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Original article (Orijinal araştırma)

Size and shape variations in wing morphology of *Anopheles maculipennis* s.s. Meigen, 1818 (Diptera: Culicidae) from northeastern Turkey

Türkiye'nin Kuzeydoğu Anadolu Bölgesi'ndeki *Anopheles maculipennis* s.s. Meigen, 1818 (Diptera: Culicidae) türünün kanat morfolojisindeki büyüklük ve şekil farklılıkları

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Abstract

Anopheles maculipennis Meigen, 1818 (Diptera: Culicidae) complex was discovered as the first sibling species complex among mosquito species and identified as a highly important malaria vector in the Middle East and Europe. *Anopheles maculipennis* s.s. is the nominotypical member species of the complex, and widely spread across the whole of Europe. Body size and shape are the most important characters of organisms and is related to numerous variables. Biological size and shape may be affected by altitude and altitudinal differences. In this study, the variation in wing size and shape of *An. maculipennis* s.s. populations collected from four sampling stations in Iğdır Province (Mürşitali, Sürmeli, Yukarıçıyırklı and Zülfikarköy) and two sampling stations in Kars Province (Kötek and Kozlu) at different altitudes and in different habitats from northeastern Turkey in 2019 were investigated. It was assumed that altitude and the environmental differences related with altitude may affect the wing (body) size or shape of *An. maculipennis* s.s. This is the first comparative geometric morphometric study of *An. maculipennis* s.s. populations in Turkey and the results indicate size and shape differences among some populations. While centroid size did not show a linear association with altitude, samples from the highest altitude population had larger wings than the other populations.

Keywords: *Anopheles maculipennis* complex, geometric morphometric, malaria, plasticity

Öz

Anopheles maculipennis kompleksi sivrisinek türleri arasında ilk ikiz tür kompleksi olarak keşfedilmiş ve Orta Doğu ve Avrupa'da çok önemli sıtma vektörü olarak tanımlanmıştır. *Anopheles maculipennis* s.s. Meigen, 1818 (Diptera: Culicidae) kompleksin nominotipik üye türü olarak bilinmektedir ve tüm Avrupa'da yaygın bir şekilde bulunmaktadır. Vücut büyüklüğü ve şekli organizmaların en önemli özelliklerindendir ve çok sayıda değişkenle ilişkilidirler. Vücut büyüklüğü ve şekli yükseklikten ve yüksekliğe bağlı farklılıklardan etkilenebilir. Bu çalışmada Türkiye'nin Kuzeydoğu Anadolu Bölgesi'nde farklı yükseklik ve habitatlarda bulunan ve 4 tanesi Iğdır İli (Mürşitali, Zülfikarköy, Sürmeli, Yukarıçıyırklı) ve 2 tanesi ise Kars iline (Kötek ve Kozlu) ait farklı örnekleme istasyonlarından 2019 yılında toplanan *An. maculipennis* s.s. popülasyonlarının kanat büyüklük ve şekil varyasyonları araştırılmıştır. Yükseklik ve yüksekliğe bağlı çevresel farklılıkların *An. maculipennis* s.s.'in kanat (vücut) büyüklüğü veya şeklini etkileyebileceği varsayılmıştır. Bu çalışma ile Türkiye'de bulunan *An. maculipennis* s.s. türleri geometrik morfometri yöntemi ile ilk kez değerlendirilmiştir ve sonuçlar bazı popülasyonlar arasında bazı büyüklük ve şekil farklılıklarına işaret etmektedir. Geometrik merkez her ne kadar yükseklikle doğrusal bir ilişki göstermese de en yüksek rakımdan toplanan popülasyon diğer popülasyonlara göre göreceli olarak daha büyük kanatlara sahiptir.

Anahtar sözcükler: *Anopheles maculipennis* kompleks, geometrik morfometri, malarya, plastisite

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Introduction

Malaria is still a particularly serious health problem in the modern world, and one of the major reasons of death of all infectious diseases. It has been endemic in Turkey for years. In recent years, while endemic malaria cases were observed in the southeastern part of Turkey, only imported cases have been reported since 2012. The case counts remained under 150 per year prior to 2012, when a peak was observed with 376 cases associated with the Syrian refugee influx; imported cases have since remained over 200 per year between 2012-2017 (WHO, 2018; Ergönül et al., 2020). Given the persistence of malaria vectors in south and southeastern part of Turkey, there will be always a threat for malaria for Turkey.

