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Pupal shape and size dimorphism in *Aedes albopictus* (Skuse, 1894) (Diptera: Culicidae)

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Abstract: *Aedes albopictus* (Skuse, 1894) is a culicid mosquito associated with the transmission of pathogens causative for dengue, yellow fever, chikungunya, Mayaro and other diseases. Several recent studies have proposed that demographic surveys of dengue vector pupae are more useful than traditional larval indices for estimating populations. Geometric morphometrics is a tool for describing phenotypic variation that has been validated for characterizing sexual dimorphism. We undertook to apply this method to describe sexual and morphological dimorphism in *A. albopictus* pupae. Two-dimensional co-ordinates were digitalized from 60 specimens in two stages using 10 landmarks in pupae and 14 in wings. Configuration matrices were aligned by generalized procrustes analysis to extract matrix configurations and centroid size (CS). A discriminant analysis (DA) was used to test group (female or male) membership significance, and non-parametric ANOVA was used for CS differences. We found significant differences (Kruskal-Wallis $P < 0.01$) among pupal cephalothorax CS and adult wings; female pupae and adults were larger than males. The DA for cephalothorax and wing specimens showed significant differences (Hotelling $P < 0.0001$) between females and males. Through cross-validation, females and males were correctly classified with greater than 90% accuracy using the conformation characteristics described. Our study is the first description of phenotypic variation of pupal shape and size in *A. albopictus* laboratory colonies, and the results can be used as an additional tool in dengue entomological survey programs. More studies are necessary to confirm the variation between natural and laboratory populations.

Keywords: Aedini, Geometric morphometrics, immature, *Stegomyia*.

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Author Contribution: ES conducted the geometric morphometrics analysis, and wrote the first manuscript draft. She also served as Daniel Castillo's thesis adviser. DC conducted lab experiments (inmature rearing) and landmarks data adquisition as part of his undergraduate thesis. JL conducted additional geometric morphometrics analysis and statistical tests, also wrote the final manuscript (including figures).

For **Spanish Abstract** see end of this article.

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INTRODUCTION

Aedes (*Stegomyia*) *albopictus* (Skuse, 1894) or "the Asian Tiger Mosquito" is a culicid associated with the transmission of several pathogens (Benedict et al. 2007). The vector competence and capacity of this species vary with locality. *A. albopictus* can serve as a primary or secondary vector of the four dengue virus serotypes (Massad et al. 2001; Hernandez et al. 2015) and can transmit several other arboviruses including the causative agents of west Nile encephalitis, yellow fever, chikungunya, Ross River and Mayaro (Bueno & Jiménez 2012; Muñoz & Navarro 2012; Rúa-Uribe et al. 2012). The species range includes Europe, Africa and the Americas. Introductions to new geographic regions are caused by importation of tires containing immature stages, especially the dessication-resistant eggs (Reiter 1998; Gratz 2004).

Adult female mosquitoes are medically important because they are hematophagous. They are distinguished from males by antenna morphology (dense, plumose in males and sparse in female) and male exposed reproductive structures (gonostylus and gonocoxite). Dengue entomological surveys are traditionally focused on the determination of culicid larval indices (house, container and Breteau) for estimating adult populations (Focks 2003). Several recent studies have proposed that pupal demographic surveys are better for this purpose, because pupal productivity in an area is associated with the number of females emerging daily under steady-state conditions (Barrera et al. 2006; Barrera 2009). Harrison (2005) stated that taxonomic identification of culicid pupae is commonly ignored in mosquito surveillance programs except to alert the participants that a major hatch is imminent. A more reliable pupal survey could be accomplished by determining the male/female ratio, which would require the ability to distinguish the sex of pupae. Geometric morphometrics is a tool for describing the phenotypic variation in organisms (Rohlf & Marcus 1993; Adams et al. 2004), and it has recently has been shown useful for characterizing sexual dimorphism in mosquito wings (Devicari et al. 2011; Virginio et al. 2015). In this study we set out to describe shape and size dimorphism in *A. albopictus* pupae to facilitate dengue vector surveys.

MATERIAL AND METHODS

Specimens source and data acquisition

A. albopictus specimens were obtained from collections in the Universidad de Carabobo campus (Hernandez et al. 2015) and maintained in the laboratory. Each specimen was individually reared from fourth instar larva to adult; pupae were photographed by transferring individual specimens to a petri dish and then returning them to a vial so that after emergence their sex could be determined via female and male morphological characters. Pupal cephalothorax images were retained from 30 females and 30 males, from which right wings were dissected and slide-mounted. For each pupal specimen a photo was created of 10 equidistant fans with MakeFan software (Sheets 2010). Ten landmarks were digitized with TPSDig (Rohlf 2008) in order to describe the cephalothoracic curve (Fig. 1A). Finally, each wing was digitalized using 14 landmarks (Fig. 1B), all type I and II following Bookstein (1991). The following landmarks were used: subcostal end (LM1), radial 1 end (LM2), radial 2 end (LM3), radial 3 end (LM4), radial 4+5 end (LM5), medial 1 end (LM6), medial 2 end (LM7), medial 3+4 end (LM8), cubital end (LM9), anal end (LM10), intersection between cubital and M3+4 (LM11), intersection between M1+2 and M3+4

