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Morphometric comparisons of the scanning electron micrographs of the eggs of *Anopheles* (*Nyssorhynchus*) *darlingi* Root (Diptera: Culicidae)

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ABSTRACT

Anopheles darlingi Root is the principal vector of *Plasmodium* in Brazil, but its biological variability is not well known. Morphometric analyses of scanning electron microscopy images of the eggs of *An. darlingi* were conducted using individuals collected in nine states of Brazil (Acre, Amapá, Espírito Santo, Pará, Paraná, Rio de Janeiro, Rondônia, São Paulo, and Tocantins). Ten attributes of the eggs (seven continuous variables and three discrete variables) were respectively measured or counted and analyzed to determine if populations from different geographical regions or biomes could be distinguished. Univariate analysis showed that the eggs from Espírito Santo were the narrowest whereas representatives from Tocantins populations had the smallest floats. Results of multivariate analyses of continuous variables showed that the first principal component (PC1), mainly represented by all four float attributes, helped to differentiate populations. The second principal component (PC2) comprised roughly the length and width of the egg. PC1 of discrete variables corresponded to the number of ribs on the float whereas PC2 was approximately equivalent to the number of discs on the micropyle. Based on those variables (continuous and discrete separately), multivariate discriminant analysis indicated that eggs from individuals collected in Tocantins were distinct from the other populations. Among sampled localities, the one from the state of Tocantins was situated within the Cerrado biome whereas the locality from São Paulo state was at the border of Cerrado, within a transition zone of the Atlantic Forest biome. Generally, the climate in the Cerrado biome was more arid than in areas of the Amazon and Atlantic Forest biomes, and the temperature had the highest range. Coincidentally, based on morphometric data, cluster analysis distinguished the population from Cerrado, Tocantins from all other populations. Results of multiple regression analysis of the variables showed no correlation between egg variables and latitude or climatic variables. We concluded that eggs were polymorphic and that some morphological patterns were regional. Although no environmental influence on the egg attributes was unequivocally detected, a potential association cannot be entirely discarded. Consequently, we hypothesize that morphological traits of the immature stages, especially from the eggs, convey evolutionary information regarding to this species.

Abbreviations: AC, Acre state; AP, Amapá state; AM, Amazonas state; CV, canonical variables; CV1, canonical variable 1; CV2, canonical variable 2; DA, discriminant analysis; DP, deck perimeter; DL, deck length; ES, Espírito Santo state; FL, float length; L, egg length; ND, number of micropylar discs; NR, number of ribs; NT, tubercle density (number of tubercles in an area of 300 μm); PA, Pará state; PCA, principal component analysis; PC1, first principal component; PC2, second principal component; PC3, third principal component; PM, micropylar disc perimeter; PR, Paraná state; RJ, Rio de Janeiro state; RO, Rondônia state; SP, São Paulo state; TO, Tocantins state; PT, tubercle perimeter average (perimeter in an area of 300 μm); W, egg width.

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1. Introduction

Anopheles (*Nyssorhynchus*) *darlingi* Root from the Argyritarsis Section (Linthicum, 1988) is largely distributed in Central and South America, extending from Southeastern Mexico to Northern Argentina and from east of the Andes to the coast of the Atlantic Ocean (Forattini, 2002). This species is the primary vector of *Plasmodium* parasites (the causative agent in malaria) in several countries in South America. Furthermore, this mosquito is associated with the malaria frontier in the Amazon region, mainly in areas undergoing intensive ecological changes in the natural ecosystems promoted by forest clearance (Castro et al., 2006).

Larvae of *An. darlingi* seem to be better adapted to habitats where chemicals and physical conditions are relatively stable (Deane et al., 1948). Additionally, they preferentially occur in partially shaded habitats, with emergent vegetation that provides some cover, pH varying from 6.5 to 7.3, and water temperature from 20 to 28 °C (Rachou, 1958). Larvae and pupae are usually found around floating debris, trunks, and emergent vegetation (Hudson, 1984; Rozendaal, 1992; Tadei et al., 1988) that give some stability to physical and chemical conditions of the water, including providing partial shade. Hiwat and Bretas (2011) have recently compiled from the published literature the commonest habitats of *An. darlingi*, showing that they can vary from large to small rivers, lakes and lagoons, flooded forest, and small pools. Additionally, Collucci and Sallum (2006) reported the presence of larvae and pupae of the species in a cemented artificial lake in the urban area of the Ribeirão Preto municipality, inland São Paulo state, Brazil.

