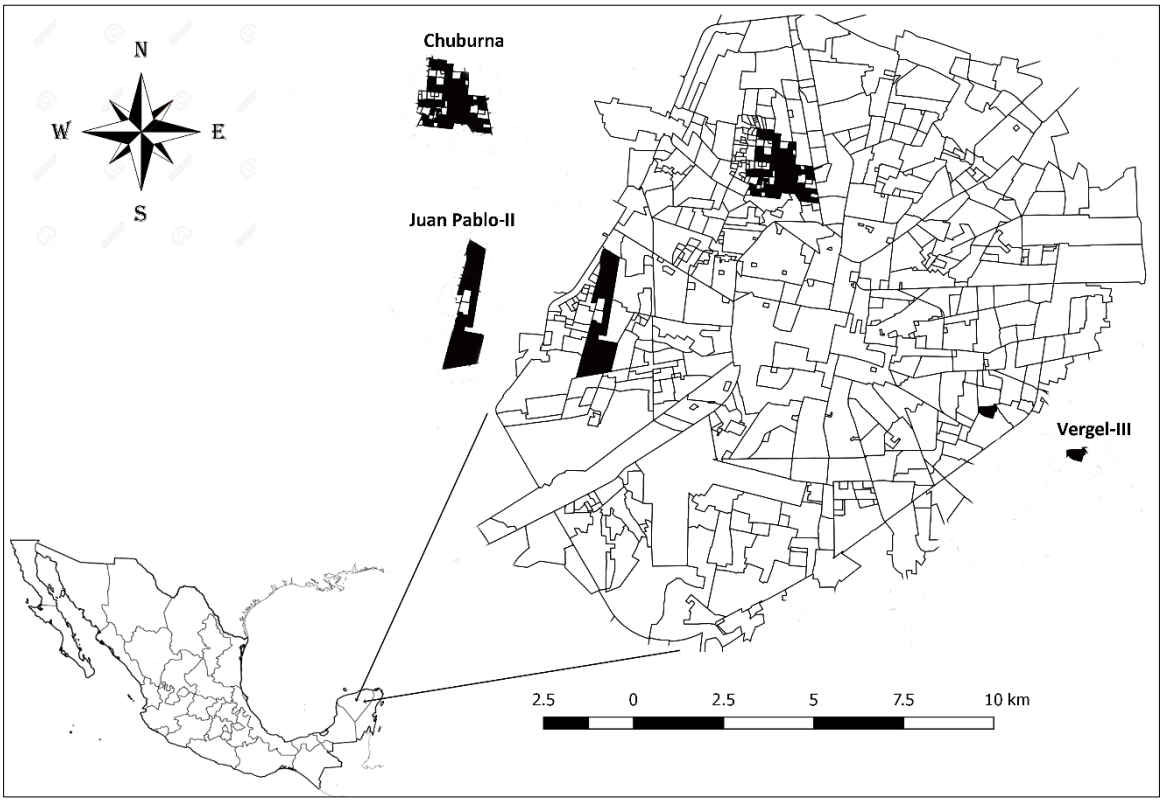

Fig 1: Study site in Merida city, Yucatan, Mexico

2.2 DNA isolation and analysis of the frequency of the Ile1, 016 mutation

To examine the presence of the Ile1, 016 mutation, 50 *Ae. aegypti* females (F_1) at each collection site were used. Deoxyribonucleic acid (DNA) was obtained from individual adults by salt technique [19]. The concentration and quality of each DNA sample were determined using a Thermo Scientific NanoDrop 2000 R spectrophotometer (Providence, RI) and visualized on 2% agarose in the ultra lum U.V trans illuminator. Genotypes at the Ile1,016 locus were detected using Real-Time PCR (Polymerase chain reaction) to identify Isoleucine 1,016 in the *Ae. aegypti* "para" gene [6, 17]. The reaction mixture consisted of 5.34 µl ddH2O, 12.5 µl of 2x IQ™ SYBR® Green Supermix, 0.72µl primer Val 1016F, 0.72µl primer Ile 1016F and 0.72µl primer Ile 1016R for a total volume of 20µl. The PCR reaction mixture was heated to 95°C for 3 min, 95°C for 10 s, 60°C for 10 s, 72°C for 30 s, 39 more times to step 2 min with 95°C for 10 s, melting curve 65°C to 95°C, increment 0.2°C per 10 sec. Genotypes were determined in a single-tube reaction using two different "allele-specific" primers, each of which contained a 3´nucleotide corresponding to one of the two alleles and a reverse primer that amplified both alleles. Allele specific primers were manufactured (Operon Inc., Huntsville, Al) with 5´tails [20] that were designed to allow discrimination between SNP alleles based on size or melting temperature.

