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# Egg number quantification for mass-rearing of *Aedes aegypti* and *Aedes albopictus*: validation by direct measurement

J. Guillermo Bond[1] Adriana R. Osorio[1] Carlos F. Marina[1], Ariane Dor[2,3], Pablo Liedo[2], Trevor Williams[4*]

[1] Centro Regional de Investigación en Salud Pública, Instituto Nacional de Salud Pública, Chiapas, Mexico.

[2] El Colegio de la Frontera Sur (ECOSUR), Unidad Tapachula, Chiapas, Mexico.

[3] Investigadora por México CONACyT - ECOSUR, Chiapas, Mexico.

[4] Instituto de Ecología AC (INECOL), Xalapa, Veracruz, Mexico.

*Corresponding author, trevor.williams@inecol.mx

## Abstract

Mass-production of *Aedes aegypti* and *Ae. albopictus* for use in vector control programs involving the sterile insect technique (SIT) requires the standardization of rearing techniques, including the production of eggs. Following published protocols, egg quantification for both species was performed based on the weight and volume characteristics of batches of 1,000 to 27,000 eggs from insect colonies that originated from natural populations in Chiapas, Mexico. The findings were validated by direct measurement of egg dimensions. On average, *Ae. aegypti* eggs were ~40% heavier and occupied ~25% larger volume than those of *Ae. albopictus* ($P<0.001$). Egg numbers were readily predicted by linear correlation with the weight and volume of eggs of both species ($P<0.001$). Volumetric measures were significantly more consistent among replicates than the weight measurements ($P <0.02$). Direct measurements revealed that the eggs of *Ae. aegypti* were ~10% longer ($P <0.001$), ~13% wider ($P < 0.001$), and also more variable in size than those of *Ae. albopictus*. These species also differed significantly in egg length:width ratios ($P <0.001$). We conclude that proxy indicators of egg numbers, such as weight and volume, should greatly assist in standardizing larval rearing procedures.

Keywords: Sterile insect technique; vector control; mosquitoes; egg size traits

## Introduction

The invasive mosquitoes *Aedes aegypti* and *Ae. albopictus* are the principal vectors of the dengue, chikungunya and Zika viruses that frequently cause major outbreaks of disease in tropical regions of the world.[1,2] Over 3.9 billion people across 129 countries are at risk of dengue infection according to the World Health Organization, and each year an estimated 40,000 people die from this disease.[3,4] In Mexico, over 23,000 cases of dengue were confirmed in 2020.[5] In the absence of effective vaccines and pharmaceuticals to combat dengue in Latin America[6], the control of vector populations is the most effective method of reducing the transmission of this disease.[7]

Current mosquito control methods include the elimination and sanitation of larval habitats, the use of insecticides targeted at larvae and adults, the use of physical barriers, such as mosquito screens on windows and doors, and bednets.[8,9] The wide-scale use of insecticides has resulted in insecticide resistance issues in both *Ae. aegypti* and *Ae. albopictus*.[10,11] In consequence, the need for novel methods of vector control that are safe for humans, non-target organisms and the environment has become increasingly recognized.[12]

The sterile insect technique (SIT) is a safe, non-polluting and species-specific method of pest control.[13] SIT involves the mass-rearing, sterilization and release of massive numbers of sterile male insects over large areas.[14,15] Given their large numbers, the sterile males outcompete wild males for copulations with wild female insects. Females that mate with sterilized males do not produce progeny.[14,15] Area-wide SIT based programs have considerable success in the control of agricultural pests and the expectations are high in the area of SIT-based vector control.[3,16]

The mass-production of sterile males for use in SIT programs requires careful standardization of insect rearing techniques, beginning with the production of eggs. In the present study, we applied methods developed for the standardization of the egg quantification process for both *Ae. aegypti* and *Ae. albopictus*,[17] based on egg weight and egg volume characteristics. We confirmed that these procedures were rapid and easy to perform compared to direct egg counting. We validated these procedures in Mexican mosquito populations by direct measurements of egg dimensions in both species.

