EGGSHELL ULTRASTRUCTURE IN FOUR MOSQUITO GENERA (DIPTERA, CULICIDAE)

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ABSTRACT. Eggshell ultrastructure in 8 species of mosquitoes from the genera *Aedes, Anopheles, Culex,* and *Toxorhynchites* is compared using transmission electron microscopy. A 3-layered organization is present. The vitelline envelope is of similar character in all genera—homogeneous, rather electron-dense, and with no substructure. The endochorion always includes a lamellate layer (with at least one lamella) and, except in *Toxorhynchites,* tubercles of varying size and shape. The exochorion is a thin layer covering the outside of the tubercles, sometimes like a sheet, but more often weblike. The ventral side of the *Anopheles* egg lacks exochorion entirely. The endo- and exochorion in *Toxorhynchites* are fused and contain numerous large empty spaces. Ultrastructural differences were found to be greater between different strains than between different species. It is suggested that species-specific characters should be chosen only after study of populations from all parts of species' ranges. Possible functional trends in the eggshells are discussed, as well as the importance of integrating ecological studies with morphology to understand how environmental and other factors act upon eggs. Egg characters suitable for phylogenetic analysis are suggested.

INTRODUCTION

Extending the work of Hinton (1981), excellent surface descriptions of the chorion of a large number of mosquito eggs have been published (Linley and Clark 1989; Linley 1990, 1991; Linley and Chadee 1990, 1991; Linley et al. 1991a, 1991b, 1991c, 1992, 1993a, 1993b, 1993c; Linley and Craig 1993; Linley and Lounibos 1993, 1994; Linley and Turell 1993, 1994). Scanning electron microscopy (SEM) has thus become the perfect tool for making detailed descriptions of the surface morphology of eggs.

Egg descriptions from SEM do not, however, address the structure of the different layers of the eggshell and how these vary among species and genera. Eggs with entirely different surface patterns may have similar or dissimilar ultrastructure of the layers in the shell (Regier and Kafatos 1985). Nath (1924) described the formation of the eggshell of *Culex* using light microscopy. His nomenclature is still valid and is used here. The term eggshell refers to the vitelline envelope and the chorion, not just to the latter. Mathew and Rai (1975) described the formation of the eggshell within the ovary for *Aedes aegypti* (L.). Pollard et al. (1986) and Sahlén (1990) showed the eggshell of *Culex* using transmission electron microscopy (TEM). However, few such studies exist and they are not comparative. I therefore chose TEM to compare the eggs of 8 mosquito species from the genera *Aedes, Anopheles, Culex,* and *Toxorhynchites.* Use of TEM has the advantage over SEM that the preparation is never dried and all artifacts due to shrinking of fragile structures are avoided (*cf.* Sahlén 1994a, 1994b). If variation in pattern

is found to be consistent among genera, a revision of the different names applied to the ultra-structural layers may be needed for phylogenetic analysis. Such a revision may also be necessary for evaluation of the pathways of functional reorganization in relation to the ecologic conditions to which the eggs are subjected.

MATERIALS AND METHODS

For this investigation eggs were used from 3 different strains of *Aedes aegypti* (L.), one strain of *Aedes togoi* (Theobald), 4 strains of *Anopheles albimanus* Wied., 2 strains of *Anopheles stephensi* Liston, 3 strains of *Anopheles gambiae* Giles (*s.l.*), one strain of *Culex quinquefasciatus* Say, one strain of *Toxorhynchites splendens* (Wied.) (London School of Hygiene and Tropical Medicine), and one strain of *Culex pipiens* L. (a laboratory strain at the Section of Entomology, Uppsala University). All eggs were taken a few hours after deposition with exception of *Toxorhynchites* eggs, which were fixed just prior to hatching. *Aedes aegypti* eggs from one of the strains were also studied after dry periods of 3 wk and 6 months.

