ORIGINAL ARTICLE

Morphometric Analysis on the Effect of Plant Material in the Water on Wing Size and Shape of *Aedes Aegypti* (Diptera: Culicidae)

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ABSTRACT

Introduction: As strong wings are integral to the survival of a mosquito; our study examined the effects of different types of plant materials on the wing size and shape of *Aedes aegypti* mosquitoes. **Methods:** Thirty eggs were hatched in water containing three different doses of either dry grass, dry leaves, or dry twigs. Eleven landmarks were first identified on the wings of *Aedes aegypti* mosquitoes before the differences in wing shapes were observed using landmark data acquisition software programmes such as tpsDig version 1.40 and MorphoJ. **Results:** The wing size of adult *Aedes aegypti* mosquitoes that had been hatched in water containing dry grass was found to be larger than those hatched in water containing dry leaves or dry twigs. The wing centroid size of these mosquitoes was also the largest followed by mosquitoes that had been exposed to dry leaves and dry twigs. Therefore, introducing dry grass at the larval stage had the most significant impact on the wing size and shape of adult mosquitoes while higher doses of dry grass at the larval stage produced adult mosquitoes with larger wings than those exposed to dry leaves and twigs. **Conclusion:** Adult *Aedes aegypti* mosquitoes that were hatched in water containing higher doses of plant materials were found to have more separated wings.
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INTRODUCTION

The mosquito is a small winged insect belonging to the order Diptera and the family Culicidae. These blood-feeding insects pose a significant health risk to humans as they can transmit infectious agents through their bites (1). *Aedes aegypti* is the primary vector of the dengue virus which causes dengue fever; a well-known disease (1-2). However, increases in vector control measures have caused Aedes aegypti populations to migrate. According to Gualberto & Demayo (2018), in order to adapt to new environments during migration, mosquito populations may have experienced adaptive changes to nutrients that have resulted in changes in wing size and shape (3) as the successful adaptation to new environments can be influenced by the nutrients consumed during the larval stage. A study by Darriet (2016) observed larvae feeding on the plant material in water for nutrients (4). As such, the plant materials chosen for this study were dry grass, dry leaves, and dry twigs. A previous study found that population growth of *Aedes aegypti* larvae is closely associated with the resources available (5).

Geometric morphometrics is the most common method of identifying variations in mosquito wings. It is increasingly used in studies that examine wing shape, especially in insects (6). An effective and low-cost tool, is it used to evaluate the correlation between wing shapes and the environment as well as to observe differences in wing size and shape (1,6,7). Gualberto & Demayo (2018) state that the wings are the most distinguishing feature for evaluating variations in the geometric morphometrics of *Aedes aegypti* mosquitoes (3). As the intersection of wing veins in Aedes aegypti mosquitoes provide clearly observable and morphologically applicable landmarks, geometric morphometrics are used to characterise wing size variations in these populations. It is noteworthy that the wings did not deform and maintained their accuracy when mounted on a slide for observation.

Characterisation of wing size and shape has been widely used to identify population structures within the mosquito species (8-10) as the wing centroid size can be used to predict the average body size of a mosquito (6,11). Dujardin (2008) states that using geometric morphometric to characterise wing size and shape could potentially predict other biological characteristics; such as flight capability and the ability to transmit viruses (11). For instance, larger wing sizes mean increased flight capability as well as an increase in the viral load capacity of a mosquito to transmit viruses (6,12). Therefore, the identification of landmarks on mosquito wings is an essential step in geometric morphometric analyses. These landmarks are data points that can be used to describe wing size, shape, and structure (8). Firstly, the wing size of each mosquito is measured using a Dino-Lite digital microscope. Wing size is measured from the centroid to each landmark (7,13). Next, in accordance with the steps in landmark data analyses of wing sizes, multivariate analysis (MVA) can be used to visualise the wing shape characterisation and variation.