## Materials and methods

### Ethical considerations

Adult female mosquitoes were fed on animal blood obtained from the municipal abattoir in Tapachula, Chiapas. The present study was performed as part of the project "Aplicación de la técnica del insecto estéril para el control de *Aedes aegypti* y *Ae. albopictus* en el sur de Chiapas, México" that was approved by the research, ethics and biosafety committees of the Instituto Nacional de Salud Pública (INSP), Mexico. The mosquito rearing procedures were performed in the installations of the Centro Regional de Investigación en Salud Pública (CRISP-INSP).

### Mosquito colonies

Two genetically diverse mosquito strains were used in the present study, *Ae. aegypti* (CGD1) and *Ae. albopictus* (CGD2) that resulted from the introgression of various populations collected along the Pacific coastal region of Chiapas, Mexico.[18,19] Larvae of both species were reared at a density of 1.5 individuals/ml in plastic trays (61x41x7.5 cm) containing 2 liters of deionized water. Larvae were fed daily with 0.53 mg/larva of rodent diet (LabDiet, Fort Worth, Texas, USA), as described previously.[20] All larvae were reared at 28±2 °C, 80±5% relative humidity under a 14:10 h (L:D) photoperiod. Pupae were separated by size using a plate separator (John W. Hock, Model 5412, Gainesville, Florida, USA). Following emergence, adults were placed in a ratio of 3:1 (females:males) in ventilated acrylic cages of 30x30x30 cm and maintained at 26±2 °C, 80±5% relative

humidity under a 14:10 h (L:D) photoperiod. Adults had continuous access to 10% (wt/vol) sucrose solution on a cotton pad within the cage.

**Egg production**

To obtain eggs, adult females were offered a meal of lamb's blood at 4 days post-emergence and for the next three consecutive days. Blood was offered through a Hemotek membrane feeding system (PS6B, Hemotek Ltd., Great Harwood, UK). At 48 h after the first blood meal, a 250 ml plastic cup (11 cm diameter x 8 cm height) was placed in each cage of adults. Each cup contained 100 ml of deionized water and a white filter paper strip (30 x 4 cm) folded in half and placed around the inside of the cup as an oviposition substrate. Filter paper strips were removed 72 h later and replaced with new paper strips. The number of eggs on paper strips was counted and eggs were then allowed to embryonate following standard procedures.[20]

**Relationship between egg numbers, egg weight and egg volume.**

Following embryonation, eggs of each mosquito species were air-dried and gently brushed from paper strips using a soft paint brush (Pinto Plano P15 No. 22, Pinto Distribuidora SA de CV, Zapopan, Jalisco, Mexico). Eggs were counted and placed in groups of 1,000, 3,000, 6,000, 12,000, 15,000, 18,000, 21,000 and 27,000 in 1.5 ml microcentrifuge tubes and weighed to a precision of ± 0.1 mg on an analytical electronic balance (Vibra AJH-620CEN, Shinko Denshi Co., Japan). After weighing egg samples, the tube was tapped three times to settle egg samples and the final height of the eggs in the 1.5 ml centrifuge tube was marked using a fine permanent marker. Eggs were then removed and water added up to the mark using a micropipette, and the volume was recorded. These procedures were performed for 3 - 6 batches (replicates) of eggs of each species following published procedures.[17] All eggs used in the study were no more than 15 days old and were collected and treated following the same procedures.

**Egg size characteristics**

To determine egg size characteristics, 50 eggs were selected at random from different batches of each species. The length of each egg was measured from the micropyle to the opposite end, while the width at the center of each egg (its widest point) was measured in millimeters. All measurements were performed to an accuracy of 0.001 mm using the image analysis software Zen 2.3 Blue Edition (Carl Zeiss) and a stereomicroscope (Stemi 508, Carl Zeiss) at x50 magnification fitted with an Axiocam Erc5s camera (Carl Zeiss). Image analysis software was calibrated against a 0.1/0.01 mm stage micrometer (Bausch & Lomb, Bridgewater, NJ, USA) prior to taking the measurements.

