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A DESCRIPTION AND MORPHOMETRIC COMPARISON OF EGGS FROM EIGHT GEOGRAPHIC POPULATIONS OF THE SOUTH AMERICAN MALARIA VECTOR *ANOPHELES (NYSSORHYNCHUS) NUNEZTOVARI* (DIPTERA: CULICIDAE)

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ABSTRACT. The egg of *Anopheles nuneztovari* is described from scanning electron micrographs of specimens collected from western Venezuela. Morphometric measurements of egg samples from 3 localities in Venezuela, one in Suriname, and 4 in Brazil are compared and relationships analyzed by multivariate statistics. Morphological characters were similar in 2 geographical groups, one Venezuelan and the other Amazonian, that were distinguishable on the basis of differences in size and density of tubercles in the anterior deck region and area of pores in the dorsal plastron. Eggs from western Brazil did not cluster with other Amazonian collections. The distinction of Venezuelan from Amazonian eggs of *An. nuneztovari* is consistent with chromosomal, ecologic, and molecular evidence for regional genetic differentiation in this species.

INTRODUCTION

Anopheles nuneztovari Gabaldón is systematically grouped in the Oswaldoi complex, Albimanus section, of the subgenus *Nyssorhynchus* and is considered to be closely related to *Anopheles rangeli* Gabaldón, Cova Garcia and Lopez and *Anopheles trinkae* Faran (Faran 1980). The distribution of *An. nuneztovari* covers an extensive area from eastern Panama, through Venezuela, Colombia, the Guyanas, and throughout much of the Amazon Basin (Faran 1980; Fig. 1). As a malaria transmitter, this species is known to be the major vector of *Plasmodium vivax* in areas of Venezuela and Colombia (Gabaldón 1981) and has been recovered infected with *Plasmodium falciparum* in Amazonian Brazil (Arruda et al. 1986). Large numbers of this species have been associated with hydroelectric projects at sites where *An. nuneztovari* has been suspected as a malaria vector (Panday 1977) and found infected with arboviruses (DeGallier et al. 1992).

Research completed more than 2 decades ago described ecologic and chromosomal differences between geographic races that led to the consideration that *An. nuneztovari* may represent a species complex. In Venezuela and Colombia, biting activity of *An. nuneztovari* peaks at 2300–2400 h in contrast to Amazonian representatives of the species, which quest for blood shortly after dusk (Elliott 1972). Chromosomally, Amazonian and Venezuelan/Colombian *An. nuneztovari* can be distinguished by a fixed inversion difference (Kitzmiller et al. 1973), and Conn et al. (1993) separated specimens from opposite sides of the Venezuelan Andes by the presence or absence of a chromocenter. *Anopheles (Nys.) goeldi* Rozeboom and Gabaldón from Amazonian Brazil was described as distinct from typical *An. nuneztovari* on the basis of differences in male genitalia (Rozeboom and Gabaldón 1941). Other genitalic differences separated *Anopheles dunhami* Causey, which was described to represent specimens from Tefé, Brazil, that were distinct from typical *An. nuneztovari* (Causey 1945). Although subsequent authors regarded *An. dunhami* and *An. goeldi* as synonyms of *An. nuneztovari,* the former taxon has recently been resurrected as a distinct species (Peyton 1993). Although we agree that *An. dunhami* is specifically distinct from *An. nuneztovari,* we do not concur with Peyton (1993) that *An. trinkae* is a junior synonym of *An. dunhami* and elsewhere will present morphologic, chromosomal, and molecular evidence to substantiate the specific status of *An. trinkae.*

Morphologic comparisons of adult characters have not revealed consistent differences that allow separation of all *An. nuneztovari* that differ karyotypically, although individuals from opposite sides of the Andes can be separated from one another, and Amazonian specimens from one Venezuelan cytotype, by properties of the male genitalia or female wing spots (Hribar 1994, 1995). Molecular studies have identified differences among geographic races of this species in sequences of nuclear genes (Fritz et al. 1994), in restriction fragment length polymorphisms of mtDNA (Conn et al., in preparation), and in allele frequencies at isoenzyme loci (Steiner et al. 1980; Fritz et al., in preparation). However, none of the molecular differences pro-

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vide unambiguous evidence that *An. nuneztovari* should be regarded as a species complex.

Scanning electron microscopy of anopheline eggs has yielded many morphologic characters that have proven useful for elucidating relationships among closely related species or geographic populations within species (Linley et al. 1993a, 1993b, 1995). During the course of field work in 3 countries, we preserved eggs for scanning electron microscopy from 8 collection sites (Fig. 1). Herein we present a formal ultrastructural description of the egg of *An. nuneztovari,* based on specimens collected near the type locality in Venezuela (Gabaldón 1940), and compare egg morphology among populations to assess the extent of geographic differentiation.

MATERIALS AND METHODS

Adult females were collected from human and animal baits at 4 sites in Brazil, 3 sites in Venezuela, and one site in Suriname (Fig. 1). Identifications of *An. nuneztovari* adults by morphologic keys (Faran 1980) were confirmed by examinations of polytene chromosomes of F_1 larvae, which further resolved populations into one of 3 cytotypes (Fig. 1; Kitzmiller et al. 1973, Conn 1990, Conn et al. 1993). Individual females oviposited in vials on damp filter paper. After 1 day to allow for embryonation, eggs were washed into alcoholic Bouin's solution. Egg batches from 3 to 7 females per collection site were available for study.

The individual egg batches from one locality at a time were dehydrated completely in an ethanol series and dried finally in a critical point drier. Single eggs were placed with a fine artist's brush in required positions on stubs (one stub per egg batch) and were then sputter-coated with gold. Examination of each series commenced immediately in a Hitachi S-510 scanning electron microscope and was completed as rapidly as possible because of the tendency of the eggs to collapse after a few hours.

For each locality, measurements were made from a set of micrographs compiled from 3 eggs of each individual female whose batch had been preserved. Exceptions were from site PR, where only 2 eggs of one female were sufficiently well preserved to be used, and from BL, where 4 eggs of 2 of the females were examined to increase the sample size derived from the 3 available egg batches. Morphologic features measured were as in Linley et al. (1993a) except that lobed tubercles, being absent in *An. nuneztovari,* were not recorded. Measurements from the micrographs were made with a digitizing tablet and SigmaScan software (Jandel Scientific, San Rafael, CA).

From the ventral views of the whole egg, 13 attributes (Table 1) were recorded or derived (percentages or ratios), and 9 attributes more were derived from micrographs (of different eggs) of the dorsal surface and micropyle (Table 2). The same acronyms as used and defined by Linley et al. (1993a) (see the Appendix) have been employed to name these attributes in the tables. Otherwise, the general descriptive terminology follows Harbach and Knight (1980) or, in some cases, Hinton (1968). The term "outer chorionic cell field" is defined by Linley (1989).

Variation in attributes among populations was examined by analyses of variance performed by the GLM procedure of SAS (SAS Institute Inc. 1985), and significant differences among means detected by the Ryan–Einot–Gabriel Welsch (REGWQ) *a posteriori* multiple comparisons procedure. Seven derived attributes were selected for multivariate analyses to detect levels of differentiation among geographic populations. Discriminant function analysis was performed with Statgraphics software (Statgraphics 1992) and principal components analysis with the procedure PRINCOMP of SAS (SAS Institute Inc. 1985) using default settings for this procedure.

RESULTS

Egg of *Anopheles nuneztovari* (Site SO = Tachira State, Venezuela)

Size: As in Table 1.

Color: Black.

Overall appearance: Boat-shaped in ventral and lateral views, frill more developed at anterior and posterior ends, which are both rounded (Figs. 2A, 2B). Lateral view shows ventral surface slightly concave, dorsal surface strongly curved, egg slightly deeper anteriorly than pos-

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Fig. 1. Locations of collection sites of *Anopheles nuneztovari,* indicated by circles and 2-letter codes, used in the present study. The gray area represents the known distribution of this species in South America from Faran (1980) with minor modifications based on more recent collection records. Venezuela (cytotype C): RS = Rio Socuavo (8°54′N, 72°38′W); Venezuela (cytotype B): EN = El Nula (7°21′N, 71°52′W), SO = Solano (7°32′N, 71°51′W); Suriname (cytotype A): VC = Victoria (5°08′N, 54°59′W); Brazil (cytotype A): CP = Capanema (1°17′S, 42°34′W), BL = Belem (1°25′S, 48°77′W), AB = Areia Branca (Porto Velho) (8°48′S, 63°54′W), PR = Puraquequara (Manaus) (3°06′S, 60°01′W).