Among mosquito species, *An. maculipennis* complex was discovered as the first sibling species complex and was previously identified as a highly important malaria vector in the Middle East and Europe (Falleroni, 1926; van Thiel, 1927). *Anopheles maculipennis* complex has eleven species in the Palearctic region. Of these eleven members, four species have been recorded in Turkey: *An. maculipennis* s.s. Meigen, 1818 (Diptera: Culicidae), *Anopheles messeae* Falleroni, 1926 (Diptera: Culicidae), *Anopheles melanoon* Hackett, 1934 (Diptera: Culicidae) and *Anopheles sacharovi* Favre, 1903 (Diptera: Culicidae), (Parrish, 1959; Postiglione et al., 1973; Kasap & Kasap, 1983; Ramsdale et al., 2001; Simsek et al., 2011). *Anopheles maculipennis* s.s. is the nominotypical member species of the complex, and widely spread across the whole of Europe, except the most northerly regions (Ramsdale & Snow, 2000). However, a recent study showed the expansion of this species to northeastern Europe and northwestern Asia (Novikov & Vaulin, 2014). This species is thought to be the only species of the complex occurring at altitudes of 1000 m and above, and often has been sampled in small open water bodies in cultivated land (Becker et al., 2010). Even if plasmodial sporozoites have been found in the salivary glands of this species (Barber & Rice, 1935), the feeding preference on domestic animals over humans make this species a moderate malaria vector (Jetten & Takken, 1994).

Variations between size and shape are particularly useful for understanding of ecological relationships because of the correlation with many biological features of different species. Insect wings show ecological responses to environmental changes such as climate and altitude (Morales et al., 2010; Stephens & Juliano, 2012; Gómez et al., 2014; Phanitchat et al., 2019; Prudhomme et al., 2019). Dispersal capacity for blood seeking, maneuverability, and transmission of *Plasmodium* sporozoites into the host are particularly important features for malaria for infected anophelines and directly associated with flight performance (Dudley, 2000). Some studies have shown a positive correlation between size and vector competence with the support of much more parasites in larger mosquitoes (Pulkkinen & Ebert, 2004; Moller-Jacobs et al., 2014). Previous studies indicated wing size and wing shape differences among *Aedes vexans* (Meigen, 1830) (Diptera: Culicidae), (Kuclu et al., 2011) and *Culex theileri* Theobald, 1903 (Diptera: Culicidae) (Demirci et al., 2012) populations.

Geometric morphometry is a powerful tool for assessing shape and size variation and the correlations between them (Dujardin, 2011). Wing size is often used as an alternative index of body size (Cowley & Atchley, 1990), and their flattened two-dimensional structure is particularly useful for landmark-based shape/size studies (Grodnitsky, 1999; Zelditch et al., 2004).

This study investigated the variation in wing size and shape of *An*. *maculipennis* s.s. populations in different altitudes and habitats from northeastern Turkey. The hypothesis was that altitude and the environmental differences related with altitude may have influence on the wing (body) size or shape of *An*. *maculipennis* s.s. More detailed information on morphological changes can improve understanding of the epidemiological patterns of medically important mosquito vectors (Dujardin, 2011).

Materials and Methods

Study sites and mosquito collection

Field studies were performed from June to September 2019 in northeastern Turkey, along habitat-climate-elevation gradients ranging from plain habitats to mid-range montane areas (848-1780 m) (Figure 1). Collection sites and coordinates information were given in Table 1.

Figure 1. Mosquito sampling sites (mapped using www.earth.google.com).

Table 1. *Anopheles maculipennis* s.s. collection site and number of specimens examined

Locality	Abbreviation	Habitat type	Altitude (m)	Latitude (N)	Longitude (E)	Specimens
Mürşitali	MA	Plain	848	40°01'15"	44°08'10"	35
Zülfikarköy	ZÜ	Plain	860	39°59'24"	44°08'44"	37
Sürmeli	SÜ	Plain-Low montane	944	40°03'56"	43°47'11"	19
Yukarıçıyrıklı	YÇ	Low montane	1,031	40°06'46"	43°34'57"	33
Kötek	KÖ	Low montane	1,350	40°13'06"	43°01'06"	23
Kozlu	KO	Mid-range montane	1,780	40°11'11"	42°56'59"	18

Active adults were collected with New Jersey light traps run between 1700 and 0700 hours at each collection site for one night per month. Additionally, two experimental collectors were collected indoor resting mosquitoes. Samples were stored at −20°C until analyzed.

