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## Chromosomal and environmental determinants of morphometric variation in natural populations of the malaria vector *Anopheles funestus* in Cameroon

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### Abstract

*Anopheles funestus* is one of the most proficient malaria vectors in the world, mainly because of its remarkable ability to populate a wide range of ecological settings across Africa. Its formidable environmental plasticity has been primarily associated to high amounts of genetic and inversion polymorphisms. However, very little is known about the morphological changes that this ecological adaptation entails. Here, we report on wing morphometric variations in karyotyped specimens of this species collected throughout a wide range of eco-geographical conditions in Cameroon (Central Africa). Our results revealed strong selection on mosquito wing traits. Variation of wing size was dependent on temperature and elevation ( $p < 0.001$ ), while wing shape did not exhibit a specific environmental pattern. On the other hand, we observed a significant correlation of wing shape variation ( $p < 0.001$ ), but not size ( $p > 0.05$ ), with regard to karyotype. This pattern was maintained across different environmental conditions. In conclusion, our findings cast strong evidence that change in morphometric traits are under natural selection and contribute to local adaptation in *Anopheles funestus* populations. Furthermore, the robust relation between chromosome polymorphisms and wing shape suggests new evolutionary hypotheses about the effect of chromosomal inversions on phenotypic variation in this malaria vector.

### Keywords

*Anopheles funestus*; morphometric variation; environmental adaptation; chromosomal inversions; Cameroon

### 1. Introduction

One of the major factors which establish the ability of an insect to become an important vector for human diseases is its “anthropophilic” behavior, e.g. its preference for feeding human blood and/or exploiting man-made resting or breeding habitats. In this sense, medically important insects, such as malaria vector mosquitoes, coevolve with to control pressures and habitat changes due to their close contact with human populations, revealing frequent genotypic and phenotypic variations (Dujardin, 2008). In the field, these variations can be based on either genetic divergence, direct environmental effects, or both. Thus, studies on phenotypic variation can provide relevant insights into the evolution of vector

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systems and help detect local populations with potentially important characters, which might affect disease transmission (Dujardin, 2008; Pigliucci, 2005). However, despite their importance, little is known about phenotypic variation and morphological plasticity in *Anopheles* species across the different habitats that they populate. This is an important limitation for studies aimed at exploring ecological adaptation in malaria vectors with direct consequences on disease epidemiology.

Morphometric traits have been employed to analyze adaptive variation in natural populations of animals and plants (Mayr, 1942). Patterns of morphological variation involving size or shape dimensions have been often interpreted with regard to their evolutionary importance (Pigliucci, 2005). In this sense, insect wings have been reported as an excellent model for studying morphological evolution in natural populations. Wing size is directly related to body size (Sokoloff, 1966) and there exists considerable evidence that size and shape are targets of natural selection (Soto et al., 2006). Moreover, they respond to environmental variation in complex ways, suggesting that the reaction norm may be part of an adaptive response (Carreira et al., 2006; Weber, 1990). Consequently, investigations on morphological traits variation necessarily require to involve the simultaneous analysis of genetic and environmental factors, which somehow cause intra-specific variation and interspecific divergence (Mackay, 2004).

Insect chromosomal polymorphism has been frequently associated with environmental adaptation (Coluzzi et al., 1979; Hoffmann et al., 2004; Krimbas and Powell, 1992; Krimbas, 1967). Natural populations of *Drosophila* and *Anopheles* species have recurrently shown clinal variation in some paracentric chromosomal inversions along latitudinal or altitudinal gradients (Balanya et al., 2003; Collinge et al., 2006; Hoffmann et al., 2004; Simard et al., 2009). Numerous chromosomal rearrangements have been linked to effects on *Drosophila* morphometric traits, establishing additional variation on which selection may be acting (Colombo et al., 2004; Colombo et al., 2001; Orengo and Prevosti, 2002; Santos et al., 2004). Hence, the observations of adaptive environmental clines where chromosomal polymorphisms and morphometric traits running in parallel suggest that both might be related and subject to similar evolutionary forces (Orengo and Prevosti, 2002). In Africa, malaria transmission is primordially ensured by three anopheline species, *Anopheles gambiae*, *An. funestus* and *An. arabiensis*, which are widely distributed across sub-Saharan Africa. The ability of those malaria vectors to thrive the wide range of habitats present in Africa has been associated to the richness of chromosomal polymorphisms (Ayala et al., 2010; Coluzzi et al., 2002; Pombi et al., 2008). However, to date, no study has examined how this ecological plasticity has modeled their phenotypic traits, contributing to increasing their local population fitness.

The purpose of this study was to investigate the association of chromosomal polymorphism and environmental conditions in phenotypic variation (wing size and shape) of *An. funestus* in Cameroon, Central Africa. In this sense, natural populations of adult females *An. funestus* were sampled across nine distinct eco-geographical zones displaying large variation in environmental conditions. First, we compared patterns of wing size and shape among ecological zones. Second, we related wing traits to environmental variables and we elucidated the contribution of each variable in morphometric variation. Finally, we studied the effect of chromosomal inversion polymorphisms and wing morphology. Our findings revealed significant effect of local environmental conditions on wing morphology. Chromosomal polymorphism was associated to wing shape variation across populations. These outcomes are discussed in a context of environmental adaptations and their impact on malaria epidemiology and vector control strategies.

