

Synthesis and function of the fibrous layers covering the eggs of *Siphonurus lacustris* (Ephemeroptera, Siphonuridae)

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Abstract

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Ultrastructural analysis (transmission and electron scanning microscopy) of the eggs of the mayfly *Siphonurus lacustris* (Eaton) showed that they are wrapped in a thick coat composed of a network of tightly entwined filaments. Groups of twisted filaments form slightly uplifted buttons that are scattered on the coat surface. After experimentally induced egg deposition, egg–water interaction promotes marked cohesion of the eggs and their firm adhesion to the substrate. Egg masses include numerous gametes; the covering of those located close to the substrate greatly extends to anchor the whole mass. Eggs removed from the coat reveal a slightly punctuated smooth chorion and tageniform micropyles (three to five). The coat increases egg size by about 20%. The lack of female reproductive accessory glands in Ephemeroptera transfers the synthesis of the adhesive coats to the follicle cells, which are typically competent for insect egg shell deposition (vitelline envelope and chorionic layers). This covering results from electron-dense granules that give rise to filaments progressively organized to form superimposed layers variously orientated around the egg. In addition to egg adhesion to the substrate, a trophic function and protection from shear stress are postulated for this covering.

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Introduction

The egg chorionic pattern of representative genera of numerous families of Ephemeroptera has been the object of numerous papers. Information is available on some chorionic features as an aid to understanding the phylogeny of this insect group and to ascertaining the function of attachment structures (Degrange 1960; Koss 1968; Koss and Edmunds 1974). Consequently, egg decorations have been utilized as markers in taxonomic studies, thanks also to the morphological details revealed by ultrastructural investigation (Kopelke and Müller-Liebenau 1981a,b, 1982; Malzacher 1982, 1986; Gaino and Mazzini 1984, 1988; Alba-Tercedor and Sowa 1987; Gaino *et al.* 1987, 1989; Mazzini and Gaino 1990; Gaino and Bongiovanni 1992; Thomas *et al.* 1999a). Among the external morphological features, particular attention has been paid to attachment structures, the variety of which has been conveniently categorized by

Koss and Edmunds (1974), who provided a useful reference model for further descriptions. The actual adhesive function attributed to the attachment structures has been repeatedly proved by observing eggs after their immersion in water. These tests revealed that egg–water contact induces remarkable changes in the organization of the attachment devices whose plasticity responds to the need for a firm egg anchorage to the substrate (Gaino and Flannagan 1995; Gaino and Mazzini 1987; Provonsha 1990).

Even though the majority of mayfly representatives have eggs with a decorated chorion, some species show a smooth surface usually surrounded by a compact mass of material forming an adhesive layer (Degrange 1960; Koss and Edmunds 1974). This coat can be organized to form an adhesive elastic membrane (Kosova 1967; Gaino and Bongiovanni 1993). Ribbed eggs may present adhesive material filling the spaces inside ribs, thus suggesting the possible involvement of this component in improving egg adhesion in

absence of differentiated attachment structures able to perform such a function (Gaino and Mazzini 1990a; Mazzini and Gaino 1985).

However organized, mayfly attachment chorionic devices and coats seem to be adapted to accomplish the same adhesive function: to prevent eggs from being dragged away into an environment unsuitable for their further development. Nevertheless, the involvement of these external structures in such a function is poorly investigated. In this work, the eggs of *Siphonurus lacustris* (Eaton) are studied and the origin and changes in its cover after deposition in water are described.

Materials and Methods

Nymphs of *Siphonurus lacustris* (Eaton) were collected in the Berlino stream (near Rossiglione, Genova, Italy, at approximately 300 m elevation, in June 1999), and reared in laboratory. Specimens were differently processed for transmission and scanning electron microscopy investigation (TEM, SEM).