Molecular identification of *An. maculipennis* s.s.

Schaffner et al. (2001) descriptions and keys were used first to identify *An. maculipennis* complex by morphology. Multiplex polymerase chain reaction (PCR) was then used for identification of complex members (Proft et al., 1999). Sequences from the isolates belong to some *An. maculipennis* s.s. species were deposited in the NCBI GenBank database under accession numbers MZ666139-48.

Wing preparation and data acquisition

The wings of specimens were separated from the thorax and fixed on labeled slides with Entellan (Merck KgaA, Darmstadt, Germany). To avoid errors in the analyses of within-individual correlation, only the left side was used. A stereoscopic zoom dissection microscope was used to photograph and digitize the wings. A single experimenter scored all the wings to avoid inter-rater bias, and to evaluate repeatability of the measurements. Procrustes ANOVA was performed with MorphoJ software (Klingenberg, 2011) on individuals for 19 landmarks, which were scored two times for each specimen.

The tpsUtil (Rohlf, 2019a) was used to build a data file (tps) from wing images. Using TpsDig (Rohlf, 2018), 19 landmarks were digitized and analyzed (Figure 2). The position of these landmarks was chosen based on the clarity of the intersection of the wing lines to prevent visual error. The generalized least squares Procrustes superimposition method was used to scaling, translation and rotation of the landmark configurations against the consensus configuration (Bookstein, 2007). Tps-Relw (Rohlf, 2019b) was used to analyze the coordinates and compute the eigenvalues for each principal warp. The consensus configurations per wing for each female mosquito were subjected to relative warps analysis by defining the variability in the shape space using the scores acquired for each individual landmark; this is technically a principal component analysis (PCA). The relative warps correspond to the principal components and define a shape space in which individual landmarks are replaced.

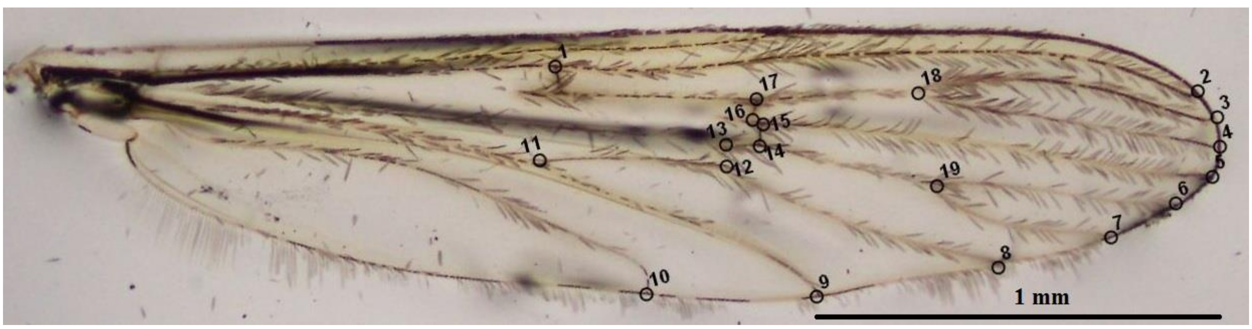

Figure 2. Positions of landmarks on the wing of *Anopheles maculipennis* s.s.

MorphoJ was used for canonical variates analysis to compare the different altitude populations using group membership information via landmark data. Squared Mahalanobis distances were provided from the computed CVA to measure the intraspecific phenetic correlations. Pair-Mahalanobis distances between populations were used for a non-parametric permutation (10,000 runs) after a Bonferroni correction test to analyze the statistical significance of wing shape differences. The matrix was used to produce a dendrogram using the unweighted pair-group method with arithmetic average (UPGMA).