## Statistical analysis

Mean weight and mean volume values were compared between *Ae. aegypti* and *Ae. albopictus* by t-test using the Statview package (v. 5.0, SAS Institute Inc., USA). The relationships between egg numbers, weight and volume were determined by calculating Pearson's coefficient of correlation using the Statview package. Variation among replicates was determined by calculating the coefficient of variation (CV) and used to compare variability in volumetric and weight measurements by paired t-test. Egg length and width values, and the ratio of length:width were not normally distributed and were compared between species by the non-parametric Kruskal-Wallis test in the R-based Jamovi package (Jamovi v. 1.6, retrieved from www.jamovi.org).

## Results

On average, *Ae. aegypti* eggs were approximately 40% heavier than those of *Ae. albopictus*. This resulted in significant species differences in the weight of all groups of eggs, from 1,000 to 27,000, which ranged in weight (± SE) from 9.23 ± 0.20 to 248.17 ± 1.62 mg in the *Ae. aegypti* samples compared to 5.67 ± 0.14 to 168.43 ± 3.31 in the *Ae. albopictus* samples (Table 1). The number of eggs present in the sample was readily predicted by the linear correlation with the weight of eggs for *Ae. aegypti* ($y = 0.0092x + 1.5958$, $R^2 = 0.9993$, $P < 0.001$) and *Ae. albopictus* ($y = 0.0063x – 5.4347$, $R^2 = 0.9932$, $P < 0.001$) (Fig. 1A).

The eggs of *Ae. aegypti* had a ~25% larger volume than those of *Ae. albopictus* (Table 2). Groups of 1,000 – 27,000 eggs occupied mean (± SE) volumes of between 19.75 ± 0.15 and 430.60 ± 0.67 µl in *Ae. aegypti* and between 15.15 ± 0.17 and 310.73 ± 0.32 µl in *Ae. albopictus*. The volume of groups of eggs was also an excellent linear predictor of the number of eggs present in samples of *Ae. aegypti* eggs ($y = 0.0156x + 8.4927$, $R^2 = 0.9964$, $P < 0.001$) and *Ae. albopictus* eggs ($y = 0.0111x + 11.094$, $R^2 = 0.9984$, $P < 0.001$ (Fig. 1B).

In terms of the precision of the volume and weight measurements, reflected in the variation present among replicates, the mean (± SE) coefficient of variation (CV) for the volume measurements (0.66 ± 0.21%) was significantly lower than the corresponding value for weight measurements (2.12 ± 0.56%) of *Ae. aegypti* eggs (paired $t = 3.07$, d.f. = 7, $P = 0.018$). A similar situation was present in measurements of *Ae. albopictus* eggs, with a significantly lower mean CV value for volume measurements (0.84 ± 0.26%) compared to weight measurements (2.45 ± 0.59%) (paired $t = 3.10$, d.f. = 7, $P = 0.017$).

The eggs of *Ae. aegypti* were significantly longer, by approximately 10% (Kruskal-Wallis $H = 74.232$, d.f. = 1, $P < 0.001$) and significantly wider, by approximately 13% (Kruskal-

Wallis H =71.899, d.f. = 1, P < 0.001) than the eggs of *Ae. albopictus* (Fig. 2A,B). The eggs of *Ae. aegypti* were also more variable in size with a maximum - minimum range of 0.094 and 0.040 mm around the length and width values, respectively, compared to a range of 0.042 and 0.14 mm, respectively, in *Ae. albopictus*. The length:width ratios of *Ae. albopictus* eggs (median 3.468 [interquartile range: 0.024]) were significantly higher than those of *Ae. aegypti* (median 3.332 [interquartile range: 0.098]) (Kruskal-Wallis H = 37.901, d.f. = 1, P < 0.001), and the higher variation in the dimensions of the eggs of *Ae. aegypti* was once again evident compared to those of *Ae. albopictus* (Fig. 3).