The valine allele specific primer was Val1, 016 (5´ [GCGGGCAGGGCGGCG GGGGCGG GGCC] ACAAATTGTTTCCCACCCGCACCG G-3´) and the isoleucine allele specific primer was Ile1, 016 (5´-[GCGGGC]ACAAATTGTTTCCCA CCCGC ACTGA-3´). Brackets indicate the portion of the primer added for melting curve PCR. The reverse primer was Ile1, 016r 5´-GGATGAACCS AAATTGGACAAAAGC-3´ [7].

2.3 Life-table of *Ae. aegypti*

All biological parameters were evaluated by simultaneously comparing New Orleans (control) versus Vergel-III, Juan Pablo-II and Chuburna strains; were reared under identical conditions, including initial larval density, feeding, temperature and illumination regimens. Eggs were induced to hatch for approximately 24 hours and then used for the bioassays.

The development of each immature stage from the first instar to the fourth instar and up to the pupal stage was observed daily. Each strain was evaluated in ten replicates of 25 newly emerged larvae and were then randomly transferred to plastic trays (28x23x11 cm) with 1.5 L dechlorinated water and 0.5 g of goldfish food (Biopro®, Monterrey, N. L., Mexico). New food supplement was added every two days. Life-table parameters were calculated according to the equations described [21]. The number of larvae that survived (n_x) were counted and recorded. Mortality, expressed as a mortality rate (q_x) was calculated as follows: $q_x = d_x/n_x$, where x = age interval (day), d_x = number dying during the age interval x to $x + 1$, and n_x = number of survivors at start of age interval x.

Each emergent pupa was separated into a container with approximately 100 ml of water. The container was identified with a label carrying the date and time of pupation. After 72 hrs., the containers were examined for pupal mortalities, and the number of dead pupae was noted. Mosquitoes that died in the process of emergence were also recorded as a part of pupal mortality.

2.4 Feed and fecundity of *Ae. aegypti*

Mated four-day old females (F_2) were blood-feed on anesthetized mice (Animal Ethic authorization: CEI082015-CIR-UADY). Batches of 30 freshly fed females by study site were killed by freezing and weighed in an analytical balance (Velab® ve-204). The amount of blood ingested by females was inferred as the weight ratio before and after the blood meal, as previously described [22]. The remaining females were allowed to develop their eggs and were kept in 30 cm^3 screened cages at 27 ± 1°C, 70% RH, and fed with sucrose solution (10%) by mean of cotton pads. After the blood intake, females reached the state of gravid at 4 days. Then, females were dissected in a drop of l0% phosphate-buffered saline (PBS). Ovaries were removed from the abdomen and separated using dissecting needles. The number of mature oocytes (eggs) were recorded for each female using a microscope at the magnification of 32X.

2.5 Morphometric parameters

Reared under identical conditions, 50 females (F_2) of each strain with four-day old were used for the morphometric analysis. Females were killed by freezing and then were dissected and measured. The head, wings and legs were carefully separated from the mosquito body and mounted on glass microscope slides [16]. Fourteen metric characters on the mosquito specimens were measured (Table 1) using a stereoscopic microscope (Carl ZEISS Microlmaging®, Germany) with an eyepiece between 10X and 32X. A micrometer (100 divisions = 10 mm) was calibrated using a micrometric lamina (0.001 mm). All measurements were carried out by the same person in order to avoid between-individual variations.

2.6 Data analysis

Statistical analyses were performed using the IBM SPSS Statistics version 22 software for Windows (IBM Corporation, Armonk, NY), and results were considered significant when $P \leq 0.05$. A 2 x 2 contingency table and the chi-square analysis was used to test the relationship of frequency of the Ile1, 016 mutation by neighborhoods in Merida city. The allele frequencies for Ile1016 were calculated using the equation [6]:

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

Table 1: Mean and standard deviation (±) of 14 metric characters of *Ae. aegypti* from Merida, Yucatan, Mexico.