For TEM fixation, the eggs were transferred to a solution of 2.5% glutaraldehyde in 0.1 mol × dm^{-3} phosphate buffer, pH 7.4, at 4°C. Eggs were cut into 2 parts with a small microsurgical knife to allow the fixative to penetrate all tissues. The eggs were left in this fixative overnight, after which they were rinsed in phosphate buffer and postfixed for 2 h in 1% osmium tetroxide in the same buffer. They were rinsed again in phosphate buffer and dehydrated in alcohol (15–30 min each in a series of 50–100%, 0.5% uranyl

acetate added to the 70% solution), transferred to acetone, infiltrated overnight with Agar 100, and mounted in Agar 100 for ultrathin sectioning. The sections were stained with 4% uranyl acetate for 20 min and Reynold's lead citrate for 5 min, and examined in a Philips CM10 transmission electron microscope. Figures were drawn from resulting TEM micrographs. Nomenclature follows Nath (1924), Harbach and Knight (1980), Margaritis (1985), Pollard et al. (1986), and Kambysellis (1993).

RESULTS

The fine structure of all layers is homogeneous if not otherwise stated. Even in complexly built layers with numerous different protrusions and enclosed empty spaces, the substance forming these structures is homogeneous.

Aedes *(Fig. 1):* The vitelline envelope (VE) is 1.65–1.85 μm thick in the 3 strains of *Ae. aegypti* and 1.45–1.56 μm in *Ae. togoi.* Eggs of *Ae. aegypti* that were 6 months old had a VE 2.25–2.40 μm thick. There were no particular differences in the organization of the layers between the different strains and species. The endochorion (EN) consists of a thin lamellate layer (L), 0.072–0.083 μm thick with 3–5 lamellae, and numerous tubercles of varying height (2.8–6.6 μm for the large ones and 0.25–0.48 μm for the small). The exochorion (EX) is a thin (0.08-μm) layer covering the tops of all the tubercles, connecting them to each other. The EX covers the whole egg, and because the tubercles are connected only between their top surfaces, the layers enclose some empty spaces in the chorion.

Anopheles *(Figs. 2–5):* The VE is 0.85–1.28 μm thick with the greatest range of variation found between the 3 strains of *An. gambiae* (0.85–1.28 μm), whereas *An. albimanus* and *An. stephensi* have a less variable VE (0.94–0.98 and 1.09–1.17 μm, respectively). The EN is complex with an L consisting of 3–5 lamellae, 0.06–0.09 μm thick. Tubercles, 1.9–4.3 μm high, composed of several subunits (Figs. 2 and 3), are attached to the L. Ventrally they make up the surface structures (Fig. 3). The rest of the egg is covered with a thin (0.1-μm), almost unbroken EX layer. Some of the tubercles on the ventral side and at both poles are not attached to the L, but rather to the EX. The floats are composed of the same kind of subunits as the tubercles (Figs. 4 and 5), but here the units have fused, forming a thick continuous layer. This layer is not attached to the L in fixed material, although inner ridge-shaped protuberances come down from it at regular intervals (Fig. 4), 4 protuberances per float ridge. It is unclear if these

protuberances reach down to the VE in living eggs. The EX is very thin (0.06 μm) on the surface of the floats. On both the dorsal and ventral surface, small endochorionic protrusions appear between the tubercles. On the ventral side the protrusions are free and clearly visible (*cf.* Fig. 3); they are obscured by the EX on the dorsal side. Another thin layer (9 nm) is found between the L and the EX, which covers the tops of the small protrusions on the ventral side (Fig. 2). Its origin is unclear but it may be exochorionic. Large empty spaces are enclosed in the chorion everywhere but on the ventral side.

Culex *(Fig. 6):* The VE is 0.65–0.95 μm thick in both species. The EN is an L (0.1 μm thick) with 5–7 lamellae, and tubercles 0.8–2.3 μm high. The EX is a thin (0.02–0.06-μm) sheath on the surface of the tubercles, and connects the tubercles in the form of strings or sheaths about halfway up from the L. Between the bases of the tubercles is empty space. For a more detailed description of the eggshell in *Cx. pipiens,* see Sahlén (1990).