## 2. Materials and Methods

### 2.1 Study sites and mosquito sampling

Wing morphometric trait variations in *An. funestus* were compared between nine locations in five different bioclimatic domains across Cameroon (Figure 1). Latitudinal survey covered most bioclimatic domains present in that country, from the northern arid savannas to the evergreen rainforest in the south (Olivry, 1986). To reduce possible local effects and increase the number of specimens, mosquitoes were collected from 2-7 villages in each zone (average distance between villages 7.83 km). Adult females *An. funestus* were captured by day-time spraying aerosols of pyrethroid insecticides inside human dwellings (Service, 1993). Anopheline mosquitoes were identified using morphological identification keys (Gillies and de Meillon, 1968). Ovaries from half-gravid *An. funestus* females were dissected and stored in Carnoy fixative solution (3 parts of 100% ethanol: 1 part glacial acetic acid by volume) for subsequent cytogenetic analysis. Carcasses were stored individually in labeled tubes containing a desiccant and kept at -20°C.

### 2.2 Mosquito PCR identification and karyotyping

Genomic DNA was extracted from the body of adult mosquito females using the protocol described in Morlais et al. (2004). DNA was then resuspended in sterile water in individual tubes. Morphological identification of *An. funestus* s.s. (hereafter *An. funestus*) was confirmed by molecular identification (Cohuet et al., 2003; Koekemoer et al., 2002). Polytene chromosomes obtained from the ovaries of half-gravid females *An. funestus*, were squashed and stained according to standard protocols (della Torre, 1997). The preparations were examined under a phase-contrast microscope, and paracentric chromosomal inversions were scored according to the *An. funestus* cytological map (Guelbeogo et al., 2005; Sharakhov et al., 2004).

### 2.3 Environmental data

A set of seven eco-geographical variables (EGVs) was used to describe the average environmental conditions in each zone (source: *LocClim* database developed by the Food Agriculture Organization – FAO, [http://www.fao.org/sd/2002/EN1203a\\_en.htm](http://www.fao.org/sd/2002/EN1203a_en.htm)): elevation (in m), rainfall (in mm), temperature (in °C), evapotranspiration (in mm), relative humidity (water vapor pressure in %), mean number of hours of sunlight per day (hours), and wind speed (in m.s<sup>-1</sup>). Climate data are yearly means, averaged over the past 30 years, obtained from interpolation of field stations data. Computational operations linked to geo-analysis requirements were performed by using the software ArcGIS 8.3 (<http://www.esri.com/software/arcgis/index.html>).

### 2.4 Morphometric Analysis

**2.4.1 Sample processing**—A digital image of each mosquito female wing, left and right (dorsal view) was taken through a binocular microscope (Leica MZ6). Both wings were removed and mounted on microscope slides under cover slips. Morphometric measurements were taken from both wings of each female, except when only one undamaged wing was available. Twelve morphometric measurements, as recommended by Bookstein (1991), were scored from the digital images of each wing (Figure 2) using the free software COOw (<http://www.mpl.ird.fr/morphometrics/>). All measurements were taken by the same person for more consistency (Bookstein, 1991).

**2.4.2 Repeatability**—Random measurement error is common in morphometric analysis, and it can cause serious statistical problems (Arnqvist and Martensson, 1998). To detect this kind of error, we repeated measures of all individuals twice, and we quantified their

repeatability by the ratio between the individual variance and the total variance. For this purpose, we used the free software VAR 1.4 (<http://www.mpl.ird.fr/morphometrics/>).

**2.4.3 Size and Shape**—For comparing overall wing size between zones, we used the isometric estimator known as “centroid size” (CS) derived from coordinates data: it is defined as the square root of the sum of squared distances between the center of the configuration of landmarks and each individual landmark (Bookstein, 1991). Variation in shape was examined by using geometric morphometrics based on generalized least squares Procrustes superimposition methods (Rohlf, 1990; Rohlf and Slice, 1990). Procrustes methods analyze shape by superimposing configurations of landmarks of two or more individuals to achieve an overall best fit. The first 11 Relative Warps (RW), principal components of shape variables or “Partial warps” (PW), representing > 95 % of the total shape variance, were retained for further analysis. The free software MOG (<http://www.mpl.ird.fr/morphometrics/>) was used to compute size and shape variation.

## 2.5 Statistical Analysis

One-way ANOVAs were employed to infer size variation across zones, EGVs and karyotypes. Furthermore, pairwise wilcoxon signed-rank tests were performed to construct a matrix based on differences among zones. To analyze shape variation with regard to zones, EGVs and karyotypes, we performed a MANOVA using the first 11 RWs. Computational statistics were performed by using the free software R (<http://cran.r-project.org/>).