Metric characters	Control group	Populations with kdr mutations			
(Length in millimeter)	New Orleans	Juan Pablo-II	Vergel-III	Chuburna	CV
Antenna	1.50±0.09	1.44±0.10	1.47±0.09	1.51±0.09	0.07
Proboscis	1.67±0.09	1.68±0.08	1.68±0.10	1.69±0.09	0.05
Palpi	0.10±0.02	0.09±0.03	0.09±0.02	0.09±0.03	0.27

Abdomen	2.45±0.19	2.58±0.19	2.48±0.18	2.45±0.16	0.08
Wing	2.74±0.16	2.77±0.05	2.70±0.17	2.73±0.15	0.06
Fore femur	1.57±0.09	1.63±0.06	1.62±0.10	1.63±0.06	0.07
Mid femur	1.68±0.13	1.72±0.09	1.70±0.12	1.70±0.10	0.07
Hind femur	1.73±0.13	1.77±0.08	1.72±0.13	1.72±0.12	0.07
Fore tibia	1.56±0.08	1.58±0.03	1.58±0.08	1.64±0.08	0.06
Mid tibia	1.74±0.10	1.76±0.08	1.78±0.13	1.80±0.12	0.07
Hind tibia	1.84±0.11	1.86±0.09	1.84±0.13	1.88±0.12	0.06
Fore tarsus	1.90±0.15	1.98±0.06	1.95±0.08	2.04±0.06	0.07
Mid tarsus	2.18±0.14	2.29±0.11	2.23±0.14	2.29±0.14	0.07
Hind tarsus	3.19±0.17	3.38±0.15	3.24±0.20	3.32±0.19	0.08

CV: Coefficient of variation

The Kaplan-Meier test was used to analyze time in days from newly hatched larvae to adult form for each strain and the survival curves were compared using the log-rank test. To compare the number of eggs and blood ingested at each strain of *Ae. aegypti*, data were submitted to a normality test. Means with a normal distribution were compared by one-way Analysis of Variance (ANOVA) test. Significant results of ANOVA test were followed by a post-hoc Dunnett test for multiple comparisons of means.

The 14 metric characters examined were analyzed with multivariate statistics such as principal component analysis, followed by discriminant function analysis. The measurements were not transformed to preserve the possible influence of differences in the body sizes among strains of *Ae. aegypti*. Bartlett's sphericity test was used to test if k samples were from populations with equal variances. If the samples were from populations with unequal variances, then is rejected the null hypothesis of the test ($P \leq 0.05$), and therefore a principal component analysis was deemed to be appropriate. Principal component analysis was conducted to simplify subsequent analysis of the data. The test is an ordination technique that reduces the number of dimensions (variables) in a set of data, finds linear combinations of the more correlated variables that explain most of the variance, and eventually assigns a component score to each original individual. The analysis generates a set of principal components by weighting all the available variables. The first component explains the most variation; the second explains the next most variation, and so on [23]. Discriminant function analysis which concerns with classification and aims to obtain a small number of useful discriminating variables [24] was carried out based on the results of the principal component analysis to assess the degree of discrimination between control group and populations with kdr mutations [16, 25]. The ability of this function to identify the metric characters of *Ae. aegypti* associated with kdr mutations was indicated as the percentage of individuals correctly classified from the sample that generated the function.

3. Results

3.1 Frequency of the mutations Ile1, 016

From all mosquitoes genotyped, we did not find Ile1, 016 homozygotes. The frequency of the heterozygotes (Ile1, 016/Val1, 016) was 34.67% (52/150) and the frequency of the kdr homozygous (Val1,016) was 65.33 % (98/150). There was a significant association between kdr mutations and neighborhoods ($X^2 = 6.06$, *d. f.* = 2, $P = 0.04$). The frequency of the kdr homozygous (Val1, 016) in all neighborhoods was high, being 76% in Juan Pablo-II, 85% in Chuburna and 87% in Vergel-III.

3.2 Life-table of *Ae. aegypti*

Overall, of the 1,000 newly hatched larvae, 89% (890/1,000) reached adulthood. Survival rate was similar among the control group (New Orleans) and *Ae. aegypti* with kdr homozygous. According to the log-rank test, the estimated hazard curve did not differ significantly between the mean for survival time per strain ($X^2 = 2.389$, *d. f.* = 3, $P = 0.496$). The fulltime of development from the first instar up to the adult stage was 15 days for the New Orleans and Vergel-III strains, and 19 and 20 days for the Chuburna and Juan Pablo-II strains, respectively. A mortality of 75% (24/32) in early stages (I-II) of the control group (Table 2) was observed. In *Ae. aegypti* with kdr homozygous (Val1,016), the mortality was present in late stages, ranging from 52% to 73%.

Overall, the percentage of emerged females (48.09%) and males (51.91%) was similar, with slight tendency of males by study site. In the Juan Pablo site, there was more females (55.16%) than males (44.84%) (Table 2).