Toxorhynchites *(Fig. 7):* The VE is 1.22–1.31 μm thick. Only a single layer appears where the L is in the other genera. This layer is 0.2 μm thick. Connected to this "single lamella" L are other equally thick strands of endochorionic material, stretching outward in an irregular pattern, sometimes interconnected. The EX is very intricate, in cross-section shaped like a piece of thin lace. This layer covers the entire surface of the egg, the EN strands and the EX lace both forming the "bubbly" tubercles. No clear line can be drawn where the EN ends and the EX begins. Both the EN and the EX have a large number of enclosed empty spaces.

DISCUSSION

The organization of eggshell layers on the generic level: In all 4 genera the VEs are similar. They are always homogenous, rather electron-dense layers, with no apparent substructures. This conforms with other studies, both of mosquitoes (Mathew and Rai 1975, Pollard et al. 1986, Sahlén 1990), and also of dragonflies (Sahlén 1994a, 1994b). Margaritis (1985) described the VE of *Drosophila melanogaster* Meigen as being formed from fused vitelline bodies, with only specializations to facilitate hatching. In the VE of the species examined here no structure was found that facilitates hatching. Margaritis (1985) also stated that the VE of *D. melanogaster* gradually thinned down to about one-fourth of its original thickness during oogenesis. However, in *Ae. aegypti* the VE was thicker in eggs that were 6 months old than those that were only 3 wk old. As the embryonic
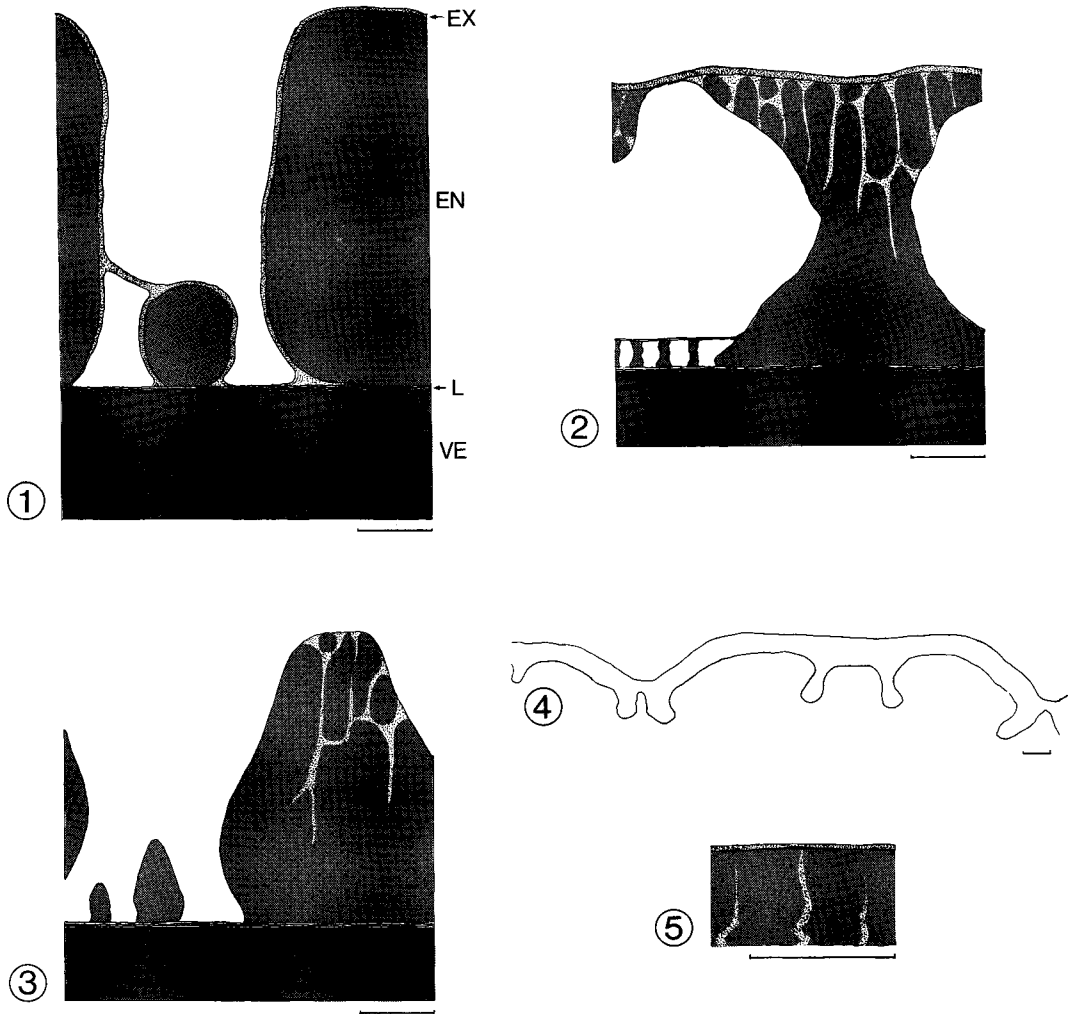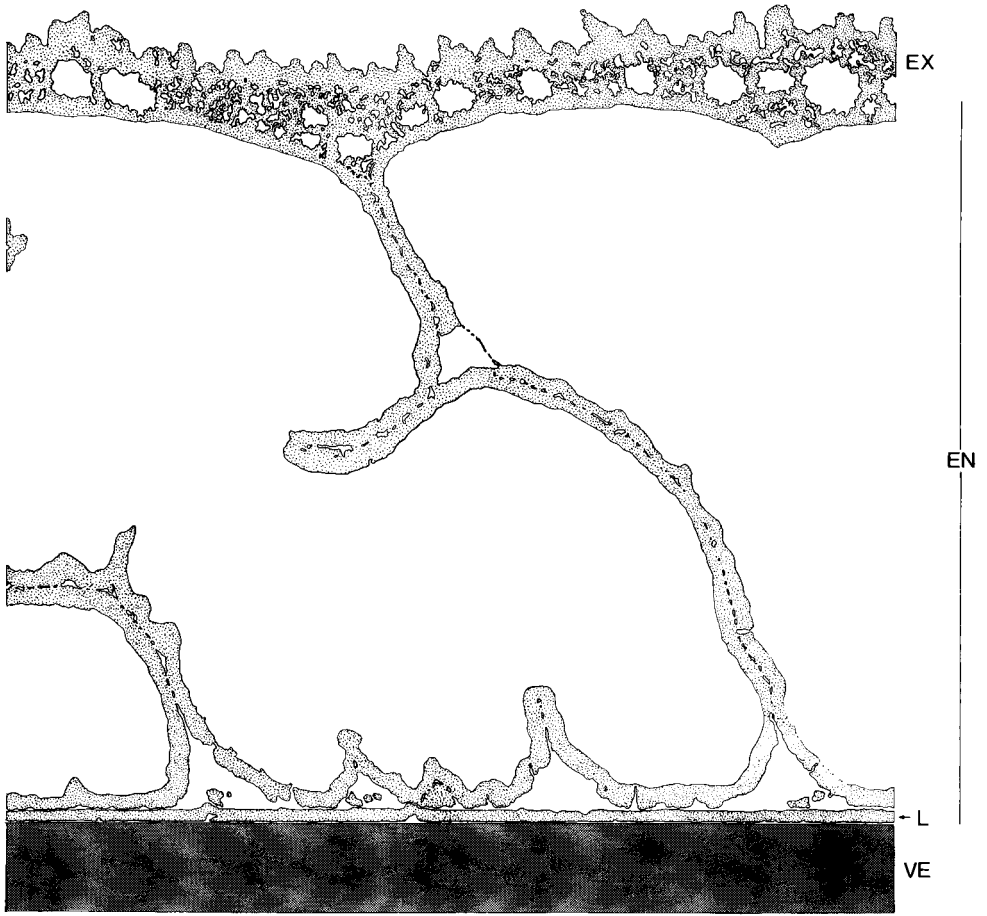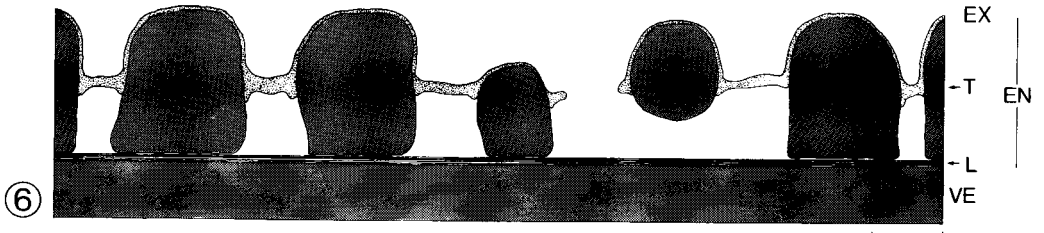
Figs. 1–5.   Schematic drawings of the eggshell. Fig. 1. *Aedes aegypti.* Fig. 2. *Anopheles gambiae,* dorsal side with exochorion covering tops of tubercles with several subunits and small tubercles under another thin sheath. Fig. 3. *Anopheles gambiae,* ventral side without exochorion. Fig. 4. *Anopheles albimanus,* float in cross section showing 4 protuberances per float ridge. Fig. 5. *Anopheles albimanus,* detail of float. Vitelline envelope (VE), endochorion (EN), and exochorion (EX) marked only in Fig. 1. Scale = 1 μm.