## 3. Results

### 3.1 Macro-environmental survey

A total of 265 *Anopheles funestus* females were karyotyped and processed for morphometric analysis (Table 1). An average of 29 mosquitoes were collected in each zone, with a range of 18 (Zone I) to 59 specimens (Zone C). Most of the specimens were processed from both wings (66%), and 34% of mosquitoes were analyzed only from one wing. Our results revealed a high level of precision and repeatability in the collection of landmarks, which guaranteed the reliability of the wing size and shape analyses (Supplementary Table 1).

Size differences between ecological zones were highly significant in the general ANOVA ( $F=14.85$ ,  $p<0.001$ ). Pairwise comparisons inferred two groups: mosquitoes collected in highland areas in the central part of the country (Zones E, F and G) were significantly different from those collected in the northern (Zone A, B and C) and southern zones (Zones H and I) (Wilcoxon signed-rank test,  $p<0.01$ ). Zone D, located close to the highlands was intermediate (Table 1, Figure 3). Wing sizes in northern savannah areas (A, B and C) were not significantly different from those in the southern rainforest biome (H, I) (Wilcoxon signed-rank test,  $p>0.05$ ). That reveals the important role of the Adamaoua Highlands in the ecological diversification of this species, as it was already observed in other animals (Bauer et al., 2006). Before proceeding to analysis of wing shape variation, an analysis of the correlation between wing size and shape was carried out (allometry). The correlation coefficient between centroid size (CS) and the 11 RWs was highly significant ( $F=6.63$ ;  $p<0.001$ ), which means that changes in wing size will carry with it changes in wing shape. Among the zones, wing shape varied significantly ( $F=2.24$ ,  $p<0.001$ ).

Association between 7 eco-geographical variables (EGVs) and wing morphology were analyzed by ANOVA. The overall effect of EGVs in wing size (CS) variation was highly significant ( $F=25.74$ ;  $r^2=0.33$ ,  $p<0.001$ ). However, only temperature and elevation were highly significantly associated to CS variation (Table 2). In Cameroon, elevation and

temperature are highly correlated (Spearman test,  $p < 0.001$ ). Therefore, wing size variation might be explained solely by temperature effect. Rainfall and wind speed revealed marginally significant values (Table 2). Similar procedure was employed to study wing shape variation, using the first 11 RWs. Again, temperature was highly significantly associated to variation in wing shape (MANOVA test  $F = 9.06$ ,  $p < 0.001$ ). However, the rest of the EGVs were also highly statistically associated to wing shape variation ( $P < 0.005$ , Table 2). As a result, and in agreement with the analysis of wing shape variation across zones, we were not able to infer a key role of any EGV on wing shape variation (Table 2).

### 3.2 Chromosomal polymorphism and wing traits

Mosquito karyotyping was carried out for the independent (non-overlapping) and common inversions on chromosome 3. Two inversions were scored on the right arm (3Ra, 3Rb) and one on the left arm (3La). These inversions have been involved in local environmental adaptation and speciation in different populations of *An. funestus* throughout Africa (Ayala et al., 2010; Cohuet et al., 2005; Costantini et al., 1999; Guelbeogo et al., 2005).

Chromosomal polymorphisms are not randomly distributed in Cameroon (Ayala et al., 2010). Latitudinal variation in inversion frequencies was observed in all three inversion systems (Table 1). The three inversions were absent in the northern dry savannahs (Zones a and B) and gradually increased in frequency southwards until they were fixed in the humid southern rainforest areas (Zones H and I) (Table 1). In Cameroon, clinal latitudinal inversion frequencies have been previously reported in *An. funestus* (Ayala et al., 2010; Cohuet et al., 2005) and *An. gambiae* (Simard et al., 2009; Wondji et al., 2005).

When comparing wing traits among karyotype forms (Homozygote standard – Heterozygote – Heterozygote inverted), significant differences were observed. Wing size was studied separately for each chromosomal rearrangement and alternative karyotype. Inversion 3Rb and 3La revealed highly significant wing size differences between standard and inverted homozygotes, while inversion 3Ra was marginally significant (Pairwise Wilcoxon rank sum test, 3Ra  $p = 0.043$ ; 3Rb  $p = 0.002$ ; 3La  $p < 0.001$  after Bonferroni correction). Because highland specimens (Zones E and F) are bigger than those from other zones in Cameroon, they were removed from the analysis. As result, the distinction of size between standard and inverted homozygotes in all three inversions disappeared (Pairwise Wilcoxon rank sum test,  $p > 0.05$ ). On the other hand, highly significant differences were observed when comparing heterozygotes with both homozygotes for inversions 3Ra and 3Rb (Pairwise Wilcoxon rank sum test,  $p < 0.001$ , after Bonferroni correction). Most of heterozygotes occur in the Adamaoua Highlands. Hence, this divergence could be due to environmental conditions rather than genetic background. Statistical analysis for 3La heterozygotes was not carried out due to the low number of specimens ( $n = 3$ ).

