Anopheles aquasalis Eggs from Two Venezuelan Localities Compared by Scanning Electron Microscopy

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Anopheles (Nyssorhynchus) aquasalis, *is the main coastal vector of malaria from northeastern Venezuela to southeastern Brazil. Several authors have argued that* An. aquasalis *is a highly polymorphic species while others indicated that it is a complex of closely related species. This investigation compared the morphology of* An. aquasalis *eggs from Sinamaica (Zulia State) and Yaguaraparo (Sucre State), the west and east of Venezuela, respectively. We were able to separate eggs from the two localities using discriminant analyses based on ratios and percentages of anterior and posterior tubercles measured by scanning electron microscopy. The results of this work suggest that* An. aquasalis *has high intraspecific variation.*

Key words: *Anopheles aquasalis* - scanning electron microscopy - eggs - Venezuela

Anopheles (Nyssorhynchus) aquasalis Curry is the main coastal vector of malaria from eastern Venezuela to southern Brazil (Berti et al. 1993). Its coastal distribution ranges from Nicaragua to southern Brazil, Ecuador (although there are no collection records from this country in the Smithsonian's collection), Trinidad and Tobago and the Lesser Antilles (Knight & Stone 1977, Faran & Linthicum 1981, Zimmerman 1992). Taxonomically, this species belongs to the Oswaldoi Series in the Albimanus Section of *Anopheles*, subgenus *Nyssorhynchus* (Faran 1980, Faran & Linthicum 1981, Harbach 1994).

Given the latitudinal range of *An. aquasalis*, one might expect that different populations exist with morphological and behavioral variations according to local conditions. The following are variants described as species related to *An. aquasalis: An. emilianus* Komp, *An. guarujaensis* Ramos, *An. guarauno* Anduze, *An. delta* Anduze (Knight & Stone 1977), and *An. deltaorinoquensis* Cova-Garcia, which are now regarded as synonyms (Faran 1980).

An. aquasalis has been examined for polymorphisms in proteins (Steiner et al. 1981, Vele 1993, Rangel et al. 1995), polytene chromosomes (Kitzmiller & Chow 1971, Moncada & Conn 1992), mitochondrial and ribosomal DNA (Conn et al. 1993) and external morphology (Cova-Garcia 1964, Faran 1980, Gabaldón & Escalante 1986, Linley et al. 1993b). Some authors suggested that *An. aquasalis* is a single entity, although Steiner et al. (1982) and Conn et al. (1993) suggested that *An. aquasalis* could represent a complex of species.

Cova-Garcia (1964) and Gabaldón and Escalante (1986) reported differences in egg morphology with light microscopy, and Moncada and Conn (1992) and Conn et al. (1993) recommended that the morphology of the eggs should be investigated by means of scanning electron microscopy (SEM) to determine the intraspecific variation in *An. aquasalis.* Accordingly, Linley et al. (1993b) described the eggs of *An. aquasalis* in populations from Brazil (Rio de Janeiro), Suriname (Paramaribo) and Venezuela (Moron and Caño Rico), representing the first SEM description of egg morphology of *An. aquasalis*. The objectives of the current investigation were twofold: (1) to provide comparative SEM descriptions of *An. aquasalis* eggs from two additional localities in Venezuela, Sinamaica, Zulia State (nonvector population) and Yaguaraparo, Sucre State (vector population), in the northwest and northeast, respectively; (2) to conduct statistical analyses of chorionic measurements in order to determine egg differences between the two populations, thus contributing more information about egg morphology of *An. aquasalis* in Venezuela.

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MATERIALS AND METHODS

Sources of specimens - Blood fed females of *An. aquasalis* were collected from animal bait in two localities of Venezuela during the dry season: Sinamaica (SI), Zulia State, 11°04'27"N 71°52'17"W and Yaguaraparo (YA), Sucre State, 10°34'27"N 62°49'37"W. Females were identified to species with the keys of Cova-Garcia and Sutil (1977) and Faran (1980).

Females were transferred individually to glass vials containing damp filter paper at the bottom to maintain a high relative humidity. Vials were covered with tulle, fixed with rubber bands and transported to the laboratory in a humidified cooler. Females were kept at 80% rh, 25-27°C until they laid their eggs.