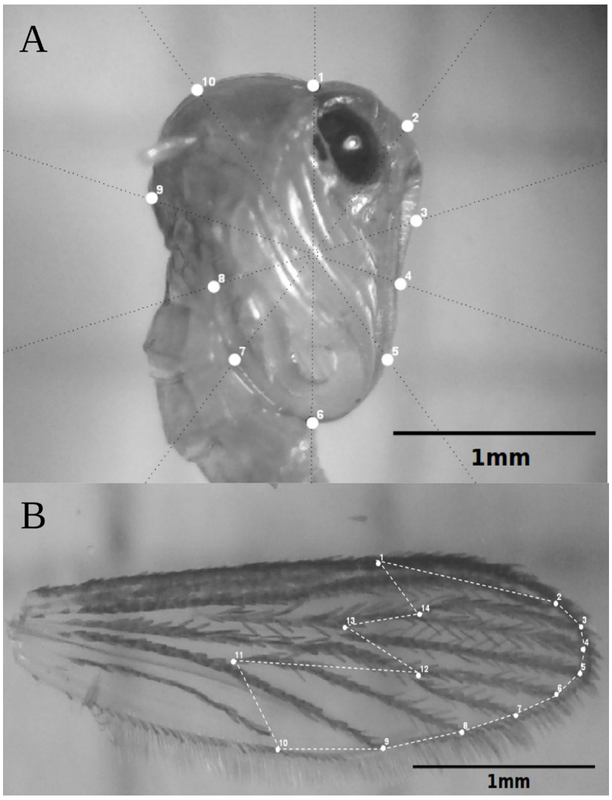

Figure 1. A - Cephalothorax of *Aedes albopictus pupa* showing the landmarks (1-10) disposition; landmarks 2-5 and 7-10 were treated as sliding; B - Right wing *A. albopictus* showing the landmarks (1–14) disposition. The polygon enclosed by the points conform the configurations analyzed.

(LM12), intersection between radio-sectorial and radial 3 (LM13), and intersection between radial 2 and radial 3 (LM14).

Geometric morphometric analysis

From each configuration matrix (pupal and adult) the coordinates were aligned by generalized procrustes analysis using tpsRelw software (Rohlf 2016) for extracting the matrix configurations and centroid size (CS). In particular, the pupal coordinates were analyzed with sliding semilandmarks. The semilandmarks were thus allowed to slide along their curve or surface in order to remove the effects of the arbitrary spacing by "optimizing" the position of the semilandmarks with respect to the average shape of the entire sample (Gunz & Mitteroecker 2013). Later, each stage aligned matrix was performed in MorphoJ software (Klingenberg 2011). A discriminant analysis (DA) was used to identify group (female or male) membership significance with Hotelling's test (Webster & Sheets 2010). Finally, CS dimorphism differences were analyzed with PAST software (Hammer & Harper 2011) by means of the non-parametric Kruskal-Wallis test (P 0.05) with Bonferroni correction.

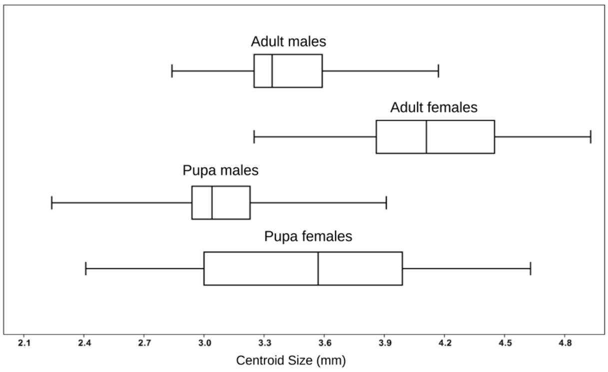

Figure 2. Box-plot diagram showing the centroid size sex differences between adult and pupa of *Aedes albopictus.*

RESULTS

Centroid size

We found significant differences (H = 7.400, df = 1, P<0.01) between sexes for pupal cephalothorax measurements (Fig. 2). Female specimens were larger (mean 3.498mm ± SD 0.106) than males (3.088mm ± 0.600). The female wing size (Fig. 2) was significantly (H = 29.39, df = 1, P < 0.001) larger (4.105mm ± 0.080) than males (3.406mm ± 0.054).

