

# Egg envelopes of *Baetis rhodani* and *Cloeon dipterum* (Ephemeroptera, Baetidae): a comparative analysis between an oviparous and an ovoviviparous species

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## Abstract

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A comparative ultrastructural investigation on the eggshell (vitelline and chorionic envelopes) has been carried out in the nymphs of two mayfly species encompassed into the Baetidae, namely *Baetis rhodani* and *Cloeon dipterum*. During oocyte differentiation in the meroistic telotrophic ovarioles, gametes are connected to discrete nurse cells by trophic cords. In *B. rhodani*, each ovariole contains several eggs arranged in sequence, whereas in *C. dipterum* each contains a single egg. Follicle cells are competent for vitelline and chorionic envelope synthesis. *Baetis rhodani* is an oviparous species and the chorion is fairly thick, formed by an alveolate endochorion and a fibrillar exochorion delimited by a honey-comb roof. *Cloeon dipterum* stands out among Ephemeroptera for being ovoviviparous. In *B. rhodani*, ovulation starts in the older nymphs with dark wing-pads, whereas in *C. dipterum* choriogenesis ends in the imaginal stage. Here the chorion is very thin and laid eggs hatch almost immediately, allowing the larvulae to move out. The maturation of a single egg per ovariole is synchronized with the achievement of the adult stage. The acquisition of ovoviviparity has led to remarkable changes in the ovariole organization along with a simplification of the eggshell structure.

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## Introduction

The adults of Ephemeroptera are short-lived insects, and this characteristic shifts oogenesis and spermatogenesis to their long-lasting aquatic stages, which are the only trophic ones (Soldán 1979).

Mayfly ovaries consist of telotrophic, meroistic ovarioles whose growing oocytes, surrounded by a monolayer of follicle cells, are connected by intercellular bridges or nutritive cords to their nurse cells, which are located in the trophic chambers (Büning 1993, 1994; Gottanka and Büning 1993).

Follicle cells are competent for synthesis of both eggshells (including vitelline and chorionic envelopes) and fibrous coats (Gaino and Mazzini 1990; Gaino and Rebora 2001). The programmed death of these follicle cells after they have

fulfilled this specific function is achieved by apoptosis (Nezis *et al.* 2002; Gaino and Rebora 2003). Eggshell has relevant mechanical and physiological functions: namely, it allows sperm entry; provides elasticity for easy oviposition; protects the embryo from environmental hazard; ensures an adequate gas exchange for the developing embryo (Carvalho dos Santos and Gregorio 2003; Iconomidou *et al.* 2000).

Mayfly chorionic decorations have been utilized for assessing phylogenetic relationships (Koss and Edmunds 1974; Gaino *et al.* 2001; Dominguez and Cuezco 2002) along with the possible role they perform in enhancing egg adhesion to the substrate after oviposition in water (Gaino and Flannagan 1995).

Ephemeroptera typically include oviparous species, apart from some representatives belonging to the Baetidae, namely

*Cloeon* and *Callibaetis*, which are ovoviviparous (Degrange 1959, 1960; Gibbs 1979; Soldán 1979). In particular, *Cloeon dipterum* and *Cloeon simile* produce eggs that may develop parthenogenetically (Harker 1997).

In the investigation on the structure and development of the female reproductive system in some European species, Soldán (1979) outlined the main changes occurring in the ovaries of oviparous mayfly during oogenesis, and argued that the marked differences evidenced in *C. dipterum* were linked to the ovoviviparity of this species.

Therefore, the Baetidae encompass both oviparous and ovoviviparous species; the eggs of these last contain mature embryos and hatch immediately on oviposition (Degrange 1959), even though in older adults, which can live for 10–14 days, newly hatched larvae can be observed in the dilated oviducts (Soldán 1979).

Recent investigation of the vitellogenesis of *C. dipterum*, focused attention on the yolk composition in older nymphs, subimagines and imagines, and stressed that, in this last stage, the vitelline envelope and chorion are nearly completed (Ikigame *et al.* 2000). These authors even raised the question of presence of an actual telotrophic ovary in this species, based on the lack of observation of a nutritive cord. In addition, the gross anatomy of the ovary showed a bridge between the paired lateral oviducts (Takahashi and Tsutsumi 2001, 2002), a feature so far exclusive to this species because, as reported by Brinck (1957), the ovaries are always paired and separate.