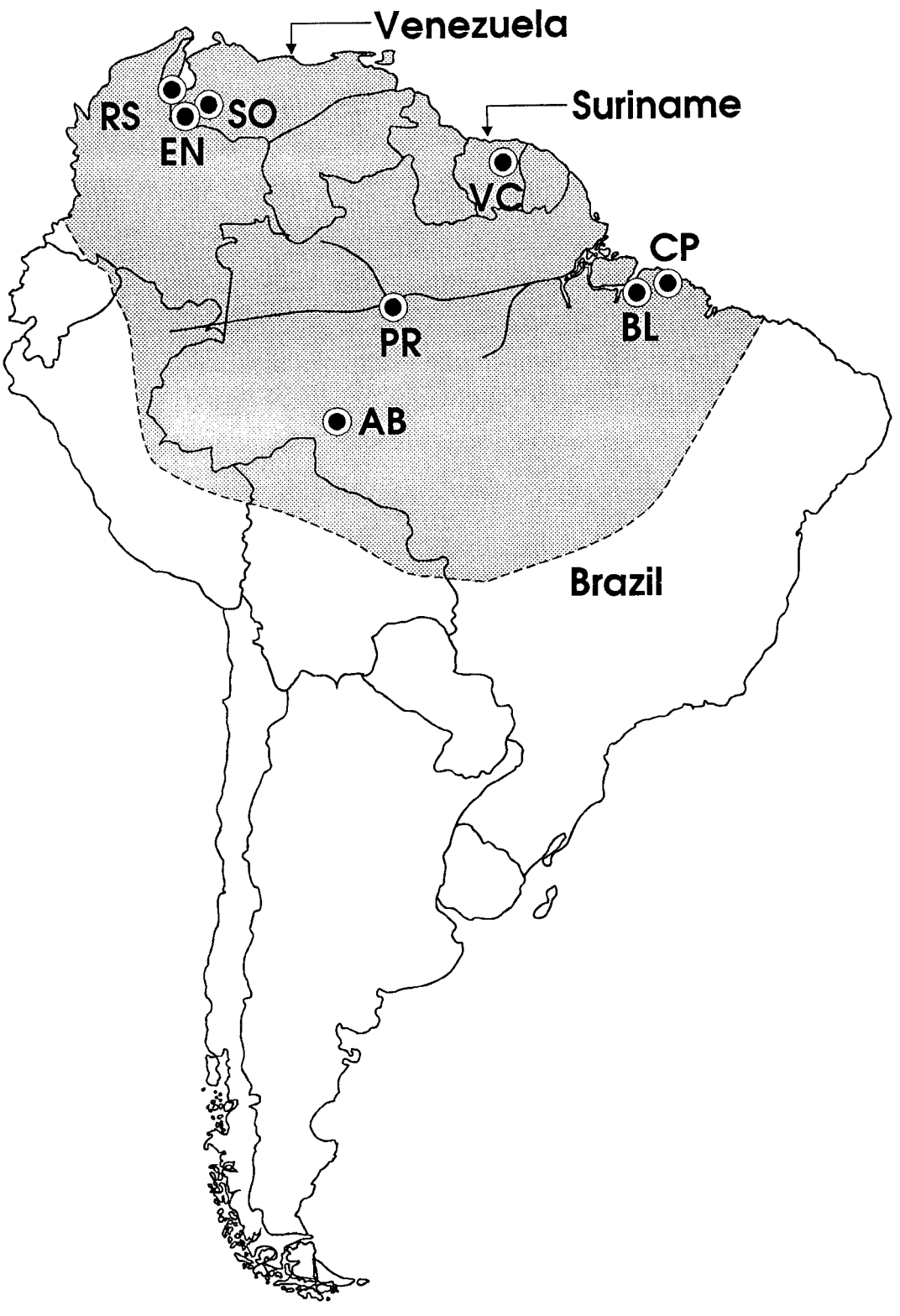
Venezuela
Suriname
RS
SO
EN
VC
CP
BL
PR
AB
Brazil

Table 1. Attributes[a] of eggs of *Anopheles nuneztovari* measured from 3 eggs of each of 5 females (populations EN, SO, VC, AB), of 7 females (population RS), of 6 females (populations PR,[b] CP), and of 3 females (population BL[c]) (mean ± SE[d] for population).

Attribute	EN (n = 15)	RS (n = 21)	SO (n = 15)	PR (n = 17)
Linear dimensions[e]				
Egglen	428.3 ± 5.9a	420.7 ± 3.9a	425.0 ± 7.5a	432.1 ± 6.8a
Eggwid	169.3 ± 3.4b	179.6 ± 1.6ab	175.5 ± 2.8ab	185.7 ± 3.9a
Lenwidrat	2.54 ± 0.05a	2.35 ± 0.03b	2.43 ± 0.5ab	2.34 ± 0.04b
Float attributes				
Mnfltlen	283.7 ± 5.5ab	293.5 ± 3.0ab	289.6 ± 6.5ab	298.8 ± 6.3a
Fltpcn	66.2 ± 0.8cd	70.0 ± 0.4a	68.1 ± 0.7abc	69.0 ± 0.9ab
Mnribs	26.5 ± 0.5ab	26.5 ± 0.3ab	27.1 ± 0.4a	27.4 ± 0.4a
Fltlenprib	10.7 ± 0.2a	11.1 ± 0.2a	10.7 ± 0.2a	10.9 ± 0.1a
Deck dimensions[f]				
Arwhlegg	558.71 ± 17.87a	563.07 ± 8.10a	561.30 ± 15.9a	590.81 ± 17.83a
Artotdk	335.03 ± 12.22ab	281.64 ± 9.00c	292.27 ± 12.9bc	303.13 ± 14.37abc
Totdkpcn	60.03 ± 1.2a	49.81 ± 1.0bc	51.88 ± 1.2bc	51.03 ± 1.4bc
Anterior deck tubercles				
	(n = 15)	(n = 21)	(n = 15)	(n = 17)
Anttbden[g]	41.3 ± 0.7e	14.5 ± 0.5de	16.6 ± 0.9cde	21.1 ± 0.7a
	(n = 10)	(n = 14)	(n = 10)	(n = 12)
Mnanttbar	7.81 ± 0.44b	9.3 ± 0.32a	8.26 ± 0.38ab	5.98 ± 0.22c
Mnanttbfm[h]	0.592 ± 0.013a	0.575 ± 0.023a	0.628 ± 0.024a	0.570 ± 0.009a

[a] Attributes defined in the Appendix.
[b] Only 2 eggs measured from one of the females.
[c] Four eggs measured from 2 of the females.
[d] Means within rows followed by the same letter do not differ significantly ($P > 0.05$ by REGWQ of SAS).
[e] All linear measurements in μm.
[f] All area measurements in μm²/100.
[g] Number in an area of 400 μm².
[h] Form factor = 4π(area/perimeter²).

Table 2. Attributes[a] of dorsal surface and micropyle of eggs of *Anopheles nuneztovari*. Measurements derived as indicated by superscripts. All area measurements μm² (mean ± SE[b] for population).

Attribute	EN	RS	SO
Dorsal plastron cells[c]	(n = 25)	(n = 35)	(n = 25)
Celardoplas	321.9 ± 15.5bcd	345.9 ± 7.4ab	287.2 ± 8.4d
Nopordoplas	7.9 ± 0.6b	8.6 ± 0.5ab	8.5 ± 0.4ab
Porardoplas	24.4 ± 2.2c	31.7 ± 1.7c	32.2 ± 2.5c
Porarpcndoplas	7.4 ± 0.5d	9.0 ± 0.4cd	11.0 ± 0.7bc
Micropyle[d]	(n = 10)	(n = 14)	(n = 13)
Totarmic	745.8 ± 35.4a	687.6 ± 23.4a	753.5 ± 20.9a
Colarmic	511.4 ± 31.5ab	481.5 ± 20.4ab	511.9 ± 19.4ab
Dskarmic	234.5 ± 9.2a	206.2 ± 5.7ab	241.6 ± 7.2a
Dskarpcn	31.8 ± 1.3a	30.2 ± 0.9a	32.2 ± 1.0a
Nosect	7.6 ± 0.2ab	6.8 ± 0.3ab	7.2 ± 0.3ab

[a] Attributes defined in the Appendix.
[b] Means within rows followed by the same letter do not differ significantly ($P > 0.05$ by REGWQ of SAS).
[c] Five cells measured from one egg of each female.
[d] One micropyle measured from each of 2 eggs from each female.

Table 1. Extended.

