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

Fabio Almeida[b,*], Lincoln Suesdek[b,c], Maysa T. Motoki[a], Eduardo S. Bergo[d], Maria Anice M. Sallum[a]

[a] *Departamento de Epidemiologia, Faculdade de Saúde Pública, Universidade de São Paulo, Avenida Dr. Arnaldo 715, CEP 01246-904, São Paulo, Brazil*
[b] *Laboratório de Parasitologia, Instituto Butantan, Avenida Vital Brasil 1500, CEP 05503-900, São Paulo, Brazil*
[c] *Programa de Pós-Graduação em Medicina Tropical da Universidade de São Paulo, Avenida Doutor Enéas de Carvalho Aguiar 470, CEP 05403-000, São Paulo, Brazil*
[d] *Superintendência de Controle de Endemias, Departamento de Controle de Vetores, Rua Rui Barbosa, 1672, CEP 14810-095, São Paulo, Brazil*

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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.

* Corresponding author. Tel.: +55 11 2627 9785.
E-mail addresses: fabiodealmeida@hotmail.com, fabiodealmeidafa@gmail.com (F. Almeida).

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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

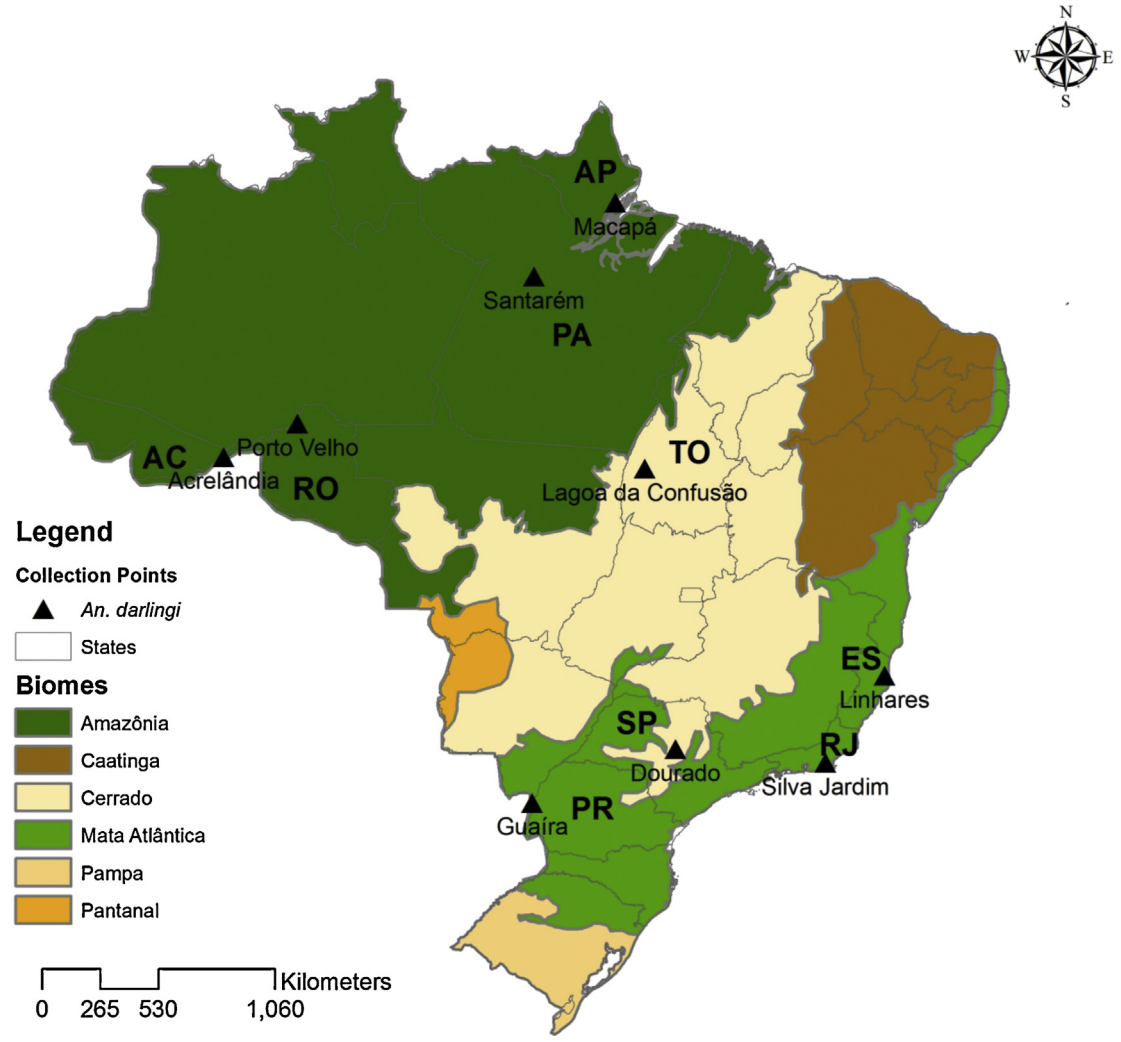

Fig. 1. Map of location sampled on ecoregions from Brazil.

were carried out to verify if attributes had Gaussian distribution. Univariate statistical analysis by ANOVA was used on all egg attributes to access the significance of observed differences. Furthermore, ANOVA and Kruskal–Wallis tests were performed to investigate if the differences in four climate variables (rainfall, maximum temperature, minimum temperature, and relative humidity) of collection sites were significant.

Climatic data of the locations where *An. darlingi* samples were collected for day and month were kindly provided by Instituto Nacional de Meteorologia (INMET; http://www.inmet.gov.br).

2.5. Multivariate analysis