The centroid sizes of the wings were used as an estimator of the body size of *An. maculipennis* s.s via a Mann-Whitney U-test, performed in Past 3.10. Centroid size was defined as the square root of the sum of the squared distances between the center of the configuration of all landmarks and each individual landmark (Bookstein, 2007). Differences in average wing CS between each location were analyzed with a non-parametric permutation (10,000 runs) and a Bonferroni correction test. A linear regression between size and altitude were tested for each altitude. Additionally, the contribution of size to wing shape (residual allometry) was estimated by multivariate regression of the shape and log-transformed centroid size variation based on the Procrustes coordinates with 10,000 permutations in MorphoJ.

Results

For the geometric morphometrics analyses, 165 female mosquito specimens were measured (Table 1). The PCR results belonging to the *An*. *maculipennis* s.s. are given in Figure 3. The results of the Procrustes ANOVA testing individual variation relative to measurement error were summarized in Table 2. Individual differences for size and shape calculated for significantly more variance than error (P < 0.0001).

Table 2. Procrustes ANOVA for *Anopheles maculipennis* s.s. used to calculate the measurement errors

Data	Effect	SS	MS	df	F	P
Centroid size	Individual	934	934	1	20.9	<0.0001
	Residual	14656	44.7	328		
Shape	Individual	0.0152	0.000449	34	15.8	<0.0001
	Residual	0.317	0.0000284	11252		

SS, sum of squares; MS, mean square; df, degrees of freedom; F, F statistic; P, probability.

Size variation

The size differences were significant between some populations (Table 3). While centroid size did not show a linear association with altitude, samples from Kozlu, located at the highest altitude, had larger wings than other populations (Figure 3). The relationship of overall shape variation with size was 1.04% and not significant (P = 0.78).

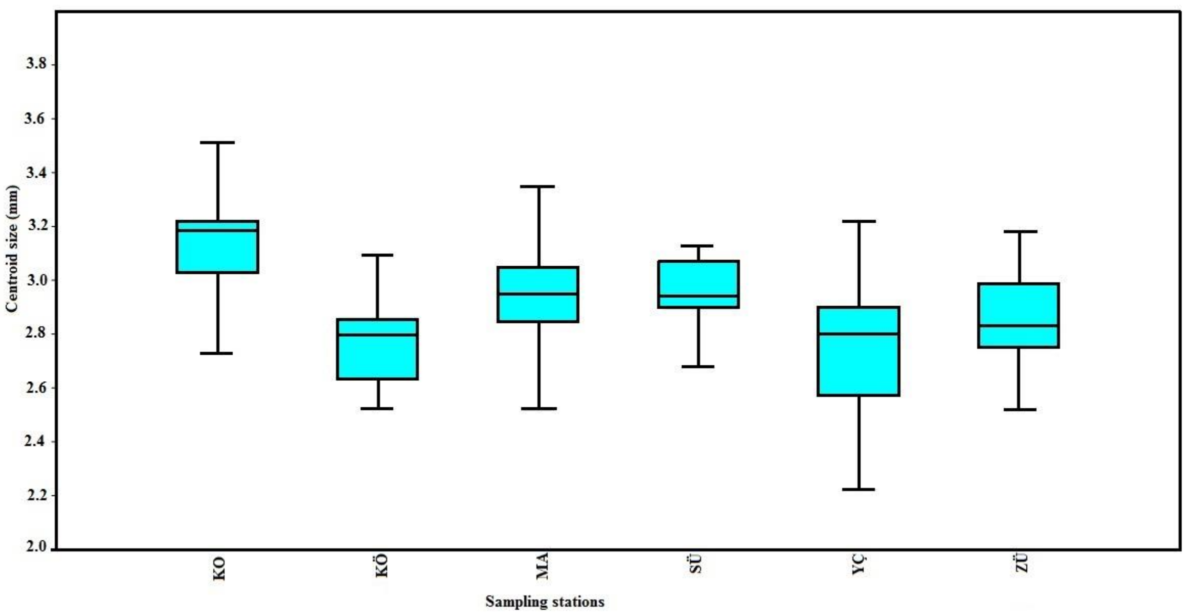

Figure 3. Centroid sizes of each *Anopheles maculipennis* s.s. population from different altitudes: Zülfikarköy (ZÜ) 848 m, Mürşitali (MA) 860 m, Sürmeli (SRM) 944 m, Yukarıçıyrıklı (YÇ) 1,031 m, Kötek (KÖ) 1,350 m and Kozlu (KO) 1,780 m.