Preparation for scanning electron microscopy - Eggs of 44 females from each locality were given 24 hr to embryonate after oviposition. Those from Zulia State were air-dried and individually placed in the required position on double-faced adhesive tape adhered to stubs, using an extra fine brush (000). Silver tincture was placed on the edges of the adhesive tape in order to ensure electric and thermal conductivity; and the samples were covered with platinum-palladium in an Eico Engineering Model 1B.2 sputter coater. The samples were observed using a Philips Model XL-20 scanning electron microscope operated between 10 and 15 kV. Two permanent records were kept: an image on the hard disc system and photographic records. Eggs from Sucre State were transported in small vials and fixed 24 hr after oviposition in alcoholic Bouin's (ethyl alcohol 80%, 150 ml; concentrated formalin, 60 ml; glacial acetic acid,15 ml and picric acid crystals, 1g). Complete dehydration was initiated with 80% ethanol using two changes (10 min each) to remove picric acid and increasing the ethanol concentration by 5% to 100% (10 min each). Subsequently, the samples were placed on stubs and coated as above.

Data collection and analyses - Length and width of eggs (ventral view), width of the deck at the anterior and posterior ends, and length of the floats and ribs on each float were measured from eggs (n=22 for each locality and female, at random) which were placed on slides in Canada balsam and phenol to maintain their shape. After hardening of the mounting medium, samples were covered with Hoyer's medium and observed under a Wild Model M11 light microscope equipped with an ocular micrometer.

Unstandardized values of all variables were taken into consideration since these attributes possess different dimensions (areas, ratios, and percentages). Evidence from previous studies on mosquitoes (Steinwascher 1984) indicated that the size of eggs may partially depend on the size of the female, which at the same time is influenced by the conditions during larval development (food supply, crowding, temperature). To avoid such influences, we used derived characters (percentages and ratios) in the discriminant analysis (Linley et al. 1993a).

Nine different attributes of the deck tubercles were studied (eggs=13 for each locality and female, at random) and five characters of the micropylar apparatus (eggs=9 for each locality and female, at random). Micrographs taken and attributes studied were similar to those previously made by Linley et al. (1993b) for eggs of *An. aquasalis*. SEM measurements were taken from digital images (stored on the hard disc) using the Philips Analysis Program 2.02.007.

Discriminant analyses were carried out to investigate differences between the SI and YA populations with Statgraphics Plus Version 6.1 Software (Statistical Graphics Corporation, Rockville, MD). The descriptive morphological terminology is based on Harbach and Knight (1980).

RESULTS

The general description of the eggs of *An. aquasalis* coincides with that indicated by Linley et al. (1993b); thus, emphasis is placed on the comparison between these two populations.

Comparisons of the measures between SI and YA populations - The mean size of eggs (length and width) was larger in YA than SI. Floats in YA occupied on average 82% of the length of the egg, while in SI they covered only 70.8%. Anterior tubercles were larger than middle and posterior tubercles (Table I), and were surrounded by smaller, more abundant tubercles (less than 1μm^2) (Fig. 1). Anterior tubercles from SI (Fig. 1a, b) were smaller than those from YA (Fig. 1c, d) (Table I). Middle tubercles were rounder as indicated by larger form factors (Table I). Posterior tubercles were irregular with few, surrounding smaller tubercles, some of which were connected to "large" tubercles by means of bridges (Fig. 2a, b). Middle and posterior tubercles from YA were larger than those from SI, and tubercle density had higher in eggs from SI than those from YA (Table I).