The principal component analysis was used in an exploratory fashion to access the variance and covariance of the egg attributes. For this purpose, a set of seven continuous variables (seven attributes) and a set of three discrete variables (three attributes) were analyzed (sets analyzed separately). Only individuals with complete data were included in the analyses; the remaining individuals with missing data were excluded from statistics. Analyses were carried out for a minimum of seven eggs from each locality.

Discriminant analysis (DA) was carried out to verify segregation of all nine previously determined populations. The procedure started with a set of observations in which all groups and both continuous and discrete variables were normalized to unequally scaled attributes to zero mean and unit variance. This analytical procedure ensured equal weight to all attributes, employing a model that allowed prediction of a group only when the attributes were known. Moreover, the results of discriminant analyses show relationships among groups according to the attributes used to predict them (Johnson and Wichern, 1999).

Cluster analysis using neighbor-joining amalgamation was used to investigate population structure based on 10 morphometric attributes.

2.6. Correspondence between environment and egg morphology

Multiple regression analysis was done to investigate the correlation between egg attributes and the latitude of each collection site (continuous and discrete variables analyzed separately). Another analysis (cluster analysis) using Euclidean distances was performed to verify the degree of similarity between localities in relation to the environment, using the four variables related to climate.

ANOVA and Kruskal–Wallis tests were performed to investigate if the differences in the four climate variables for the collection sites were significant. An $\alpha = 0.05$ was used for considering significance.