For TEM analysis, the ovaries dissected from nymphs were fixed in Karnovsky's medium (Karnovsky 1965), rinsed in 0.1 M cacodylate buffer (pH 7.2) at 4 °C, and postfixed in 1% osmium tetroxide in cacodylate buffer. Selected material was rinsed, dehydrated in a graded ethanol series, and embedded in Epon-Araldite (Epon Araldite, Sigma-Aldrich Chemie Schnellendorf, Germany). Sections were cut with a Reichert ultramicrotome, mounted on Formvar-coated copper grids (Formvar, Sigma-Aldrich Chemie), and stained with conventional uranyl acetate and lead citrate. Specimens were observed with a Philips EM 400 transmission electron microscope.

For SEM analysis, a part of the eggs was dissected from adults (emerged from reared nymphs) and immediately fixed in the above reported medium; a part was obtained by slightly pressing the female abdomen on a water-filled culture dish, the bottom of which was filled of glass coverslips. Eggs gently settled on the coverslips arranged on the bottom of the culture dish. Two hours after settlement egg aggregates were fixed in Karnovsky's medium. For SEM examination, selected material was dehydrated following the above described procedures for TEM study, critical-point dried using liquid CO₂ in a Bomar apparatus (Bomar, Pobish CPD 750), attached to specimen holders by silver conducting paint, and coated with gold-palladium in a Balzer Union evaporator (Balzers, SCD 004). Specimens were observed with a Philips EM 515 scanning electron microscope.

The basic descriptive terminology used by Koss and Edmunds (1974) is followed in the present paper.

Results

The eggs of *Siphonurus lacustris* (Eaton) dissected from the abdomen are about 200 µm long and 140 µm width (average

from 10 eggs). When observed under SEM, they are covered with a compact mass of material giving them an irregular shape (Fig. 1A). This external coat appears fibrous and includes scattered slightly uplifted buttons (10–15 µm in diameter) (Fig. 1B). High magnifications reveal that the coat consists of a loose collection of single filaments that are tightly adherent and organized to form a uniform covering of the entire egg (Fig. 1C). Each button derives from the coiling of the terminal part of the filament clusters (Fig. 1D) and consists of a stalk, which continues with the rest of the filaments, and of an apical part with a granular appearance (Fig. 1D).

Observations under SEM of an egg aggregate 2 h after being laid in water give evidence of the influence of the water on the fibrous coat. Upon entering the water, eggs do not separate from one another but form a mass of closely adherent elements (Fig. 1E), the external coat of which has a remarkable adhesive role. The fibrous layer allows eggs to stay in close contact and the entire mass to be anchored to the substrate. Indeed, the immediate coat of the eggs extends to the substrate and firmly adheres to it (Fig. 1F). This feature proves that the whole filamentous coat, not only the buttons, is involved in egg mass adhesion.

In order to test the strength of this adhesion, flat and thin devices were used to remove settled egg masses from the substrate. Adhesion of the egg masses was also tested by immersion of the coverslips with settled egg masses in running water. No detachment has been recorded during this treatment.

Eggs removed from their coat revealed a slightly punctuated smooth chorion (Fig. 1G) and disclosed the tageniform micropyles with the suprachorionic sperm guide (Fig. 1G, inset). The number of micropyles varied from three to five. They were located in the equatorial region of the egg. On occasion, two micropyles were situated side by side.

In an experimentally broken egg mass, the chorion revealed its organization in two layers (Fig. 1H).

TEM analysis carried out on the ovarioles gave evidence of the relationship between developing egg and the surrounding follicle cells (Fig. 2A), which are competent for egg envelope synthesis.

