Effect of different larval rearing temperatures on the productivity ($R_o$) and morphology of the malaria vector Anopheles superpictus Grassi (Diptera: Culicidae) using geometric morphometrics

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Received 26 May 2008; Accepted 5 March 2009

ABSTRACT: Temperature affects both the biology and morphology of mosquito vectors. Geometric morphometrics is a useful new tool for capturing and analyzing differences in shape and size in many morphological parameters, including wings. We have used this technique for capturing the differences in the wings of the malaria vector Anopheles superpictus, using cohorts reared at six different constant temperatures (15°, 20°, 25°, 27°, 30°, and 35°C) and also searched for potential correlations with the life tables of the species. We studied wing shape in both male and female adults, using 22 landmarks on the wing in relation to ecological parameters, including the development rate. The ecological zero was calculated as 9.93° C and the thermal constant as 296.34 day-degrees. The rearing temperature affects egg, larval, and pupal development and also the total time from egg to adult. As rearing temperatures increased, longevity decreased in both sexes. In An. superpictus, $R_o$ value and productivity correlated with the statistically significant gradual deformations in the wing shape related to size in both sexes. These deformations directly linked to differences in immature rearing temperatures. Analysis using PCA and UPGMA phenograms showed that although wings of females became narrower dorsoventrally as the temperature increased, they became broader in males. Comparisons of the wing landmarks indicated the medial part of the wing was most affected by larval rearing temperatures, showing relatively more deformations. Algorithmic values of the life tables were determined in correlation with the results of geometric morphometrics. Comparisons of centroid sizes in the cohorts showed that overall wing size became smaller in both sexes in response to higher rearing temperatures. Journal of Vector Ecology 34 (1): 32-42. 2009.

Keyword Index: Anopheles superpictus, geometric morphometrics, temperature, life tables, relative warps.

INTRODUCTION

Malaria is one of the most important vector-borne diseases in the world. Anopheles superpictus Grassi, 1899 is a vector of the human malarial, Plasmodium vivax and P. falciparum. This species is widely distributed throughout the southern Palearctic region, especially the Mediterranean Basin (Jetten and Takken 1994, Alten and Çağlar 1998), and although present in Turkey, its direct role in malaria transmission has not been established. Between 1989 and 2003, malaria cases were reported in 108 countries (Anonymous, 2008) and its incidence is affected by a series of factors including the density of Anopheles spp., the distribution and abundance of the parasite, socio-economical instabilities, and climatic conditions (Martens and Hall 2000). In the last decade, global warming scenarios have initiated a number of studies focusing on the effects of increasing ambient temperature on both the morphology and the biology of various species (Tun-Lin et al. 2000, Patz et al. 2000, Carvalho et al. 2002, Epstein 2002, Debat et al. 2003, Bahoy and Lindsay 2003, Mourya et al. 2004, Alten et al. 2007). Temperature is a critical factor in insects, directly affecting the length of the aquatic life span, mortality, and development rates, which can govern phenotypic alterations including the changes in morphology plasticity (Maharaj 2003, Debat et al. 2003).

Geometric morphometrics is a powerful tool for capturing the shape characteristics in several morphological aspects, particularly head, wings, and genitalia (Zelditch et al. 2004). This method involves examining the structures from which Cartesian coordinates can be taken. Readers unfamiliar with the techniques are directed to published studies by Bookstein (1991), Rohlf (1999), and especially to O’Higgins (2000), Zelditch et al. (2004), and Adams et al. (2004), which cover the technical aspects of these methods in detail. Insect wings are very appropriate structures for such studies because their 2-D flattened shape bears several useful landmarks (Grodnitsky 1999, Zelditch et al. 2004). Several authors have employed insect wings, particularly in 2-D morphometrical studies in systematics and phylogeny (Rohlf 1993, Debat et al. 2003, Klingenberg 2003, Gumiel et al. 2003, Aytekin et al. 2007).