From the bulk of the data, it emerged that *C. dipterum* stands out among the other mayflies for several peculiarities, including the end of choriogenesis occurring in the imagines, even though this event is poorly documented.

This paper extends previous studies on the oogenesis of *C. dipterum*, by paying particular attention to the unknown ultrastructural aspects of the pathways of egg envelope synthesis. The concomitant analysis of this process in an

oviparous baetid, namely *Baetis rhodani*, allowed us to compare the egg maturation pattern in oviparous and ovoviviparous species, and to show that several aspects of oogenesis and egg envelope organization reflect these different reproductive strategies.

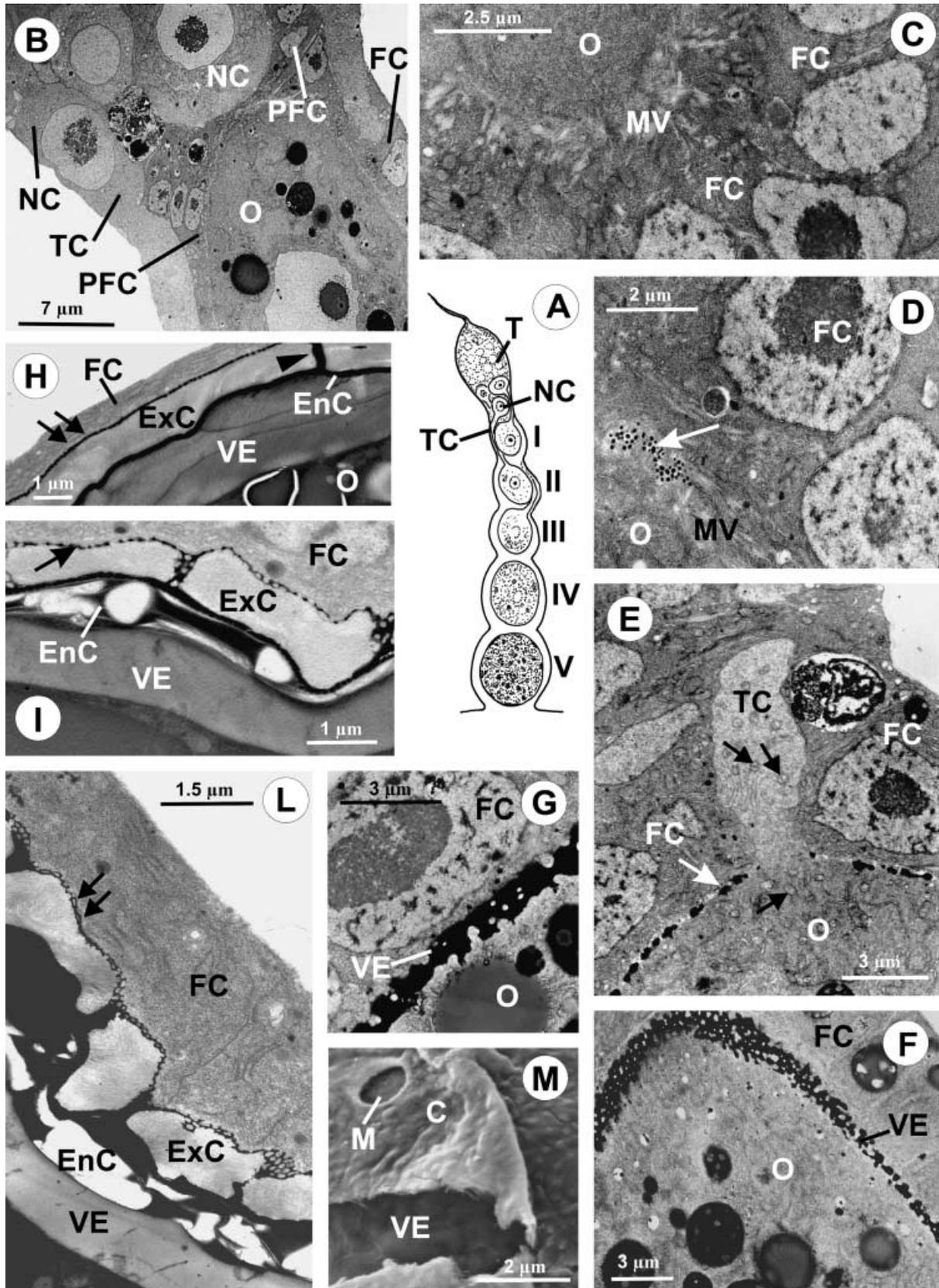
## Materials and Methods

Specimens of *B. rhodani* (Pictet 1843) and *C. dipterum* (Linnaeus 1761) belonging to different developmental phases (nymphs with clear wing-pads, nymphs with dark wing-pads, subimagines, imagines) were collected in the Tescio Stream (Bastia Umbra, Perugia, Italy) and in a cement pond (base dimensions 4 × 4 m with rain water 1–3 m deep), close to Perugia (Umbria, Italy). Decapitated specimens were dissected under a stereomicroscope to remove the ovaries and the eggs. The egg surface was investigated by scanning electron microscopy (SEM), whereas the fine architecture was highlighted by transmission electron microscopy (TEM). Immediately after dissection, selected material was fixed in Karnovsky's medium (1965) in cacodylate buffer, pH 7.2, for 1 h, repeatedly rinsed in the same buffer and post-fixed in 1% osmium tetroxide for 1 h.

For SEM observations, the specimens were dehydrated using an ethanol gradient, followed by critical-point drying in a CO<sub>2</sub> Pabisch critical-point drier. Specimens were mounted on stubs with silver conducting paint, sputter-coated with gold-palladium in a Balzers Union Evaporator and observed with a Philips EM 515 SEM.

For TEM analysis, the tissue dehydrated in the graded ethanol series was embedded in Epon-Araldite mixture resin. Thin sections, obtained with a Reichert ultramicrotome, were collected on formvar-coated copper grids, then stained with uranyl acetate and lead citrate. They were examined with Philips EM 400 and EM 208 electron microscopes.