Table 2: Life-table attributes of *Ae. aegypti* associated with the Ile,016 mutation in Merida, Yucatan, Mexico.

Immature stage	New Orleans	Juan Pablo-II	Vergel-III	Chuburna
Number of death (%)				
Larvae I	3 (9.38%)	2 (7.41%)	4 (18.18%)	8 (27.59%)
Larvae II	21 (65.63%)	10 (37.04%)	2 (9.09%)	6 (20.69%)
Larvae III	2 (6.25%)	2 (7.41%)	5 (22.73%)	4 (13.79%)
Larvae IV	4 (12.50%)	7 (25.93%)	8 (36.36%)	9 (31.03%)
Pupae	2 (6.25%)	6 (22.22%)	3 (13.64%)	2 (6.90%)
Total	32 (100%)	27 (100%)	22 (100%)	29 (100%)
Fulltime of immature development (mean ± in days)				
1st Larvae to Pupae	13.12±0.33	18±0.33	13.25±0.29	17.16±0.25
1st Larvae to Adults	15.12±0.33	20±0.29	15.16±0.29	19±0.25
Number of emerged adults (%)				
Female	98 (44.95%)	123 (55.16%)	102 (44.74%)	105 (47.51%)
Male	120 (55.05%)	100 (44.84%)	126 (55.26%)	116 (52.49%)
Total	218 (100%)	223 (100%)	228 (100%)	221 (100%)

3.3 Feed and fecundity of *Ae. aegypti*

Overall, there was a significant statistical difference among blood ingested per females at each study site (F = 13.551, *d. f.* = 3, P = 0.000). *Aedes aegypti* females of Juan Pablo-II ingested less blood (3.98±0.75 µg) compared with Chuburna (4.24±0.79 µg), Vergel-III (4.79±0.67 µg) and New Orleans (5.34±1.32 µg). The post hoc Dunnett t-test treated New Orleans as a control group and compared all other strains against it (Juan Pablo-II, Vergel-III and Chuburna). This result revealed that there was a statistical difference between blood ingested per *Ae. aegypti* females of New Orleans versus Juan Pablo-II ($P \leq 0.05$) and Chuburna ($P \leq 0.05$) (Figure 2A).

Overall, there was a significant statistical difference among mean number of eggs per females at each study site (F = 4.872, *d. f.* = 3, P = 0.003). *Aedes aegypti* females of Juan Pablo-II developed a lower mean of eggs (99.27±23.01) compared with females of New Orleans (106.97±23.36), Vergel-III (115.03±15.85), and Chuburna (116.06±14.76). When the mean of eggs from *Ae. aegypti* females was analyzed with the post hoc Dunnett t-test, there was no a significant statistical difference from the control group (New Orleans) and *Ae. aegypti* with kdr homozygous ($P \geq 0.05$, Figure 2B). A significant difference was found among number of eggs of *Ae. aegypti* females of Juan Pablo-II versus Chuburna ($P \leq 0.05$) and Vergel-III ($P \leq 0.05$) strains.