Similar analyses were performed for wing shape morphometry among chromosomal inversions and karyotypes. Pairwise comparisons revealed significant differences among karyotypes for each inversion. However, main dissimilarities were observed between standard and inverted forms for the three chromosomal rearrangements across Cameroon, even when the Adamaoua highlands' populations were not included in the analysis (Pairwise Wilcoxon rank sum test  $p < 0.001$ , after Bonferroni correction). Besides, wing shape of 3Ra and 3Rb heterozygotes remained slightly different from standard homozygotes (Pairwise Wilcoxon rank sum test, 3Ra  $p = 0.03$ ; 3Rb  $p = 0.035$  after Bonferroni correction) and were not significantly different from inverted homozygotes (Pairwise Wilcoxon rank sum test  $p > 0.5$ ). The low number of heterozygotes for 3La inversion ( $n = 3$ ) made it impossible to carry out robust statistical analysis with this karyotype.

To reduce potential environmental determinants in wing trait variation, we further focused our analysis on Zone C, where both homozygotes (standard and inverted) for the three



inversions systems (3Ra, 3Rb and 3La) occur in sympatry. In this area, the three inversions appear in almost absolute linkage disequilibrium (*G-test*,  $p < 0.001$ ). We computed the Hardy-Weinberg Equilibrium test using the software GENEPOP v4 (Raymond and Rousset, 1995; Rousset, 2008). All inversions showed strong and significant deficit in heterozygotes (Exact Test,  $F_{IS} > 0.8$ ,  $p < 0.001$ ). Due to low numbers of heterozygotes in the 3 inversions, they were removed from further analyses, and we only performed comparisons between specimen homozygotes for all 3 inversions, e.g. 19 and 34 specimens for standard and inverted homozygotes, respectively. At first glance, the results did not reveal significant levels of wing size variation between homozygotes in Zone C (MANOVA test,  $F = 0.43$ ,  $p > 0.05$ ). However, when we analyzed wing shape, highly significant variation was observed between standard and inverted homozygotes (MANOVA test,  $F = 13.35$ ,  $p < 0.001$ ). To confirm these differences in wing shape between standard and inverted homozygotes, we extended the wing shape variation analysis to the rest of the ecological zones. We compared standard individuals from zones A, B and C to inverted homozygotes collected in zones C, D, E, F, G, H and I. We did not observe any significant wing shape difference within standard (MANOVA test,  $F = 1.20$ ,  $p > 0.05$ ) and inverted homozygotes (MANOVA test,  $F = 1.28$ ,  $p > 0.05$ ). However, differences in wing shape between standard and inverted homozygotes was highly significant (MANOVA test,  $F = 36.04$ ,  $p < 0.001$ ). These results revealed that the pattern of wing shape variation, but not size, depends upon the chromosomal background and karyotype of *An. funestus* specimens.

## 4. Discussion

This study is the first of its kind to demonstrate environmental and genetic effects associated with phenotypic variation in an *Anopheles* species. We showed i) that wing size variation in *An. funestus* from Cameroon was almost exclusively mediated by temperature effects and independent of the genetic background; and ii) that chromosomal polymorphism is associated with wing shape variation, but not size. The statistical analysis suggested that this latter pattern is stout and maintained across different environmental conditions.

### 4.1 Wing size variation

Clines in body size have repeatedly been observed in many insects, such as in fruit flies and mosquitoes, and were commonly related to eco-geographical variables such as latitude, temperature or elevation (Capy et al., 1993; Collinge et al., 2006; Weeks et al., 2002) or to biological processes such as larval crowding and/or availability of limited resources during larval development (Aboagye-Antwi and Tripet, 2010; Manoukis et al., 2006). It is not clear why wing size evolves in response to temperature or elevation. It has been suggested that natural selection could act on the ratio between the surface area and the volume of the body, and hence on the rate of heat exchange, a theory known as Bergmann's rule (Mayr, 1963). Although this feature is typical for endothermic animals, it has been also reported for ectothermic animals, like insects (Partridge et al., 1994; vanVoorhies, 1996).

Several hypotheses could explain wing size variation among *An. funestus* populations. First, larval development conditions affect final adult size in mosquitoes (Aboagye-Antwi and Tripet, 2010; Briegel, 1990a, 1990b; Lyimo et al., 1992). Water temperature during mosquito larval development may affect the amount of food eaten, therefore, the efficiency of assimilation and growth efficiency. In *Drosophila*, larval development at low temperature reduces growth rate but increases final adult size (French et al., 1998). If we consider a direct relationship between body size and wing size to be common in *Diptera* (Briegel, 1990a; Briegel et al., 2001; Sokoloff, 1966), it could explain why larger wings were found in the colder regions of the Adamaoua highlands. Second, biotic processes affecting larvae are directly correlated to final adult size in *Anopheles* (Lyimo et al., 1992; Manoukis et al., 2006). For instance, larval crowding affects adult size negatively. However, (adult)