BL (n = 11)	CP (n = 18)	VC (n = 15)	AB (n = 15)
410.8 ± 5.2a	422.8 ± 6.9a	426.1 ± 5.8a	424.4 ± 5.8a
175.2 ± 2.7ab	182.5 ± 3.2a	175.0 ± 2.3ab	174.6 ± 2.3ab
2.35 ± 0.03b	2.33 ± 0.04b	2.44 ± 0.04ab	2.44 ± 0.04ab
277.1 ± 3.1bc	282.1 ± 5.1ab	277.8 ± 5.0abc	260.3 ± 3.3c
68.0 ± 0.7abcd	67.0 ± 0.3bcd	65.2 ± 0.6d	61.4 ± 0.6e
27.1 ± 1.3a	26.1 ± 0.6ab	24.5 ± 0.5bc	23.4 ± 0.5c
10.5 ± 0.5a	10.9 ± 0.2a	11.4 ± 0.2a	11.2 ± 0.3a
543.16 ± 9.35a	567.64 ± 14.36a	555.64 ± 12.39a	561.29 ± 10.97a
262.37 ± 11.71c	277.88 ± 9.49c	300.74 ± 11.49abc	342.11 ± 9.18a
48.09 ± 1.5c	48.94 ± 1.2bc	54.00 ± 1.3b	60.94 ± 2.00a
(n = 9)	(n = 18)	(n = 15)	(n = 15)
19.7 ± 1.7ab	22.4 ± 0.6a	19.5 ± 0.7abc	17.3 ± 0.4bcd
(n = 6)	(n = 12)	(n = 10)	(n = 9)
7.24 ± 0.65b	5.47 ± 0.19c	5.87 ± 0.31c	7.46 ± 0.19b
0.612 ± 0.015a	0.581 ± 0.018a	0.602 ± 0.017a	0.656 ± 0.022a

teriorly, float quite large and deep, midlaterally positioned (Fig. 2B).

Dorsal (lower) and lateral surfaces: Entire dorsal and lateral surfaces covered with mostly hexagonal, occasionally pentagonal, plastron-type cells (Hinton 1968), length 19.9–31.0 μm ($\bar{x}$ = 24.2 ± 0.5 μm, n = 30), width 12.5–21.7 μm ($\bar{x}$ = 16.5 ± 0.5 μm), area as in Table 2, cell boundaries indistinct (Figs. 3E, 3F, and 4). Individual cell fields with short pillars (mostly invisible), supporting buttonlike tubercles connected by a continuous thin sheet except where perforated by pores in central, raised, blisterlike area (Figs. 3E, 3F, and 4). Pores more or less round, occasionally oval, central ones coalesced to form larger, more complex openings (Figs. 3F and 4), diameter (widest point) 0.8–6.1 μm ($\bar{x}$ = 2.8 ± 0.2 μm, n = 60), other attributes as in

Table 2. Extended.

PR	BL	CP	VC	AB
(n = 25)	(n = 15)	(n = 30)	(n = 25)	(n = 25)
347.9 ± 8.9ab	362.4 ± 20.8a	340.5 ± 9.0abc	313.0 ± 9.6bcd	297.0 ± 12.3cd
10.4 ± 0.6ab	10.3 ± 0.9ab	10.3 ± 0.9ab	11.2 ± 0.8a	11.0 ± 0.5a
68.2 ± 3.7a	50.5 ± 5.3b	44.8 ± 2.7b	56.7 ± 3.0b	55.7 ± 4.6b
19.5 ± 0.8a	13.7 ± 0.8b	13.1 ± 0.6b	19.7 ± 0.6a	19.0 ± 1.5a
(n = 11)	(n = 8)	(n = 12)	(n = 13)	(n = 10)
732.4 ± 26.6a	728.0 ± 11.3a	639.9 ± 24.0a	673.9 ± 28.8a	726.0 ± 27.4a
525.4 ± 20.9ab	517.2 ± 17.0ab	431.0 ± 18.1b	479.9 ± 22.4ab	542.5 ± 22.7a
207.0 ± 10.4ab	210.8 ± 8.9ab	209.9 ± 8.9ab	194.0 ± 11.6b	183.5 ± 8.6b
28.3 ± 1.0ab	29.1 ± 1.5ab	32.7 ± 0.9a	28.8 ± 1.3ab	25.3 ± 1.0ab
7.6 ± 0.2a	6.8 ± 0.2ab	7.3 ± 0.2ab	7.7 ± 0.4a	6.5 ± 0.3b

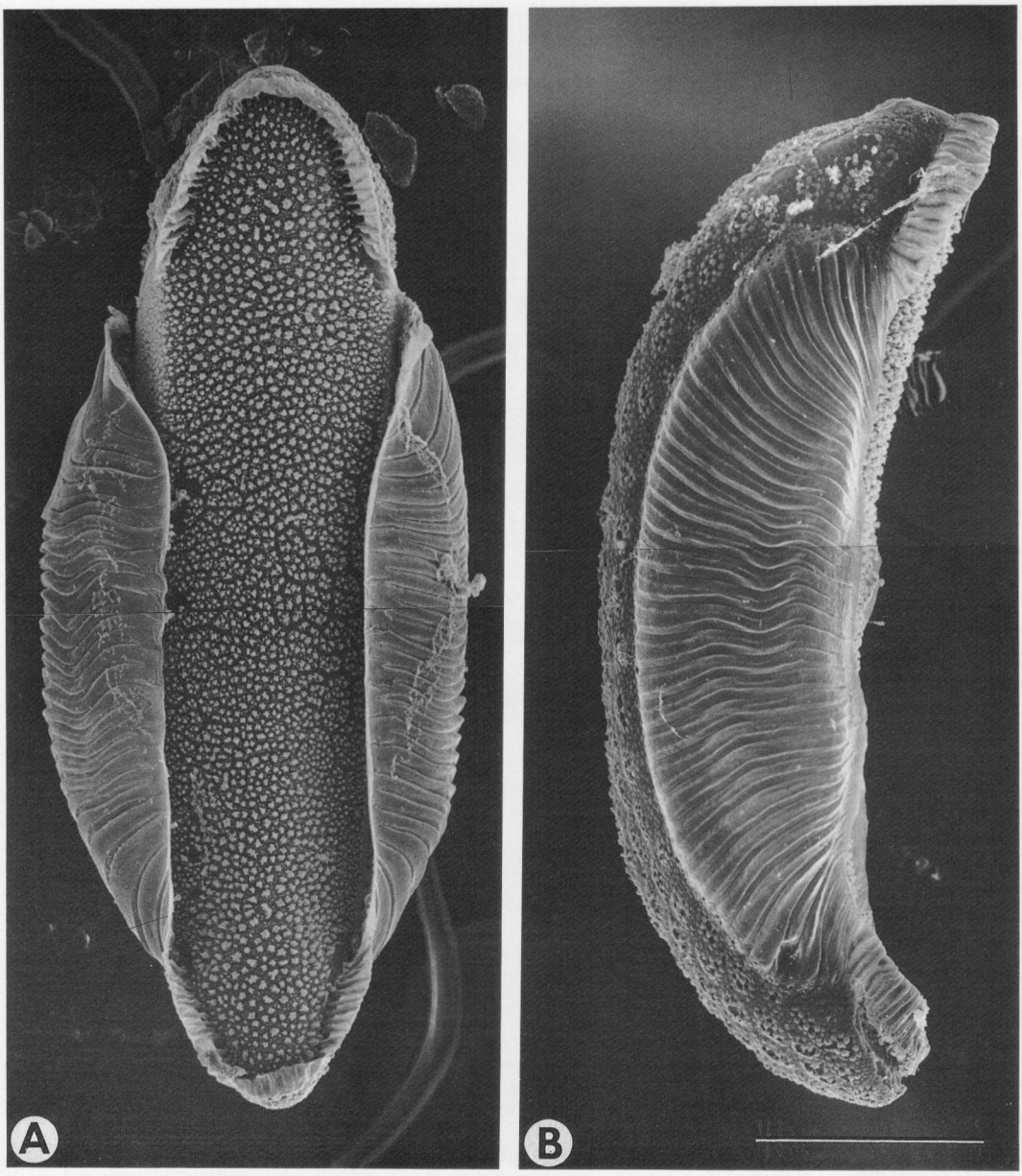

Fig. 2. Eggs of *Anopheles nuneztovari* from site SO. A. Entire egg, ventral (upper) view, anterior end at top. B. Entire egg, lateral view, ventral surface at left, anterior end at top. Scale = 100 μm.

Table 2. Float fairly long, about two-thirds length of egg, other measurements as in Table 1.

Ventral (upper) surface: Deck large, continuous, widest at anterior end, narrowed in middle (Fig. 2A), frill more developed anteriorly and posteriorly (Figs. 2, and 5A, 5D). Outer chorionic cell boundaries not visible, deck surface entirely covered by large, prominent, irregularly formed tubercles, with interspersed smaller ones (Figs. 2A and 3A–3C). Tubercles larger in area on anterior and posterior deck regions than in middle (Figs. 3A–3C), structurally complex, with buttressed sides rising to domed, smooth tops (Fig. 3D). Ventral surface devoid of plastron-type cells and lobed tubercles.

