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Wing Shape as an Indicator of Larval Rearing Conditions for *Aedes albopictus* and *Aedes aegypti* (Diptera: Culicidae)

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ABSTRACT Estimating a mosquito's vector competence, or likelihood of transmitting disease, if it takes an infectious bloodmeal, is an important aspect of predicting when and where outbreaks of infectious diseases will occur. Vector competence can be affected by rearing temperature and inter- and intraspecific competition experienced by the individual mosquito during its larval development. This research investigates whether a new morphological indicator of larval rearing conditions, wing shape, can be used to distinguish reliably temperature and competitive conditions experienced during larval stages. *Aedes albopictus* (Skuse) and *Aedes aegypti* (L.) (Diptera: Culicidae) larvae were reared in low intraspecific, high intraspecific, or high interspecific competition treatments at either 22 or 32°C. The right wing of each dried female was removed and photographed. Nineteen landmarks and 20 semilandmarks were digitized on each wing. Shape variables were calculated using geometric morphometric software. Canonical variate analysis, randomization multivariate analysis of variance, and visualization of landmark movement using deformation grids provided evidence that although semilandmark position was significantly affected by larval competition and temperature for both species, the differences in position did not translate into differences in wing shape, as shown in deformation grids. Two classification procedures yielded success rates of 26–49%. Accounting for wing size produced no increase in classification success. There seemed to be a significant relationship between shape and size. These results, particularly the low success rate of classification based on wing shape, show that shape is unlikely to be a reliable indicator of larval rearing competition and temperature conditions for *Ae. albopictus* and *Ae. aegypti*.

KEY WORDS geometric morphometrics, classification, vector competence, larval competition

The likelihood that a mosquito, or any vector, will show disseminated infection after taking an infectious bloodmeal, and thus be able to transmit disease, is its vector competence (Hardy et al. 1983, Eldridge and Edman 2000). A mosquito's vector competence can be influenced by its environment, including the larval rearing environment. For example, inter- and intraspecific competition among larval *Aedes albopictus* (Skuse) and *Aedes aegypti* (L.) (Diptera: Culicidae) produced adult *Ae. albopictus* with significantly higher rates of infection and dissemination with Sindbis virus (i.e., higher vector competence; Alto et al. 2005). Effects on *Ae. aegypti* were similar, although not significant. Alto et al. (2008) found similar effects of larval competition on vector competence for dengue virus in *Ae. albopictus* and *Ae. aegypti.* Greater competitive stress during larval stages produced adult *Ae. albopictus* that were significantly more competent as dengue vectors, with both a higher proportion of infected individuals as well as a higher proportion with disseminated infections compared with those from low competition conditions, with no difference between inter- and intraspecific competition treatments. For *Ae. aegypti*, the results were not significant, but they again showed a trend similar to that of *Ae. albopictus*, with high competition treatments leading to increased dengue infection and dissemination rates. Increased interspecific larval competition with *Ae. albopictus* also has been shown to produce adult *Aedes triseriatus* (Say) that show increased rates of infection and dissemination for LaCrosse virus (Bevins 2008).

Vector competence is one important component of a mosquito population's vectorial capacity; vector competence is the average rate at which infected bites occur after an infected host has been introduced (Anderson and Rico-Hesse 2006). It would therefore be desirable to have a way of sampling a natural population of adult mosquitoes and accurately estimating each individual's vector competence as a step toward estimating vectorial capacity. Some indicator of the larval competition conditions under which an adult mosquito developed would be one contributor to estimates of vector competence. Such a measure could help to predict when and where outbreaks of vector-borne illness might occur.

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The most straightforward index of an adult mosquito's larval competition environment would be adult size; the negative relationship between larval competition and adult size is well known (Livdahl 1982; Agnew et al. 2000; Gimnig et al. 2002; Alto et al. 2005, 2008; Jirakanjanakit et al. 2007). However, other factors in the larval rearing environment, most notably temperature, also affect the relationship between size and competition. Larval rearing temperature has an inverse relationship with size for various mosquito species (Rueda 1990, Lyimo et al. 1992, Tun-Lin et al. 2000, Alto and Juliano 2001). Furthermore, larval rearing temperature is inversely related to adult infection rate for both Rift Valley Fever and Venezuelan equine encephalitis (Turell 1993) and inversely related to adult body titer of Chikungunya virus (Westbrook 2010). This means that an adult mosquito may be large because of low competition (yielding lower vector competence) or low temperature (yielding greater vector competence), or it may be small because of high competition (yielding greater vector competence) or high temperature (yielding lower vector competence). Other stressors acting on larvae (e.g., pesticide exposure) also can alter adult size and may affect vector competence (Muturi et al. 2011). Therefore, a method that can distinguish both an adult mosquito's larval competition environment as well as its larval rearing temperature would be desirable. Here, we evaluate geometric morphometric analysis of wing shape and size as a way to classify adult mosquitoes into their larval rearing conditions, testing specifically whether wing shape gives a reliable classification.

Geometric morphometrics (Bookstein 1991, 1996a,b; Zelditch et al. 2004) is a method of analyzing shape that accounts for the spatial relationships among the variables in the analysis, which are landmark points on the structure in question (Rohlf 1999). As such, geometric morphometrics retains the geometry of the morphological structure (Adams et al. 2004). Landmarks are plotted on images of the object, resulting in sets of coordinates for each individual structure, that can then be used in a variety of statistical methods to determine whether between-group differences exist. Using geometric morphometrics also allows for the visualization of shape change between groups and can aid in the interpretation of results in ways that are not possible using traditional morphometrics (Rohlf 1999). In addition, this method is relatively easy and inexpensive and could thus be readily used in the field. Here, for two species of *Aedes*, we determine whether wing shape can be used to discriminate among adults that developed under different larval competition and temperature conditions. If this can be done, wing shape could be used as an indicator of vector competence for field-collected individual adults.