mosquitoes abundance has been negatively related to elevation (Tchuinkam et al., 2010; Zhou et al., 2007). Unfortunately, we do not have any information on larval density of *An. funestus* in Cameroon but high density is not expected because these mosquitoes typically breed in large bodies of water such as in lakes and semi permanent swamps (Coetzee and Fontenille, 2004). Therefore, we cannot conclude on the implication of biological process in the variation of wing size. A third hypothesis relates wing size and altitudinal environments based on air conditions in high elevations. On a developmental or evolutionary timescale, flying insects in high altitudinal environments may compensate for reduced air density by altering wing length and/or wing area relative to body size (Dillon et al., 2006). Insects with greater wing area relative to body size decrease the induced velocity necessary to sustain flight, therefore minimizing the energetic cost in reduced air density conditions (Dillon et al., 2006). Some morphological data support this hypothesis. Wing length in *Drosophila robusta* is greater at higher elevation whereas thoracic dimensions remain constant (Stalker and Carson, 1948). Similarly, mountain honeybees have longer wings and a greater wing area but invariant body mass relative to their lowland counterparts (Hepburn et al., 1998). Unfortunately, in our analysis we only measured mosquito's wings and no other body traits. Consequently, we could not verify this hypothesis, something which remains open for future studies. The last hypothesis is related to chromosomal inversion background. Chromosomal inversion polymorphism has been associated with a large number of traits (Hoffmann and Rieseberg, 2008; Hoffmann et al., 2004). In *Drosophila*, several authors have sought relationships between body (or wing) size and chromosomal inversions karyotype, leading to diverse results (Bitner-Mathe et al., 1995; Fanara et al., 1997; Orengo and Prevosti, 2002; Prevosti, 1967; Santos et al., 2004; Yadav and Singh, 2006). Moreover, in seaweed flies, Day and Gilburn related the male body size with one inversion (Day and Gilburn, 1997). In general, body size is affected only by some inversions, and negatively or positively associated with one of the homozygotes. Moreover, several studies have revealed a positive effect of heterosis (heterozygotes) in body size (Prevosti, 1967; Yadav and Singh, 2003). In our study, both homozygotes (standard and inverted) for the different inversions systems did not show significant wing size variation at local or country scale. Instead, heterozygotes for inversion 3Ra and 3Rb were mostly found in the highlands and they exhibited significantly bigger wings than either homozygote. Our data therefore suggest that chromosomal polymorphisms do not play a significant role in determining wing size, but heterosis does. These results should be tested experimentally in the laboratory under controlled environmental conditions. However, unfortunately, conventional approaches based on formal laboratory crosses are not possible with *An. funestus* because of scarcity of laboratory colonies

Some studies have correlated mosquito body size with malaria parasite transmission efficiency: larger *An. gambiae* females were shown to live longer, to bite more often and to utilize the blood meal more efficiently than smaller ones (Aboagye-Antwi and Tripet, 2010; Manoukis et al., 2006; Takken et al., 1998). Thus, variations in body size may have a direct impact on vector capacity and malaria transmission dynamics, because larger mosquitoes will be able to transmit the parasite more efficiently. Accordingly, it might be expected that in the highlands of Cameroon, mosquitoes might be able to transmit malaria better than in the lowlands. However, many other local factors are known to affect transmission intensity and vector capacity of mosquito populations (Cohuet et al., 2010).

#### 4.2 Wing shape variation

In morphometrics, the shape of a configuration of landmarks is represented by their relative positions as contained in their coordinates. Since each form can be explained by the change in linear dimensions, it is obvious that size and shape are not independent attributes. In this study we analyzed the relationship between size and shape (called 'allometry'). Our results

showed a significant correlation between both traits. As shown above, environmental changes affected the size, which in turn should produce passive shape changes (allometric effects). However, wing shape did not follow the same environmental pattern than wing size, showing higher environmental diversity across Cameroon and within ecological zones. It was therefore not possible to attribute the variations in shape to allometric effects only.

Although latitudinal and/or temperature clines were described in many animal species in size-related traits, the relationship between shape and environmental conditions is still unclear (Huey et al., 2000; James et al., 1997). Birdsall et al., (2000) concluded that wing shape in *D. melanogaster* is poorly affected by developmental temperature. However, there is circumstantial evidence suggesting that developmental and evolutionary temperature-related cell size divergence have contrasting effects on wing shape in *D. subobscura* (Calboli et al., 2003). Although hypothetical functional explanations for subtle wing shape variation are missing for environmental variables, apparently wing shape would affect aerodynamics in insects. Because wing shape largely determines the high energetic costs of flight, it may be expected that inter- or intraspecific differences be partly due to selection. One of the environmental variables considered in this study was wind speed, which could be presumed to have a direct effect on insect flight. However, again, no significant pattern in wing shape variation could be linked to variations in this EGV. One study in dragonflies revealed important modifications in wing shape between migrant and non-migrant populations (Johansson et al., 2009). In mosquitoes, migration is not a well-known phenomenon. *Anopheles funestus* breeds in semi-permanent or permanent sites whose distribution is patchy, such as dams, agricultural irrigation schemes, and marshes (Coetzee and Fontenille, 2004). Migration therefore, does not appear to be a major biological attribute of *An. funestus*. However, low levels of neutral genetic differentiation between geographically distant *An. funestus* populations in Cameroon (Ayala et al., 2010; Cohuet et al., 2005) suggests that intensive gene flow occurs across long distances may be facilitated by some levels of migratory patterns. This area of research clearly deserves further attention, and the evolutionary significance of long distance migration, and the physiological, morphological and behavioral traits that favor it in *An. funestus* and other major malaria vectors needs to be clearly assessed.