In this study, geometric morphometrics was employed to detail subtle changes in wing morphology of An. superpictus in relation to cohorts reared at different constant temperatures as larvae. We correlated larval rearing temperature with biological parameters including thermal constant, survival rate, development time, fecundity, and longevity.
MATERIALS AND METHODS

Maintenance of cohorts

The colonies used in this study were maintained at 27±1° C, 65±5% RH, and a 12:12 (L:D) regime at the Ecological Research Laboratories of Hacettepe University (ESRL). Colony material originated from field material collected from the Magrali village in Sanliurfa Province, southeast Turkey, in June 2001. Larval and adult rearing followed the methods of Kasap and Kasap (1983). Dawn and dusk phases were supplemented with automatically dimmed fluorescent bulbs activated between 06:00-07:00 and 18:00-19:00 h.

Five replicates of 1,000 An. superpictus eggs were transferred into standard 17 x 12.5 x 3 cm cups containing 400 ml distilled water on the day of laying. The cups were placed in separate climate chambers, programmed at six different temperatures (15°, 20°, 25°, 27°, 30°, and 35° C), and exposed to a 12:12 (L:D) photoperiod at a constant relative humidity of 65±5%. Two different control groups were utilized: one in the insectarium at 27° C with relative humidity of 65±5% and 12:12 h light and dark regime (27 L), and the other in a temperature controlled chamber at 27°C (27 D), to control for the possible stress caused by the chambers. Egg hatching rates and incubation periods were recorded daily.

The first 200 1st instar larvae to hatch in each cup were used to continue the experiment. Although larvae were not fed on the first day following hatching, on subsequent days, larvae were fed daily with 0.01-0.04 mg of powdered Tetramin® administered evenly over the water surface of each cup. Development and survival rates of immature stages were monitored daily. Dead larvae or pupae were removed from experimental cups and rearing medium was replaced every day to prevent scum formation.

Fifty of the emerging adults in each temperature cohort (25 females and 25 males) were placed in 20x20x20 cm cloth cages with plastic cups containing distilled water and fish food (0.04 mg) provided for oviposition. Cups were lined with filter paper and replaced daily. Defibrinated rabbit blood was presented via artificial membrane methods for 1 h each day. The days taken for males and females to emerge, and the numbers of females that blood-fed each day were noted. Horizontal life table parameters were calculated from daily records of mortality and fecundity of each of the six temperature cohorts and the two controls. Experiments to determine adult longevity and life table parameters were replicated six times for each temperature. All individuals were used for morphometrical analysis. Calculation of predictive population parameters based on horizontal life tables are summarized in Table 1. To determine the effect of larval rearing temperature on life table parameters in An. superpictus, a consensus tree of the UPGMA phenogram was conducted by SAHN clustering in Ntsys 2.10 (Rohlf 2000).

Geometric morphometrics

The right wings of 105 female and 94 male An. superpictus were mounted on slides in Entellane and carefully numbered according to sex and temperature cohort. Wings were photographed with a Leica MZ-7.5 dissection microscope and a DC-300 digital camera system. All specimens were scored by a single experimenter (S.A.). Photographs were first entered into tps-UTIL1.28 (Rohlf 2007a). Two-dimensional Cartesian coordinates of 22 landmarks from wings (Figure 1) were digitized by tps-DIG1.40 (Rohlf 2007b). All wings were digitized twice in order to reduce the measurement error (ζ) (Arnqvist and Mårtensson 1998), with the second measurements taken after removal of the wing to take the positioning error into account (Arnqvist and Mårtensson 1998, Alibert et al. 2001). To keep the digital errors minimal, no analogous systems were used during the procedure.