Study Species. *Ae. albopictus* is native to Asia and the islands of the Western Pacific and Indian Oceans, but it has recently been introduced into Africa, the Middle East, Europe and North and South America (Gratz 2004). *Ae. albopictus* can transmit at least 22 arboviruses (Moore and Mitchell 1997) and is second only to *Ae. aegypti* in its importance as a vector of dengue (Benedict et al. 2007). *Ae. albopictus* probably acts as a maintenance vector of dengue in rural areas of Southeast Asia, where dengue epidemics often occur (Gratz 2004). *Ae. albopictus* is also important because it is an aggressive diurnal biter and could serve as a bridge vector for various viruses (Moore and Mitchell 1997).

Ae. aegypti is native to Africa, but it is now found throughout most tropical and subtropical regions (Tabachnick and Powell 1979, Kamgang et al. 2011). Its geographic distribution and population densities have increased because of uncontrolled urbanization creating more larval habitat in the form of artificial containers (Gubler 1998). *Ae. aegypti* is an efficient vector because it bites during the day and spends its entire life around human habitation (Morrison et al. 2008). Furthermore, *Ae. aegypti* often feeds on several people to acquire a full bloodmeal (Gubler 1998). *Ae. aegypti* is the most competent, and most important, vector of dengue virus (Rueda et al. 1990) and it can transmit yellow fever, chikungunya virus, and other arboviruses (Morrison et al. 2008).

Materials and Methods

Rearing Larvae and Wing Preparation. *Ae. albopictus* and *Ae. aegypti* from Tampa, FL, colonies (three–six generations in the laboratory) were reared from eggs in plastic containers containing 200 ml of water, 1.0 g of live oak (*Quercus virginiana* Mill.) leaves, 0.01 g of dried crickets [*Gryllodes sigillatus* (Walker)], and 1 µl of tree hole water inoculum. Each container was randomly assigned to one of five competition treatments and one of two temperature treatments, chosen based on a preliminary run of this experiment. The competition treatments (described by initial numbers of *Ae. albopictus:Ae. aegypti* first-instar larvae) were 15:0, 30:0, 15:15, 0:30, and 0:15, and the temperature treatments were 22 and 32°C. There were 10 replicates of each treatment combination. Containers were kept in temperature-controlled environmental chambers with a photoperiod of 14:10 (L:D) h until all individuals eclosed as adults or died. Adults were identified for sex and species and then dried at 50°C for >24 h. Work with mosquitoes was done under protocols 01-2010 and 02-2007 of the Institutional Animal Care and Use Committee.

The right wings were gently removed from adult females, for 248 *Ae. albopictus* and 143 *Ae. aegypti* wings in total. If the right wing was damaged, the left was used. If both wings were damaged, the female was excluded from the study. The wings were then photographed with a Sony Power HAD image capturing system and loaded into TPSdig version 2.14 (Rohlf 2009) for landmark digitization. Nineteen landmarks and 20 semilandmarks were plotted on images of the mosquito wings. Landmarks were placed at intersections of wing veins or intersections of veins and the wing margin (Fig. 1). Semilandmarks were placed along the posterior edge of the wing (Fig. 1). Semilandmarks are placed along curves that do not have features that could otherwise be designated with traditional landmarks (Zelditch et al. 2004). The po-
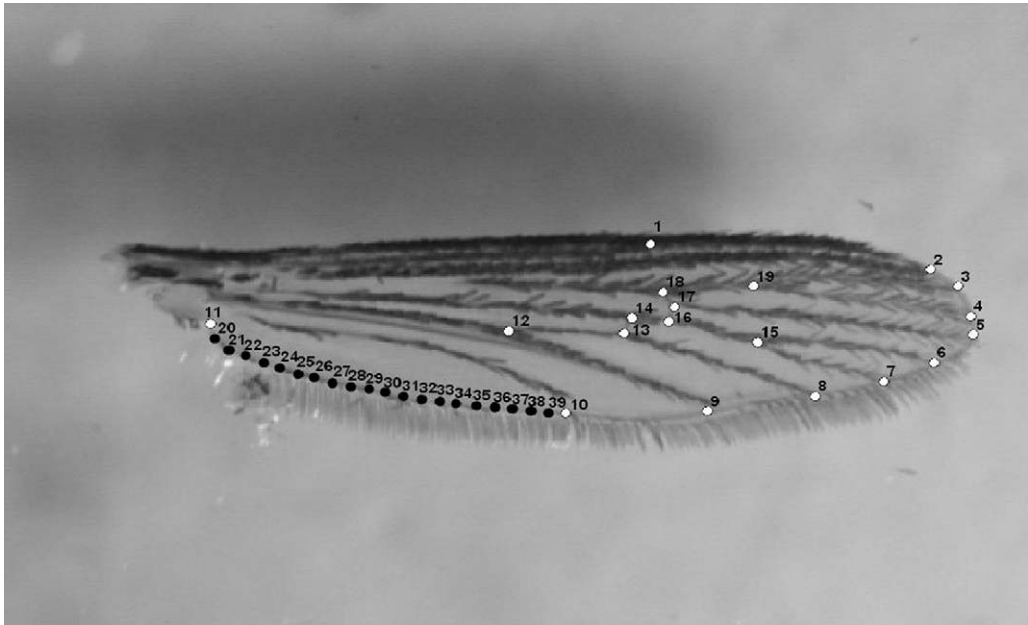
Fig. 1. Landmarks plotted on right wings from *Ae. albopictus* and *Ae. aegypti* females. Landmarks 1–19 (white) are traditional landmarks, and 20–39 (black) are semilandmarks along the posterior edge.