**Discussion**

In the present study, we confirmed that the weight and volume of eggs were closely correlated with the number of eggs present in samples for both *Ae. aegypti* and *Ae. albopictus*, with little variation among replicates, thereby validating the technique developed by Zheng et al.[17] for the quantification of very large numbers of mosquito eggs. Of the two metrics, volumetric measures had higher repeatability than the weight measurements but required greater operator skill with regard to the precise and consistent use of the micropipette. The eggs of *Ae. aegypti* were consistently heavier and occupied a larger volume than those of *Ae. albopictus*, a finding that was confirmed by direct measurements of egg dimensions. Although larger, *Ae. aegypti* eggs were also more variable in size than those of *Ae. albopictus*. Previous studies also reported that *Ae. aegypti* eggs were larger than those of *Ae. albopictus*.[23,24]

The *Ae. aegypti* colony used in the present study was collected at several sites along the Pacific coast of Chiapas, southern Mexico. The eggs from this colony were heavier and occupied a larger volume than those reported for a colony from Juazeiro, Brazil.[17] In contrast, eggs from an *Ae. aegypti* colony in India[23] and Florida, USA[24] were larger than those of our Mexican colony, whereas eggs from colonies in Australia[21] and Maranhão or Sao Paulo, Brasil[22] were smaller (Table 3). Although we cannot rule out a genetic component to egg size, these differences were likely influenced by different larval diets, the density of larvae during rearing, and the type of blood meal consumed by adult females in each colony.

Eggs of *Ae. aegypti* had significantly lower length:width ratios than *Ae. albopictus* in the present study, although the opposite tendency was reported in a population from Florida, USA,[25] and no marked species differences were observed in a scanning electron microscope study on eggs had been fixed in glutaraldehyde and dried in a critical point drier in India.[23]

Variation in egg size and weight characteristics has also been attributed to differences in adult female body size and particularly blood meal volume in *Ae. aegypti*.[25] Egg size had no significant influence on larval survival or development time in males, whereas female development rate was positively correlated with egg size.[25] However, egg size is likely constrained by the tradeoff against the number of eggs that each female can produce from a given blood meal. That said, genetic variation among females has been identified as more influential that egg size on male survival and female growth rate in *Ae. aegypti*.[25] Larger females consumed larger blood meals and produced more eggs than smaller females, although the probability of insemination was not related to body size.[26]

Given the need to standardize rearing densities for mass production of insects in SIT programs, and given the laborious nature of direct counting, the use of proxy indicators of egg numbers, such as weight and volume used in the present study should greatly assist in standardizing larval rearing procedures. This approach, originally developed in the IAEA laboratories in Austria,[17] was rapid, repeatable and more accurate than an image analysis based method developed previously.[27] However, as egg morphometric traits are likely influenced by diet, environment and possibly genetic variation, it will be necessary to calibrate this technique for each colony or insect strain used in mass rearing for SIT programs.

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**Data availability statement:** The authors confirm that the majority of the data supporting the findings of this study are available within this article.

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Table 1. Mean weight (± SE) of *Aedes aegypti* and *Ae. albopictus* eggs measured in different quantities of counted eggs

| Number of eggs | Weight of eggs (mg) *Ae. aegypti* (N) | *Ae. albopictus* (N) | *t* | P |
|---|---|---|---|---|
| 1000 | 9.23 ± 0.20 (6) | 5.67 ± 0.14 (6) | 14.615 | < 0.0001 |
| 3000 | 27.52 ± 0.47 (6) | 15.53 ± 0.10 (6) | 24.734 | < 0.0001 |
| 6000 | 57.27 ± 0.42 (3) | 31.17 ± 0.23 (3) | 54.554 | < 0.0001 |
| 12000 | 113.97 ± 1.57 (3) | 64.80 ± 0.75 (3) | 28.240 | < 0.0001 |
| 15000 | 143.83 ± 0.58 (3) | 81.30 ± 0.21 (3) | 100.854 | < 0.0001 |
| 18000 | 167.87 ± 3.55 (3) | 112.03 ± 2.93 (3) | 12.130 | 0.0003 |
| 21000 | 193.23 ± 1.41 (3) | 130.57 ± 2.87 (3) | 20.280 | < 0.0001 |
| 27000 | 248.17 ± 1.62 (3) | 168.43 ± 3.31 (3) | 21.661 | < 0.0001 |