Early in ovarian development, follicle cells are interlocked apically with the plasma membrane of the oocyte via a few microvilli (Fig. 2B), where electron-dense amorphous material is located to produce an infant form of the vitelline envelope (Fig. 2C). In a more advanced phase of oogenesis, the vitelline envelope consists of a coat about 0.6 µm thick formed by the coalescence of discrete units (Fig. 2D). Synthesis of chorionic layers gives rise to an initial narrow and wavy band adjacent to the vitelline envelope that, even though it has not yet developed a continuous layer, shows most of its constitutive plates fused together (Fig. 2D). Afterwards, the band grows to form a homogeneous coat, the endochorion (about 100 nm thick). It has a striated appearance and supports the outermost electron-opaque chorionic sheet, the exochorion (about 180 nm in thickness, Fig. 2E). As a consequence, the chorion consists of a

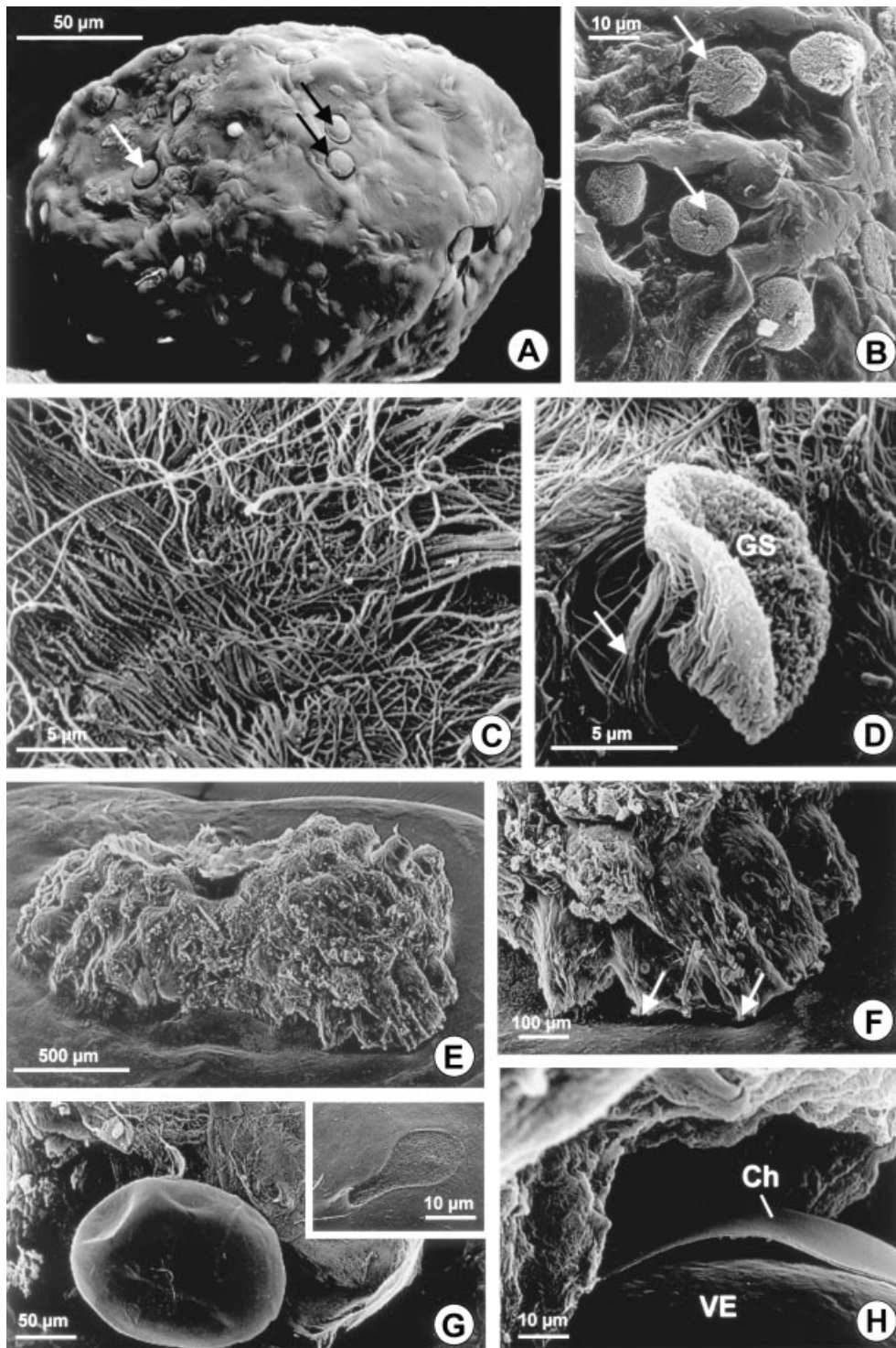


Fig. 1—SEM view of the egg of *Siphonurus lacustris*. —**A**. The adhesive coat including scattered buttons (arrows) envelops the underlying egg. —**B**. Some buttons (arrows) raise slightly from the coat surface. —**C**. Detail of the coat formed by filaments. —**D**. A closer view of a button showing its stalk (arrow) formed by twisted filament bundles that merge to form the apical granular surface (GS). —**E**. An egg mass adherent to the substrate.

—**F**. Detail of the egg mass of **E**. Note the coat of each egg located close to the substrate and extending to adhere to it (arrows). —**G**. An egg experimentally removed from its coat. Note its slightly punctuated smooth chorion and the tageniform micropyle (inset). —**H**. An experimentally broken egg showing its double-layered chorion (Ch) raising from the vitelline envelope (VE).