sition of an individual semilandmark is only informative relative to the positions of the surrounding semilandmarks, so the semilandmarks must be interpreted as a group. The arrangement of the group of semilandmarks can show changes in the bowing of the curve and nothing more. This procedure resulted in a set of landmark coordinates for each wing. Landmark coordinate data were transformed into Integrated Morphometrics Package (IMP) files by using Coord-Gen (Sheets 2006a), semilandmark positions were optimized using TPSrelw version 1.46 (Rohlf 2008), and centroid sizes (=square root of the sum of the squared distances between each landmark and its centroid, or center of the configuration of landmarks) for all individuals were calculated. The combined landmark data then had the nonshape variation removed using the generalized least squares Procrustes superimposition method (Bookstein 1991) to translate, scale, and rotate each individual's configuration against the reference (=average) configuration. The resulting coordinates were then used to calculate partial and uniform warp scores, which describe to what extent, and in what direction, the reference configuration would have to change to match exactly the shape of that individual (Zelditch et al. 2004). These partial warp scores were the measures of shape that were used as variables in many of the subsequent analyses.

Effects of Competition. First, a preliminary analysis tested whether the competition treatments stressed the larvae. If competition and temperature affect life-history traits such as survivorship, time to eclosion, and wing size, then it would be meaningful to test for effects of competition and temperature on wing shape. If the competition treatments were effective, one would expect a decrease in survivorship, an increase in median days to eclosion, and a decrease in wing size with increased inter- or intraspecific density (Livdahl 1982, Russell 1986, Hard et al. 1989). For each species, treatment effects on these three variables were tested using analysis of variance (ANOVA). Because competition significantly affected these life-history traits (Table 1), analyses on wing shape and size were then performed.

Morphometric Differences between Treatment Groups. Partial warp scores were used in canonical variate analyses (CVAs) to describe differences in shape among treatment groups. CVAs were performed using CVAGen (Sheets 2006b), including a preliminary analysis to determine whether shape differed between the two species regardless of treatment. Because of software limitations, all CVAs were done without the inclusion of centroid size as a dependent variable. CVAGen also was used to visualize differences in landmark and semilandmark position between groups.

Because a CVA cannot test for statistically significant differences between groups, a multivariate ANOVA (MANOVA) also must be performed (Zelditch et al. 2004). For standard MANOVAs on shape data, the appropriate number of degrees of freedom must be calculated, but there is no best method of doing so when semilandmarks are used (Zelditch et al. 2004). To circumvent this problem, randomization MANOVAs were used. These do not require the calculation of new degrees of freedom. This process shuffles the observations and randomly assigns them to different temperature and competition treatments. The shuffling procedure is repeated

Table 1. ANOVA results for effects of competition and temperature on survivorship, median days to eclosion, and centroid size

Effect	df	Survivorship		Median days		Centroid size	
		F	P	F	P	F	P
Ae. albopictus							
Competition	2	47.14	**<0.001**	63.36	**<0.001**	0.64	0.533
Temp	1	1.33	0.255	23.19	**<0.001**	9.89	**0.003**
Competition × temp	2	3.06	0.055	0.72	0.490	0.36	0.699
Error df	49						
Ae. aegypti							
Competition	2	51.25	**<0.001**	20.97	**<0.001**	0.84	0.437
Temp	1	3.2	0.079	35.41	**<0.001**	9.01	**0.004**
Competition × temp	2	0.98	0.383	3.73	**0.031**	3.67	**0.033**
Error df	49						

Effects significant at $\alpha = 0.05$ are shown in bold.

many times, producing hundreds or thousands of randomized data sets (permutation). Then, a MANOVA is performed on each random permutation, as well as on the original data. For each effect in the model (temperature, competition, and interaction, in this case), if <5% of the permutations yield a P value smaller than that obtained for the original data set, the effect is deemed significant. Even though this method does not analyze every possible permutation of the data set, it does take a large, random sample of all possible permutations while remaining computationally feasible. The randomization MANOVA was performed using SAS 9.2 (SAS Institute 2008). The macros for randomization and analysis processes were modified from a randomization wrapper for ANOVA (Cassell 2002). Each randomization test created 1,000 permutations. A preliminary randomization MANOVA was done to determine whether the species differed in shape overall, and subsequent analyses were run for each species both with and without centroid size.

Classification. To determine whether shape and size variables can be used to discriminate among individuals based on rearing conditions (i.e., to predict the conditions from which an individual came), two classification procedures were performed. The first was a jackknife classification procedure that accompanied each CVA and was performed with CVAGen, which was run without centroid size. In this analysis, each observation was held out of the data set to develop the discriminant function, and then classified based on the resulting function. Percent correct assignment was then assessed. The second procedure used the discriminant function procedure (PROC DISCRIM) in SAS 9.2 (SAS Institute 2008) to create linear discrimination functions and to run cross-validation. Discriminant function analysis in SAS was run both with and without centroid size.

Within each procedure, individuals were classified into two sets of treatment conditions. The first run classified individuals into temperature and competition treatments. However, it is possible that only the magnitude of competition (i.e., total larval density) is important, rather than type of competition (i.e., inter- versus intraspecific competition). Therefore, the second run classified individuals into temperature and density treatments. In this second run, the high intra-

and high interspecific groups were termed the "high-density" treatment, and the low intraspecific treatment was the "low-density" treatment.