development of these eggs advances to a fully formed larva before the resting period (Wigglesworth 1972), it is apparent that no thinning, but rather a thickening of the VE takes place in this species. Mathew and Rai (1975) reported the newly formed VE of *Ae. aegypti* to be only 1.2 μm thick, and because no apparent variation between strains exists (below), it would seem as if the VE thickens continually during the entire egg period. A thick VE can act as a protective barrier against desiccation for the resting embryo/larva. This has been suggested to take place in overwintering eggs of the dragonfly *Sympetrum sanguineum* (Müller) (Sahlén

1994b). The dry period of *Ae. aegypti* corresponds well to the relative dryness inflicted by a cold winter period.

The L in all species but *Toxorhynchites* is a thin layer consisting of 3–7 lamellae. If we assume the L to be part of the EN, one should see its counterpart in other insect eggshells as well—unless it is a specialization evolved only in mosquito eggshells. Margaritis (1985) stated that the EN of *D. melanogaster* is composed of an inner part with holes and pillars, a solid outer part, and a "roof network." Thus, there are no lamellae in the eggshell of *D. melanogaster.* But, between the VE and the EN there is a thin

Figs. 6 and 7. Schematic drawings of the eggshell. Fig. 6. *Culex quinquefasciatus.* Fig. 7. *Toxorhynchites splendens.* Vitelline envelope (VE), endochorion (EN), and exochorion (EX) marked on right. In the upper part of the *T. splendens* eggshell only approximate boundaries for the EN and the EX are given. Scale = 1 μm.

layer known as the innermost chorionic layer, ICL. The ICL is very thin, and consists of several crystalline layers (Margaritis 1985). There is thus a resemblance between the ICL of *D. melanogaster* and the L of mosquito eggs. Further studies are needed to determine if the structures are indeed homologous, which I believe they are. In the primitive insect order Odonata, the whole EN consists of lamellae (Sahlén 1994a, 1994b), and therefore the L in mosquitoes might also belong to the EN rather than the ICL.

The L of *Toxorhynchites* has only one thick lamella, but it is present in the same location, and takes up the same amount of stain as the L of the other species. As an L seems to appear in all the other mosquito eggs, we can assume the one-layered L in *Toxorhynchites* to be either a primitive trait or a specialization. On the other hand, an L with several sublayers seems to be a general feature in culicid eggshells (Mathew and Rai 1975, Pollard et al. 1986, Sahlén 1990).

All tubercles of varying sizes correspond well to the pillars in the EN of *D. melanogaster* (*cf.* Margaritis 1985). If the L is endochorionic, then the question arises of whether the tubercles are of endochorionic or exochorionic origin. According to Mathew and Rai (1975) and Pollard et al. (1986) the tubercles are excreted in the form of droplets from the follicle cells after the formation of the L and before the final layers (the EX) are deposited. The tubercles take up about the same amount of stain as the L, whereas the EX generally takes up less. If we assume that the general organization of the eggshell (Margaritis 1985) can be applied to mosquitoes, then the tubercles must belong to the EN. The only problem here is that only a very thin layer remains as the EX. However, this may be a particular specialization that has evolved in Culicidae.