The landmark configurations were scaled, translated, and rotated against the consensus configuration by the GLS Procrustes superimposition method (Bookstein 1991, Rohlf, 1993, 1999, Alibert et al. 2001). The coordinates were analyzed using tps-RELV1.34 (Rohlf 2007c) to calculate the eigen values for each principal warp. Consensus configurations and relative warps for each specimens were

![Figure 1. Location of the 22 landmarks on the wing of An. superpictus female.](Image)
Table 1. Definitions and formulas for various life table and demographic parameters (from Southwood and Henderson 2000).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Definition</th>
<th>Formula</th>
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<tbody>
<tr>
<td>x</td>
<td>Age interval in days</td>
<td></td>
</tr>
<tr>
<td>$l_x$</td>
<td>Proportion of females surviving to start of the age interval</td>
<td></td>
</tr>
<tr>
<td>$m_x$</td>
<td>Number of female eggs laid by average female at age $x$</td>
<td>$m_x = E_x \cdot s$</td>
</tr>
<tr>
<td>Oviposition period</td>
<td>Amount of time prior to eggs being laid (days)</td>
<td></td>
</tr>
<tr>
<td>Female longevity</td>
<td>Life-span of female (days)</td>
<td></td>
</tr>
<tr>
<td>Male longevity</td>
<td>Life-span of male (days)</td>
<td></td>
</tr>
<tr>
<td>Net reproductive rate ($R_0$)</td>
<td>Per generation contribution of newborn females to the next generation</td>
<td>$\sum_{x=0}^{\beta} l_x m_x$</td>
</tr>
<tr>
<td>Intrinsic rate of increase ($r_m$)</td>
<td>Rate of natural increase in a closed population</td>
<td>$I = \sum_{x=0}^{\beta} e^{-x} l_x m_x$</td>
</tr>
<tr>
<td>Mean generation time (T)</td>
<td>Time required for a newborn female to replace herself $R_0$ fold</td>
<td>$(\log R_0) / r$</td>
</tr>
<tr>
<td>$b$</td>
<td>Birth rate</td>
<td>$b = \ln (1+\beta)$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$1/\beta = \sum_{x=1}^{\infty} L_x e^{\beta(x-1)}$</td>
</tr>
<tr>
<td>$d$</td>
<td>Death rate</td>
<td>$d = b - r_m$</td>
</tr>
</tbody>
</table>
conducted. The variability in wing shape was assessed using the scores obtained for each individual on the first two relative warps (technically a PCA). The relative warps correspond to the principal components and define a shape space in which individuals are replaced (Alibert et al. 2001). To better visualize the shape variation, we added only the deformations on the positive and negative extremes of the first two PCAs on the graph. A Canonical Variates analyses (CANOV AR) was also conducted on landmark data by IMP CV AGEN6n to compare the groups raised at different temperatures, using group membership information and Barlett’s test examined differences amongst groups. The size morphometry of *An. superpictus* was investigated by using the centroid sizes of the front wings as an estimator with the nonparametric Kruskal-Wallis test (Sokal and Rohlf 1995). Centroid size is the square root of the sum of squared distances of a set of landmarks from their centroid, i.e., the square root of the sum of the variances of the landmarks about that centroid in x- and y- directions (Bookstein 1991).

The coordinates of the landmarks obtained from tps-DIG were manipulated in Morpheus (Slice 1998) to make comparisons among different rearing temperatures by means of spline plots. The results were exaggerated twice for a better visualization. Males were not generally illustrated if they gave exactly the same appearance as the females. We also used the same data to construct an UPGMA phenogram for females, males, and both sexes, using Ntsys 2.1o (Rohlf 2000).

**RESULTS**

**Development and survival of immature stages**

Rearing temperature significantly affected the mean duration and survival of immature stages of *An. superpictus*. Although egg hatching rates were nearly identical at each temperature (15°, 20°, 25°, 27°, 30°, and 35° C), incubation periods significantly differed in each temperature cohort. As the temperature decreased, the period of incubation increased (Kruskal-Wallis test, H=5614.61 DF=6, P<0.0001). Times from larvae to adult were also significantly affected by increasing temperature (Kruskal-Wallis test, H=447.77 DF=115, P<0.0001). Survival rates from the 1st instar to the adult was higher at the optimum temperature (27° C) when compared with the increasing and decreasing temperatures (Table 2). As all the 15° C cohort died before reaching the 3rd instar, no life table was constructed for this temperature regime. To calculate the ecological zero and the thermal constant, the development rate at 35° C was calculated as the shortest adult emergence time (11.82±0.75 days). The highest adult rate calculated as (70%) at 27° C from the egg stage was taken into account (Bar-Zeev 1958) (Table 2). The ecological zero was calculated as 9.93° C and the thermal constant as 296.34 day-degrees.