Relationship of Shape to Centroid Size. Multivariate analyses of covariance (MANCOVAs) were performed using SAS 9.2 (PROC GLM) for each species to determine the relationship between size and shape for the treatment groups. First, we tested whether the relationship between size and multivariate shape was the same for all treatment groups. We then determined whether size was significantly related to multivariate shape. We also compared the results of the different classification procedures to determine whether including size resulted in better discrimination of individual adults for larval competition and temperature conditions.

Results

Effects of Competition. The interaction of competition level and temperature was marginally nonsignificant for *Ae. albopictus* survivorship (Table 1; Fig. 2a). For both species, the effect of competition treatment was significant (Table 1; Fig. 2a and b). For *Ae. albopictus* in both temperatures, low intraspecific competition resulted in the greatest survivorship, followed by the high interspecific and then high intraspecific treatments (Fig. 2a). In *Ae. aegypti*, only competition affected survivorship (Table 1), with low intraspecific competition yielding the greatest survivorship, and both high intra- and high interspecific treatments produced equally low levels of survivorship (Fig. 2b).

Median days to eclosion for *Ae. albopictus* was significantly affected by competition and temperature, but not the interaction (Table 1). Increased competition resulted in an increase in median days to eclosion (Fig. 3a). Median days decreased from 33.8 ± 1.182 at 22°C to 25.75 ± 1.182 at 32°C. For *Ae. aegypti*, the interaction between competition and temperature was significant (Table 1). In both temperatures, high inter- and high intraspecific competition resulted in a longer time to eclosion than the low intraspecific treatment, and this trend was significant at 22°C but not at 32°C (Fig. 3b).
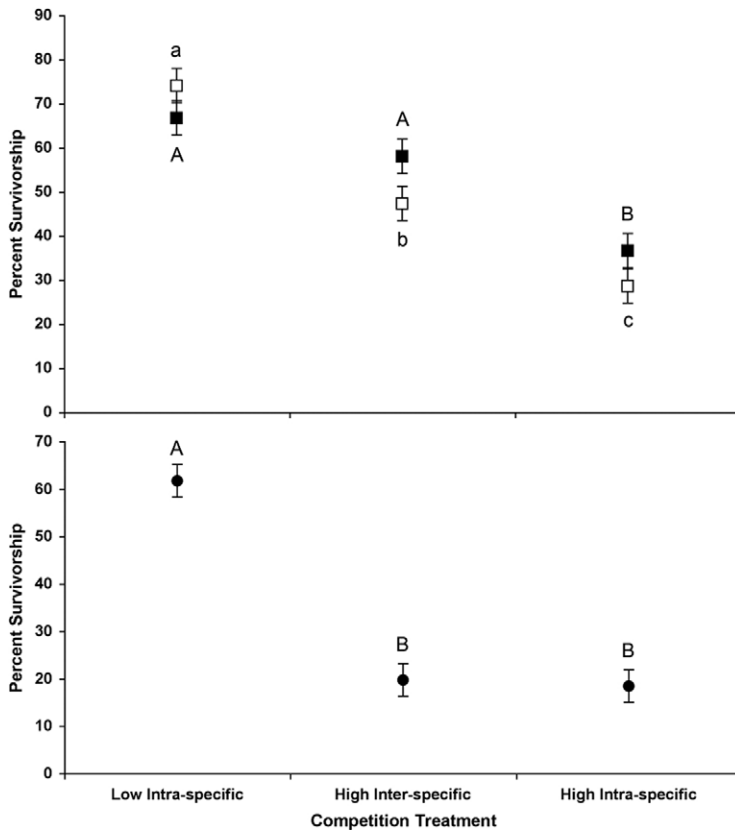
Fig. 2.   Percentage of survival (average ± SE) for each competition treatment for *Ae. albopictus* by temperature (a) and *Ae. aegypti* (across both temperatures; b). Filled squares in panel a represent the 22°C treatment, and open squares represent the 32°C treatment. Means with different letters are significantly different.

Centroid size for *Ae. albopictus* was significantly affected only by temperature (22°C, 728.34 ± 7.412; 32°C, 695.38 ± 7.412). There was a significant inter-action for *Ae. aegypti* (Table 1). At 22°C, both the low intraspecific and high interspecific treatments pro-duced larger females than the high intraspecific treat-ment (Fig. 4), although this trend was not significant. Even though increased competition (increased larval density) is expected to produce smaller adults, the high inter- and intraspecific treatments at 32°C pro-duced larger females than the low intraspecific treat-ment (Fig. 4), although the trend was again not sig-nificant. This result further illustrates that size cannot be used to determine larval rearing conditions. The relationship between size and rearing conditions dif-fered between the two species, and the relationship was either counter-intuitive or nonexistent. Overall, the competition treatments were successful at stress-ing the larvae, but the phenotypic responses to density and temperature were complex and multifaceted.

Morphometric Differences Between Treatment Groups. The two species were well separated via shape regardless of treatment (Fig. 5). The CVA yielded a distinct separation of two clouds of points along the first CVA axis, suggesting that some aspect of wing shape strongly separates *Ae. albopictus* and *Ae.*

aegypti. The difference in shape between the average *Ae. albopictus* and the average *Ae. aegypti* wing (Fig. 6) indicates that most of the difference occurs in the positions of the semilandmarks along the posterior edge of the wing, with some less pronounced differ-ences in the center of the wing. The accompanying randomization MANOVA yielded a highly significant species effect ($P < 0.001$). A jackknife classification was able to assign 96.16% of the individuals to the correct species. Because the consistent differences in landmark position indicate the two species have dif-ferent wing shapes, subsequent morphometric analy-ses were done for each species separately.