Anterior end, micropyle: Elevated frill slight-

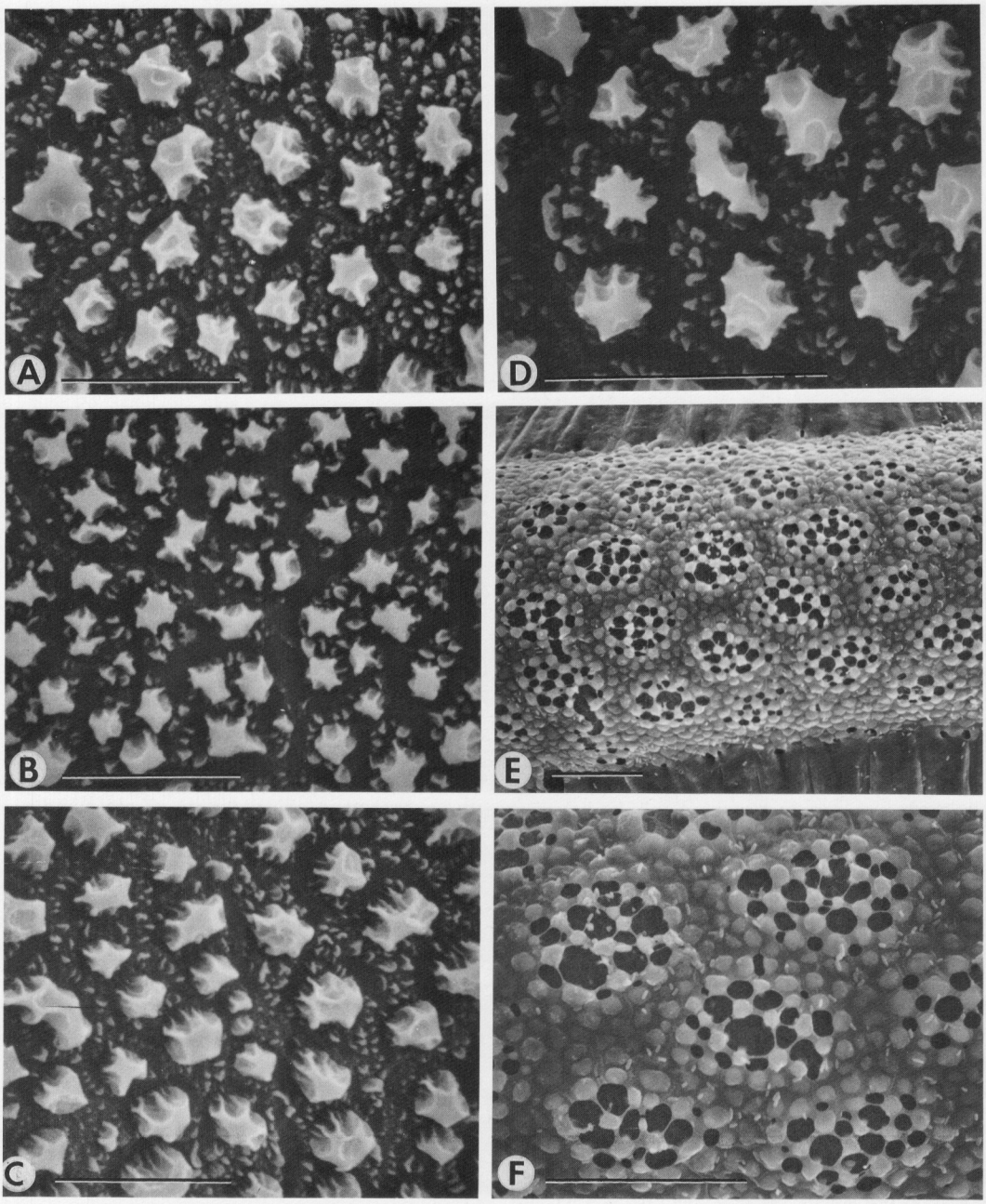

Fig. 3. Eggs of *Anopheles nuneztovari* from site SO. A. Outer chorionic tubercles, middle of anterior deck. B. Tubercles, middle deck. C. Tubercles, posterior deck. D. Detail, anterior deck tubercles. E. Chorionic cells (plastron), middle of dorsal surface. F. Chorionic cells detail, middle of dorsal surface. Scale = 10 μm (A–D); = 20 μm (E, F).

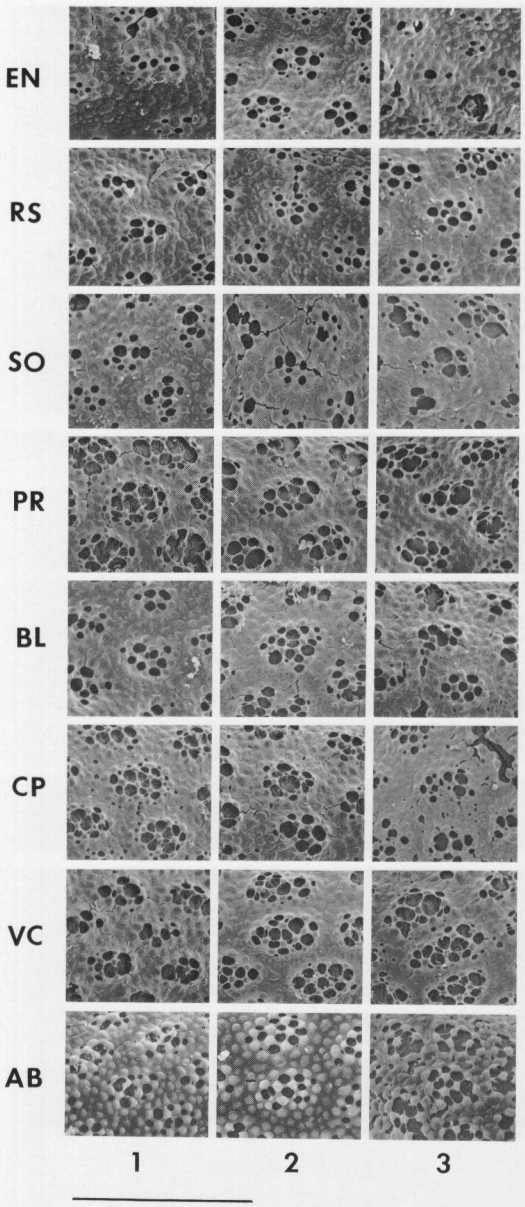

Fig. 4. Cells of the dorsal (lower) plastron of eggs of *Anopheles nuneztovari* from 8 geographic populations (labelled at left); one egg from each of 3 females (numbered at bottom) per site. Scale = 50 μm.

ly flared outwardly in some views (Figs. 5A, 5B), inside surface with pronounced ribs (Fig. 5C), tubercles appearing uniform in size across width of deck (Fig. 5A). End-on view shows micropylar collar separated from lower frill margin (Fig. 5B), plastron-type cells immediately around micropylar apparatus not so raised centrally, pores fewer. Outer margin of micropylar collar irregular, but inner margin tending to be heptagonal. Collar surface smooth, inner margins with indentations separated by radial micropylar rays, but many with scarcely any indentations or a faint ring visible at origin of rays (Fig. 5F). Disk surface slightly rough, micropylar orifice a depression within a central mound (Fig. 5F). Quantitative attributes of micropylar apparatus are given in Table 2.

Posterior end: Rounded, frill well developed, slightly flared outwardly (Figs. 5D, 5E), little change in form of dorsal plastron cells approaching posterior end, except pores reduced in number at end of egg (Fig. 5E).

General Visual Appraisal and Brief Comparison of Attributes

From the low-power ventral views of 4 eggs from each locality (Figs. 6 and 7), it is apparent that, as represented by 8 geographic populations covering a vast area, the eggs of this species are quite uniform morphologically, certainly with respect to general form and appearance and attributes visible at low magnification. In the EN population the floats appear to be less substantial, and the eggs consequently seem rather larger in relation to width (Fig. 6). The deck area seems proportionately larger in EN and AB eggs (Figs. 6 and 7).

Despite the superficial impression of similarity, a systematic consideration of measured attributes reveals many significant differences among populations (Table 1 and 2). None of the 8 groups differed in egg length, but egg width of EN was significantly smaller than that of PR or CP. Hence, the mean length/width ratio for EN was significantly greater than the ratios for Amazonian populations PR, BL, and CP, as well as for Venezuelan RS (Table 1). Significant differences in float lengths were detected between the longest (PR) and shortest (AB), and among more populations for float length as a percentage of total egg length (Table 1). The mean number of ribs in floats was significantly higher for SO, PR, and BL than for VC or AB, but float length per rib did not differ significantly among sites (Table 1).

Although there were no significant differences in whole egg area among groups, deck areas of EN and AB were significantly greater than those of RS, SO, BL, and CP, and deck area as a proportion of total area was greater for EN and AB than for all other groups (Table 1), confirming visual impressions from low-power micrographs (Figs. 6 and 7).