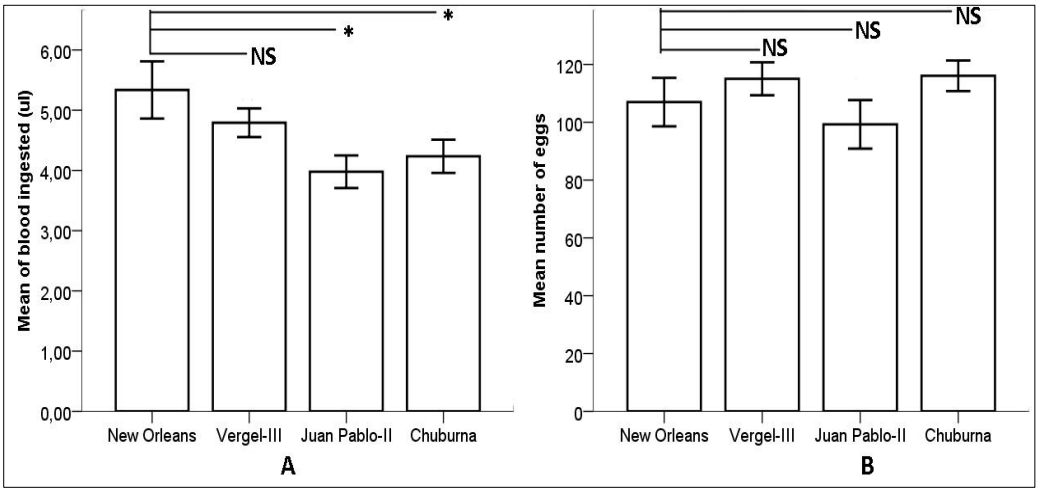

Fig 2: Comparison of control group (New Orleans) and natural population of *Ae. aegypti* with the Ile,016 mutation in Merida, Yucatan. (A) One-way ANOVA test was used to compare the mean of blood ingested per strains of *Ae. aegypti*. (B) One-way ANOVA test was used to compare mean number of eggs per strains of *Ae. aegypti*. The Post Hoc Dunnett t-tests treat one group as a control (New Orleans), and compare all other groups against it (Juan Pablo-II, Vergel-III and Chuburna). Asterisk above the error bars (SE) indicate a significant difference ($P \leq 0.05$). NS: no significant

3.4 Morphometry of *Ae. aegypti*

Principal component analysis (PCA) was carried out to simplify the analysis of morphometric data (Table 1). The results of this analysis produced two factors that collectively explained 67% of the variation in the morphometric of *Ae. aegypti* females, with a significant Bartlett's sphericity test (P < 0.001). The first component was heavily loaded (56.43%) by variables with locomotion function, mainly characterized by legs (femur, tibia and tarsus), wings and abdomen (Table 3). The second component (10.57%) was largely influenced by the appendages of the head (proboscis, palpi and antenna).

Box and whisker plot was built with the coefficients of PCA1 (Figure 3) and it showed a significant difference between a New Orleans and compared to the *Ae. aegypti* with kdr homozygous (Val1,016) (F = 12.47, *d.f.* = 200, P = < 0.001). According the overall mean score, *Ae. aegypti* females of the kdr populations had larger body sizes compared with control group (Figure 3A). This result was confirmed by the post hoc Dunnett test that revealed a significant difference among *Ae. aegypti* females of New Orleans compared with Juan Pablo-II (P < 0.001), Vergel-III (P < 0.001) and Chuburna (P < 0.001). Although the variance explained by PCA2 was comparatively lower (~10%), its analysis showed a significant difference between control group and strains with kdr homozygous (Val1,016) (F = 6.18, *d.f.* = 200, P = < 0.001). In contrast to the first component, in PCA2, *Ae. aegypti* females of the control group had larger length the antenna, palpi, and proboscis compared with kdr homozygous (Figure 3B). The post hoc Dunnett test revealed a significant difference among females of New Orleans compared with Juan Pablo-II (P < 0.001), Vergel-III (P < 0.001) and Chuburna (P < 0.001).

Table 3: Principal component analysis of metric characters of *Ae. aegypti* to discriminate between control and populations with kdr mutations in Merida, Yucatan, Mexico.

Metric characters	Coefficients	
	PCA1	PCA2
Fore femur	[a]0.819	0.056
Mid tarsus	[a]0.808	0.214
Mid tibia	[a]0.793	0.272
Mid femur	[a]0.748	0.378
Fore tibia	[a]0.748	0.126
Hind tibia	[a]0.742	0.382
Fore tarsus	[a]0.700	0.055
Hind tarsus	[a]0.693	0.158
Hind femur	[a]0.686	0.340
Wing	[a]0.636	[a]0.501
Abdomen	[a]0.545	0.393
Palpi	-0.029	[a]0.798
Proboscis	0.411	[a]0.744
Antenna	0.205	[a]0.708

The percentage variation for each component is PCA1 (56.43%) and PCA2 (10.57%). The coefficients are sorted by decreasing magnitude at each component. [a]Scores >0.5.