Ae. albopictus. The canonical variate analysis for competition and temperature (Fig. 7) resulted in one significant axis ($\chi^2_{370} = 591.81$, $P \ll 0.0001$), along which the two temperature groups were reasonably well separated. The difference in landmark positions between the average high and average low tempera-ture wings (data not shown) comes primarily from the semilandmarks shifting noticeably along the posterior edge. Randomization MANOVA confirmed a highly significant temperature effect ($P < 0.001$), a margin-ally nonsignificant competition effect ($P = 0.064$), and a nonsignificant interaction ($P = 0.572$) for shape variables.
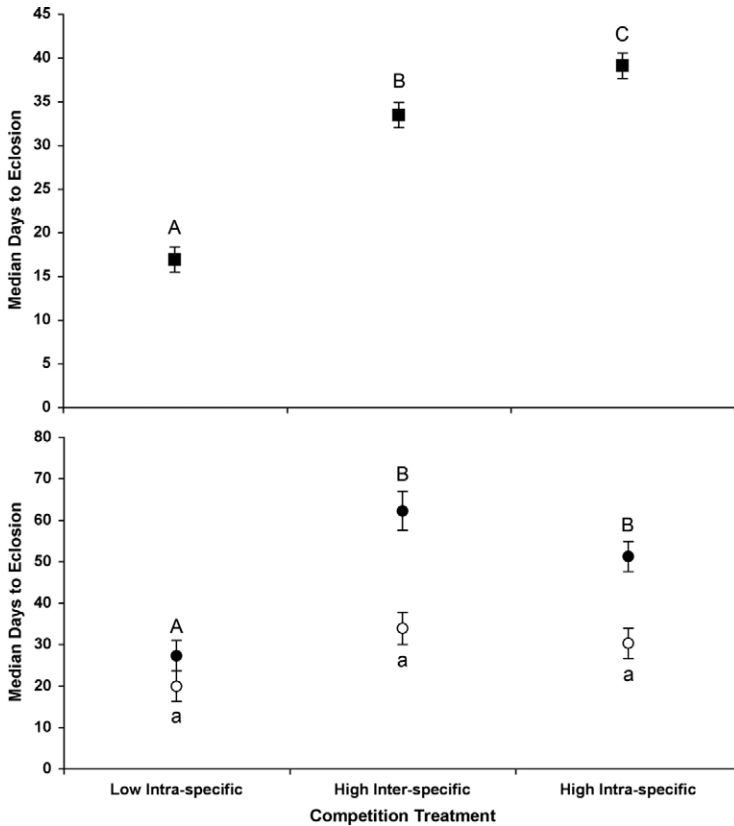
Fig. 3. Median days to eclosion (average ± SE) for each competition treatment for *Ae. albopictus* (across both temperatures; a) and *Ae. aegypti* by temperature (b). Filled circles in panel b represent the 22°C treatment, and open circles represent the 32°C treatment. Means with different letters are significantly different.

Contrasts were performed within each temperature to determine whether shape could be used to distinguish between high inter- and high intraspecific competition independent of temperature (sequential Bonferroni $\alpha_e = 0.05$). Within both temperatures, the two treatments were not significantly different (22°C: $F_{74,169} = 1.04$, $P = 0.4076$ and 32°C: $F_{74,169} = 1.08$, $P = 0.3337$). This result agreed with the visualizations of shape differences between the corresponding competition groups in each temperature (data not shown). In both cases, the only difference between the groups was a slight shift of the semilandmarks along their curve.

Because shape did not differ between the high inter- and high intraspecific competition treatments, contrasts were used to determine whether shape differed solely because of density (i.e., magnitude of competition, regardless of species composition) by
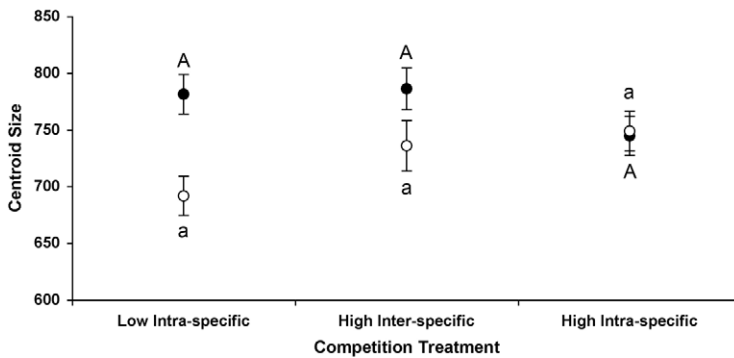

Fig. 4. Centroid size (average ± SE) for *Ae. aegypti* for all competition treatments by temperature. Filled circles represent the 22°C treatment, and open circles represent the 32°C treatment.
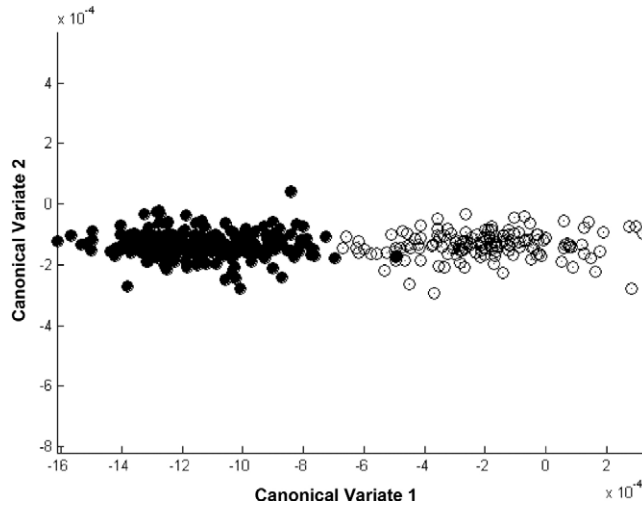
Fig. 5. CVA plot of species effect on shape. One axis was found to be significant ($P \ll 0.0001$). Filled circles represent *Ae. albopictus* and open circles represent *Ae. aegypti*.

comparing high- and low-density groups within each temperature. For both temperatures, with correction for multiple tests, the high and low densities did not differ in shape (22°C: $F_{74,169} = 0.96$, $P = 0.5662$ and 32°C: $F_{74,169} = 1.37$, $P = 0.0492$). Again, the only change between the average treatment shapes were the shifts of semilandmarks along the curve (data not shown).