Substantial differences among populations were revealed from measurements of anterior

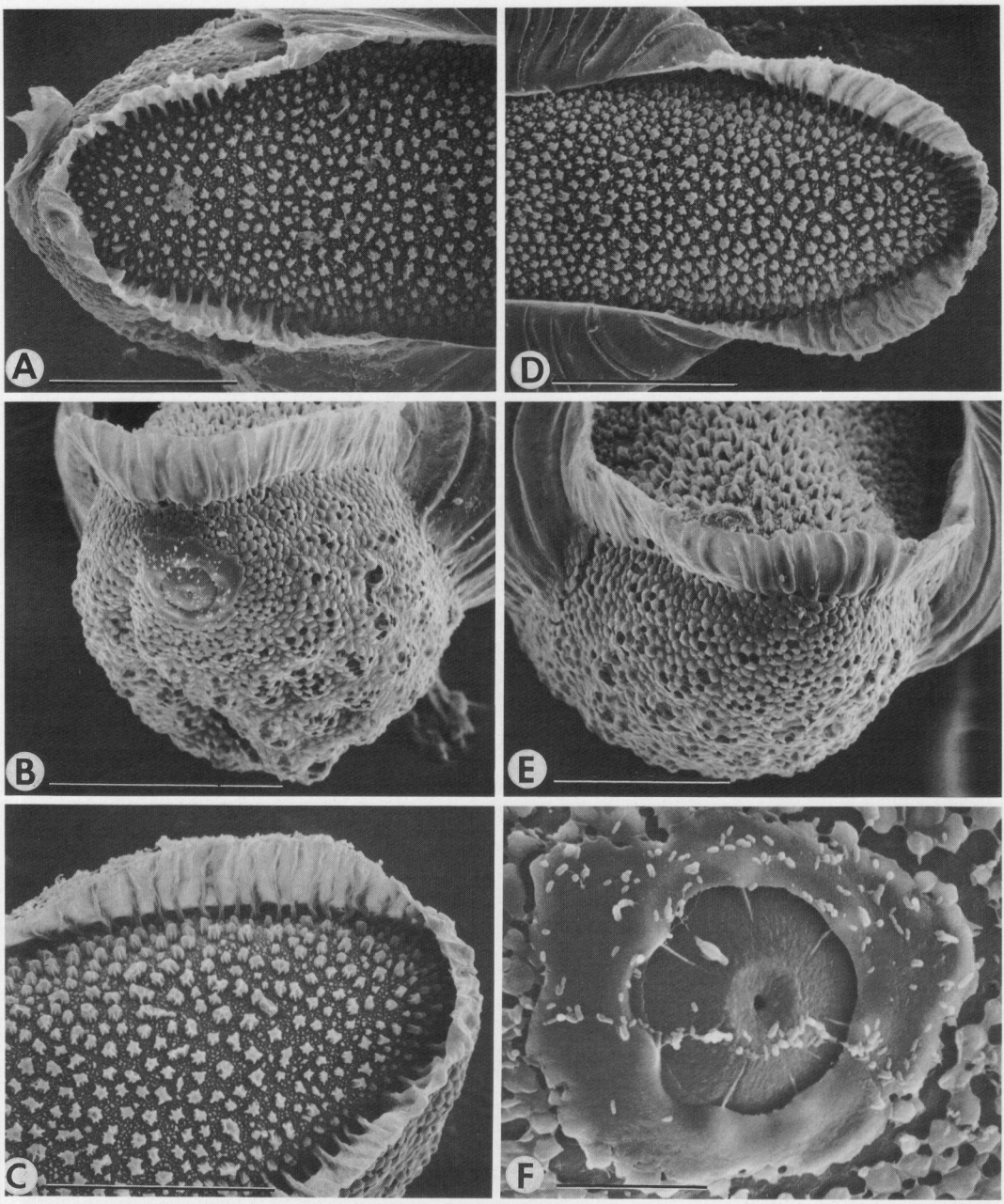

Fig. 5. Eggs of *Anopheles nuneztovari* from site SO. A. Anterior end, ventral (upper) surface. B. Anterior end, end-on view. C. Anterior end, ventral surface, showing inner side of frill. D. Posterior end, ventral surface. E. Posterior end, end-on view. F. Detail of micropylar apparatus. Oblong splotches on micropyle in B and F are contaminants, probably bacteria. Scale = 50 μm (A–E); = 10 μm (F).

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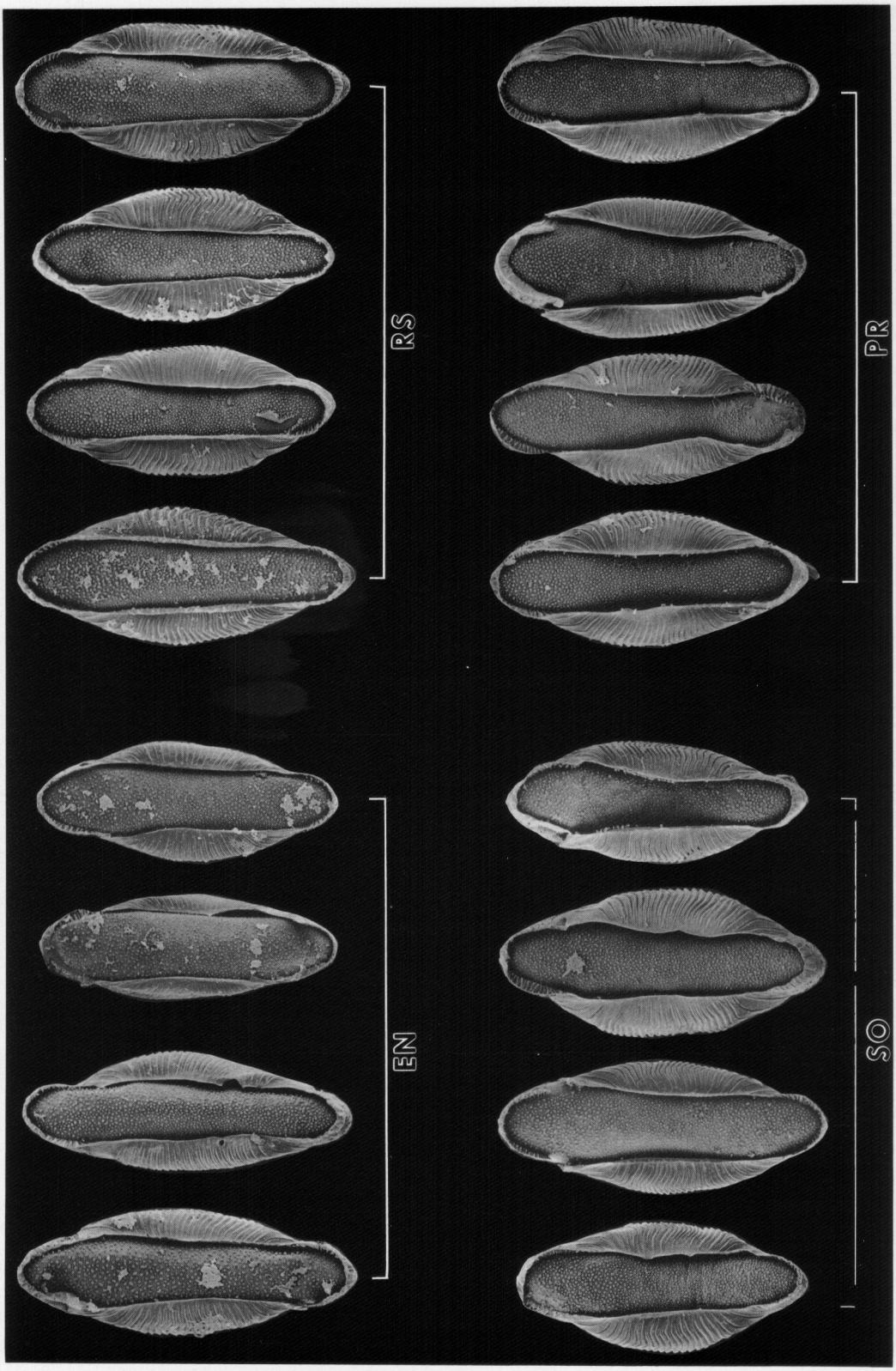
Fig. 6. Eggs of *Anopheles nuneztovari* from sites EN, RS, SO, and PR, ventral view, one egg from each of 4 of the females studied. Scale = 200 μm.