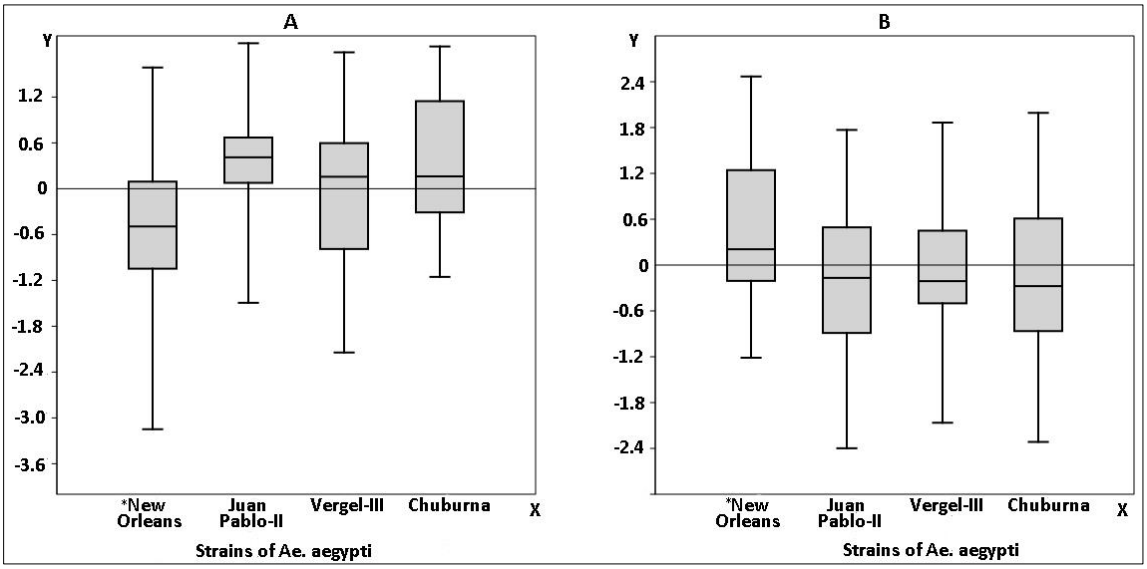

Fig 3: Box and whisker plot of the scores of principal components. A) PCA1 (56.43% explained variance) of the control group and populations with kdr mutations; B) PCA2 (10.57% explained variance). Value zero on the y-axis is the grand centroid (overall mean of the component score). *Control group

Multiple discriminant analysis was used to analyze the 11 metric characters which emerged from PCA1 because they comprised most influential characters for morphometry of *Ae. aegypti* females (Table 3). The characters used were: wing, abdomen, femur (fore, mid and hind), tibia (fore, mid and hind) and tarsus (fore, mid and hind). When these characters were subjected to discriminant function analysis, three functions were derived, being the first function most significant to discriminate between groups. The canonical correlation coefficient was 0.63; this finding suggests a good correlation between the discriminant function and the original variables, allowing differentiation among strains of *Ae. aegypti*. In addition, was estimated a low Wilks' Lambda of 0.36 and the chi-square was highly significant (X^2 = 192.59, *d.f.* = 33, $P < 0.001$), thus it is possible to accept that the means of the groups (centroids) are different. It was also observed by tests of equality of group means that the length of abdomen, fore femur, fore tibia, fore tarsus, mid tarsus and hind tarsus are statistically significant ($P < 0.001$). These results suggest that there was a difference among strains according of the morphometric data and it can be used to discriminate between control group and *Ae. aegypti* with kdr homozygous (Val1,016). The unstandardized coefficients (Table 4) showed that the variables that most influenced the separation in decreasing order were fore tarsus, fore tibia, fore femur, hind tarsus, hind tibia (with positive values), wing and hind femur (with negative values). Moreover, it was observed that 62.5% of original grouped cases were correctly classified. Standardized coefficients of the canonical discriminant functions yielded three functions per strains. The first function distinguishes mainly *Ae. aegypti* females from Vergel-III and Chuburna (whose centroids values are positive) from the New Orleans and Juan Pablo-II strains (whose centroids values are negative). Based on these results and the unstandardized score, we can assume that the long lengths of the fore tarsus, fore tibia, mid tarsus, fore femur, hind tibia, hind tarsus belong to females of Vergel-III and Chuburna compared to the New Orleans and Juan Pablo-II strains that had shorter lengths of the wing and hind femur (Table 5).

Table 4: Discriminant function estimated of metric characters of *Ae. aegypti* between control and populations with kdr mutations in Merida, Yucatan, Mexico.

Metric characters (Length in millimeter)	Tests of equality of group means			Discriminant function coefficients	
	Wilks' Lambda	F	P-value	Standardized	Unstandardized
Fore tarsus	0.762	20.382	0.000	0.749	*8.483
Fore tibia	0.862	10.450	0.000	0.470	*6.393
Mid tarsus	0.896	7.604	0.000	0.287	
Fore femur	0.864	10.241	0.000	0.222	*2.249
Hind tibia	0.976	1.607	0.189	0.189	*0.461
Hind tarsus	0.857	10.938	0.000	0.157	*1.372
Wing	0.963	2.525	0.059	-0.567	*-4.587
Hind femur	0.967	2.224	0.087	-0.503	*-4.480
Mid femur	0.976	1.605	0.190	-0.331	
Abdomen	0.923	5.443	0.001	-0.131	
Mid tibia	0.963	2.499	0.061	-0.008	
**Constant					**-15.509

*Variables that most influenced the separation between control group (New Orleans) and populations with kdr mutations.
**Constant of the unstandardized coefficient

Table 5: Standardized coefficients of the canonical discriminant functions at each strain of *Ae. aegypti* females.