Randomization MANOVA, including centroid size among the dependent variables, tested whether some combination of size and shape differed among treatments. Inclusion of centroid size produced results similar to those found excluding centroid size. Temperature remained highly significant ($P < 0.001$), competition marginally significant ($P = 0.046$), and the interaction remained nonsignificant ($P = 0.5750$). A second set of contrasts including centroid size yielded the same conclusions as the first set. The high inter- and intraspecific competition treatments did not differ

Fig. 6. Deformation grid of shape differences between an average *Ae. albopictus* and *Ae. aegypti* wing. The changes have been exaggerated 250× for easier viewing.

at either temperature (22°C: $F_{75,167} = 1.03$, $P = 0.4250$ and 32°C: $F_{75,167} = 1.08$, $P = 0.3445$). There was also no difference between high and low density at either temperature (22°C: $F_{75,167} = 1.04$, $P = 0.4061$ and 32°C: $F_{75,167} = 1.34$, $P = 0.0612$).

Ae. aegypti. Canonical variate analysis yielded two significant axes ($\chi^2_{370} = 514.39$, $P \ll 0.0001$ and $\chi^2_{292} = 350.93$, $P = 0.010$). The first corresponded to temperature and the second roughly to competition treatment (Fig. 8). There seemed to be an interaction, because the score on the competition axis for each treatment depended on the temperature treatment (Fig. 8). The randomization MANOVA confirmed a significant temperature by competition interaction ($P = 0.046$), so contrasts were performed to see which groups differed in shape. Contrasts comparing high inter- and high intraspecific competition treatments within each temperature were not significant (22°C: $F_{74,64} = 1.15$, $P = 0.2881$ and 32°C: $F_{74,64} = 1.28$, $P = 0.1576$). Only the semilandmarks slid along the posterior edge when transitioning between the two competition treatments for both temperatures (data not shown), although the movement was very slight. Contrasts between high and low density for each temperature indicated that shape was significantly different between the two densities in the 32°C treatment ($F_{74,64} = 1.85$; $P = 0.0065$), but not for the 22°C treatment ($F_{74,64} = 1.01$, $P = 0.4906$). Despite being statistically significant, the movement at 32°C was confined to slight movement of the semilandmarks along the curve; semilandmark shifts were even smaller at 22°C (data not shown).

MANOVA and contrasts including centroid size with shape variables yielded conclusions identical to the analysis of shape alone. Temperature ($P < 0.001$), competition ($P = 0.0110$), and the interaction ($P = 0.0330$) were all significant. High inter- and intraspecific competition treatments were not significantly different at either temperature (22°C: $F_{75,63} = 1.12$,
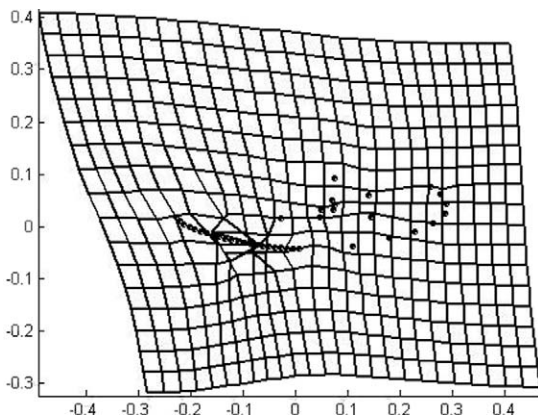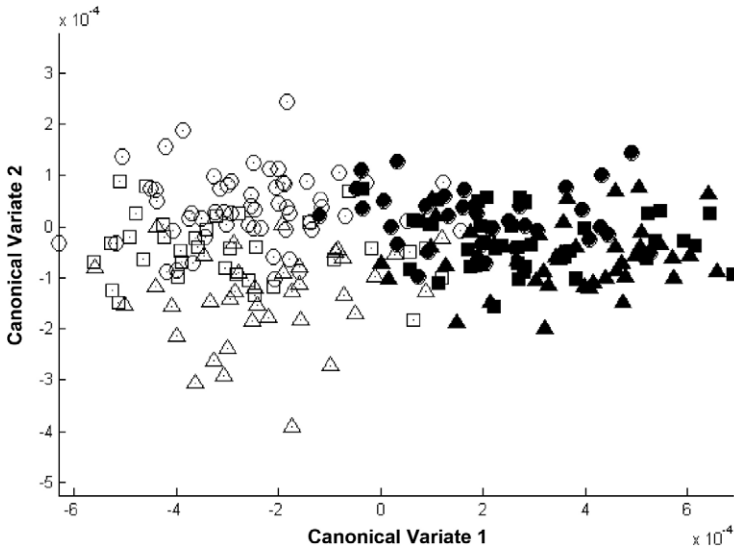
Fig. 7. CVA plot for *Ae. albopictus*, with individuals grouped by temperature and competition treatments. One axis was found to be significant ($P \ll 0.0001$). Filled shapes represent 22°C treatments and open shapes represent 32°C treatments. Circles represent low intra-specific competition treatments (15:0 [*albopictus*: *aegypti*]), squares represent high interspecific treatments (15:15), and triangles represent high intraspecific treatments (30:0).