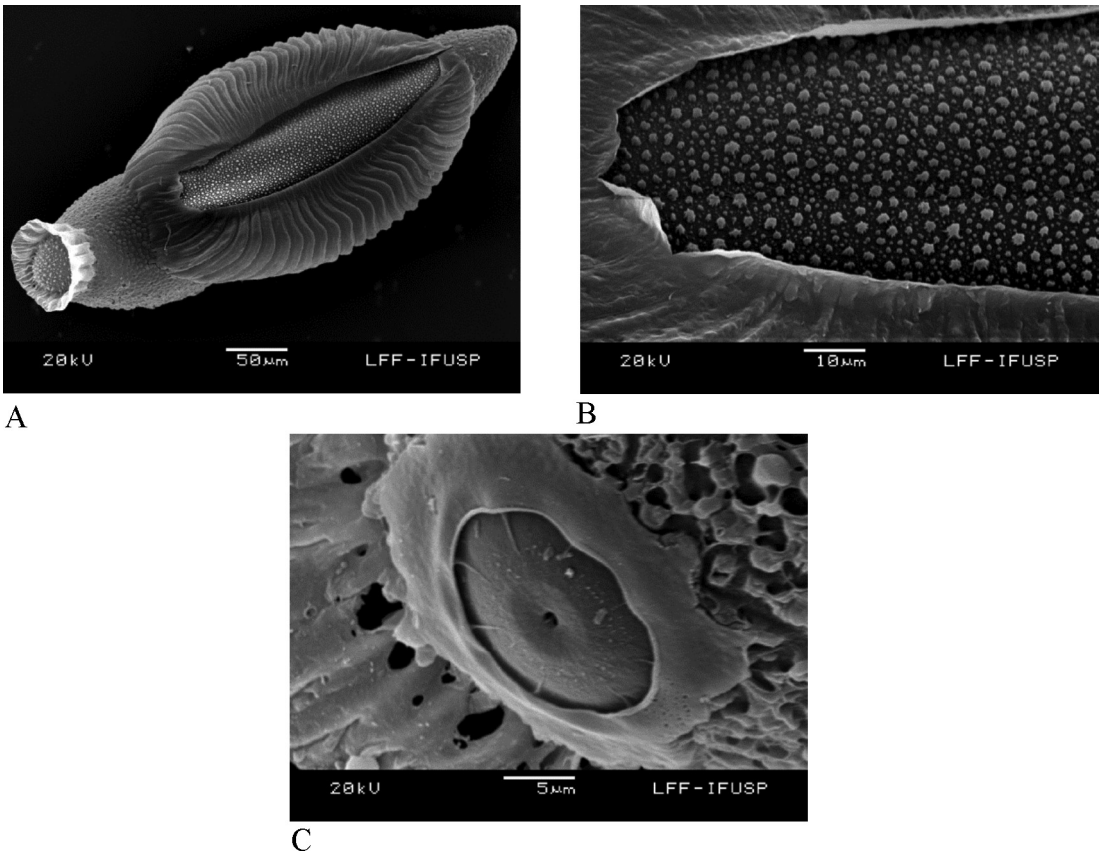

Fig. 2. Eggs of *An.* (*Nyssorhynchus*) *darlingi*. (A) Entire egg, anterior end at top, on the right, dorsal view. (B) Anterior deck tubercles, dorsal view. (C) Anterior end at top, on the right, showing micropylar collar, micropyle, and corolla, ventral view.

3. Results

3.1. Morphometrics – descriptive analysis and univariate analysis

Results of the ANOVAs showed that the length and width of the eggs were not very informative for differentiating populations (Tables S1 and S2). The main difference was that the population of Espírito Santo (ES) had the shortest egg width and second shortest egg length (Table 1, S1 and S2). Furthermore, we note that the width of the egg was more variable (31% of difference from the widest to the thinnest) than the length (17% of difference from the longest to the shortest) among populations.

The most marked difference was observed on the floats. All four attributes related to the float had the lowest values in the

Table 1
Attributes of the eggs of nine populations of *Anopheles darlingi* measured from 2 to 8 eggs from each of five females. Abbreviations: L—egg length; W—egg width; FL—float length; NR—number of ribs; DP—deck perimeter; DL—deck length; NT—tubercle density[a]; PT—tubercle perimeter average[a]; MP—micropylar disc perimeter; ND—number of micropylar discs.