Very few studies have examined the effect of plant materials on the wing size and shape of *Aedes aegypti* mosquitoes. Darriet (2016) studied the effect of plant nutrients during larval feeding on the development of adult Aedes mosquitoes (4). A study by Jirakanjanakit et al., (2007) focused on the wing geometry of Aedes aegypti mosquitoes (7). It also assessed the influence of larval density on wing geometry. As a major gap exists in the literature on the effect of plant materials on the wing geometry of adult *Aedes aegypti* mosquitoes, this study aimed to identify the effect that different types and doses of plant materials have on the wing size and shape of adult *Aedes aegypti* mosquitoes.

MATERIALS AND METHODS

Mass Rearing and Water Nutrient Content

Aedes aegypti eggs were obtained from the Vector Control Research Unit (VCRU) at Universiti Sains Malaysia (USM), Penang, Malaysia. The eggs were then mass reared according to the method used by Yeap et al., (2013). The eggs were reared in a plastic container of dechlorinated tap water and fed ground chicken liver at the larval stage. Larvae that emerged into pupae were then transferred into a separate container before being placed in a rearing cage in preparation for their emergence as adult mosquitoes. These adult mosquitoes were fed a sucrose solution. Upon random mating, gravid female mosquitoes were fed artificial blood to support the development of their eggs. The laid eggs were then dried mimicking the conditions within an insectarium before they were used in this study. Using a Hach© DR 2800™ portable spectrophotometer, an analysis of the water from each of the nine containers containing three varying doses of either dry grass, dry leaves, or dry twigs determined that nutrients; such as nitrate, manganese, and zinc; were present.As the mosquito eggs take three to seven days to hatch into larvae, readings were taken on days one, three, and five to determine the concentration of nutrients in the water. These readings provided us with the concentration of organic matter present in water that might act as nutrients for the eggs to hatch.

Wing Shape Measurement and Characterisation

Our experiments were conducted at the insectarium of Universiti Teknologi MARA (UiTM) Puncak Alam, Selangor, Malaysia, under controlled laboratory conditions of 29 ± 3°C and 75 ± 10% humidity. Thirty *Aedes aegypti* eggs were obtained from the laboratory strain and hatched in three separate containers. Each container held 200ml of dechlorinated tap water and either ground dry H*opea odorata* leaves, ground dry *Hopea odorata* twigs, or ground dry Lolium perenne grass. These species were chosen because they grow in open spaces and are usually planted on sidewalks and roadsides in residential areas. As such, they have a high possibility of falling into mosquito breeding sites in residential areas. The dry *Hopea odorata* leaves, dry *Hopea odorata* twigs, and Lolium perenne grass were each ground separately to avoid cross-contamination.

To determine the effect of dose on wing size and shape, each plant material was prepared at three different doses (1.7g/l, 2.5g/l, and 3.3g/l) as proposed by Darriet, (2016) (4). Each plant material was first weighed, according to the specified dosages, before being tightly secured in a piece of cloth and placed into each of the nine containers to act as a source of nutrients for the developing larvae. The water level of each container was regularly monitored and maintained to provide the optimum conditions for larvae growth until pupation. Larvae that had emerged into pupae were then separated into standard 30cm x 30cm x 30cm rearing cages where they emerged into adult mosquitoes.

Morphological observations of the wing sizes of the adult *Aedes aegypti* mosquitoes was performed according to the methodology adopted by Wilke et al., (2016) with slight modification (1). The wing sizes were measured using a Dino-Lite digital microscope. Dead mosquito samples were collected from all nine containers containing three varying doses of the three different plant materials. As suggested by Gualberto & Demayo (2018), the dead mosquitoes were left for two to three days before the wings were detached to avoid tearing as freshly dead adult mosquito wings tear easily (14). Only the right wing of each dead mosquito was detached and mounted onto a clean microscope slide (1,2,7). The wings were detached easily and intact from the thorax by nudging the wings with a needle (14).