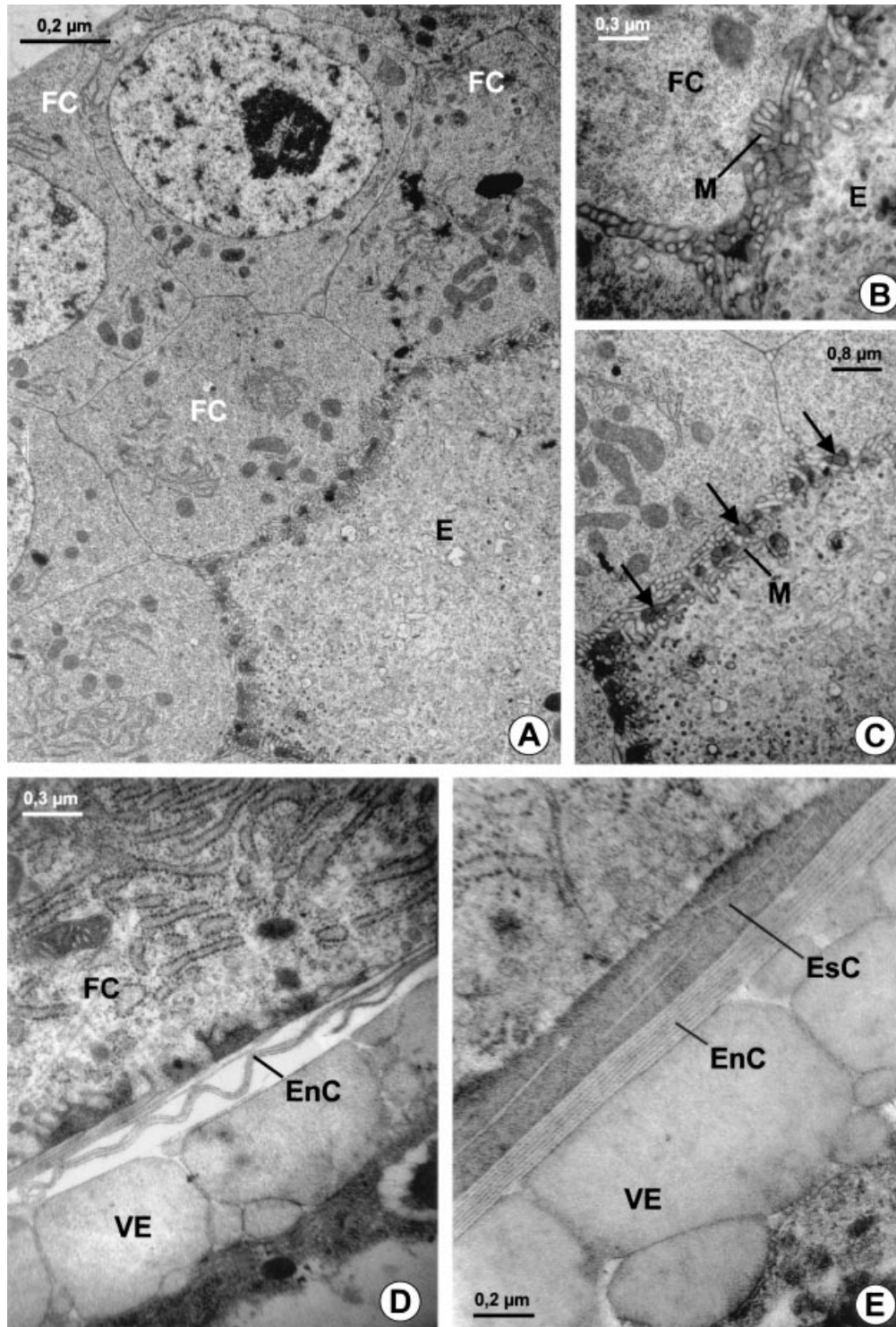


Fig. 2—TEM view of the relationship between egg and follicle cells during synthesis of vitelline envelope and chorion in *Siphonurus lacustris*. —**A**. Section through an ovariole showing follicle cell (FC) epithelium around the egg (E). —**B**. Follicle cells (FC) tightly interlocked with egg (E) via microvilli (M). —**C**. The accumulation of electron-dense material (arrows) along the microvillar interdigitations (M) marks the onset of vitelline membrane synthesis. —**D**. The thick

vitelline envelope (VE) betrays its derivation from the coalescence of several units. Note the early and waived endochorion (EnC) between the vitelline envelope (VE) and the follicle cell (FC). —**E**. The double-layered chorion results from the endochorion (EnC), having a striated appearance, and the outermost more electron-dense exochorion (EsC). Note the considerable difference in thickness between chorion and vitelline envelope (VE).

double-layered structure, as also seen in the SEM view of the broken egg (Fig. 1H).

After secretion of the egg envelope in this well-defined order, follicle cells are involved in the synthesis of the fibrous coat enveloping each egg. This coat tends to thicken close to the follicle cell junctions (Fig. 3A). At high magnification, this covering reveals that its fibrous organization depends upon the accumulation of electron-dense granules that are initially seen in the cell cytoplasm (Fig. 3B) and then gather together to form units of different sizes (Fig. 3B, inset). The coalescence of this precursor