Mean ± standard deviation									
Attributes	AC	AP	ES	PA	PR	RJ	RO	SP	TO
	$n = 22$	$n = 21$	$n = 20$	$n = 24$	$n = 19$	$n = 22$	$n = 23$	$n = 19$	$n = 18$
Linear dimensions									
L	425.23 ± 13.18	446.95 ± 17.46	429.8 ± 14.59	434.04 ± 3.23	432.11 ± 7.15	440.27 ± 8.31	432.57 ± 5.66	446.37 ± 16.75	431.11 ± 6.84
W	177.5 ± 9.09	177.19 ± 13.01	150.8 ± 9.65	161.39 ± 4.30	181.84 ± 7.62	175.09 ± 6.69	171.35 ± 3.28	168.32 ± 10.16	159.56 ± 3.13
Float attributes									
FL	274.32 ± 23.26	310.89 ± 6.71	299.54 ± 17.35	290.77 ± 5.74	288.35 ± 14.02	277.11 ± 21.98	289.69 ± 15.06	282.59 ± 13.58	212.30 ± 28.27
NR	49 ± 3.40	54 ± 2.96	51 ± 4.50	47 ± 1.12	49 ± 2.73	45 ± 5.36	53 ± 3.06	53 ± 3.31	39 ± 6.26
DP	535.09 ± 28.52	567.93 ± 25.45	545.69 ± 29.62	568.70 ± 21.81	566.54 ± 45.66	496.33 ± 37.49	506.23 ± 22.11	536.03 ± 39.01	343.00 ± 36.98
DL	257.86 ± 31.23	259.22 ± 21 27	247.73 ± 26.88	294.41 ± 15.17	261.73 ± 29.37	231.02 ± 24.30	238.62 ± 14.85	250.74 ± 24.63	163.26 ± 20.77
Anterior deck tubercles									
[a]**NT**	12 ± 1.89	14 ± 2.26	15 ± 2.02	21 ± 2.55	17 ± 1.32	17 ± 1.96	16 ± 1.17	19 ± 0.67	19 ± 0.99
[a]**PT (mean)**	5.92 ± 0.28	5.68 ± 0.26	5.77 ± 0.23	6.06 ± 0.26	5.97 ± 0.22	5.80 ± 0.21	5.77 ± 0.21	5.99 ± 3.14	5.73 ± 6.81
Micropyle									
MP	46.01 ± 3.20	47.35 ± 4.17	42.58 ± 5.14	52.42 ± 5.76	52.30 ± 3.90	44.86 ± 3.69	43.26 ± 2.99	45.79 ± 3.14	40.78 ± 6.81
ND	7 ± 0.55	7 ± 0.66	7 ± 0.79	7 ± 0.62	7 ± 1.00	7 ± 0.83	7 ± 0.60	7 ± 0.67	6 ± 0.99

[a] Number and perimeter in an area of 300 μm.