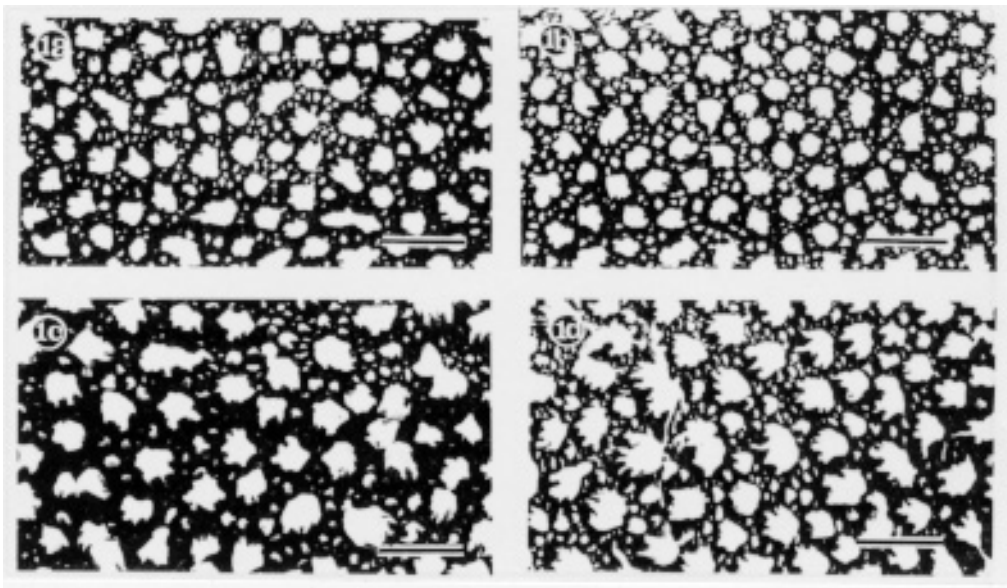
Discriminant analysis - The discriminant analysis of data on tubercles indicated that the anterior and posterior tubercles were the more important variables and provided excellent differentiation of the eggs from both localities. Summary of the discriminant analyses is indicated in Table II. The variants measured in the micropylar apparatus and the anterior/posterior deck width ratio and float attributes did not provide any significant differentiation of eggs from the two localities using discriminant analysis.

TABLE I

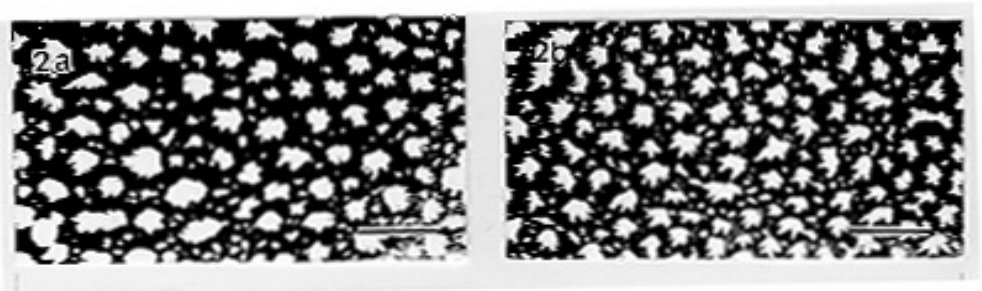
Attributes of the eggs of two populations of *Anopheles aquasalis* in Venezuela

	Mean (±SD) for population	
Attribute	Yaguaraparo	Sinamaica
Linear dimensions(n=22) [a]		
Egg length	434.91 ± 31.86	403.64 ± 30.48
Egg width	218.61 ± 26.03	182.46 ± 28.70
Lenght/width ratio	2.01 ± 0.20	2.28 ± 0.48
Anterior/posterior deck width ratio	1.26 ± 0.5	1.35 ± 0.21
Floats		
Mean float length (of the two floats)	356.07 ± 28.80	284.24 ± 28.51
Mean float length as % egg length	82.04 ± 6.01	70.81 ± 8.50
Mean number of ribs (of the two floats)	29.41 ± 4.60	21.41 ± 2.44
Mean float length/mean number of ribs	12.36 ± 2.00	13.39 ± 1.59
Tubercles (n=13)		
Anterior deck tubercle density [b]	65.16 ±19.10	92.12 ± 17.62
Mean anterior deck tubercle area [c]	1.48 ± 0.53	1.12 ± 0.22
Mean anterior deck tubercle form factor [d]	0.53 ± 0.10	0.56 ± 0.09
Middle deck tubercle density	81.96 ± 13.44	109.40 ± 18.53
Mean middle deck tubercle area	1.04 ± 0.33	0.65 ± 0.08
Mean middle deck tubercle form factor	0.59 ± 0.22	0.58 ± 0.07
Posterior deck tubercle density	82.77 ± 25.54	93.12 ± 21.08
Mean posterior deck tubercle area	1.11 ± 0.39	0.78 ± 0.20
Mean posterior deck tubercle form factor	0.50 ± 0.13	0.55 ± 0.11
Micropyle (n=9)		
Total area of micropylar apparatus	404.01 ± 78.40	409.66 ± 75.69
Collar area of micropylar apparatus	263.08 ± 51.43	265.42 ± 50.55
Disk area of micropylar apparatus	140.93 ± 30.90	144.46 ± 32.00
Disk area as % total apparatus area	34.83 ± 2.93	35.23 ± 4.25
Number of sectors in micropylar disk	6.56 ± 0.73	6.44 ± 0.73
Diameter micropyle	2.79 ± 0.63	3.26 ± 0.47

a: all linear measurements in µm; *b*: number in an area of 400 μm^2; *c*: all area measurements in μm^2; *d*: form factor = 4 xπx area/$perimeter^2$ (index of roundness); SD: standard deviation