Strains	Functions at group centroids		
	1	2	3
New Orleans	-1.083	-0.549	0.276
Vergel-III	0.065	-0.167	-0.706
Juan Pablo-II	-0.192	1.035	0.126
Chuburna	1.210	-0.319	0.305

4. Discussion

Previous studies carried out in Mexico have provided evidence that increased levels of esterases (α- and β-) and glutathione S-transferase is related with resistance to permethrin, and deltamethrin in *Ae. aegypti* populations [26, 27]. In bioassay, it has been observed that not all mosquitoes containing a mutant allele are phenotypically resistant to pyrethroid [28]. However, it has been documented that natural populations of *Ae. aegypti* from the city are resistant to deltamethrin and permethrin [6, 8, 17, 28]. Resistance to chlorpyrifos has also been reported [28]. Studies of enzymatic mechanisms and target-site insensitivity suggest that the widespread use of permethrin has conferred cross-resistance with organophosphates [26-27], currently used in mosquito-control programs in Mexico such as chlorpyrifos. In Merida, houses sprayed with deltamethrin did not reduce the presence of *Ae. aegypti*. However, bendiocarb (carbamate) spraying led to a significant reduction in *Ae. aegypti* abundance during a 3-month period with an average efficacy of 60% [29]. In our study, the frequency of kdr homozygous (Val1, 016) was high in all sites. In Chuburna, we expected to find low frequency of Ile1016 in *Ae. aegypti*, because it is an area with low dengue virus transmission and the application of insecticides is low compared with Juan Pablo-II and Vergel-III. Saavedra-Rodriguez *et al*. [17] reviewed the gene flow of insecticide-resistant genes in the Yucatan State and concluded that in areas where insecticides are no longer applied, the frequencies of resistance alleles has declined. In addition to the indoor residual spraying, the most likely explanation for the high frequency of kdr homozygous (Val1,016) is in part due to the extensive use of household insecticides (i.e., insecticide aerosol spray cans) in Yucatan and the use of deltamethrin-treated curtains in houses [4, 30].

In some cases, it is not possible to generalize the resistance effects on immature development. In *Ae. aegypti*, ~5% of larval mortality is due to genetic deficiencies irrespective of environmental circumstances [31]. In this study, the high frequency of kdr homozygous (Val1, 016) did not affect the survival rate of natural population of *Ae. aegypti*. Survival was similar among control group (New Orleans) and kdr populations; ranging from 0.87 to 0.91. In contrast with our results, Diniz *et al*. [10] found that in Brazil only 60% of *Ae. aegypti* larvae resistant to insecticide temephos reached the adult stage. It is common that the highest mortality among the immature larval forms usually occurs during the first two larval stages [32]. In this regard, the larvae mortality in the control group was 75% (24/32) in early stages (I-II), while in *Ae. aegypti* with kdr homozygous (Val1,016), the mortality was present in late stages, and ranged from 52% to 73%. This is a fitness cost disadvantage in *Ae. aegypti* because late stages such as the pupae is considered that has lower mortality and therefore is a better proxy for the abundance of emerging adults [33].

On the other hand, time of development is a primary aspect of fitness in disseminating mosquito populations. In the field, a slower development increases the chances of larvae predation, parasitism or even breeding site destruction [9, 22]. In the present study, there was a significant delay of 4 and 5 days in larval development of the Chuburna and Juan Pablo-II strains, respectively, compared with the control group. Our results are consistent with those of Brito *et al*. [22] who demonstrated that the kdr mutations (Ile1016 +Cys1534) have a negative effect on larval development time of *Ae. aegypti* from Brazil. However, in another study carried out in Thailand, *Ae. aegypti* associated with permethrin resistance did not have a negative impact on larval development, pupation success, and adult emergence [13]. Further studies are needed to quantify the fitness cost in priority areas with presence of this vector and with intensive use of chemical insecticides. Previous studies showed no significant differences between female and male of *Ae. aegypti* associated with resistance to insecticides [9, 10, 14]. These results agree with our findings, sex ratio was in accordance with the ~1:1 expected values in all groups evaluated.