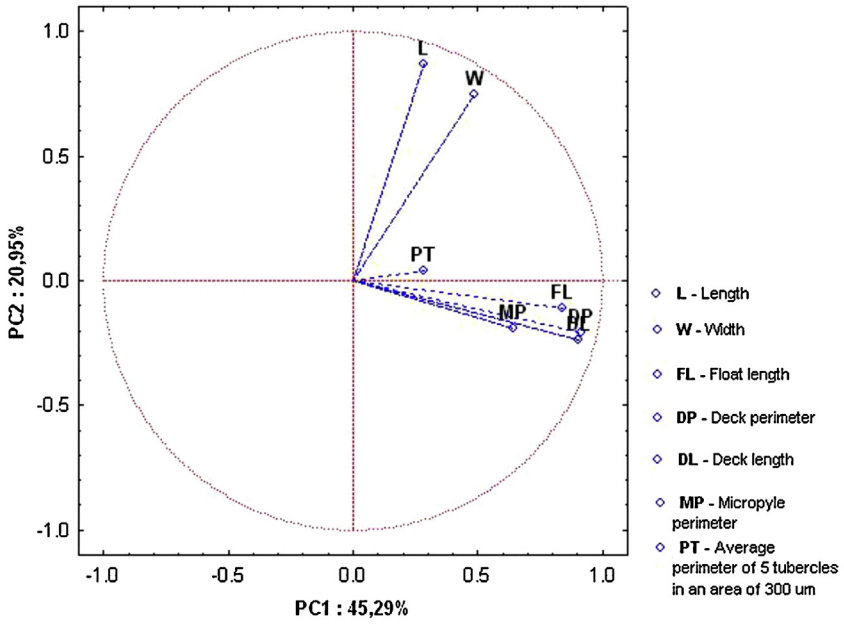

Fig. 3. Plot of PC1 and PC2 of continuous variables.

TO population (Table 1, S3–S6). In this population, the arithmetic mean of the ratio of the float length and egg length was 0.49 while in the remaining eight populations (AC, AP, ES, PA, PR, RJ, RO, SP), the arithmetic mean of the ratio varied from 0.63 to 0.74.

The population of Amapá (AP) exhibited more fulfilled floats (Table S3), and the Pará (PA) population had bigger decks (Tables S5 and S6). Moreover, the population of PA and Paraná (PR) showed bigger micropyles (Tables S7 and S8). In relation to tubercles, the population of PA had a higher concentration per area (Table S9), and TO had smaller micropyles (Table S10).

Regarding the analyses conducted to test if morphological polymorphisms were related to climate variables (climate data are in Tables S11–S15), we observed that the relative humidity was lower in the location of the State of Tocantins than in the remaining localities (Tables S15–S20). Moreover, the locality of TO had a higher daily temperature range (Tables S14 and S19). All statistical analysis related to climatic variables are in Tables S16–S20.

3.2. Cluster analysis

Regarding the cluster analysis of the 10 egg attributes, groups did not correlate with geographical location (not shown). The cluster analysis of climatic variables suggested overall differentiation between the Atlantic Forest and Amazon and showed that the TO location (Cerrado) has unique climatic conditions (Fig. S1).

3.3. Principal component analysis of egg attributes

Principal component analysis (PCA) of continuous variables showed that PC1 was responsible for 45.29% of the variation and

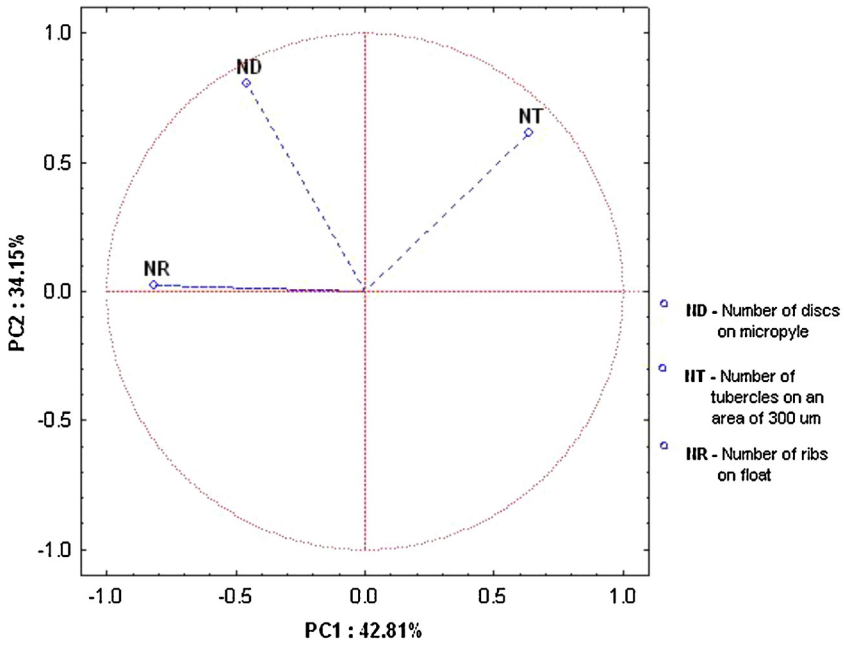

Fig. 4. Plot of PC1 and PC2 of discrete variables.