Few studies have paid attention to wing shape variation with regard to chromosomal inversions. Santos et al., (2004) showed significant differences in wing shape depending on chromosomal inversions in *D. subobscura*. Actually, shape appears as a classical polygenic character (Patterson and Klingenberg, 2007). Many genes with small additive effects on wing shape are dispersed along the *Drosophila* genome (Weber et al., 2001; Zimmerman et al., 2000). This suggests plentiful chance for gene-inversion linkage disequilibria in inversion-rich species such as *D. subobscura*. In contrast to what was observed for environmental variables, there was a consistent pattern of wing shape variation among populations of *An. funestus* carrying the same karyotype across ecological areas. Mosquitoes sharing identical full homozygote state (standard or inverted for the 3 inversions systems on chromosome 3) did not show significant differences in wing shape between localities across Cameroon. This pattern was consistently observed, even when both homozygotes occurred in sympatry (Zone C). Non-random distribution of chromosomal inversions, strong and significant heterozygote deficits, and linkage disequilibrium between inversions within natural populations of *An. funestus* have been described and interpreted as indicative of environmental adaptation and/or incipient speciation, a situation that is reminiscent of *An. gambiae* (Cohuet et al., 2005; Coluzzi et al., 2002; Costantini et al., 1999; Guelbeogo et al., 2005; Michel et al., 2005). Recently, one study in six sympatric cryptic species in the neotropical genus *Blepharoneura* (*Tephritidae*) suggests a direct link between behavioral traits exhibited by males of different species, the degree of wing shape dimorphism, and the extent of reproductive isolation (Marsteller et al., 2009). Such



divergence operating on courtship and mate recognition (e.g., prezygotic reproductive isolation) might also exist in *An. funestus* and contribute to some extent to the strong deficits in heterozygote we observed. Indeed, significant levels of genetic differentiation have been reported between sympatric populations characterized by different levels of chromosomal polymorphism (i.e., the “Folonzo” and “Kiribina” chromosomal forms described by Costantini et al., (1999) and Guelbeogo et al., (2005)) in Burkina Faso (Michel et al., 2005). Here again, further research is needed to explore mating behavior and mating preferences, and their genetic bases in anophelines mosquitoes.

### 4.3 Conclusion

In conclusion, our study demonstrated that wing morphology in *Anopheles funestus* shows significant phenotypic variation in different bioclimatic settings. In particular, *An. funestus* wing size exhibits significant variation according to temperature and elevation, suggesting this character is potentially targeted by natural selection. Because wing size is correlated to body size in mosquitoes and variations in body size were shown to impact on the overall fitness and vector efficiency, further studies aiming at unraveling the genetic bases of such plasticity are needed. Moreover, wing shape morphology analyses revealed significant variation between as well as within eco-climatic regions of Cameroon, and were shown to correlate with chromosomal polymorphisms. Differences in wing shape related to chromosomal inversions might be involved in mate recognition and therefore impact on the population structure of this vector. This has major consequences for the development and implementation of any vector control strategies, including innovative vector control using genetically altered mosquitoes for population suppression and/or replacement (Boete and Koella, 2002).