$P = 0.3263$ and 32°C: $F_{75,63} = 1.33$, $P = 0.1214$) and high- and low-density treatments were significantly different at 32°C ($F_{75,63} = 1.83$; $P = 0.0072$) but not at 22°C ($F_{75,63} = 0.99$; $P = 0.5265$).

Classification. The results of the jackknife classification procedure (Table 2) show that frequency of correct classification was consistently low for both species, although there is a moderate increase for each when individuals are classified into tempera-

ture and density conditions instead of temperature and competition conditions. The results of the discrimination and cross-validation approach (Table 2) are very similar to the jackknife procedure. For both species, success increased when classifying into temperature and density conditions. However, the effect of adding centroid size was different for the two species. Including centroid size slightly lowered the success of the procedure for *Ae. al-*
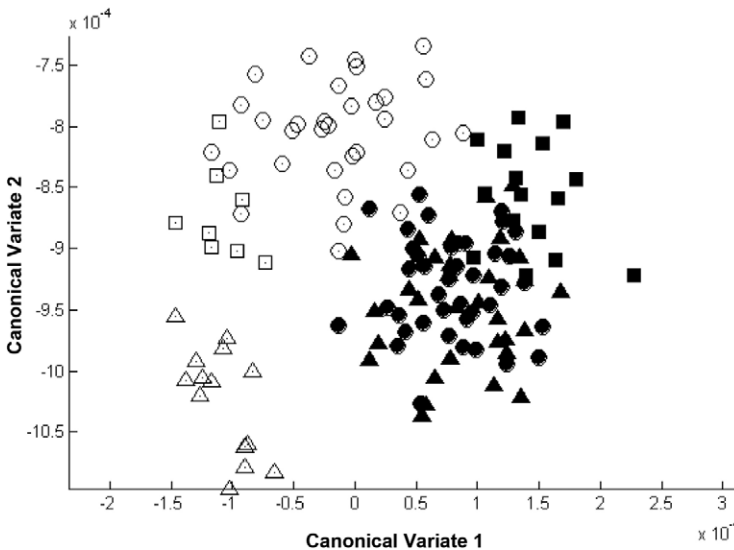
Fig. 8. CVA plot for *Ae. aegypti*, with individuals grouped by temperature and competition treatments. Two axes were significant ($P \ll 0.0001$ and $P = 0.010$). Filled shapes represent 22°C treatments and open shapes represent 32°C treatments. Circles represent low intraspecific competition treatments (0:15 [*albopictus*: *aegypti*]), squares represent high interspecific treatments (15:15), and triangles represent high intraspecific treatments (0:30).

Table 2. Results of the CVA jackknife and discriminant function classification procedures

Grouping	*Ae. albopictus*	*Ae. aegypti*
Jackknife procedure		
Temp, competition	27.82	26.57
Temp, density	46.77	39.86
Discriminant analysis		
Centroid size excluded		
Temp, competition	31.05	27.97
Temp, density	49.60	39.16
Centroid size included		
Temp, competition	30.36	32.17
Temp, density	47.77	46.85

Values are the percentage of individuals correctly assigned to their larval rearing conditions (temperature and competition or density).

bopictus, but it produced a small increase in success for *Ae. aegypti* (Table 2).

Relationship of Shape to Centroid Size. For both species multivariate analysis of shape showed nonsignificant interactions of temperature, competition, or temperature $\times$ competition with centroid size ($P \gg 0.05$; data not shown), indicating homogeneous slopes so that MANCOVA is appropriate. MANCOVA (Table 3) showed a significant relationship between centroid size and shape. However, these results should be interpreted with caution. The df for these tests have not been adjusted to account for the inclusion of semilandmarks in the analysis (Zelditch et al. 2004). This means that these tests are more likely to find the relationship between size and shape significant than a test with the appropriate df. MANCOVA showed significant effects of temperature, competition, and their interaction on wing shape in *Ae. aegypti*, whereas only temperature was significant for *Ae. albopictus* (Table 3). Even though the relationship was significant, it does not seem that accounting for size is beneficial when classifying adults into their larval rearing conditions. Including centroid size did not produce a meaningful increase in successful classification rate (Table 2), and because successful classification is the main goal of this study, we do not look further into the relationship between size and shape.

Discussion

We observed significant differences in shape variables between the two temperature treatments for

Table 3. MANCOVA results for the effects of temperature, competition, and centroid size on shape

Effect	Pillai's trace	*F*	df	*P*
Ae. albopictus				
Temp	**0.716**	**5.68**	**74,167**	**<0.01**
Competition	0.706	1.24	148,336	0.059
Temp $\times$ competition	0.607	0.99	148,336	0.525
Centroid size	**0.610**	**3.52**	**74,167**	**<0.01**
Ae. aegypti				
Temp	**0.807**	**3.56**	**74,63**	**<0.01**
Competition	**1.257**	**1.45**	**148,128**	**0.014**
Temp $\times$ competition	**1.213**	**1.33**	**148,128**	**0.047**
Centroid size	**0.767**	**2.80**	**74,63**	**<0.01**