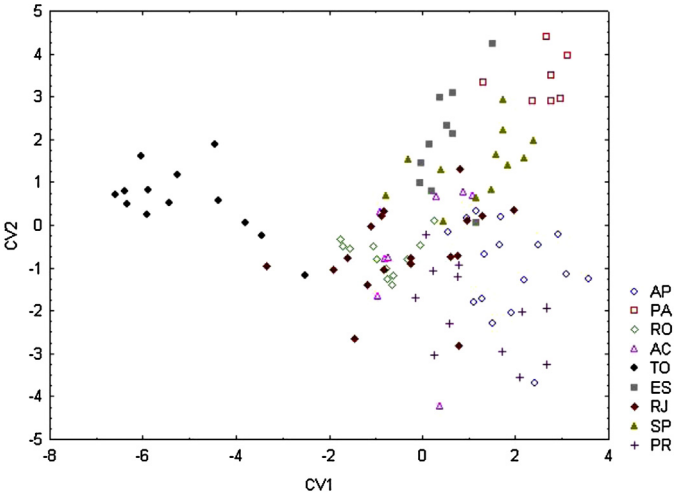

Fig. 5. Morphospace of CV1 and CV2 of continuous variables.

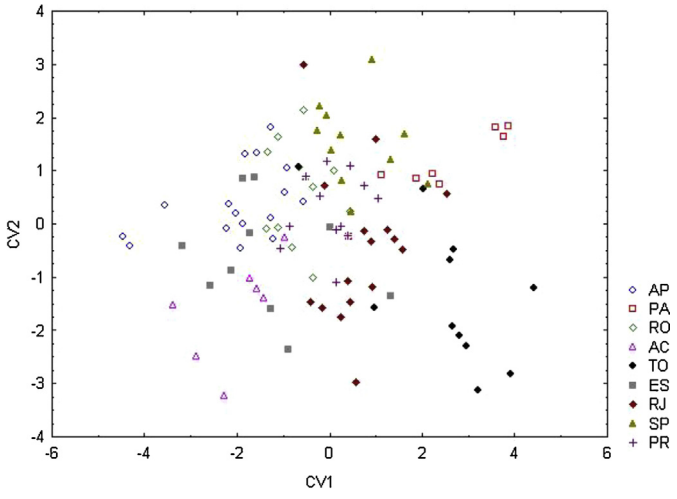

Fig. 6. Morphospace of CV1 and CV2 of discrete variables.

that the attributes that most contributed to PC1 were the float length, deck length, and deck perimeter (three attributes of the float) and the micropyle perimeter. PC2 showed 20.95% of the variation and was composed of the length and width of the egg (Figs. 2a and 3). The arithmetic mean of the perimeter of five tubercles (Fig. 2b) in an area of 300 μm and the micropyle (Fig. 2c) perimeter formed PC3, which was responsible for 15.85% of the variation (Fig. S2). Regarding the PCs of the discrete variables, PC1 was responsible for 42.81% of the variation, and the attribute that most influenced it was tubercle density, while PC2 (34.15%) was mainly influenced by the number of ribs of the floats (Figs. 2a and 4). PC3 showed 23.04% of the variation and was composed of the number of discs on the micropyle (Figs. 2c and S3). The remaining results generated from PCA of continuous and discrete variables are shown in Tables S21, S22, and S23.

3.4. Discriminant analysis

Results of the discriminant analyses of continuous variables showed representatives of TO be distinct from the remaining populations, except for RJ, by the canonical variable 1 (CV1). Furthermore, CV1 differentiated populations from RO from those from AP and PA, and PA from AC. The canonical variable 2 (CV2) distinguished PA from the remaining populations, except from ES and SP, and allowed distinction of the populations of PR from those from ES and SP (Fig. 5).