**Fig. 1**—Ultrastructural view of some phases of eggshell (vitelline and chorionic envelopes) synthesis in *Baetis rhodani*. —**A**. Sketch of a meroistic telotrophic ovariole showing the tropharium (T) and the arrangement of the maturing oocytes (I–V). The trophic cord (TC) connects each of them to the nurse cells (NC). —**B**. Section through the apical region of the ovariole showing nurse cells (NC), a trophic cord (TC), prefollicle cells (PFC) and the first oocyte (O) in vitellogenesis. The oocyte is enveloped by follicle cells (FC). —**C**. Cube-shaped follicle cells (FC) tightly interlocked with oocyte (O) via microvilli (MV). —**D**. The accumulation of electron-dense granules (arrow) along the microvillar interdigitation (MV) between follicle cell (FC) and oocyte (O). —**E**. Accumulation of electron-dense material (white arrow) at the follicle cell (FC)/oocyte (O) interface. Note the extension of the oocyte cytoplasm to form the trophic cord (TC) filled with mitochondria evident also in the oocyte apical region (black arrows). —**F**. The infant form of the vitelline envelope (VE), derived from the coalescence of the electron-dense granules that assemble at the follicle cell (FC)/oocyte (O) interface. —**G**. A gradual completion of the vitelline envelope (VE) between follicle cell (FC) and oocyte (O). —**H**. Extended follicle cells (FC) envelope the growing oocyte (O) showing the sequence of the eggshell: vitelline envelope (VE) and chorionic layers. These last consist in a uniform electron-dense endochorion (EnC) and a fibrillar exochorion (ExC) delimited by a honey-comb roof (arrows). The arrowhead points out the pillar that joins the endochorion to the outermost chorionic surface. —**I**. The endochorion (EnC) acquires a typical vacuolated appearance. FC, follicle cells; ExC, exochorion delimited by the honey-comb roof (arrow); VE, vitelline envelope. —**L**. The widening of the endochorion (EnC) as a result of the increase of the vacuolar pattern. VE, vitelline envelope; FC, follicle cell; ExC, exochorion. Arrows indicate the honey-comb roof. —**M**. SEM view of a mature egg dissected from the oviduct. The breaking off of the chorion (C) reveals the morphology of the vitelline envelope (VE) beneath. M, micropilar opening.