Fig. 7. Eggs of *Anopheles nuneztovari* from sites BL, CP, VC, and AB, ventral view, one egg from each of 4 of the females studied, except for BL where 2 of the eggs come from one female. Scale as in Fig. 6.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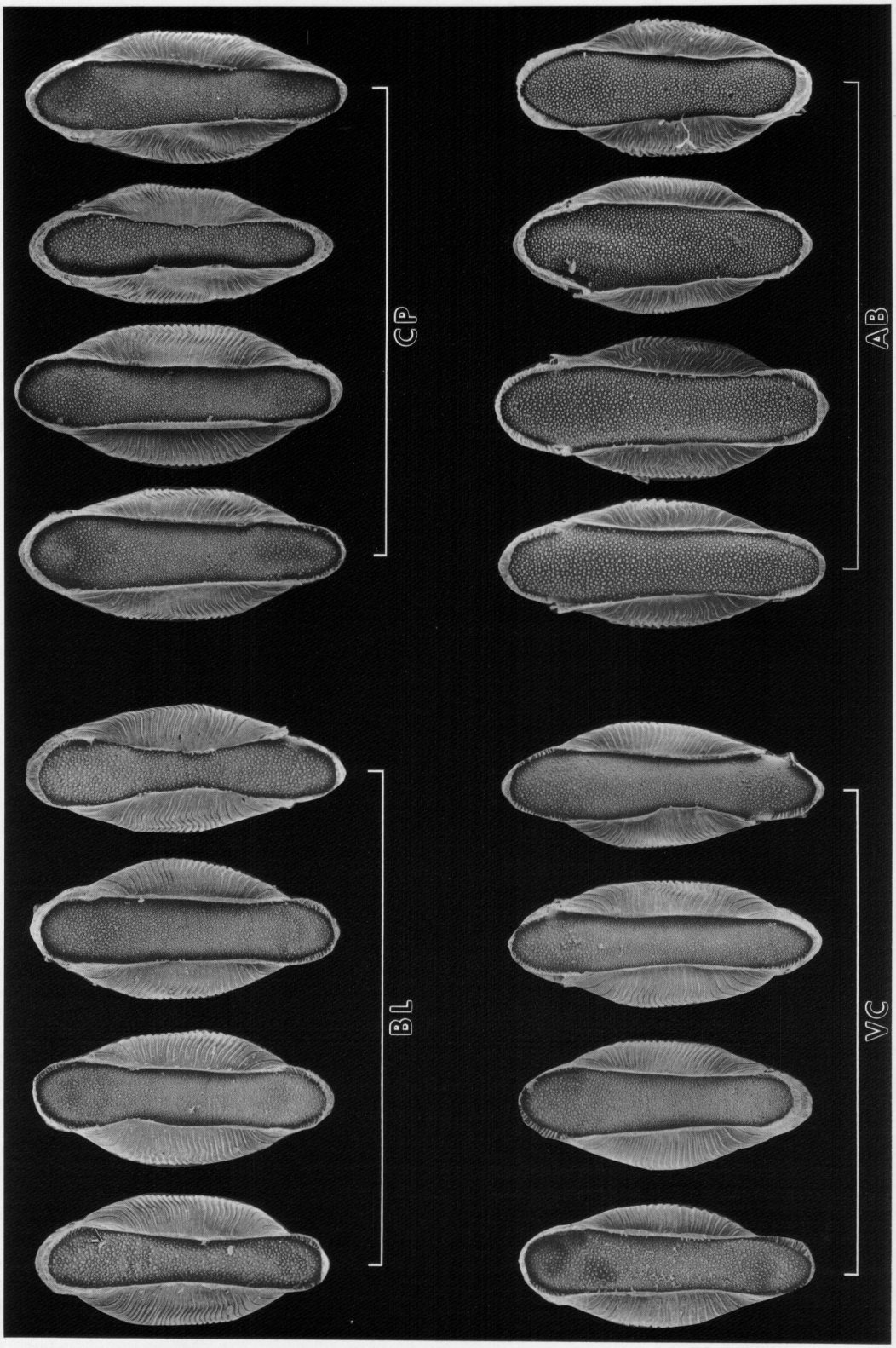
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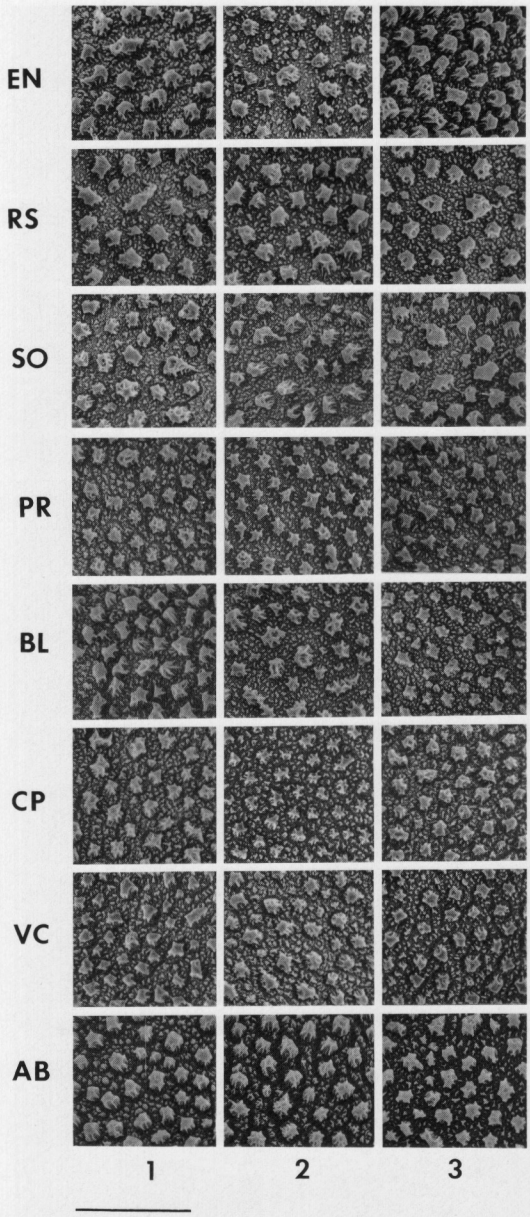

Fig. 8. Tubercles of the middle anterior deck region of eggs of *Anopheles nuneztovari* from 8 collection sites, one egg from each of 3 females (numbered at bottom). Scale = 20 μm.

deck tubercles, whose attributes were consistent within broad geographic regions (Table 1 and Fig. 8). Tubercle densities in the Venezuelan populations EN, RS, and SO were lower, and tubercle areas greater, in most cases significantly so, than in the Amazonian samples PR, BL, CP, VC, and AB (Table 1). There was no evidence of shape differences of tubercles among groups, as indicated by the form factor variable Mnanttbfm.

On the dorsal surface, differences in the area of plastron cells were detected among populations (Table 2), but these differences were not related to geographic zones. The mean number of pores in plastron cells was lower in Venezuelan compared to Amazonian *An. nuneztovari,* albeit most differences were not statistically significant, and total pore area was significantly less in all Venezuelan compared to Amazonian samples (Table 2). As indicated in terms of total pore area as a percent of cell area, plastrons in Venezuelan populations were less open in structure, usually significantly so, than from other sites, as apparent from the micrographs (Fig. 4).

At the extreme anterior end, the micropylar apparatus was relatively consistent among groups; significant differences were rare in paired comparisons (Table 2), with no indications of a geographic pattern in variability.

Principal Components

Principal components are useful as a means of identifying the combinations of attributes wherein the most important contrasts and differences reside, even though the simple structure of *An. nuneztovari* eggs limits the number of usable characters. We have followed the precepts given previously (Linley et al. 1993a, 1993b, 1995) in selecting for analysis variables that are ratios or percentages (rather than absolute measures), or are not related to egg size. Seven attributes derived from micrographs of the ventral aspect of the eggs were used: lenwidrat, fltpcn, fltlenprib, totdkpcn, anttbden, mnanttbar, and mnanttbfm (see the Appendix). When tested by regression analyses, the last 2, which were direct measures, were not related to egg length or total area.

Of the 7 components derived from the standardized (zero mean, unit variance) variables, the first 4 accounted for 79.9% of the variation and the first 2 for 51.9% (Table 3). Component 1 carried a heavy positive weighting (Fig. 9A)for area of total deck as a percentage of whole egg area (totdkpcn), with somewhat lower ones for egg length/width ratio (lenwidrat) and the ratio of float length to number of ribs (fltlenprib). The attribute float length as a percentage of total egg length (fltpcn) had the largest negative eigenvector in component one (Table 3). Geographically separated populations of *An. nuneztovari* were not clearly distinguished by the first principal component, with many points clustering on either side of the *x*-axis (Fig. 9B).

Component 2 accounted for slightly less vari-

Table 3. Partial tabulation of principal components analysis of 7 attributes of *Anopheles nuneztovari* eggs.

Principal component	Eigenvalue	% of variance explained	Attribute[a]						
			Lenwidrat	Fltpcn	Fltlenprib	Totdkpcn	Anttbden	Mnanttbar	Mnanttbfm
1	2.078	29.7	0.461	−0.496	0.388	0.531	−0.277	−0.091	0.151
2	1.555	22.2	0.099	0.173	−0.255	0.068	−0.628	0.702	−0.043
3	1.181	16.9	−0.397	−0.219	−0.315	0.197	0.073	0.091	0.801
4	0.775	11.1	−0.260	0.521	0.691	0.265	0.125	0.256	0.179

[a] Attributes defined in the Appendix.

ance than component 1 (Table 3). Two attributes contributed strongly to weightings on this axis: anterior deck tubercle area (mnanttbar) and anterior deck tubercle density (anttbden) (Fig. 9A). Venezuelan samples were distinguishable more clearly from most Amazonian sites on this axis (Fig. 9B) because of their larger, rounder tubercles and lower tubercle densities (Table 1). Eggs from site AB in western Brazil were less clearly differentiated than other Amazonian populations by component 2.

Components 3 and 4 accounted for, respectively, 16.9 and 11.1% of total variance. The heaviest weightings in these components were mostly attributable to ratios (lenwidrat and fltlenprib) and percentages (fltpcn) that related egg lengths and widths, although anterior deck tubercle form factor (mnanttbfm) yielded a heavy positive weighting in component 3 (Table 3).