Table 2. Mean development time (days ± SD), mean development rate (%), and thermal constant of *Anopheles superpictus* reared at six constant temperatures and insectary (27 L).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>15°C</th>
<th>20°C</th>
<th>25°C</th>
<th>27°C L</th>
<th>27°C D</th>
<th>30°C</th>
<th>35°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg hatching rate (%)</td>
<td>89</td>
<td>90.2</td>
<td>87.9</td>
<td>92.5</td>
<td>89.9</td>
<td>88.9</td>
<td>90.1</td>
</tr>
<tr>
<td>Egg incubation period (day) x±SD</td>
<td>6.05±0.25</td>
<td>4.01±0.09</td>
<td>2.21±0.04</td>
<td>2.01±0.09</td>
<td>2.02±0.15</td>
<td>1.4±0.0</td>
<td>1±0.0</td>
</tr>
<tr>
<td>Larvae-pupae development (day) x±SD</td>
<td>21.11±2.44</td>
<td>14.26±1.38</td>
<td>13.22±1.7</td>
<td>13.43±1.04</td>
<td>10.99±1.17</td>
<td>9.81±0.89</td>
<td></td>
</tr>
<tr>
<td>Pupa rate (%) pupae:larvae</td>
<td>70.5</td>
<td>84</td>
<td>74.5</td>
<td>79</td>
<td>44</td>
<td>44</td>
<td>21</td>
</tr>
<tr>
<td>Pupa-adult development (day) x±SD</td>
<td>2.64±2.29</td>
<td>2.02±1.31</td>
<td>1.78±1.14</td>
<td>2.14±1.07</td>
<td>1.34±1.21</td>
<td>1.01±0.75</td>
<td></td>
</tr>
<tr>
<td>Survival rate (%) adult:larvae</td>
<td>57</td>
<td>66.5</td>
<td>65</td>
<td>70</td>
<td>35</td>
<td>35</td>
<td>6</td>
</tr>
<tr>
<td>Larvae-adult development (day) x±SD</td>
<td>23.75±2.29</td>
<td>16.28±1.31</td>
<td>15±1.14</td>
<td>15.36±1.07</td>
<td>12.33±1.21</td>
<td>10.82±0.75</td>
<td></td>
</tr>
<tr>
<td>female/male rate</td>
<td>1</td>
<td>1.03</td>
<td>0.94</td>
<td>1.03</td>
<td>1.06</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Thermal constant (day-degree)</td>
<td>279.44</td>
<td>275.48</td>
<td>290.19</td>
<td>296.34</td>
<td>267.53</td>
<td>296.33</td>
<td></td>
</tr>
</tbody>
</table>

Figure 2. UPGMA conducted by the data obtained from A- Geometric Morphometrics, B- Life-table parameters (euclid distance, SAHN clustering).
Adult longevity and fecundity

Mean fecundity (± SD) and longevity (days ± SD) of *An. superpictus* reared at six constant temperatures were calculated. Age-specific life table values for females and males for each temperature regime is shown in Table 3. The mean longevity for both sexes was clearly lower at higher rearing temperatures. Adults emerging from larvae that were reared in 20°, 30°, and 35° C did not lay eggs. The average number of eggs per female varied from 59.8 at 25° C, to 45.5 at 27° C-D and 47.8 at 27° C-L. The net reproductive rate, *R₀*, was highest for the cohort reared at 25° C. The cohort maintained at 25° C was found to be significantly different (P<0.01) with the cohorts maintained at 27° C both in the insectary and in the chamber producing the fewest progenies. The mean age of reproduction, *Tᵦ*, occurred latest in life for those reared at high temperatures, and earliest in cohorts raised at 27° C. The cohorts that produced offspring earlier in life also produced more female offspring, since *Tᵦ* was negatively correlated with *R₀* among cohorts (*r*=-0.792, 0.01<P<0.05). *Tᵦ* was also correlated with female wing length (*r*= 0.851, *P*<0.01), i.e., temperature cohorts producing larger females also produced their offspring later in life. Surprisingly, no significant differences among the population were found in terms of intrinsic rate of increase (*rₘ*), finite rate of increase (*λ*), birth (b) and death (d) rates, or in female or male life expectancies (*eₓ*) among cohorts raised at different temperatures.