Table 3. Statistical significance (P < 0.05) for size (upper half) and shape (lower half). Results in bold denote statistical significance after a Bonferroni correction test

Population	KO	KÖ	MA	SÜ	YÇ	ZÜ
KO	-	**<0.001**	**0.105**	**0.013**	**<0.001**	**<0.001**
KÖ	0.591	-	**0.001**	**0.036**	1.000	1.000
MA	0.360	**0.045**	-	1.000	**0.005**	0.212
SÜ	**0.001**	**0.036**	0.777	-	**0.033**	0.671
YÇ	**0.001**	1.000	0.364	1.000	-	1.000
ZÜ	**0.001**	**0.006**	0.415	**0.001**	**0.001**	-

Shape variation

When the PCA was performed on the 19 landmarks, the first three discriminant factors explained 20.2, 14.4, and 10.1% of the total variance. Canonical variables 1 and 2, which explained 32.3 and 30.9%, respectively, indicating the distinction between populations. The distributions of the individuals along the first two canonical variables are shown in Figure 4. The wing shape differences were supported by permutation tests (10,000 rounds of permutation) of Mahalobonis distances for most of the populations (Table 3). When the shape differences between populations were analyzed by UPGM provided from Mahalobonis distances: Sürmeli, Yukarıçıyırklı and Zülfikarköy populations comprised the first group and Kötek, Mürşitali, and Kozlu populations the second group (Figure 5).

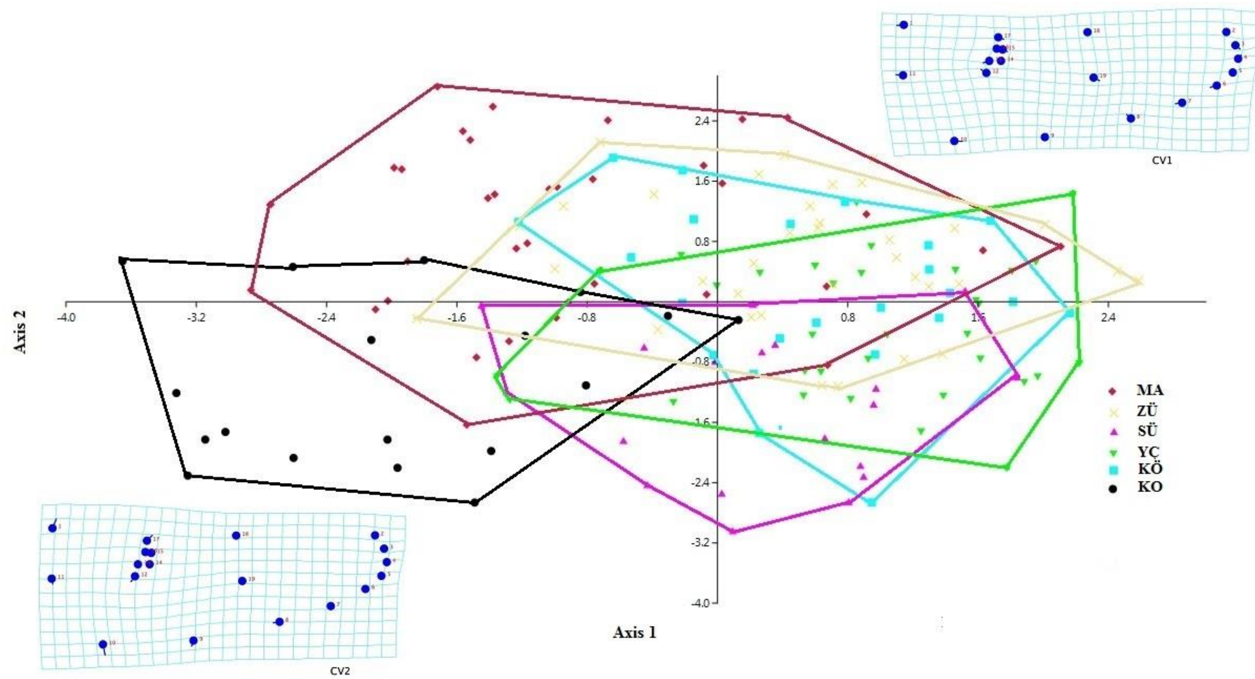

Figure 4. Distribution of the female *Anopheles maculipennis* s.s. along the first two canonical variables.