material produces filaments that, arranged in tightly interconnected bundles, form overlapping layers of various orientation (Fig. 3C). The twisting of the filament bundles and their apical enlargement into fan-shaped structures (Fig. 3D) give rise to the buttons observed at SEM level.

Eggs wrapped by the fibrous coat increase their diameter by approximately 20% in comparison with the state deprived of the fibrous coat.

Discussion

Degrange (1960) reported that the females of *Siphonurus lacustris* carry from 1712 to 2499 eggs and that in the congeneric *S. aestivalis* their number increases to 2678. These values are in keeping with the inclusion of both species among Ephemeroptera having the highest level of fecundity. This feature has been investigated by Sartori and Sartori-Fausel (1991) in *S. aestivalis*, a species in which variability in the number of eggs (from 1159 to 4675) correlated directly with the body mass. As a consequence, fecundity of maximum-size specimens may be fourfold that of minimum-size specimens. It is quite surprising that, in spite of the accumulation inside the oviducts of such a remarkable number of eggs, the covering of the eggs does not glue them together as is seen after egg deposition in water. According to Degrange (1960) female ducts must secrete some substance capable of hampering the adhesive activity of the coat. The involvement of the cells of the female ducts in the secretory activity has been observed in a leptophlebiid, thus proving that this epithelium has more function than simple protection (Gaino and Mazzini 1989).