Immersion oil was then added onto the wing to get a clearer view under the microscope. Each wing was photographed at 169x magnification to measure the size of each landmark. The eleven landmarks were identified and measured from the centroid using a Dino-Lite digital microscope. Although this study included more landmarks than the study by Yeap et al. (2011), it contained fewer landmarks than Vargas et al., (2010) as it was difficult to accurately identify some of the landmarks specified in the latter study (13,15). Wing surface area was calculated using the wing centroid size formula. The mean and variance of the wing centroid size is the square root of the sum of the squared distance between each landmark and their centroid.

Data Analysis

In order to characterise wing shapes, each wing photograph was digitised using the tpsDig version 1.40 landmark data acquisition software programme. Canonical variate analysis (CVA) was performed to identify similarities and differences between the wings. CVA uses landmark data in the morphospace (x-axis and y-axis) to describe the formal structures and coordinates in vector and insect samples thereby transforming an otherwise complex correlation structure into a much simpler and more understandable structure. This way, it is easier to observe the differences in wing shape patterns. The analysis was conducted using TpsUtil 1.29, TpsRelw 1.39, and MorphoJ software programmes (1).

RESULTS

Nutrient Concentration and Distribution

The concentration of nutrients in the water of all nine containers (three different plant materials at three different doses) was measured every other day (days one, three, and five). An analysis by a Hach© DR 2800™ portable spectrophotometer (2nd Edition) discovered significantly different concentrations of nitrate (NO_3^-), manganese (Mn), and zinc (Zn). Table 1 summarises the different nutrient concentrations found in the dry grass, dry leaves, and dry twig water samples. The dry grass sample had the highest NO_3^- content (3.500 mg/l ± 1.519) followed by the dry leaves sample (2.867 mg/l ± 1.378), and the dry twig sample (0.183 mg/l ± 0.111). Similarly, the dry grass sample had the highest Mn content as well (0.685 mg/l ± 0.040) followed by the dry leaves sample (0.233 mg/l ± 0.049), and the dry twigs sample (0.199 mg/l ± 0.141). However, the dry grass sample had the lowest Zn content (0.016 mg/l ± 0.003) while the dry leaves sample (0.032 mg/l ± 0.015) and the dry twigs sample had the highest concentration of Zn (0.070 mg/l ± 0.046). The nutrient distribution in each plant material also varied. The dry twigs sample primarily consisted of Mn (0.199 mg/l ± 0.141) followed by NO_3^- (0.183 mg/l ± 0.111) and Zn (0.070 mg/l ± 0.046). The dry leaves sample consisted mainly of NO_3^- (2.867 mg/l ± 1.378) followed by Mn (0.233 mg/l ± 0.049) and Zn (0.032 mg/l ± 0.015). This order of nutrient distribution was similar in the dry grass sample where NO_3^- (3.500 mg/l ± 1.519) followed by Mn (0.685 mg/l ± 0.040) and Zn (0.016 mg/l ± 0.003).

Table 1: The concentration of nutrient content in water for each plant material

Nutrient	Nutrient content (mg/l) ± standard deviation			Distribution of nutrient
	Twigs (A)	Dry leaves (B)	Dry grasses (C)	
NO_3^-	0.183 ± 0.111	2.867 ± 1.378	3.500 ± 1.519	C > B > A
Mn	0.199 ± 0.141	0.233 ± 0.049	0.685 ± 0.040	C > B > A
Zn	0.070 ± 0.046	0.032 ± 0.015	0.016 ± 0.003	A > B > C

Effect of Dose on Wing Size

The boxplot of landmark wing sizes (Figure 1) correlates the data between landmark wing sizes and clearly illustrates the difference in landmark wing sizes between the samples collected from dry grass, dry leaves, and dry twig samples at low, medium, and high doses. The median landmark wing sizes from the dry grass, dry leaves, and dry twigs samples were different for low and high doses. At medium doses, the median landmark wing size of dry grass and dry leaves samples were similar but different for the dry twigs samples as the boxplot data was not normally distributed. As seen in the trend of landmark sizes in the boxplot, at all three doses, wings from the dry grass sample had the largest landmark size followed by the dry leaves sample with the dry twigs sample having the smallest landmark sizes.