N = number of replicates

Table 2. Mean volume (± SE) of *Aedes aegypti* and *Ae. albopictus* eggs measured from different quantities of counted eggs

| Number of eggs | Volume (µl) *Ae. aegypti* (N) | *Ae. albopictus* (N) | *t* | P |
|---|---|---|---|---|
| 1000 | 19.75 ± 0.15 (6) | 15.15 ± 0.17 (6) | 20.641 | < 0.0001 |
| 3000 | 59.88 ± 0.24 (6) | 45.73 ± 0.29 (6) | 37.810 | < 0.0001 |
| 6000 | 100.93 ± 1.05 (6) | 81.57 ± 0.44 (6) | 17.037 | < 0.0001 |
| 12000 | 189.03 ± 0.35 (6) | 149.73 ± 0.43 (6) | 71.096 | < 0.0001 |
| 15000 | 260.00 ± 0.44 (6) | 180.67 ± 0.83 (6) | 84.667 | < 0.0001 |
| 18000 | 279.63 ± 0.65 (6) | 210.60 ± 0.52 (6) | 83.040 | < 0.0001 |
| 21000 | 330.43 ± 0.46 (6) | 241.20 ± 0.96 (6) | 83.656 | < 0.0001 |
| 27000 | 430.60 ± 0.67 (6) | 310.73 ± 0.32 (6) | 162.451 | < 0.0001 |

N = number of replicates

Table 3. Comparison of the mean dimensions (± SE) of *Aedes aegypti* eggs reported in different geographical regions.

| Size (mm) | Cairns, Australia[21] | Charters, Australia[21] | Maranhão, Brazil[22] | Sao Paulo, Brazil[22] | Chiapas, Mexico* | Gwalior, India[23] | Florida, USA[24] |
|---|---|---|---|---|---|---|---|
| Length | 0.554 ± 0.037 | 0.563 ± 0.031 | 0.580 ± 0.032 | 0.581 ± 0.040 | 0.603 ± 0.024 | 0.626 ± 0.020 | 0.670 ± 0.007 |
| Width | 0.168 ± 0.007 | 0.160 ± 0.010 | 0.167 ± 0.020 | 0.175 ± 0.020 | 0.179 ± 0.009 | 0.183 ± 0.011 | 0.186 ± 0.002 |

*Present study.

**Figure legends**

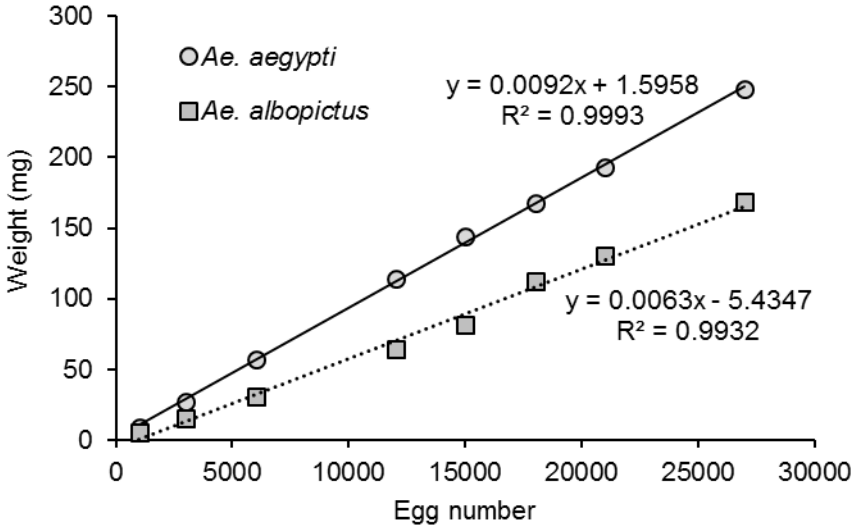
Figure 1. Linear correlation between egg number and (A) weight (mg) or (B) occupied volume (μL) for both species.