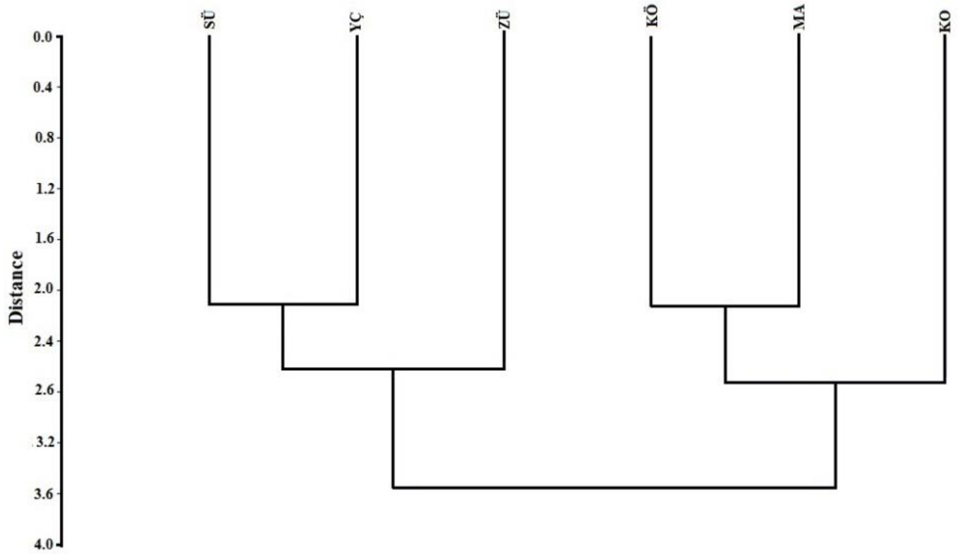

Figure 5. UPGMA conducted by the data derived from Mahalanobis distances.

Discussion

This is the first comparative geometric morphometric study of *An*. *maculipennis* s.s. populations in Turkey or elsewhere. Although the results indicate different levels of wing size/shape differences between populations, it may not be possible to draw any conclusions concerning the effect of month on mosquito phenotypes. Environmental and climatic features could change depending on the month. Also, sampling size may be limiting for discriminant analyses. Additional studies should be considered with larger sample sizes according to month and altitude.

Although the results indicate different levels of wing size/shape differences between populations, there was not an obvious difference between populations due to altitude. Similar to the present results, *Ae. vexans* populations from northeastern Anatolia (Kuclu et al., 2011), *An*. *sacharovi* populations from southeast Anatolia (Yurttas & Alten, 2006), and *Phlebotomus papatasi* (Scopoli 1786) (Diptera: Psychodidae), populations from Şanlıurfa Province of Turkey (Belen et al., 2004) did not show a clear difference related to altitude. Nevertheless, *C. theileri* populations collected from nine altitudes from 808 to 2,130 m in northeastern Anatolia appeared to have a clear phenotypic difference related to altitude (Demirci et al., 2012). However, while centroid size did not show a linear association with altitude in the present study, samples from Kozlu, located at the highest altitude, had larger wings than other populations.

Several species show geographical clines in body size with larger individuals presenting in higher latitudes/altitudes and this adaptation pattern was first explained by Bergmann (1847), with the explanation that small surface area to volume ratios may be more suitable for not losing heat in cold areas (Blanckenhorn & Demont, 2004). Previous studies conducted with *Anopheles gambiae* Giles 1902 (Diptera: Culicidae) revealed a positive correlation between mosquito body size and malaria transmission due to the fact that larger females have a longer lifespan, feed more often and use the blood meal more effectively compared to smaller individuals (Takken et al., 1998; Manoukis et al., 2006; Aboagye-Antwi & Tripet, 2010; Christiansen-Jucht et al., 2014). It might be expected that the larger mosquitoes from Kozlu (1,620 m) populations might be more effective malaria vectors than the other populations. Nevertheless, there are numerous other regional factors known to affect the vector and pathogen transmission capacity of mosquito populations (Cohuet et al., 2010).