An analysis of variance (ANOVA) of the different plant materials is also shown in the upper left of Figure 1. As an integrated analysis of all three doses puts the p-value at 0.013($p < 0.05$), therefore, there is a statistically significant difference in mean landmark sizes between the dry grass, dry leaves, and dry twigs samples at low, medium, and high doses. As the ANOVA of the plant materials at low doses (A) and medium doses (B) was 0.001($p < 0.05$), there was also a significant difference in mean landmark size. As the p-value was 0.041 ($p < 0.05$) at high doses (C), there was also a significant difference between the dry grass, dry leaves, and dry twigs samples. However, the differences between the plant materials at low and medium doses were more significant than at high doses. The differences between the dry grass, dry leaves, and dry twig samples were also identified and which plant material differed from the other is shown in the upper right of Figure 1. The Tukey post hoc test was used to analyse the differences between the plant materials. The dry grass and dry twigs

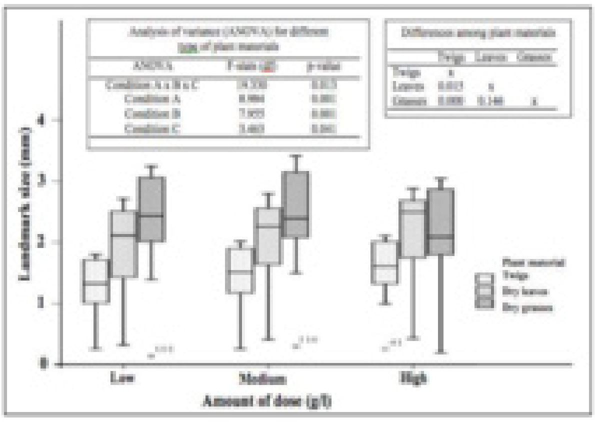

Fig.1: Boxplot showing data distribution of landmark sizes. ANOVA of different plant materials at different doses (upper left) and difference in wing size according to plant material via Tukey post hoc test (upper right). *Note: A (low dose), B (medium dose), and C (high dose).

samples had the most significant differences (p = 0.000) in landmark sizes. There was also a significant difference between dry leaves and dry twigs samples (p = 0.015) as well as between dry grass and dry leaves samples (p = 0.146).

Effect of Dose on Morphometric Wing Characteristics

Figure 2 shows the morphometric wing characteristics of adult Aedes aegypti mosquitoes exposed to low, medium, and high doses of dry grass, dry leaves, and dry twigs during the larval stage. Morphometric wing characterisation is a tool that is presently used in mosquito identification. Canonical variate analysis (CVA) was used to perform a discriminant analysis to identify the degree of similarity and differences in wing shape among mosquitoes in a morphospace. A regression analysis of the CVA graph points against variations in mosquito wing shapes was used to visualise differences between the wings being compared. The data set of landmark positions on each wing shape were used to produce a canonical analysis. The data was then split into two groups; the x-axis (CV1) and the y-axis (CV2). The dots on the CVA graph represented the sum of the canonical correlation of data variables in the analysis. Figure 2A shows the CVA of different plant materials at low doses and indicates only marginal dissimilarity between the examined wings. For the low doses, the wing shape CVA was correctly segregated between dry grass, dry leaves, and dry twig samples. However, there was an overlap along the CV1 and CV2 axes for all three plant materials. Figure 2B displays the CVA of different plant materials at medium doses and shows obvious difference between wing shapes. The wing shape CVA between dry grass, dry leaves, and dry twig samples was successfully separated along the CV1 and CV2 axes. However, there was a small overlap between dry grass and leaf samples. Figure 2C shows the CVA of different plant materials at high doses and shows a clear difference between wing shapes. The wing shape CVA revealed that the structuring within wings from dry grass samples was the most segregated while wings from dry leaves and twig samples overlapped.