Tubercles, anterior deck, different females. Fig. 1a, b: Sinamaica. Fig. 1c, d: Yaguaraparo. Bar = 10 µm.



Tubercles, posterior deck. Fig. 2a: Sinamaica. Fig. 2b: Yaguaraparo. Bar = 10 µm.

TABLE II
Summary of discriminant analysis

Discriminant analysis	Eigenvalues	Chi-square	df	p
Micropylar apparatus	0.3848038	4.2322601	6	0.64528
Ratio and percentages measured by optical microscopy	0.8521945	24.654846	4	0.67831
Tubercles	6.6260463	34.536688	14	0.00172

df: degree of freedom; p: probability

DISCUSSION

The discriminant analysis differentiated the two localities studied, supporting the idea of intraspecific variation, as observed by Steiner et al. (1981), Moncada and Conn (1992) and Rangel et al. (1995). Linley et al. (1993b) described the eggs of *An. aquasalis* from Venezuela (CR and MO) as having shorter floats, larger decks not tapered posteriorly, and a greater width in relation to egg length. Also, the tubercles of the anterior deck seen under high magnification differentiated populations. Our results agree with these observations. Attributes that contributed more to differentiation included the anterior and posterior tubercles, with the anterior tubercles contributing more to the differentiation of the two analyzed populations.

The populations of *An. aquasalis* analyzed here (SI and YA) and by Linley et al. (1993b) (MO and CR) (Fig. 3) span the Venezuelan coast from east to west. Intraspecific differences among the four populations are basically in the length and width of the egg, the floats, and anterior tubercles. This characteristics could be suggested as diagnostic markers in eggs taxonomy of *An. aquasalis* by SEM. However, these characters did not show evidence of morphological sharp discontinuities between Venezuelan samples of malaria vectors (YA) and nonvectors (MO, CR and SI) populations. This results and the more or less continuous coastal habitat of *An. aquasalis* and the apparent geographical continuity of the Venezuelan coast, (with no important geographical barriers since the Mio-Pliocene), provides biogeographical support to the hypothesis that in Venezuela *An. aquasalis* is a species with high intraspecific variation. The same could be said about *An. aquasalis* from Trinidad, which is a continental island sharing the same geological origin as the Venezuelan Cordillera de la Costa (coastal range). From its close proximity and recent isolation (change of sea level in the latest glaciation) one would not expect to find any major differences in the coastal Venezuelan and Trinidad

Fig. 3: map of Venezuela showing location of *Anopheles aquasalis* populations analyzed by scanning electron microscopy.

populations of *An. aquasalis*, such as those reported by Conn et al. (1993) among Venezuelan (MO, SF)/ Trinidadian (PS) and Brazilian (MJ, RJ) populations.

Also, our results and the reports of Conn et al. (1993) and Moncada and Conn (1992) on Sucre State populations (YA, Santa Fe and Guayana) compared with west and central populations (SI, MO, CR and Puerto Cabello) might suggest that Cova-Garcia's interpretation of the egg morphology (*An. emilianus*) was mistaken.

This investigation supports the hypothesis of intraspecific variation in *An. aquasalis* and demonstrates the usefulness of studying the morphology of anopheline eggs with SEM. Similar studies should be done throughout the range of the species to determine the continuity of structural variants and confirm the existence of morphological differences that would allow the characterization of different populations, including vector competence studies. It is recommended that future analyses take into consideration the effects of seasonal variation (wet and dry seasons) and different habitats (eggs from fresh and brackish water populations) on egg morphology. Finally, polymorphism in proteins and mitocondrial and ribosomal DNA studies must be done including SI, YA populations and compare with the anothers Venezuelan and Sudamerican localities.

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