The presence of an adhesive material coat on Ephemeroptera eggs had raised some problems on its function and origin. In this insect group, female accessory reproductive glands are absent (Brinck 1957), and follicle cells have been considered responsible for adhesive layer deposition after the secretion of the egg envelopes (Koss 1970). This assumption has been confirmed by ultrastructural investigation in the leptophlebiid *Habrophlebia eldae* whose chorion is decorated with prominent ridges (Gaino and Mazzini 1984) including mucous material (Mazzini and Gaino 1985) secreted by the follicle cells (Gaino and Mazzini 1989, 1990a). As the eggs pass through the oviduct, the coat secreted by follicle cells is enriched with a fine layer of

fibrous material produced by the oviduct cells (Gaino and Mazzini 1989).

As in all insect groups, follicle cells overlying the eggs of *Siphonurus lacustris* are competent in egg envelope deposition. The thick fibrous coat wrapping the eggs of this species represents the final product of their metabolism. Our experiments with laid eggs proved that the entwined filaments of the coat are responsible for sticking the egg to the substrate, thereby confirming previous observations with light microscopy (Degrange 1960; Koss 1968). It is worth stressing that this adhesive role is not limited to the filaments of the buttons but involves the whole fibrous coat that interacts directly with the bottom and promotes the mutual cohesion among eggs.

Egg attachment structures of insects laying their eggs in water show various levels of differentiation and complexity, as shown, for instance, by Odonata (Andrew and Tembhare 1992; Sahlén 1995) and Plecoptera (Rościszewska 1991a,b, 1995, 1996). In contrast, the chorion of Trichoptera is smooth and their eggs are frequently laid into spumaline masses of marked diversity in shape and morphology (Bicchierai and Gaino 1997). On this account, the egg aggregates of *S. lacustris* are similar to the egg masses of Trichoptera from which they differ because each egg maintains its envelope and only the outermost surface keeps the eggs close to one another. This feature is consistent with the fact that in Ephemeroptera the function of the reproductive accessory glands, which secrete spumaline in Trichoptera, is transferred to the follicle cells and, consequently, at ovulation discrete eggs have already their own covering.

Trichoptera laying eggs into spumaline show a thick vitelline envelope and a thin chorion (Bicchierai and Gaino 1997). Likewise, the egg shell of *S. lacustris* is characterized by a vitelline envelope almost twice as thick as the double-layered chorion. This organization contrasts with the marked development of the mayfly chorion whose thickness is usually increased by complex decorations, which also are of taxonomic value.

Even though the adhesive role seems to be prevalent, the thick covering of *S. lacustris* could also protect eggs from both particulate matter sedimentation and mechanical stress. As a matter of fact, the protective role of extracellular layers (jelly coats) has been recently documented for echinoderms whose embryos may not develop normally if the gametes are exposed to shear stress (Thomas *et al.* 1999b). In addition, we believe that, as for Trichopteran spumaline, the fibrous network enveloping the eggs of *S. lacustris* could fulfil a trophic function allowing the newly hatched larvae to feed on it.

In sum, follicle cells of Ephemeroptera have an intense metabolism because they have functions that in other insects are performed by other organs. The secretion of suprachorionic coats increases egg size of about 20%. Their adhesive properties, shear stress protection, and trophic function can work together to enhance survival of the species in its environment.