Figure 2. Box and whisker plot of (A) egg length or (B) egg width of both species from direct measurements. Horizontal line indicates median, box indicates interquartile range, whisker indicates range and outlier points. Values next to boxes indicate median [interquartile range].

Figure 3. Box and whisker plot of egg length:width ratio of both species from direct measurements. Horizontal line indicates median, box indicates interquartile range, whisker indicates range and outlier points. Values next to boxes indicate median [interquartile range].

Figure 1.

A.


Ae. aegypti
Ae. albopictus
y = 0.0092x + 1.5958
R² = 0.9993
y = 0.0063x - 5.4347
R² = 0.9932
Weight (mg)
Egg number
0
50
100
150
200
250
300
0
5000
10000
15000
20000
25000
30000

B.

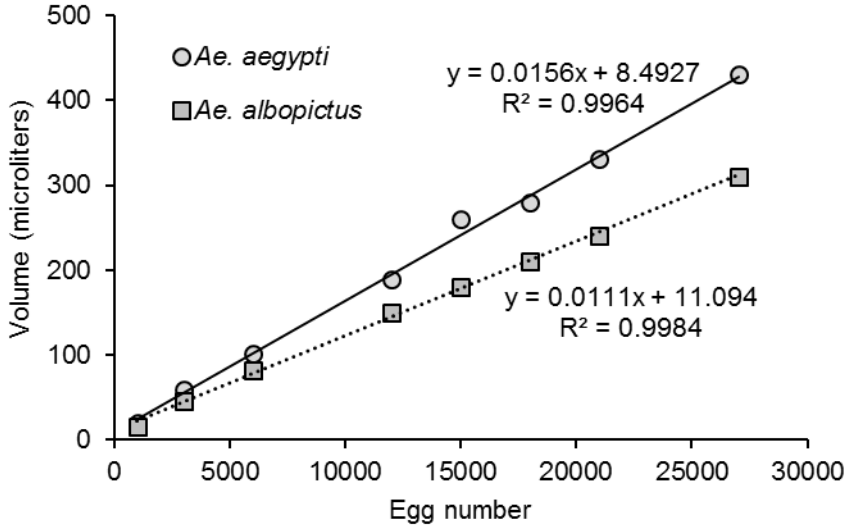
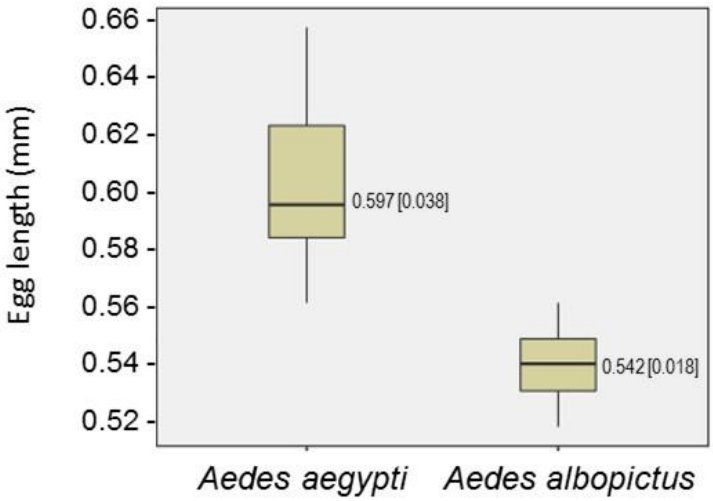

Ae. aegypti
Ae. albopictus
y = 0.0156x + 8.4927
R² = 0.9964
y = 0.0111x + 11.094
R² = 0.9984
Volume (microliters)
Egg number
0
100
200
300
400
500
0
5000
10000
15000
20000
25000
30000

Figure 2.