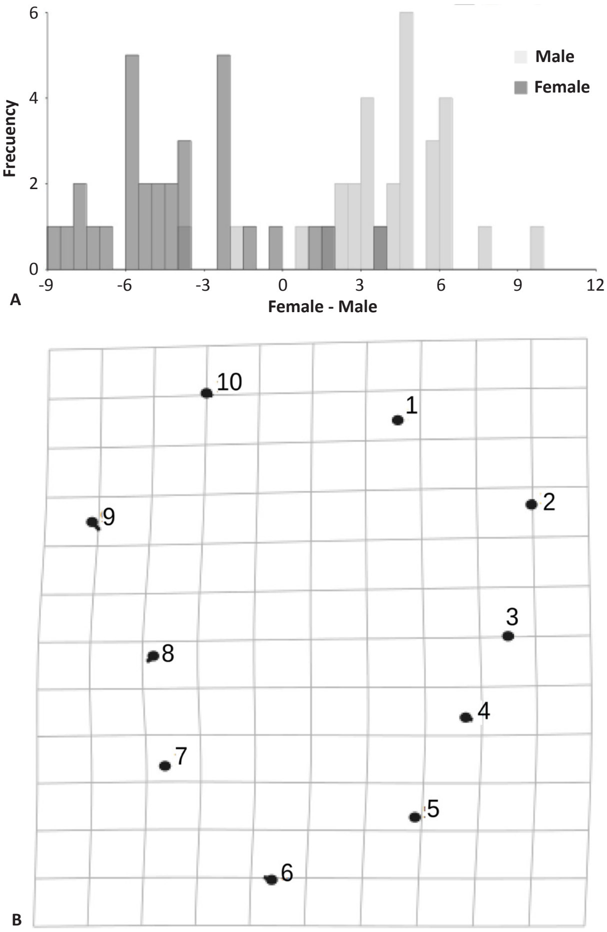

Figure 3. Discriminant analysis for 60 *Aedes albopictus* pupae specimens: A - histogram with the values of the discriminant scores for groups (females and males) and B - the thin-plate spline deformation grid with the differences between females and males.

Shape differences

The DA histogram for cephalothorax specimens showed (Fig. 3A) significant size differences between females and males (Procrustes distance: 0.022; Mahalanobis distance: 2.841; T-square: 121.10, P<0.0001). In the cross-validation DA, the females were 90% and males 93.3% correctly classified into a posteriori groups (sex). Figure 3B shows the thin-plate spline deformation grid between females and males; the LM8 (located in the mesothoracic wing) displaced posteriorly to the first abdominal segment, and LM9 (located over the postscutal area) displace anteriorly to the mesothoracic wing. Figure 4A shows the DA histograms for wing measurements, where the configuration for females and males differs significantly (Procrustes distance: 0.053; Mahalanobis distance: 4.239; T-square: 269.34, P < 0.0001). In the cross-

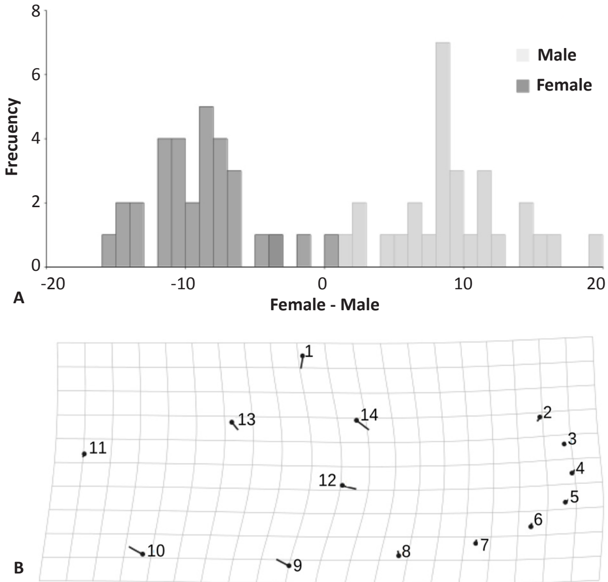

Figure 4. Discriminant analysis for 60 *Aedes albopictus* wing specimens: A - histogram with the values of the discriminant scores for groups (females and males), and B - the thin-plate spline deformation grid with the differences between females and males.

validation DA, the females were 96.7% and males 96.5% correctly classified. Figure 4B shows the thin-plate spline deformation grid between female and male wings: LM9 and LM10 displace to the wing basis, LM12, LM13 and LM14 displace to the wing apex and LM1 displaces to the posterior wing margin.