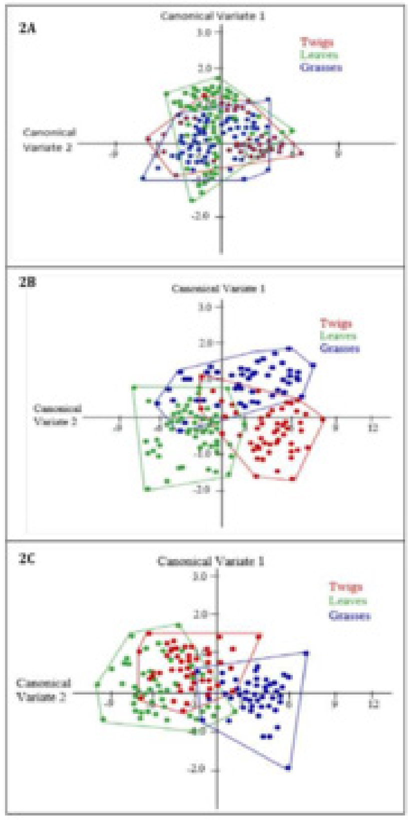

Fig. 2: The morphometric wing characteristics of adult Aedes aegypti mosquitoes exposed to low (A), medium (B), and high doses (C) of dry grass, dry leaves, and dry twigs during the larval stage.

DISCUSSION

Plant material was one of the factors affecting the size and shape of *Aedes aegypti* mosquito wings. The plant materials used in this study were dry grass, dry leaves, and dry twigs. Dry grass was found to have the most profound effect on wing size followed by dry leaves. It was the least affected by dry twigs. Our findings indicate that adult *Aedes aegypti* mosquitoes that were exposed to low, medium, and high doses of dry grass at the larval stage had the largest landmark size measurements compared to dry leaves and dry twigs. Wings from dry grass samples also had the highest wing centroid sizes, especially in high doses. In contrast, wings from dry twig samples had the smallest wing landmark size and wing centroid size. There was a significant difference in wing landmark size and wing centroid size between dry grass, dry leaves, and dry twig samples at low, medium, and high doses. Wing centroid sizes were slightly larger when exposed to high doses of dry grass, dry leaves, and dry twig than medium or low doses. Suwanchaichinda & Paskewitz (1998) assessed the correlation between larval nutrient and adult body size (16). The larvae were divided into three groups; low, medium, and high diet; and fed a mixture of finely ground fish food, dogfish, and yeast at low, medium, and high doses. Their study discovered that larvae from the high diet group had the longest wing length as adults. Our study found that larvae supplemented with high doses of dry grasses had the largest wing sizes as adults. Reiskind et al., (2009) state that leaf species can also affect the wing length and size of adult mosquitoes, especially in the case of the *Aedes* mosquito (17). However, our study found that mosquitoes exposed to dry leaves as larvae had smaller wing sizes than those exposed to dry grass. Well-fed larvae emerged into adult Aedes aegypti mosquitoes with larger body sizes and wing sizes than poorly-fed larvae (18). These same results were observed in our study. Adult mosquitoes that were exposed to high doses dry grass, dry leaves, or dry twigs as larvae were larger than those exposed to medium and low doses. As wing size corresponds to body size in adult mosquitoes, wing size can be used to estimate the body size of adult mosquitoes (19-21).