Under optimum conditions of development (i.e., food, temperature, humidity and without intra- or inter-specific competition), adult *Ae. aegypti* females are able to ingest up to 6.6 µg of human blood [34]. In the present study, there was a statistically significant difference between blood ingested per *Ae. aegypti* females of control group (New Orleans) compared with Juan Pablo-II and Chuburna strains (Figure 2A). Our results are consistent with previous studies by Brito *et al*. [22] who found a significant reduction of the relative amount of ingested blood of *Ae. aegypti* associated with pyrethroid resistance. A similar situation was observed by Martins *et al*. [9] working with *Ae. aegypti* females after pyrethroid selection. Interestingly, reduced blood intake has also been observed in *Ae. aegypti* associated with temephos resistance [12]. In the present study, we found no evidences that the kdr mutations interfered with egg laying. This was surprising, since the number of eggs is generally directly related to the amount of ingested blood. These results are in accordance with those of Plernsub *et al*. [13] who did not find differences between mean numbers of eggs laid by *Ae. aegypti* females associated to permethrin resistance and the control group. Conversely, Brito *et al*. [22] reported that fewer *Ae. aegypti* females associated to kdr mutations laid eggs in smaller amounts compared to susceptible strain. Previous studies have shown that deltamethrin resistance caused reproductive disadvantage in the selected strains of *Ae. aegypti* [9, 11]. These authors found a significant reduction in fecundity and fertility in comparison to their control. Early studies, also had confirmed lower oviposition rates in a malathion-resistant strain of *Ae. aegypti* [35]. A significant decrease in the fecundity rates of *Ae. aegypti* after treatments with pyrethroids [36] such as cyfluthrin and fenfluthrin has also been reported [37].

Wing length is often used in mosquitoes as an estimator of body size [34]. Plernsub *et al*. [13] found that the mean wing length of *Ae. aegypti* females associated to permethrin resistance was significantly smaller than control group. Diniz *et al*. [10] also found small *Ae. aegypti* females associated to temephos resistance. However, Jaramillo *et al*. [14] suggest that the wing centroid size, rather than wing length is a better estimator of global size, because it detects size changes in all

directions, not only along the largest axis of wing. These authors found that wing shape was strongly correlated with resistance levels to lambda-cyhalothrin in field population of *Ae. aegypti*. Additionally, most of the *Ae. aegypti* that exhibited higher resistance included the smaller individuals. Diniz *et al*. [10] reported that wing shape also varied significantly between *Ae. aegypti* females associated to temephos resistance and the control group. To the best of our knowledge, this is the first work that evaluates the effect of site targeted mutation Ile016 over body size of *Ae. aegypti* females. According to the score of principal component analysis, *Ae. aegypti* females with kdr homozygous (Val1,016) had larger body sizes compared with control group. These results suggest that there was a difference according of the morphometric data, where the variables that most influenced the separation are fore tarsus, fore tibia, fore femur, hind tarsus, hind tibia, wing and hind femur. The legs are the first structures to come in contact with residual insecticides and probably have modified their cuticular wall to reduce the entry of insecticides. Thickening of the integument as a mechanism of resistance to insecticides is recognized phenomenon in insects [38, 39], and recently Balabanidou *et al*. [39] have observed that the overall cuticle thickness of legs of *Anopheles gambie* was significantly higher in the pyrethroid resistant strains than in the control group, and that the internalization of deltamethrin was ~50% slower. Lilly *et al*. [38] also found that the Bed bug *Cimex lectularius* associated to resistance with lambda-cyhalothrin has a higher mean cuticle thickness compared with the control group and cuticle thickness positively correlated with increasing time-to-knockdown.

5. Conclusions

Our study revealed that the Ile1, 016 mutation is highly present in natural populations of *Ae. aegypti* from Merida, Yucatan, which is associated with the resistance to pyrethroids. Furthermore, it clear that the adaptive disadvantages in populations with kdr homozygous (Val1, 016), were particularly reflected in the reproductive parameters, especially in delay of the larval development, higher mortality in late stages, and decrease in blood ingested. Conversely, they have a larger body sizes, probably as a strategy that can minimize deficits related to the survival and reproduction of the *Ae. aegypti* females. This has epidemiological relevance because larger *Ae. aegypti* females ingest twice the volume of blood during feeding and can be more efficient vectors in terms of their physiologic capability to acquire arbovirus infection orally. The absence of bioassays and biochemical tests to evaluate behavior and physiological status are limitations of the present study. However, we provide data on the presence of genetic mutations in natural populations of *Ae. aegypti* from Merida, Yucatan, Mexico, which could help to ensure more effective and efficient use of available resources for vector control.

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