A consensus UPGMA was drawn from the life table data (Table 3) and these parameters showed exactly the same clustering pattern as that obtained with that using the morphometric data (Figure 2B).

Geometric morphometrics

When a PCA was conducted on the 22 wing landmarks of *An. superpictus*, it was shown that the first PC summarized 58% of the total variance in females (n=105) and 48.5% in males (n=94). Main deformations centered on the medial of the wing in both sexes on landmarks 13-19 and 21-22 (Figure 3 females; males are not illustrated). The deformations showed a gradual change when the temperature increased (Figure 4 females; males are not illustrated). No statistically important differences were noted in either sexes between those reared at 27° C in the chambers or the insectaria (P>0.05). The UPGMA phenogram of the examined temperatures showed the same clustering patterns in males and females with 30° and 35° C showing a distinct pattern, whereas the insectary and chamber clustered together with 99.9% similarity.

CANOVAR analysis also showed similar grouping patterns and three statistically significant groups were obtained in females (Figure 5) and males. MANOVA Axis 1 Lambda=0.0024 *X²*=488.6344 df=200 *P*=2.22045e-016, Axis 2 Lambda=0.0407 *X²*=259.4055 df=156 *P*=3.8561e-007, Axis 3 Lambda=0.1556 *X²*=150.7148 df=114 *P*=0.0121058 were calculated for females and MANOVA Axis 1 Lambda=0.0034 *X²*=488.1369 df=200 *P*=2.22045e-016, Axis 2 Lambda=0.0510 *X²*=255.9243 df=156 *P*=7.912e-007, Axis 3 Lambda=0.1530 *X²*=161.4670 df=114 *P*=0.00230805
Figure 3. Distribution of the females of *An. superpictus* along the first two PCs. Principal component analysis of tangent space coordinates derived from GPA of the original coordinates that was conducted for the 22 landmarks digitized from the wings. Horizontal axis, PC1; vertical axis PC2. Numbers indicate each individual. Deformations are shown at the end of each axis. Links used to better visualize the wing.
Figure 4. Thin-plate spline deformation grid showing the difference between mean of the *An. superpictus* wings reared in different constant temperatures (females, magnified x2, vectors are also shown). A- Difference between 20 and 25° C. B- Difference between 20 and 27° C. C- Difference between 20 and 30° C. D- Difference between 20 and 35° C. E- Difference between 25 and 35° C. F- Difference between 30 and 35° C.
Figure 5. Distribution of the females of *An. superpictus* along the first two CVs. Canonical Variates Analysis of tangent space coordinates derived from GPA of the original coordinates that was conducted for the 22 landmarks digitized from the wings. Horizontal axis, CV1; vertical axis CV2. Numbers indicate each individual.

in males. A consensus tree for both sexes was constructed (Figure 2A). When centroid sizes were compared, wing size decreased significantly as the temperature increased in females (ANOVA, $F_{(5, 99)} = 127.35, P < 0.001$) (Figure 6A) and males (ANOVA, $F_{(5, 104)} = 230.73, p < 0.001$) (Figure 6B).

**DISCUSSION**

Shape and size variation generally correlates with the biology of many species. This is especially important in understanding ecological relationships. It is well known that when mosquito larvae are raised in an optimum crowded environment and feeding optimally, adult body size is directly related to rearing temperatures. As histogenesis/metabolic rate is a limiting factor, larvae reared at lower temperatures gave rise to larger adults, whereas high temperatures produced relatively smaller mature forms. This situation has been observed both in wild and laboratory populations (Clements 1963).