Discriminant Functions

When discriminant analysis was applied to the same 7 variables to facilitate the separation of groups, the first 3 functions proved to be significant, and the first 2 captured 87.4% of the differences among populations (Table 4). The 3 Venezuelan collections and Amazonian site AB were centered on the positive side of the first function, whereas the 4 other Amazonian sites were negative (Fig. 10). For function 2, an equal number of sites appeared on either side of the centerline, but site AB was far removed from the others. Examining the centroids for each site, interpopulational differences are found primarily between the Venezuelan groups (EN, SO, RS), site AB in western Amazonia, and the remaining, more central and easterly Amazonian sites (VC, PR, CP, BL).

DISCUSSION

Although relatively simple in structure, the egg of *An. nuneztovari* differs quite clearly at the stereomicroscopic level from *An. rangeli* and *An. trinkae,* the 2 species with which it is associated in the *An. nuneztovari* "sister group" (Faran 1980). *Anopheles rangeli* and *An. trinkae* are easily separated from one another in the egg stage (*An trinkae* referred to as *An. dunhami* in Linley and Lounibos 1993), and the distinct anterior crown present on both of them, as well as the structure of the very large floats, immediately set them apart from *An. nuneztovari.* Eggs of the closely related *An. dunhami* have neither the anterior crowns found in *An. rangeli* and *An. trinkae* nor the raised, blisterlike pores found in the dorsal plastron area of *An. nuneztovari* (Fig. 4; Lounibos et al., in preparation). Distinctions from other species of the Oswaldoi complex of *Nyssorhynchus* may be more difficult to identify based on the line drawings and low-power micrographs of Rozeboom (1938, 1942), Kumm (1941), and Causey et al. (1944). However, the floats of *An. nuneztovari* appear generally to be the least developed of all the species with which there might be confusion, and they are not positioned so ventrally, leaving the deck relatively more open.

Eggs from site SO were chosen for detailed descriptions because this site is geographically closest to San Carlos, Cojedes State, Venezuela, the type locality of *An. nuneztovari* (Gabaldón

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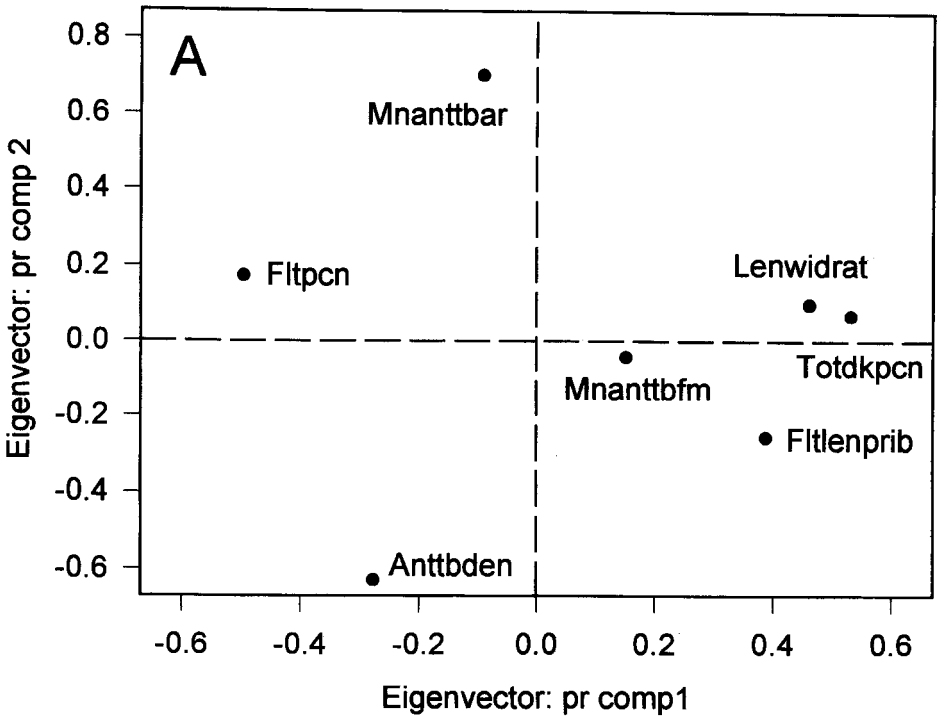
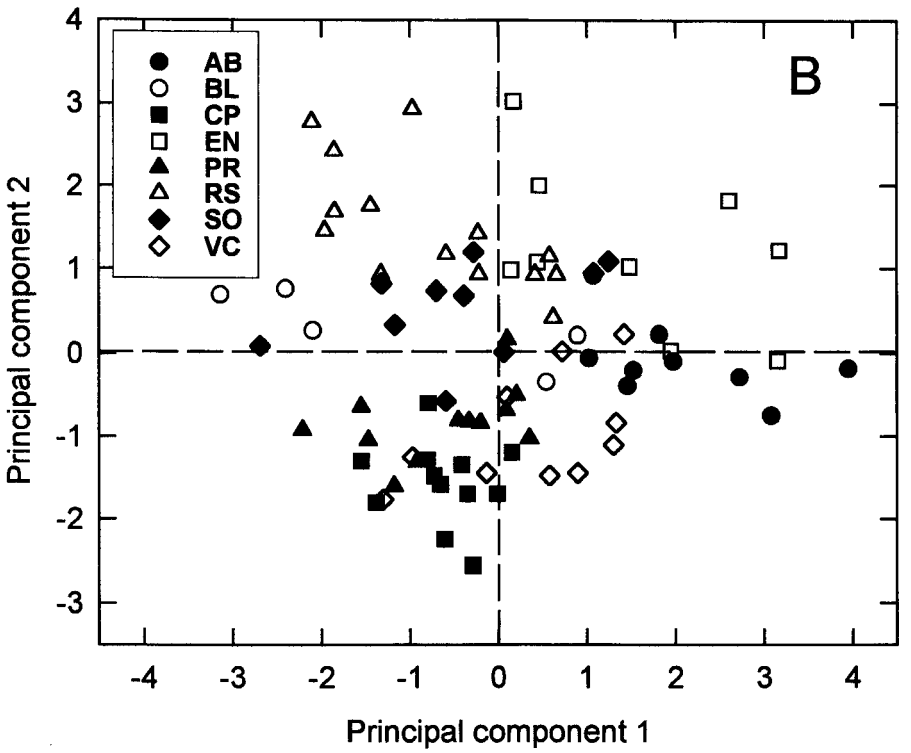
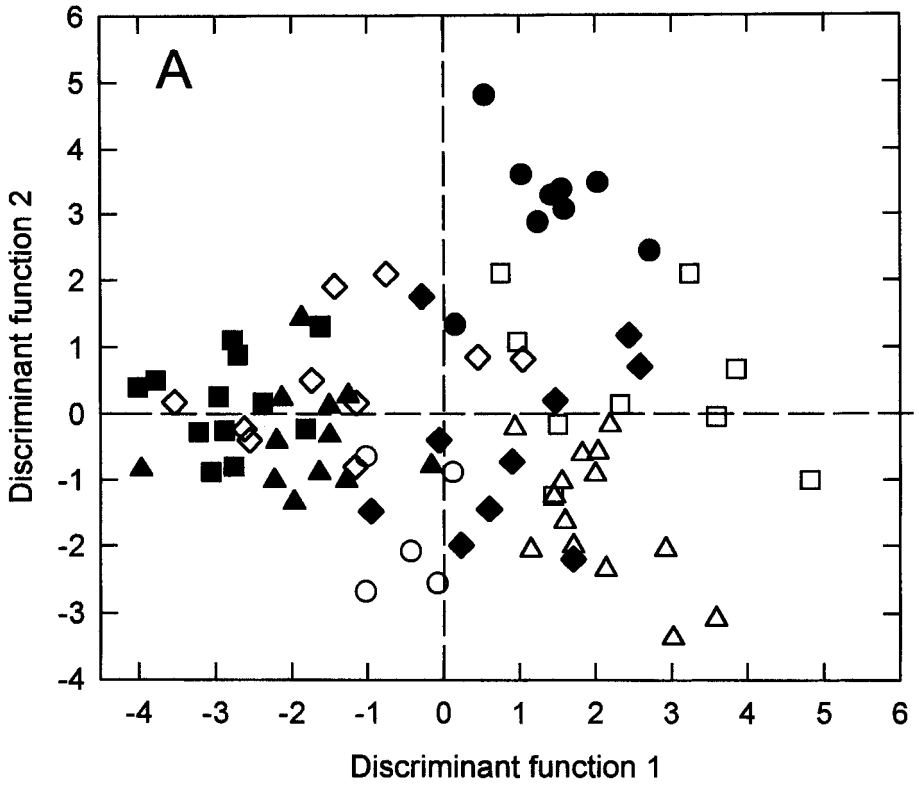
Fig. 9. A. Plot of the eigenvectors of the first 2 principal components based on 7 attributes of the eggs of *Anopheles nuneztovari.* B. Plot of the individual components of 81 eggs from 8 collection sites.