However, other studies conducted with *Aedes albopictus* (Skuse 1895) (Diptera: Culicidae), and *Aedes aegypti* (Linnaeus 1762) (Diptera: Culicidae), (Noden et al., 2016; Reiskind & Lounibos, 2009) did not show a correlation between body size and longevity, and studies conducted with *Anopheles coluzzii* Coetzee & Wilkerson, 2013 (Diptera: Culicidae), (Vantaux et al., 2016) and *Ae*. *aegypti* (Zeller & Koella, 2016) showed a negative correlation between body size and longevity.

The size of adult mosquitoes is primarily associated with temperature (Bar-Zeev, 1958; Lyimo et al., 1992; Zeller & Koella, 2016) and larval diet, which are associated with various factors such as food availability, larval habitat quality, larval density and competition (Nasci & Mitchell, 1994; Renshaw et al., 1994; Gimnig et al., 2002; Strickman & Kittayapong, 2003; Schneider et al., 2007; Moller-Jacobs et al., 2014; Shapiro et al., 2016; Vantaux et al., 2016). Larval development conditions might thus be an explanation for larger size. Unfortunately, there is no data on larval habitats, so the relationship between larval habitats and size changes is unknown.

In this study, although mean wing shape comparisons revealed significant differences between populations such as the Sürmeli, Yukarıçıyrıklı, and Zülfikarköy populations comprised the first group, Kötek, Mürşitali, and Kozlu populations comprised the second group, a clear-site/altitude specific population differentiation is not evident in this species. Various factors such as genetic, biological, ecological, environmental and physiological influences may be associated with shape differences between populations, similar to size differences. Although geographical clines associated with latitude or temperature were described in many animal species for size-related features, the relations between shape and environment are still not particularly clear (James et al., 1997; Huey et al., 2000). There is conditional confirmation suggesting that developmental and evolutionary temperature-connected cell size variation have opposite effects on wing shape in *Drosophila subobscura* Collin, 1936 (Diptera: Drosophilidae) (Calboli et al., 2003). Variations in wing shape could have relation with flight performance and be an indicator for an adaptation related to flight dynamics. A previous study conducted between migrant and non-migrant populations in dragonflies showed important wing shape modifications (Johansson et al., 2009). A previous morphometrics study revealed that thinner wings could be more adaptive for *Aedes albifasciatus* (Macquart, 1838) (Diptera: Culicidae) populations to avoid disturbance in a windy environment (Garzón & Schweigmann, 2018). Other studies conducted with *Ae*. *aegypti* populations in multiple geographical locations (coastal, residential and cultivated areas) of Samut Songkhram, Thailand (Chaiphongpachara & Laojun, 2020) and with *Anopheles darlingi* Root, 1926 (Diptera: Culicidae) in five major Brazilian ecoregions (Motoki et al., 2012) revealed differences in wing shapes between all geographical areas and ecoregions due to environmental factors such as wind current and weather. A previous study conducted with *Ae*. *albopictus* populations from Black Sea and Aegean coastal populations in Turkey indicated some shape and size differences between some populations and also a high-level significant difference was found between Aegean and Black Sea coastal populations in wing shapes (Demirci et al., 2021). A study conducted on sandflies comparing island and mainland locations in Thailand showed differences between populations with greater morphological variations in island populations (Sumruayphol et al., 2017). Contrasting ecosystems results variation in mosquito populations (Vicente et al., 2011; Motoki et al., 2012). Studies have also indicated that vector competence differences, such as transmission and infection rates, may exist in different geographical locations (Bennett et al., 2002; Goddard et al., 2002).

It is known that the wing traits are not only affected by environmental conditions but also genetics and changes at the molecular level (Fusco & Minelli, 2010). Ayala et al. (2011) showed the effect of environmental conditions and chromosome polymorphisms on phenotypic variation of a very important African malaria vector, *Anopheles funestus* Giles, 1900 (Diptera: Culicidae) in different eco-climatic regions of Cameroon.

In conclusion, these results highlight the adaptability and plasticity of this mosquito species even over short distances, however, the reasons for observed shape and size variations in wing morphology need more exploration. The investigation of phenotypic and genetic differences in *An. maculipennis* s.s. populations could shed light on malaria transmission dynamics. It is necessary to conduct further studies to determine the drivers of these variations and if they have a genetic basis or a phenotypic reflection in different environments.

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