The lace pattern of both the EN and the EX of *Toxorhynchites* eggs has no counterpart in any other mosquito egg. However, Mazzini and Gaino (1985) showed somewhat lace-shaped areas in the eggshell of *Habrophlebia fusca* (Curtis) (Ephemeroptera). The cavities in this shell were filled with a mucouslike substance, which also covered the surface of the egg. Thus, it is possible that some corresponding substance also fills the cavities of the *Toxorhynchites* eggshell, but is lost due to preparation or the age of the eggs, or is not present at all because of differences in ecology.

In *Anopheles* the tubercles are composed of several subunits (*cf.* Figs. 2 and 3). The subunits are most likely formed by the fusion of secreted droplets prior to their arrival in final position during oogenesis. Subunits should be visible if the droplets were coated with a different kind of material, and this material should remain after the fusion. According to Mathew and Rai (1975) the secretion of tubercles in *Ae. aegypti* alternates with a fibrous mesh surrounding the droplets. It is therefore tempting to speculate that the droplets forming the tubercles in *Anopheles* are also surrounded by a fibrous mesh, and that a thin layer of this material remains permanently around each of the subunits. However, no trace of fibrous mesh was found in any of the eggs examined, which indicates that the substance present in *Anopheles* tubercles is different from that described by Mathew and Rai (1975). Any fibrous substance present may also be temporary and disappear from the mature egg.

The ventral side of the *Anopheles* egg is remarkable because it lacks EX altogether (Fig. 3). The rest of the egg has a normal thin EX. There are no previous reports of any insect eggs lacking the EX, but on the other hand, not many studies have been published that use TEM to study eggshells in cross section. It is possible that other dipteran species also might lack the EX. For example, flies of the genus *Calliphora* have a special enclosed "naked" area between the hatching lines on one side of the egg (Hinton 1981). However, in the figures provided by Hinton (1963, 1981) structures that may correspond to an EX can be distinguished.

Variation within and between species: Only small differences in thickness, structure, or electron density of the eggshell layers were found between different mosquito strains. Sometimes differences were greater between strains than between species. Apparent differences in external pattern have been sued as a means to identify certain strains and species (Causey et al. 1944, Kalpage and Brust 1968, Dahl 1988). However, within a strain of a given species, the qualitative characters are not always stable. Dahl (1988) found only an 80% likelihood of identifying eggs of *Cx. pipiens* and *Culex torrentium* Martini to the correct species. The present study also indicates that there is a certain amount of plasticity in the characters. Therefore, it is necessary to study species-specific qualitative characters in widely separated populations before any definite features for species separation can be chosen.

Possible functional trends and their evolutionary relationships: When examining eggshell structures it is important to bear in mind the ecology of the eggs and to understand which environmental and other factors are acting upon them. Here I discuss eggs laid on water versus eggs laid in dry environments. No phylogenetic analyses have been made for the family Culicidae as a whole. The only attempts so far deal with representatives from a single genus or sub-

Table 1.   Ecologic and morphologic characters of the mosquito eggshell suitable for phylogenetic analysis. Sources are: 1, present study; 2, Lincoln (1965); 3, Harbach and Knight (1980); 4, Beament and Corbet (1981); 5, Dahl (1988); 6, Sahlén (1990); 7, Linley and Chadee (1991).

Location	Character description	Source
General	Eggs in rafts or single, attached to each other or not	2, 4
	Continual variation in tubercle size and shape over the egg	6
	Thickness of VE	1
	Number of lamellae in EN	1
	Fusion of EX and EN	1
	Location and shape of empty spaces in chorion	1, 2, 4, 6
	Methods for water uptake	4
Anterior end	Micropylar disc flat or corolla-shaped	1, 3
	Absence or presence of special large tubercles	3, 4, 5
Posterior end	Absence or presence of special large tubercles	3
Ventral surface	Absence or presence of EX	1
Dorsal surface	EX (and EN?) extended into long protuberances	7
Lateral sides	EN and EX forming floats	1, 3

genus (Pape 1992; *cf.* also Rohlf 1977). Morphometric analysis of several populations of *Anopheles aquasalis* Curry using surface morphology have been carried out by Linley et al. (1993b). Among other insect groups comparisons using SEM have been published sporadically (e.g., Kambysellis 1993, for *Drosophila* and *Scaptomyza*).