A.


0.66
0.64
0.62
0.60
0.58
0.56
0.54
0.52
Egg length (mm)
0.597 [0.038]
0.542 [0.018]
Aedes aegypti
Aedes albopictus

B.

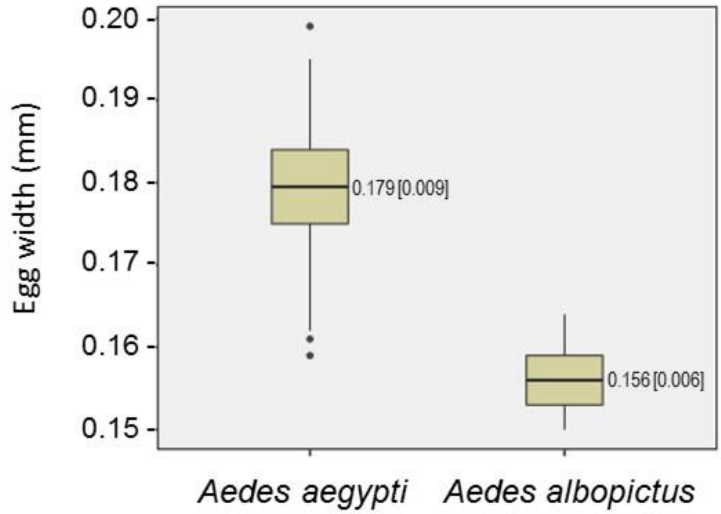
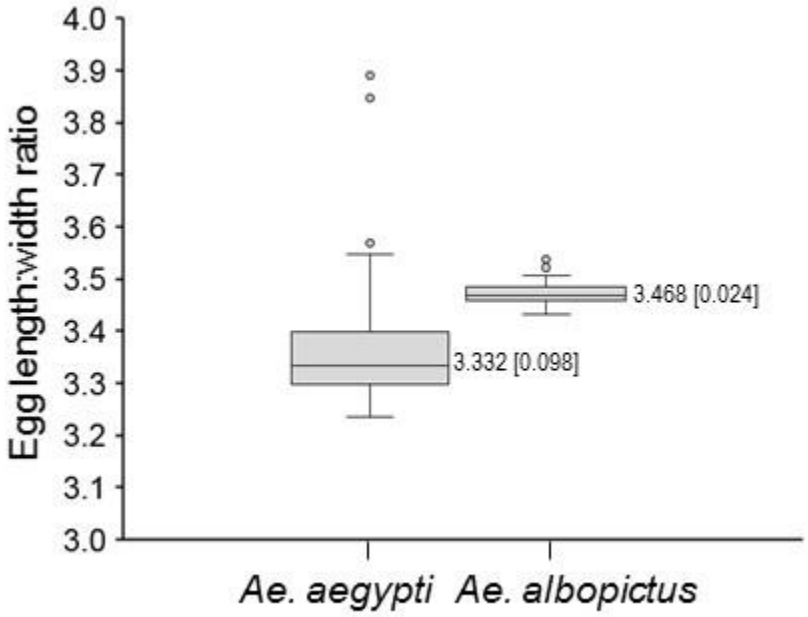

0.20
0.19
0.18
0.17
0.16
0.15
Egg width (mm)
0.179 [0.009]
0.156 [0.006]
Aedes aegypti
Aedes albopictus

Figure 3.


Egg length:width ratio
4.0
3.9
3.8
3.7
3.6
3.5
3.4
3.3
3.2
3.1
3.0
8
3.468 [0.024]
3.332 [0.098]
Ae. aegypti
Ae. albopictus