In identifying and quantifying biodiversity through the computation of morphospecies for disparity estimates, geometric morphometrics appears powerful enough to assess morphological variation at all taxonomic levels, including the intraspecific, subspecific, and clinistic levels (Alibert et al. 2001). The tool is also very useful for estimating the effects of different ecological conditions in both wild (Rohlf 1993) and laboratory populations (Debat et al. 2003, Griffiths et al. 2004). Like size, shape can be measured in many different ways (Zollikofer and Ponce de Leon 2005), and landmark coordinates allow a detailed examination of many minor changes that are more difficult to consider using other types of morphometric data (Slice 2005).

The shape and size variations revealed in *An. superpictus* populations reared in six different fixed temperatures (15, 20, 25, 27, 30, and 35° C) support changes in life table characteristics. The same clustering patterns obtained from geometric morphometric results both in females and males correlated with those obtained from the life table data. Although no effects of size on the shape variation were noted, we observed that rearing at high temperatures causes smaller individuals and smaller wings. Similar results were noted in *An. quadrimaculatus* (Lanciani 1992) and in *Ae. punctor* (Packer and Corbet 1989). In *Drosophila birchii* and *D. mercatatum*, Griffiths et al. (2004) noted temperature-related changes in wing size, wing shape deformations, and impacts on development rate as shown in this study.

Researchers new to geometric morphometrics are often confused by unfamiliar terminology. Relative warps, used in this study, are linear combinations of the partial warps and affine components computed to decompose total shape variability into uncorrelated, variance-maximizing variables. In more familiar terms, the relative warps are the...
Figure 6. Centroid sizes of each group reared in different constant temperatures in *An. superpictus*. A- Females  B- Males.
principal components of sample variability in shape space with respect to the partial warp and affine scores (Slice 2005). There is currently only one previous study that shows the shape deformations on the relative warps of mosquito wings (Rohlf 1993) that focuses on interspecific differences. Most entomological studies using geometric morphometrics are generally on fixed shape changes across a taxonomic gradient. Even in fossil Hymenoptera, it is possible to define useful key characters in wing shape deformations (Aytekin et al. 2007), thus proving these characters are evolutionarily fixed. This makes the high level of deformations detected due to rearing temperatures in one generation of An. superpictus so surprising. This could be related to several ecological, morphological, and physiological factors specific to Diptera in general. Rapid deformations in wings would surely affect the rate and distance of insect dispersion and would exert pressure on energy required for flying. This could then impact other ecological parameters such as the success of finding mates, the kinematics of flying, dispersal, and pressure from predators, etc.

All the size changes and deformations in the wing shape of An. superpictus males and females are correlated with the biology of the species. Considering the life table characteristics (Table 2), we conclude that immatures at rearing temperatures of 25 to 27°C are optimal for An. superpictus. No differences in mean wing centroid sizes, changes in the life table parameters, nor deformations in shape variations were noted between those reared in the insectarium (27-L) or in the climate chambers (27-D) at 27°C. Estimates of development times and survival rates calculated in this study are comparable with other published data (Bar-Zeev 1958, Bahoy and Lindsay 2003, Maharaj 2003). Among the examined temperatures, no adult emergence was observed at 15°C while the fastest development occurred at 35°C. We also found that at 27°C, An. superpictus completes its development in an average of 17.36±1.07 days. The ecological zero is calculated from egg to adult as 9.93°C.

Temperature, altitude, humidity, rains, floods, wind, and other climatic factors can effect malaria infections (Patz et al. 2000). One of the possible implications of the global warming scenario is the change in the dispersion of the vector species (Lindsay and Birley 1996, Patz et al. 2000, Epstein 2002). Temperature may also affect the vectorial capacity and changes in the population densities of the species (Olejnicek and Gelbic 2000). From this study, we conclude that temperature is a crucial factor in the evolution and ecology of An. superpictus, and because of this, increased temperatures could cause some possibly unexpected effects on the dispersal of malaria in Turkey and across the species range in the future.

Acknowledgments

We thank Dr. Yvonne Linton for her very valuable comments. This study is a part of an MSc thesis submitted to Hacettepe University.

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