Of the investigated species all but those of *Aedes* lay their eggs on the surface of water. *Aedes* prefer dry or damp ground, but sometimes water is used directly. The egg morphology of *Aedes* is the least differentiated in this study (by no means implying that *Aedes* is placed at the base of a future phylogeny), with the shape of the egg and the layers having no apparent specializations. The shell is adapted to withstand long dry periods but is not significantly thicker than that of *Anopheles*. In overwintering eggs of the dragonfly *Sympetrum sanguineum* the VE is about 5 times thicker than on nonoverwintering species (Sahlén 1994b), and may act as a barrier against desiccation. But in *Aedes* the VE has no such function and subsequently desiccation of the whole egg takes place (Beckel 1958). The VE of *Aedes* is therefore just as thin as that of *Anopheles, Culex,* and *Toxorhynchites.* However, the surface patterns vary greatly and might provide us with clues to the relationships between the different species (Linley 1990; Linley et al. 1991a, 1991b, 1991c, 1992, 1993c; Linley and Craig 1993; Linley and Turell 1993).

In *Culex* the corolla is clearly derived from the micropylar disc present in *Aedes* (*cf.* Harbach and Knight 1980). The corolla enables the egg to stand up on the water surface (Beament and Corbet 1981, Sahlén 1990). The structures

of the shell are very thin and a special type of plastron respiration is present (Christophers 1945, Lincoln 1965, Hinton 1968, Beament and Corbet 1981). Also, the secretion of an oviposition pheromone from the posterior end of the egg has been reported (Iltis and Zweig 1962, Laurence and Pickett 1985). Secretion of such a pheromone has thus far not been reported from any other type of culicid egg and therefore is a specialization. The assembly of eggs into rafts by the ovipositing female, as described by Christophers (1945) and Beament and Corbet (1981), is also a specialization. Egg assembly also occurs in *Mansonia* (Lincoln 1965), and as the egg of that genus has several structures (e.g., corolla and chorionic pattern) in common with that of *Culex,* these 2 genera are probably related.

Anopheles eggshells have the general shape of *Aedes* eggshells, but have several specializations, viz., the floats, specialized tubercles on the ventral side (*cf.* Linley et al. 1993a, 1993b) and the absence of EX from that area. Both the floats and the "naked" area are special traits for this genus, and the absence of similar structures from other eggs in the family indicates that they cannot be primitive characters.

Of the genera examined, *Toxorhynchites* is completely different from the others. The shape is oval with a small corolla surrounding the micropyle. This small corolla enables the egg to float upright on the water surface, just like its larger counterpart in *Culex* (*cf.* Beament and Corbet 1981). However, other morphologic features do not imply a close relationship. Possibly the ability to use the corolla as a floating device evolved more than once within the family. Although the VE of *Toxorhynchites* is of the same

type as in the other species, the EN and EX are fused and entirely different (Fig. 6), even by external morphology (Harbach and Knight 1980). The differences could indicate a far-reaching specialization, but considering the adults (not taking blood meals) and larvae (carnivorous) (Laird 1988), at least some of the egg traits are more likely to be primitive. Future phylogenetic research will show whether the branch leading to present-day *Toxorhynchites* diverged early from the rest of the culicids. I would consider that likely on the basis of the eggshell morphology.

Based on the material above, a number of characters suitable for phylogenetic analysis appear (Table 1). Among these possible characters the purely morphologic ones are those that can best be analyzed with further TEM and SEM studies. As for the ecologic characters, studies of egg-laying behavior are, with the exception of *Culex pipiens* (Beament and Corbet 1981), virtually nonexistent. Therefore, much remains to be done before any suggestion of mosquito phylogeny can be made. Not only are characters needed from eggs in many other species, but also the larval, pupal, and adult characters must be taken into account.

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