Regarding discrete variables, CV1 succeeded in separating populations as follows: (1) AC from PA, TO, SP, and RJ; (2) PA from AP; and (3) AP from SP. CV2 separated AC from the PA and SP populations (Fig. 6).

3.5. Correlation with latitude

The multiple regression analyses carried out to verify correlation between each egg attribute and geographical latitude of the correspondent collection locations revealed no correlation between any morphological attributes and geographical latitudes (Table S24).

4. Discussion

Several studies have shown the taxonomic importance of the external morphology of eggs in identifying species of the genus *Anopheles* (Nagaki et al., 2010, 2011; Sallum et al., 2010) and distinguishing populations of a species (Linley et al., 1993a, 1993b, 1996). In the present study, morphometric analyses of scanning electron microscopy imaging, employing continuous and discrete variables derived from egg attributes from nine populations of *An. Darlingi*, were used to either distinguish or group populations. Considering the general morphology of the eggs, individual polymorphisms were observed on the corolla (Fig. 7A) and on the floats (Fig. 7B). However, these visually distinct variations were not geographically related because they were observed in individuals from distinct localities.

Descriptive statistical analyses clearly separated populations from Tocantins (TO) and Pará (PA) from the remaining studied populations. The major differences were found in characteristics of the floats and tubercles on the anterior deck. The most important attribute of the float was the ratio of the egg length and float length measured in the dorsal view, which was higher in the TO population. Furthermore, both the number and perimeter of the tubercles in the anterior deck were higher in the PA population, distinguishing it from the remaining populations. In contrast, no obvious difference in the external characteristics of the eggs was useful for distinguishing populations of *An. darlingi*. Variations in the length and width of the insect eggs depended on the size of the female, climate variables (e.g., temperature and humidity), and environmental factors (e.g., food availability) (Steinwascher, 1984). Furthermore, morphological polymorphisms have been associated with geographical distance among populations of *Culex quinquefasciatus* from India (Suman et al., 2009).

Our data showed no clear correspondence between environmental features and egg attributes. The main climatic dichotomy (Amazon and Atlantic Forest + Cerrado) was not correlated with egg variability. Latitudinal position was also not a determinant of egg morphology. TO might be an exception because of the unique climatic conditions of that location (the driest and the highest temperature range), but this possibility is only speculative. Apparently, local and microenvironmental conditions are more influential than climate for egg morphological variability.

Results of the morphometric analyses of the eggs did not corroborate the population structures resulting from the geometric morphometric analyses of 18 landmarks of the wing venation of females (Motoki et al., 2012). Rather, *An. darlingi* populations are structured in a way that reflects the major Brazilian ecoregions where the species occurs. For instance, populations from the coastal and inland Atlantic Forest are more similar to each other than they are to either the Cerrado or Amazonian populations.