DISCUSSION

Sexual size dimorphism

Since pupae do not feed, the larval period critically influences pupal size and development (Castro et al. 1994; Consoli & de Oliveira 1994; Couret et al. 2014). Pupal size has been reported to be useful as a sex indicator (Cantrell 1939; Hayes 1953; Moorefield 1953), although this has been challenged for species where male/female size ranges overlap (Penn 1949; Vargas 1968). Recently, Mikery-Pacheco et al. (2015) used cephalothorax and abdomen lengths to perform a sieving device for sexing *A. albopictus* males and females. They considered pupae morphometrics sufficient to support such a device with good separation efficiency, although smaller females can pass through the sieve. In our investigation the relationship between size and dimorphism was established by means of the isometric or centroid size, an estimator used in geometric morphometrics for calculating the global dimensions of a biological structure. The isometric size can express variations in longitudinal, lateral or oblique measurements (Bookstein 1991; Dujardin 2008). In medical and forensic entomology, the isometric size has been used for size estimation (Belen et al. 2004; Calle et al. 2008; Vásquez & Liria 2012; Nuñez-Rodríguez & Liria 2017).

The CS analysis indicates that female pupae are larger than males, consistent with evidence of adult sexual dimorphism in *A. albopictus* and other mosquitoes (Devicari et al. 2011; Oliveira et al. 2012; Virginio et al. 2015). Lounibos (1994), however, considered adult size to be a direct effect of feed and density conditions during larval instars. In adults, larger females are related to life history components. Haramis (1985) and Nasci & Mitchell (1994) reported positive relationships between vector size and longevity that are associated with vector capacity. Armbruster & Hutchinson (2002) found that wing length and pupal mass were reliable indicators of fertility in *A. albopictus* and *A. (Finlaya) geniculatus* (Oliver, 1791) females. In contrast, male size is mainly related to survival and spermatic capacity (Xue et al. 2010).

Sexual shape dimorphism

Moorefiled (1951) and Vargas (1968) observed sexual dimorphism in size and shape of the ninth pupal abdominal segment (genital segment) in various mosquito species; Moorefiled (1951) considered the genital segment variations to be useful taxonomic characters for species recognition. Harrison (2005) described morphological characters for separating *A. albopictus* pupae from others species inhabiting artificial containers. The characteristics used were paddle shape, seta 1-P length, and the presence of a thick fringe of long filamentous spicules on the lateral and mesal edges of the paddles.

In relation to wing shape, our results are similar to those of Devicari et al. (2011) and Virginio et al. (2015) who observed wing shape dimorphism in *A. albopictus* and other culicid species. Several studies indicate that mosquito wing conformation has a polygenic basis, being weakly influenced by heritable epigenetic factors (Jirakanjanakit et al. 2007; Dujardin 2008; Morales-Vargas et al. 2010). The observed sexual shape dimorphism in *A. albopictus* corroborates studies suggesting that wing conformation is sex-specific (Devicari et al. 2011; Virginio et al. 2015), and that differences can be attributed to various genes that participate in the expression of wing shape waving additive and/or differential effects on the phenotype (Carreira et al. 2011).

To our knowledge this is the first description of phenotypic variation in pupal shape and size in *A. albopictus* laboratory colonies. The results may be useful for creating new tools for dengue entomological surveys once potential variations between natural and laboratory populations have been characterized, along with the effects of variations in larval density.

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Resumen: *Aedes albopictus* (Skuse, 1894) es un culicido asociado con la transmisión de patógenos causantes de dengue, fiebre amarilla, chikungunya, Mayaro, y otras enfermedades. Ciertas investigaciones sugieren la importancia de los estudios demográficos en pupas de vectores de dengue, respecto a la estimación poblacional basada en indices larvarios. La morfometría geométrica es una herramienta que describe la variabilidad fenotípica relacionada con la caracterización del dimorfismo sexual. Nosotros utilizamos este método para describir el dimorfimo sexual en pupas de *A. albopictus*. Se digitalizaron coordenadas 2D en 60 especímenes en dos fases: 10 hitos en pupa y 14 en alas. Las matrices de configuraciones fueron alineadas mediante análisis generalizado de procrustes para extraer matrices de conformación y tamaño centroide (TC). Se utilizó el Análisis Discriminante (AD) para evaluar la significancia en la membresía de grupos (hembras o machos), y una prueba no-paramétrica para el TC. Se encontraron diferencias significativas (Kruskal-Wallis $P < 0.01$) entre el TC de pupas y alas; las hembras fueron más grandes que los machos. El AD para los especímenes de pupas y alas mostraron diferencias (Hotelling $P < 0.0001$) entre sexos. En la reclasificación, las hembras y machos fueron correctamente asignados en un 90% con base en la conformación. Este estudio es el primero que describe la variación fenotípica en la conformación de pupas y adultos en *A. albopictus* mantenidos en laboratorio, y los resultados podían ser usados como herramientas adicionales en programas de vigilancia entomológica. Son necesarios investigaciones adicionales para confirmar la variación entre poblaciones naturales y de laboratorio.

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