## Results

Sketches, as models, of the telotrophic meroistic ovarioles of *B. rhodani* and *C. dipterum* are reproduced in Figs 1(A) and 2(A), respectively.

### *Baetis rhodani* (Fig. 1A–M)

This species has two ovaries, each housing numerous ovarioles ordered in several rows along the lateral oviducts.

In the *nymphs with clear wing-pads*, each ovariole contains five eggs showing different phases of maturation from the apical region of the ovariole towards the basal region. This sequential arrangement allows the gradual acquisition of the eggshell layers up to their final completion to be recorded.

Each ovariole is surrounded by a thin tunica externa that continues into a short, terminal filament. The tropharium includes nurse cells, characterized by an electron-translucent cytoplasm with scattered organelles, which are intermingled with a few spindle-shaped prefollicle cells (Fig. 1B). Some images show the occurrence of a trophic cord that represents the continuity between sibling nurse cells and the oocyte of the lower region of the ovariole (Fig. 1B). In early vitellogenesis, the first oocyte is enveloped by cube-shaped follicle cells with a large nucleolated nucleus (Fig. 1C). Their cytoplasmic membrane extends into microvilli that markedly interdigitate with those of the oocyte (Fig. 1C). No precursor material of the vitelline envelope is evident at the oocyte–follicle cell interface. The vitelline envelope precursors, in the form of electron-dense granules, appear at the interface between the second oocyte and its follicle cells (Fig. 1D).

An infant form of the vitelline envelope, as a result of the coalescence of this material to form irregular blocks, gradually organizes around the third oocyte, except for the region where the trophic cord persists (Fig. 1E). In the nutritive cord, the same organelles of the oocyte cytoplasm are evident (Fig. 1E).

The vitelline envelope progressively increases in thickness and originates an electron-dense coat (about 0.7  $\mu\text{m}$  thick) (Fig. 1F) around the fourth oocyte, leading to an irregularly indented configuration around the fifth oocyte (Fig. 1G).

In the *nymphs with dark wing-pads*, the eggs are ovulated progressively. Concomitantly with the gradual darkening of the wing-pads, eggs are ovulated in such a way that in the nymph that is about to emerge most ovarioles contain two or three eggs in a late stage of maturation, and the oviducts begin to be filled with some mature eggs.

During egg maturation the vitelline envelope becomes a uniform electron-translucent coat (about 1–2  $\mu\text{m}$  thick) which supports the chorion (Fig. 1H). This last initially consists of two main layers: the endochorion, in the form of a thin electron-dense layer, and the exochorion, with a fibrous appearance and bordered with an honey-comb network (Fig. 1H). Some images reveal that the electron-dense endochorion gradually splits to originate an alveolate pattern,

where translucent regions are interposed among the initial electron-dense areas (Fig. 1I). The endochorion undergoes further modifications that give rise to a thicker layer (about 1  $\mu\text{m}$  thick) with a harmonic permeation of electron-dense and electron-translucent regions (Fig. 1L). On the whole, both endochorion and exochorion measure about 2–3  $\mu\text{m}$  in thickness.

In the *subimago*, egg maturation proceeds and some eggs still enveloped by the follicle cells coexist with the ovulated eggs.

In the *imago*, ovulated eggs accumulate in the lateral oviducts, which greatly enlarge to contain them.

In the ovulated eggs, the alveolate structure of the exochorion facilitates its detachment from the vitelline envelope in such a way that during egg manipulation the chorion breaks down and reveals the coat of the vitelline envelope beneath (Fig. 1M).

### *Cloeon dipterum* (Fig. 2A–I)

In the *nymphs with clear wing-pads*, each ovariole consists of a tropharium where the nurse cells are connected to the cytoplasmic extension of the single developing oocyte, thereby forming a trophic cord that interrupts the enveloping coat of the follicle cells (Fig. 2B). The oocyte cytoplasm contains numerous inclusions, with both electron-translucent and electron-dense appearance, that push the nucleolated nucleus to the apical region of the oocyte. Follicle cells are elongated in shape and their microvilli interdigitate with those of the oocyte surface (Fig. 2C).

In the *nymphs with dark wing-pads*, electron-dense granules interspersed among flocculated material gradually accumulate at oocyte/follicle cell interface (Fig. 2D).