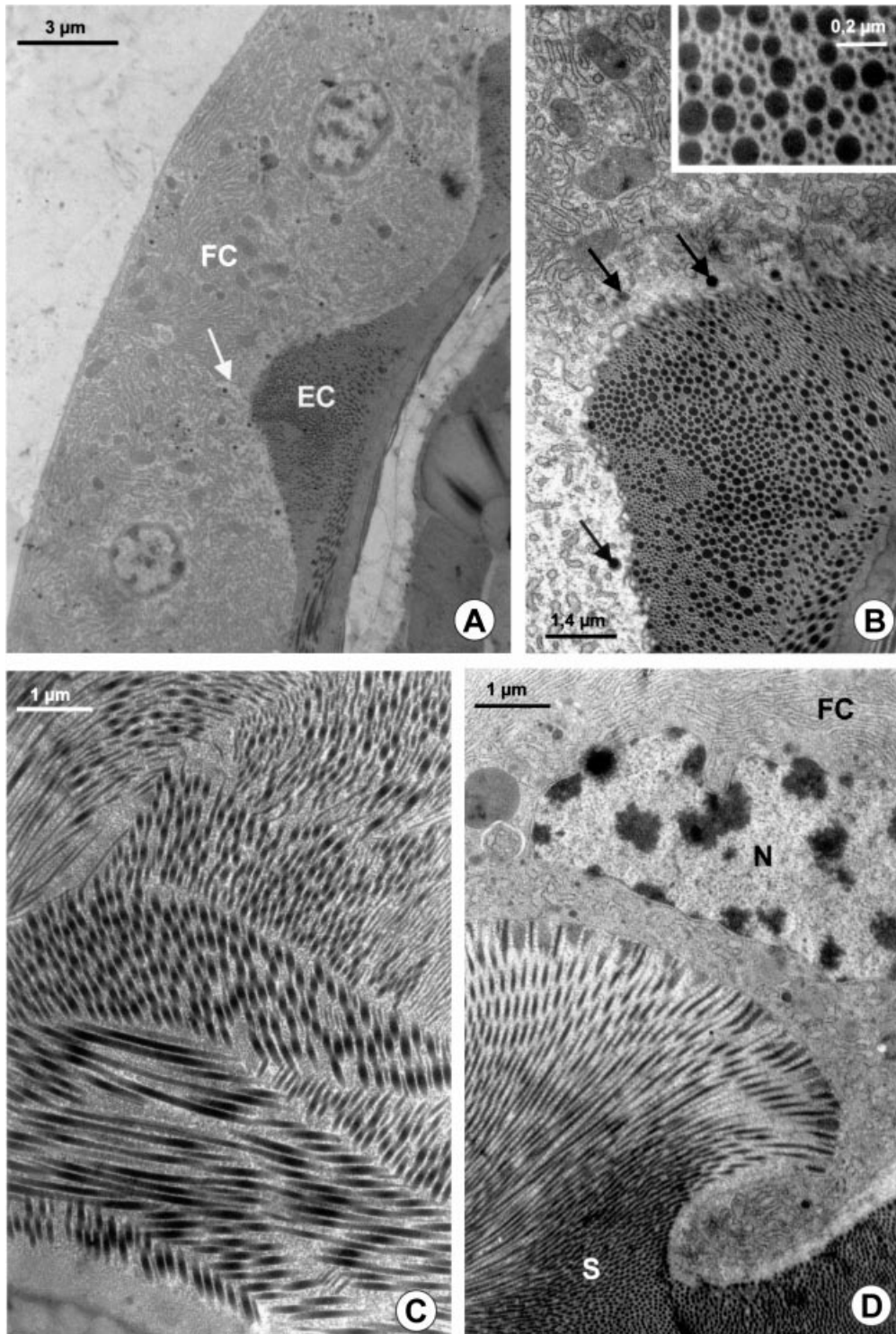


Fig. 3—TEM view of the internal organization of the follicular cells active in synthesis of the fibrous egg covering in a follicle of *Siphonurus lacustris*. —**A**. A fragment of the follicle cell epithelium (FC) partially penetrated by the material forming the fibrous egg coat (EC). Note that the coat tends to be thick close to follicle cell (FC) junctions (arrow). —**B**. Electron-dense granules (arrows) accumulate to

form the fibrous coat. The inset shows that the fibrous coat derives from various sized electron-dense units. —**C**. The fibrous coat results from several overlapping filament bundles orientated in various directions. —**D**. A detail of a follicle cell (FC) close to the nucleus (N) and enveloping a button. Filament bundles twist to form the stalk (S) of the button and then enlarge into a fan-shape configuration.

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