Fig. 10. A. Plot of the first 2 individual discriminant functions, based on 7 attributes, of 81 eggs of *Anopheles nuneztovari* from 8 collection sites. B. Group centroids, by collection site, of the data plotted in A.


A
Mnanttbar
Fltpcn
Lenwidrat
Totdkpcn
Mnanttbfm
Fltlenprib
Anttbden
Eigenvector: pr comp 2
Eigenvector: pr comp1
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.6 -0.4 -0.2 0.0 0.2 0.4 0.6


B
AB
BL
CP
EN
PR
RS
SO
VC
Principal component 2
Principal component 1
4
3
2
1
0
-1
-2
-3
-4 -3 -2 -1 0 1 2 3 4


A
Discriminant function 2
Discriminant function 1
6
5
4
3
2
1
0
-1
-2
-3
-4
-4 -3 -2 -1 0 1 2 3 4 5 6

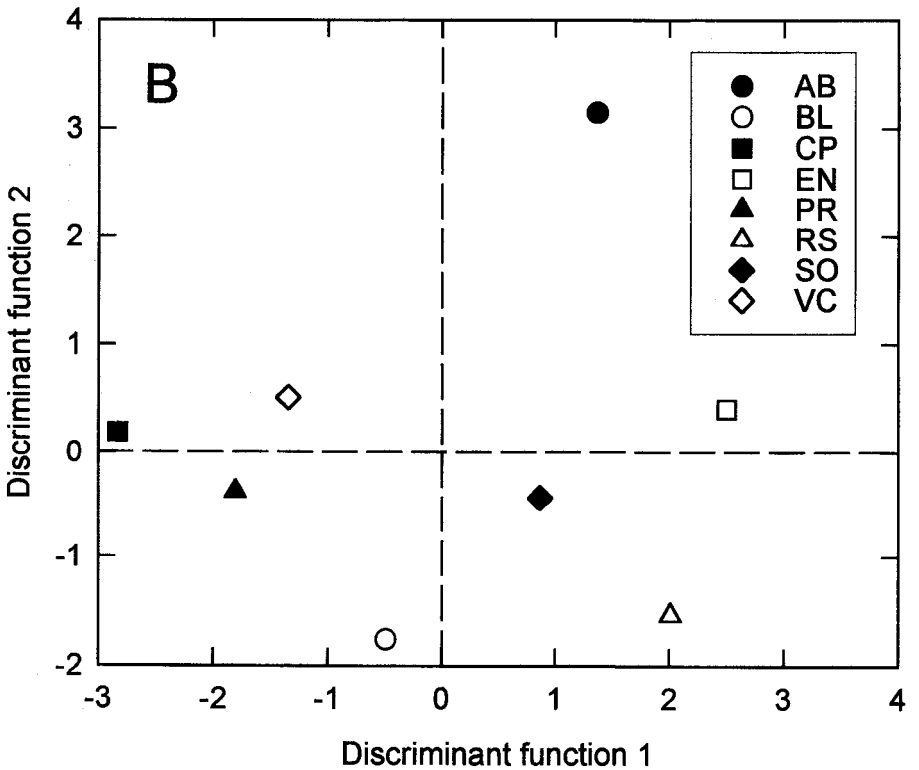

B
Discriminant function 2
Discriminant function 1
AB
BL
CP
EN
PR
RS
SO
VC
4
3
2
1
0
-1
-2
-3 -2 -1 0 1 2 3 4

Table 4. Partial tabulation (nonsignificant functions omitted) of discriminant analysis of 7 attributes of *Anopheles nuneztovari* eggs.

Discriminant function	Eigenvalue	Percentage	Chi-squared	df	*P*
1	3.998	58.3	248.65	49	<0.001
2	1.995	29.1	131.99	36	<0.001
3	0.593	8.6	52.46	25	0.001

1940). Attempts by 2 of us (L.P.L. and J.C.) to collect this species in Cojedes State in 1991 were unsuccessful.

An important aspect of the present study is that 7 of the 8 *An. nuneztovari* populations examined here have been compared at molecular levels of organization. Based on analyses of polymorphisms of mtDNA found in *An. nuneztovari* from 12 localities in South America that included all of our sites except AB, Conn et al. (in preparation) concluded that this species consists of 5 distinctive lineages. All Venezuelan and Colombian haplotypes occurred in one mtDNA lineage and nucleotide diversity was considerably higher in the 4 exclusively Amazonian lineages. Comparisons of DNA sequences of the ITS2 intergenic spacer region led to identification of 3 geographic groups: Colombia, Venezuela, and Bolivia; Suriname and northern Brazil; and eastern and central Brazil (Fritz et al. 1994). Allele frequency differences at several isoenzyme loci discriminated *An. nuneztovari* from 10 different sites, and genetic divergences calculated from these differences at the extremes of variation within this taxon are consistent with the interpretation that *An. nuneztovari* could be a species complex (Fritz et al., unpublished). However, the absence of either morphologic or molecular characters that clearly indicate lack of gene flow may favor an interpretation that *An. nuneztovari* is polymorphic within its vast range and that the Venezuelan and Amazonian populations have been isolated from each other relatively recently (Conn et al., in preparation).

Multivariate analyses of egg characters indicated that the Amazonian populations PR, BL, CP, and VC generally cluster together (Figs. 9B and 10B), and that their separation from the Venezuelan populations EN, RS, and SO on the 2nd principal component was largely attributable to differences in tubercle density and size (Table 1 and Fig. 9A). Another attribute confirming the similarity of Amazonian eggs was the amount of open (pore) area in cells of the dorsal plastron, where total pore area as a percent of cell area was significantly higher in the 5 Amazonian populations than in the 3 Venezuelan ones (Table 2 and Fig. 4). However, eggs from site AB in the western Amazon region did not cluster with other Amazonian populations (Figs. 9B and 10B), primarily because of differences in characters relating lengths or areas of parts to the whole egg (e.g., fltpcn, totdkpcn; Table 1). Further work is needed to determine whether *An. nuneztovari* from western Brazil may be more related to Bolivian collections, which did not group with Brazilian specimens in ITS2 sequence (Fritz et al. 1994).

The current study is the 4th in a series to apply multivariate analyses of egg attributes to resolve differences among anopheline populations or among species groups or species in complexes (Linley et al. 1993a, 1993b, 1995). Five species are recognized in the *Anopheles* (*Anopheles*) *quadrimaculatus* complex, and discriminant function analysis of 13 egg attributes allowed 97.7% of specimens collected in Florida to be assigned correctly to species, in spite of considerable character overlap between closely related species pairs (Linley et al. 1993a). Four species of the *Anopheles* (*Anopheles*) Hyrcanus group collected in Malaysia were separated by principal components analyses performed on 13 egg characters (Linley et al. 1995).

In a study that is the most similar to the present one, interpopulation variation in *Anopheles* (*Nyssorhynchus*) *aquasalis* Curry was examined through relationships of 9 attributes of eggs from 4 localities that extended across the range of this species in South America (Linley et al. 1993b). Eggs of *An. aquasalis* from southern Brazil were somewhat more differentiated from eggs of this species from coastal Venezuela than were eggs of *An. nuneztovari* from the extremes of its range (e.g., EN vs. CP), although the multivariate results of the 2 studies are not directly comparable because of differences in number of sites and attributes examined. Some of the same attributes (e.g., lenwidrat, fltpcn, anttbden, mnanttbar) were useful for separating populations of both species. Diagnostic isoenzyme loci and relatively high nucleotide divergence among populations of *An. aquasalis* (Conn and Fritz, unpublished) indicate that this species may be more highly differentiated than *An. nuneztovari* at the extremes of its ranges. Reappraisals of the systematic status of these polymorphic species should consider the clinal nature of both morphologic and molecular variation in these taxa.

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APPENDIX

Definitions of abbreviations (acronyms) of measured or calculated attributes of eggs of *An. nuneztovari.*

Anttbden—anterior deck tubercle density
Artotdk—area of total deck (anterior + posterior)
Arwhlegg—area of whole egg (ventral view)
Celardoplas—mean chorionic cell area, dorsal plastron
Colarmic—collar area of micropyle
Dskarmic—disk area of micropyle
Dskarpcn—disk area as % of total apparatus area
Egglen—egg length
Eggwid—egg width (widest point, across floats)
Fltlenprib—mean float length/mean number of ribs
Fltpcn—mean float length as % of egg length
Lenwidrat–length/width ratio
Mnanttbar—mean anterior deck tubercle area
Mnanttbfm—mean anterior deck tubercle form factor
Mnfltlen—mean float length (of the 2 floats)
Mnribs—mean number of ribs (of the 2 floats)
Nopordoplas—mean number of cell pores, dorsal plastron
Nosect—number of sectors in micropylar disk
Porardoplas—mean individual pore area, dorsal plastron
Porarpcndoplas—total pore area as % of cell area, dorsal plastron
Totarmic—total area of micropylar apparatus
Totdkpcn—area of total deck as % of whole egg area