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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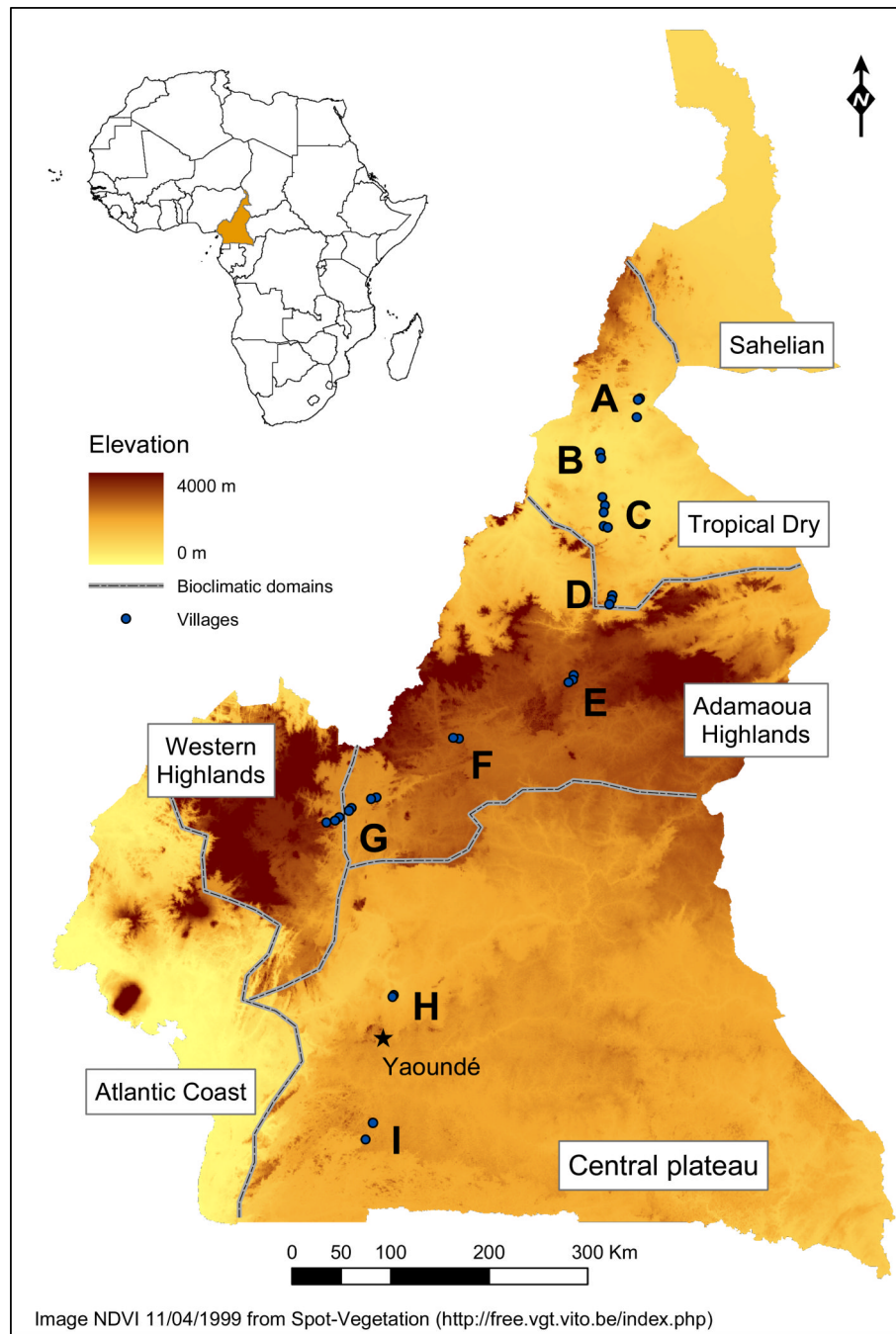
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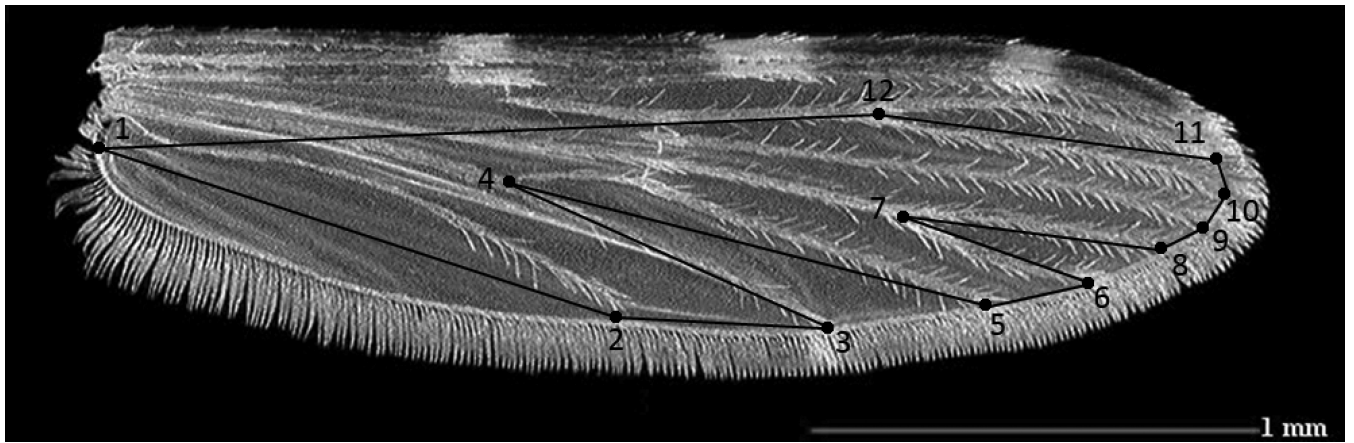
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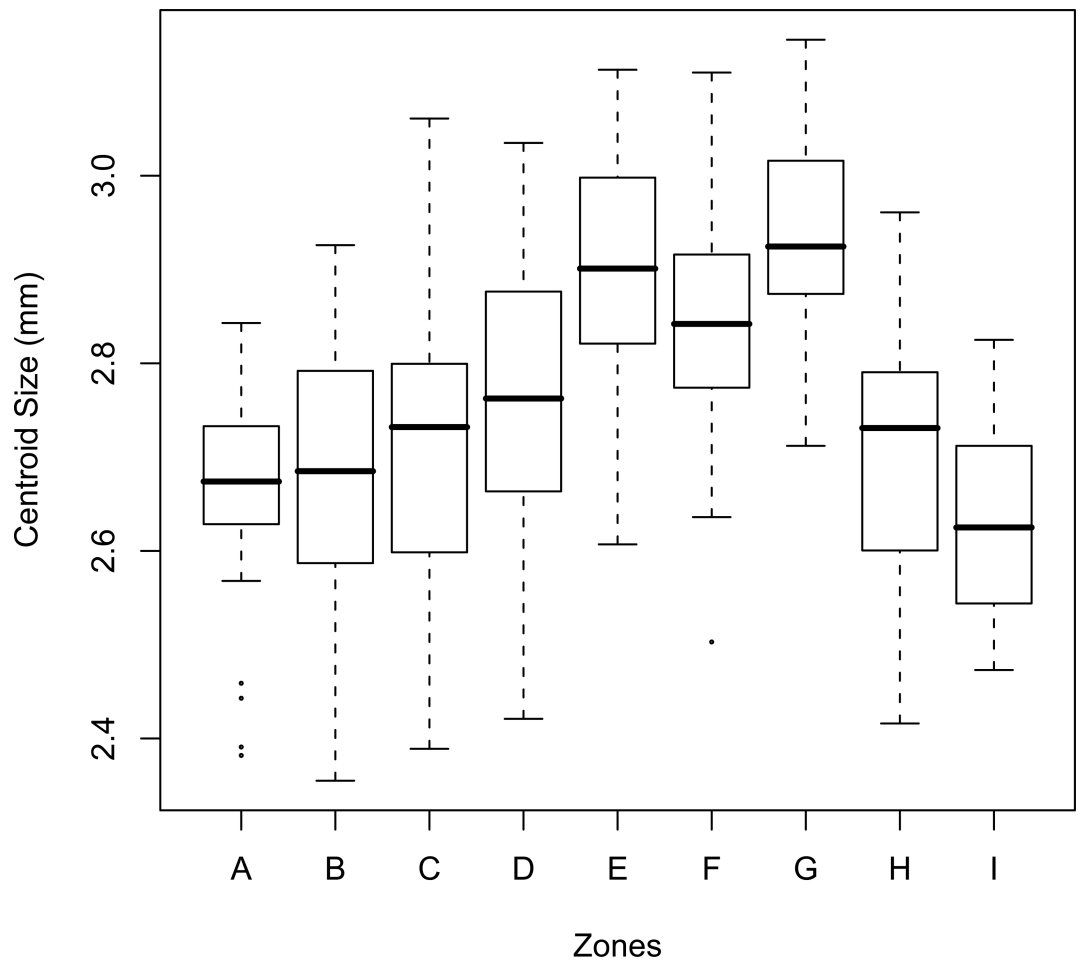