Adult mosquitoes prefer breeding sites with poor circulation and high organic content. More adult mosquitoes are produced in poorly circulating stagnant water rich in organic content than those with low organic content (22-23). Our study found that dry grass water samples had more organic contents than dry leaves and dry twig water samples. Dry grass was also found to have the highest concentrations of nitrate (NO_3^-) and manganese (Mn) and the lowest concentration of zinc (Zn). As such, the wings of adult Aedes aegypti from this group were the largest as the $NO_3^-$and Mn present in the water acted as organic nutrients. The wings of adult Aedes aegypti from the dry twigs group were smaller as the water contained the lowest concentrations of NO_3^- and Mn but the highest concentration of Zn. Therefore; it is safe to conclude that Zn does not positively affect the wing size of adult mosquitoes, as larvae prefer NO_3^- and Mn as nutrients. Studies have shown that the physicochemical and chemical characteristics of a breeding site; such as vegetation; may also affect larval survival and development (5,24). The decomposition of plant materials in water was also a concern in our study as different types of organic materials; such as dry grass, dry leaves, or dry twigs; release different types of nutrients that ultimately affect the wing size of adult mosquitoes. The nitrogen released by these organic materials also increases the longer it remains in contact with water due to the generation of energy in the form of adenosine triphosphate and oxygen consumption (25-26). However, the amount of nitrogen released by the decomposition of these organic materials differs at each stage (27) as it is aided by the presence of dissolved oxygen and microbes in the water. Thomas et al., (1970) found that the flow rate of water aided in the decomposition of organic matter (28).

Mosquitos are highly selective and prefer to choose a breeding sites that meets a specific set of criteria. This is because the breeding site will support their survival and population dynamics (5,29,30). Dom et al., (2016) states that *Aedes* mosquitoes prefer to breed and survive in polluted water (5) as its ambient humidity and temperature is favourable.The study concluded that *Aedes* mosquitos can breed in a variety of habitats, be it clear water, turbid water, or polluted water. Our study discovered that between water containing dry grass, leaves, and twigs, dry leaves and dry grasses, the dry grass samples appeared to be more turbid than the others due to the decomposition of the dry grass. This resulted in the emergence of adult mosquitoes with larger wing sizes. The water from the dry twig samples were the clearest and the adult mosquitoes from these samples had smaller wing sizes. Therefore, turbid waters resulted in adult mosquitoes with larger wings. Apart from the type of plant material, many other factors; such as larval density, vector competence, resource availability, and environmental characteristics such as temperature and humidity; also affect the wing size of adult *Aedes* mosquito. A previous study summarised that high larval densities at breeding sites significantly affect the body mass of immature mosquitoes (7,31) as it indirectly increases larval competition for resources. Based on the results of our study, larvae that were fed high doses emerged as adult mosquitoes with larger wing sizes than those fed low and medium doses. This was because high doses provide more resources, thereby, reducing larval competition. However, as the availability of food resources decreased over time, larval densities increased. As higher larval densities increase vector competition, the body and wing size of the adult mosquitoes will decrease (32-33). Dutra et al., (2016) found that larval densities result in significant variations in the wing shapes of adult mosquitoes (34).

CONCLUSION

Despite the clear correlation between larval density and larval competition and their effects on mosquito body size, other environmental factors; such as temperature; are also primary factors that affect the body and wing size of adult mosquitoes. A study by Lyimo et al., (1992) found that decreasing the temperature, from 30 °C to 24 ºC, increased the body size of adult mosquitoes (35). Vargas et al., (2010) found that climatic variations are also one of the causes for the variations in size (15) as wing shape characterisation is driven by the size of the wing which is primarily affected by relative humidity. A recent study stated that there is a significant correlation between rearing season and variations in wing size and shape. It discovered that wing centroid size significant increased when mosquitos were reared at the onset of the dry season (36) as adult mosquitos are able to reproduce and survive better during the dry season. In our study, plant materials; namely dry grass, dry leaves, and dry twigs; were studied as the only factor affecting wing size and shape. These materials also served as nutrients for larvae development and resulted in significant difference in adult wing sizes. Our study found that adult mosquitoes that had been exposed to dry grass during the larval stage had larger landmark wing sizes and larger wing centroid sizes followed by those exposed to dry leaves and dry twigs.

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