Effects significant at $\alpha = 0.05$ are shown in bold.
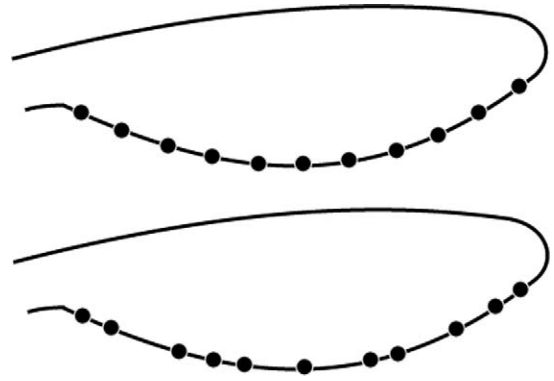
Fig. 9. Two hypothetical and identical wings with semilandmarks placed equidistantly (top) and nonequidistantly (bottom) along the posterior edge. Despite differences in individual semilandmark positions, both sets of semilandmarks describe the same curve.

both species, and between the high- and low-density treatments at 32°C for *Ae. aegypti*. However, the classification success remained very low regardless of the procedure used. To understand these results, it is important to remember what each part of the analysis can tell us. The randomization MANOVA found differences in shape variables that arise because of significant shifts in landmark position. The randomization MANOVA cannot determine which landmarks or semilandmarks moved or the manner in which they moved. To understand the nature of the movement, it is necessary to visualize changes in landmark position using deformation grids. In each grid, the majority, if not all, of the movement of landmarks and semilandmarks was among the semilandmarks along the posterior edge of the wing.

Semilandmarks as a set can only provide information about the bowing of the curve that they delineate (Zelditch et al. 2004). If the bowing changes, that is, if the curve bends more or less, between groups, then the shape of the configuration has changed in that area. If we look more closely at the way in which the semilandmarks in the current study move, we see that they shift along the curve and not perpendicular to it in either direction. This means that the movement of the semilandmarks is not describing a change in the shape of the curve, a problem arising from the common practice of equidistant spacing of semilandmarks (Gunz et al. 2005). Consider two hypothetical and identical wings shown in Fig. 9. The semilandmarks along the posterior edge are merely arranged differently along the same curve in both wings. This is the kind of movement that was deemed significant using the randomization MANOVA. Although the positions of the semilandmarks changed going from high to low temperature, or from high to low density, the actual shape of the wing did not. Thus, competition and temperature seem to have no effect on actual wing shape, leading to very low classification success. With an error rate from 50 to 75%, this method would not give investigators confidence that larval rearing conditions have been correctly identified.

If all of the analyses were redone excluding the semilandmarks, the results are unlikely to change. The inclusion of semilandmarks does not obscure movement of traditional landmarks (Zelditch et al. 2004). They serve as an additional way to capture shape change separate from landmarks, particularly for morphological areas where landmarks are absent. If there was no movement of traditional landmarks in the presence of semilandmarks, there should be no movement of traditional landmarks excluding semilandmarks.

The low classification success was not improved by the inclusion of centroid size in the analysis. For both procedures where centroid size was included, successful classification rate increased only slightly or even decreased compared with excluding centroid size. Even though the relationship between centroid size and shape is significant, reliable classification is the main goal of this study and accounting for size was ultimately not beneficial.

Although our findings were not useful for discriminating among larval rearing conditions, they are consistent with previous work on effects of environmental variables on insect wing shape. For *Ae. aegypti*, Morales Vargas et al. (2010) found that altering larval temperature produced no significant changes in adult wing shape. In *Drosophila birchii* Dobzhansky & Mather, the latitude at which populations were found also had no effect on wing shape (Griffiths et al. 2005). However, the results from the study are inconsistent with other work on the effect of larval rearing temperature on adult wing shape for both mosquitoes and *Drosophila*. In *Anopheles superpictus* Grassi, differences in wing shape caused by larval temperature were quite large (Aytekin et al. 2009), whereas the differences in our study were only in semilandmark position and not in overall shape. Similarly, variation in larval temperatures has produced significant differences in *Drosophila simulans* Yutaka wing shape (Debat et al. 2003).

Geometric morphometrics has already proven to be a valuable tool for entomological problems. It has been used to investigate physiological changes between different populations of triatomines (Jaramillo et al. 2002, Schachter-Broide et al. 2004) and tsetse flies (Bouyer et al. 2007). This method also is frequently used to solve taxonomic problems, where it can provide support for proposed changes in taxonomic status or call into question previously accepted classifications (Baylac et al. 2003, Mutanen 2005). It also can serve as an inexpensive, reliable, one-step way to identify members of otherwise morphologically similar species (Villemant et al. 2007, Henry et al. 2010).

The results from this experiment show that wing shape by itself is not a useful means of discriminating larval competition and temperature conditions for *Ae. albopictus* or *Ae. aegypti* from Florida, despite finding significant effects of competition and temperature on semilandmark position. However, this approach could be successfully used in future studies under different circumstances. For example, there are many more mosquito species across different genera that can act as vectors for other diseases (Jupp et al. 1981, Rosen

et al. 1985, Turell et al. 2001), and a similar morphometric shape analysis on them might prove productive. Indeed, Aytekin et al. (2009) has shown that differences in wing shape of *An. superpictus* caused by larval temperature are readily detected by morphometric analysis. If larval rearing conditions of this species influence vector competence, then wing shape may be a useful indicator of vector competence in *An. superpictus.* Alternative approaches may involve morphometric analyses on the shape of a mosquito's entire body, not just the wings. Analyzing shape in three dimensions by using geometric morphometrics presents some difficulties (Spencer and Spencer 1995, Bookstein 1996b, Zelditch et al. 2004), but it has been done (Dean et al. 1996, O'Higgins and Jones 1998, Hildebrand et al. 1999). The problem of uninformative semilandmarks also requires a solution, because mosquito wing shape is probably influenced by the proximal, posterior curve of the wing (Fig. 1). Gunz et al. (2005) suggest a statistical solution to the problem of semilandmark placement on curves, and this approach may be useful in future work examining environmental effects on wing shape.

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