**Figure 1.** Topographic Map of Cameroon showing sampling zones (A to I) and villages in each zone (dots). Dotted lines delimit biogeographical domains (Olivry, 1986).



**Figure 2.**  
Selected landmarks on *Anopheles funestus* mosquito wing.



**Figure 3.** Boxplot representing wing size (CS, centroid size) variation in different sampling zones. Expand: Median, percentile and outliers.

Table 1

Chromosomal inversion frequencies and wing size details for *Anopheles funestus* specimens collected in Cameroon

Zone *	Bioclimatic Domain	Temperature (°C)	Rainfall (mm)	Elevation (m)	Number of villages	Number of mosquitoes	Inversion Frequency (%)			Wing Size (mm)
							3Ra	3Rb	3La	
A					3	27	0.0	0.0	0.0	2.66 ± 0.12
B	Dry tropical	27	1,000	285	2	33	1.5	0.0	3.0	2.67 ± 0.15
C					5	59	63.6	68.6	66.9	2.70 ± 0.16
D	Cliff	24	1,330	610	3	24	87.5	97.9	95.8	2.76 ± 0.15
E	Highlands	23	1,500	870	3	21	76.2	73.8	100.0	2.90 ± 0.14
F	Highlands	22	1,860	780	2	26	71.2	80.8	100.0	2.84 ± 0.12
G	Highlands	22	1,860	780	7	22	63.6	72.7	100.0	2.93 ± 0.12
H	Rainforest	25	1,700	570	2	35	100.0	100.0	100.0	2.70 ± 0.13
I					2	18	100.0	100.0	100.0	2.63 ± 0.10

\* Zone denomination refers to Figure 1. Elevation: mean elevation (in meters); Rainfall: mean yearly rainfall (in mm); Temperature: mean yearly temperature (in °C).

**Table 2**

Statistical correlation between eco-geographical variables (EGVs) and wing morphometric traits (size and shape).

EGV	Wing size <sup>1</sup>		Wing shape <sup>2</sup>	
	F-statistic	pvalue	F-statistic	pvalue
Elevation	18.21	<0.001	6.352	<0.001
Evapotranspiration	2.06	0.152	7.889	<0.001
Sunlight exposure	0.00	0.982	5.774	<0.001
Rainfall	4.71	0.031	7.779	<0.001
Wind speed	5.14	0.024	6.322	<0.001
Temperature	14.16	<0.001	9.060	<0.001
Water Vapor Pressure	3.27	0.072	2.549	0.005

<sup>1</sup>Wing size was estimated by centroid size (CS) measures. Statistical significance was studied by ANOVA test.

<sup>2</sup>Wing shape was estimated by the first 11 relative warps (RW) which represent >95% of total wing shape variance. Statistical significance was studied by MANOVA test