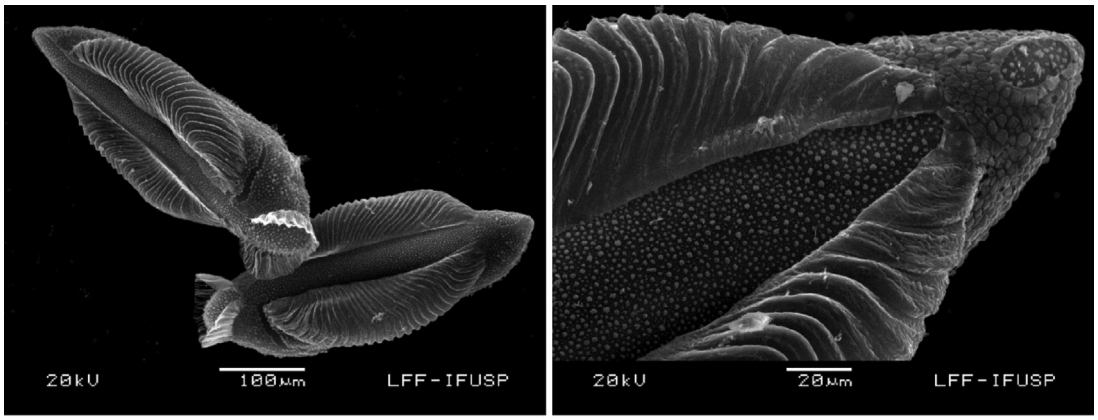

Fig. 7. Eggs of *An.* (*Nyssorhynchus*) *darlingi*. (A) Entire egg, anterior end at top, on the left, dorsal view, showing a discontinuity in the middle dorsal surface of the corolla. (B) Entire egg, posterior end at top, on the right, dorsal view, showing the frill encircling the deck at the posterior pole, dorsal view.

Considering that the present study included representatives of most populations used for the wing geometric morphometrics published by Motoki et al. (2012), we expected to obtain somewhat similar results. However, the single similarity observed was the smaller size of the floats found in the Cerrado (TO) population that clearly distinguished it from the remaining groupings, including from the SP population situated in the southern limit of the Cerrado. Consequently, we cannot strongly affirm that smaller floats are a unique condition for the Cerrado, mainly because few populations from that biome were examined in the current study. However, differences in the morphology of the floats may indicate that adaptive processes could have occurred in that population, which likely occupies specific larval habitats. Morphological variations in the floats might promote a higher rate of successfully hatching eggs and thus a higher probability of obtaining adults.

Morphological variabilities in the floats have previously been observed in other *Anopheles* species. Those variabilities have been associated either with the presence of the *An. strodei* species complex (Sallum et al., 2010) or with seasonal variations (Bates, 1941). Accordingly, representatives of *An. sacharovi* from the early spring show distinct float conformation in comparison to individuals from the summer (Bates, 1941; Bates and Hackett, 1939). The functions of the floats have not been addressed more recently. Earlier hypotheses compiled by Hinton (1968) suggested that the floats are buoyancy structures and also important to maintain aeration around the egg surface. However, Hinton suggests that these hypotheses are somewhat contradictory because of the presence of eggs that do not possess floats but that stay on the water surface and because aeration is maintained by the intricate structure of the exochorion.

Furthermore, interactions between biotic and abiotic factors as well as the stability and duration of the immature habitats are key factors that guarantee the reproductive success of any *Anopheles* population (Rejmánková et al., 2013), including *An. darlingi*. Consequently, it is plausible to suppose that variation in the egg floats may be related to the survival of the eggs in the habitat of a specific population. Moreover, the chemical and physical characteristics of the larval habitats may vary depending on biotic and abiotic factors. Finally, it would be important to investigate morphological variability in the egg floats of populations along a gradient of vegetation following transects that include representatives from the Atlantic Forest, Cerrado, and Amazon biomes, and also from the biome transition zones. In those zones, interactions among climate traits, vegetation, and topography could explain species differences (Gosz and Gosz, 1996) and also variability in biological traits, including morphology.

Authors' contributions

MAMS and ESB designed the experiment and collected representatives of nine populations with help from MTM to obtain representatives from the ES population; MTM prepared eggs for scanning electron microscopy and measured egg traits; FA and LS performed the analyses; and FA, LS, and MAMS wrote the manuscript.

Authors' information

FA is a postdoctorate from the Instituto Butantan, Brazil, and fellow of FAPESP (Grant# 2012/17717-2); MTM was a doctoral student from the Departamento de Epidemiologia, Universidade de São Paulo and recipient of a FAPESP doctorate fellowship, process 07/07573-5; LS is a research scientist from the Instituto Butantan, Brazil; ESB is a researcher from the Superintendência de Controle de Endemias (SUCEN), São Paulo; and MAMS is a professor in the Departamento de Epidemiologia, Universidade de São Paulo, Brazil.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actatropica.2014.07.010.

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