Differences in populations of *An. darlingi* from the north and south of Brazil are corroborated by the morphological traits of the eggs (Causey et al., 1944; Galvão, 1938; Galvão et al., 1937; Root, 1926), polytene chromosome banding patterns (Kreutzer et al., 1972), physiological conditions (Rosa-Freitas et al., 1992), behavior (Forattini, 1987; Hiwat and Bretas, 2011), and genetics (Conn et al., 2006; Malafronte et al., 1999; Pedro and Sallum, 2009). Despite these differences, *An. darlingi* has been considered a monotypic species (Lounibos and Conn, 2000; Manguin et al., 1999).

The exochorion of eggs of *Anopheles* species is morphologically polymorphic (Hinton, 1968). The egg of *Anopheles sacharovi* Favre usually does not possess lateral floats; however, when females are exposed to low temperature, their eggs develop rudimentary floats (Bates and Hackett, 1939). Similarly, eggs from early spring, when the temperature is still low, show either rudimentary or small floats (Bates, 1941). An influence of temperature on egg external morphology has also been documented for other species of *Anopheles*, including *Anopheles gambiae* (Deane and Causey, 1943) and *Anopheles walkeri* Theobald (Hurlbut, 1938), among others (see Hinton, 1968, for details). Additionally, morphological heterogeneities in a single egg batch have also been described in the frill and deck structure of eggs of *An. gambiae* (Gillies and De Meillon, 1968) and of *Anopheles strodei* (Galvão, 1938). Recently, Linley (1992), using scanning electron microscopy, has provided the first highly detailed description of the *An. darlingi* egg, and morphological comparisons have been carried out based on three females collected in Puerto Ayacucho, Amazonas State, Venezuela. Generally, the egg morphology was consistent with the light microscopy-based description by Root (1926); however, Linley (1992) has considered that the crown may be smaller and anteriorly tapered, as found by Root (1926) and Causey et al. (1944).

Considering that *An. darlingi* is a primary vector of human *Plasmodium* parasites in South America and that its population variability remains poorly known, we investigated whether the morphological traits of the egg can vary in accordance with the population's geographical origin. Eggs from several populations from Brazil, including individuals from a rural area in the vicinity of the type locality, in Rio de Janeiro, were employed to address morphological polymorphisms that might differentiate populations. Our major objectives were to (1) address the morphological variability of the eggs in nine populations of *An. darlingi* and (2) test if the degree of phenetic similarity of the eggs corroborates patterns of population structure as evidenced by the wing shape (described by Motoki et al., 2012).

2. Materials and methods

2.1. Mosquito collection

A total of 45 field-collected adults, five females from each of nine populations of *An. darlingi* (Fig. 1) were blood-fed and kept in the laboratory for 48 h. One wing was removed to induce oviposition (Sallum et al., 2010). Thirty-six hours after the oviposition, 20–25 eggs of each female were taken from the water and fixed in alcoholic Bouin's solution. The remaining eggs were kept in separate vials to obtain progenies linked with pupae and fourth-instar larval exuviae. Males and females were identified using the key proposed by Forattini (2002). Females of four populations were captured in partially deforested rural areas within the Amazon biome; three populations contained representatives of the Atlantic Forest biome; and two populations were from two distinct localities of the Cerrado biome. Regarding representatives from Cerrado, five females were from a northern locality whereas the remaining five females were from a locality situated in the southern limit of the biome (Fig. 1).

2.2. Scanning electron microscopy

Eggs were dehydrated in ethanol with concentrations at 70%, 80%, 90%, and 100%. This procedure also removed the Bouin's solution. Subsequently, the eggs were transferred to a critical point drying apparatus of a liquid/gas system. The dried eggs were positioned on stubs on a copper conductive tape, covered with carbon and then gold in a sputter coater, and observed and imaged in a Jeol 6460LV scanning electronic microscope as described by Sallum et al. (2010).

2.3. Data acquisition

Morphological attributes were measured and counted in two to eight eggs from each individual female. Scanning electron micrographs were employed to assess 10 morphological attributes. Among them, seven formed a set of continuous variables (length and width of the eggs, length of the float, perimeter and length of the deck, perimeter of micropylar disc, average perimeter of five tubercles measured in a 300 μm area) whereas three were discrete variables [number of ribs on the float, number of discs on the micropyle, and number of tubercles measured in an area of 300 μm (tubercle density)]. All statistical analyses of the continuous variables were performed separately from the analyses carried out for discrete variables. Morphological attributes employed in the study included characteristics from the dorsal surface (Fig. 2A), from the tubercles of the anterior deck (Fig. 2B), and from the ventral surface of the anterior area of egg (Fig. 2C). The length and width of the entire egg were measured dorsally, with a Wild stereomicroscope connected to a digital micrometrical ocular Wild MMS 235® (Heerbrugg, Switzerland). The remaining attributes were measured using a Zeiss LSM Image Browser software.

2.4. Univariate analysis of egg attributes

Range, mean, and standard deviation of the means of 10 attributes were calculated. Normality and homoscedasticity tests