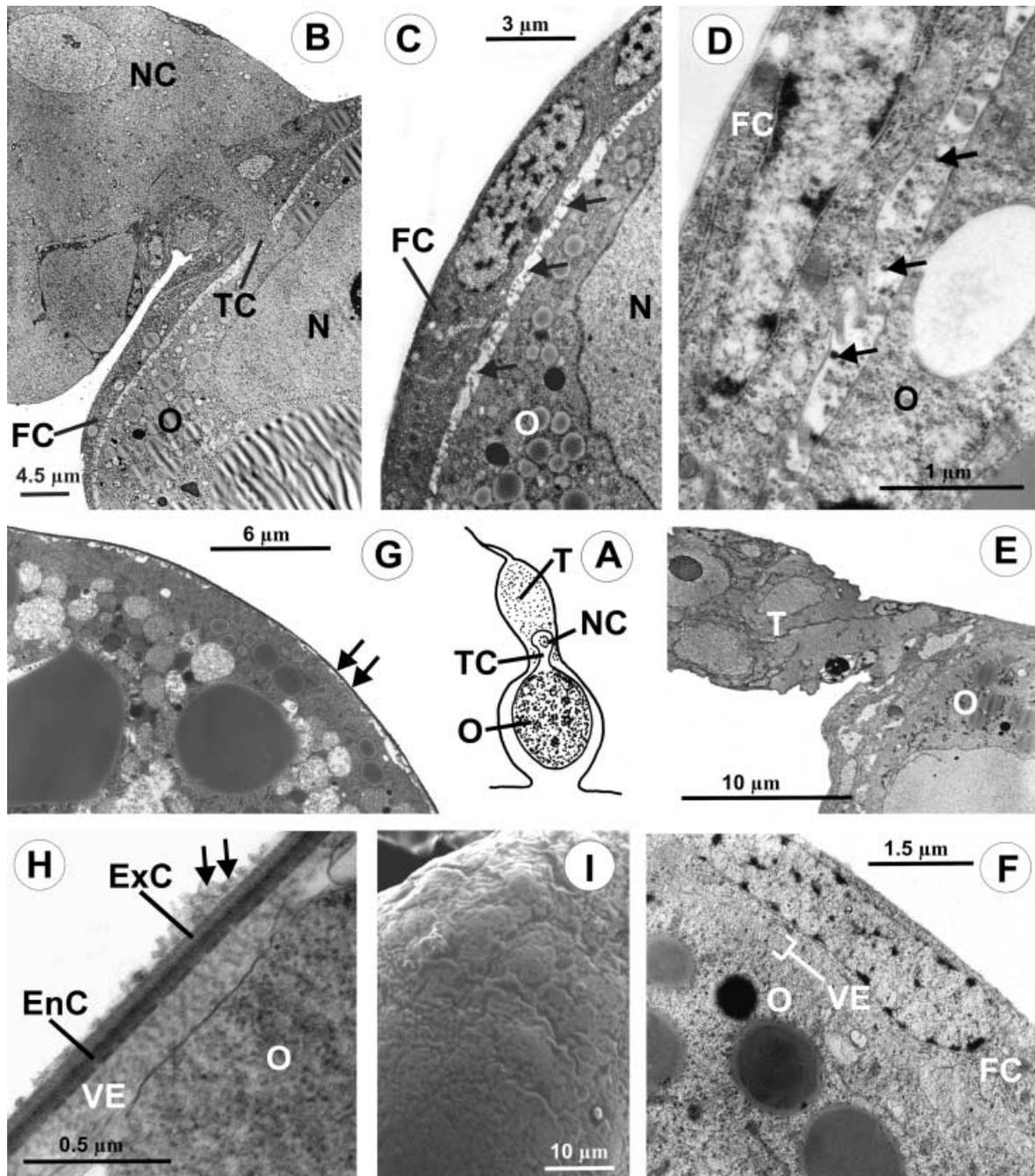
In the *subimago*, the apical region of the ovariole becomes thin and irregularly bordered, a feature consistent with its progressive degeneration (Fig. 2E). The vitelline envelope acquires a more homogeneous texture even though its granular appearance makes difficult to distinguish this layer from the cortical cytoplasm of the oocyte beneath (Fig. 2F).

In the *imago*, ovulated eggs are delimited with a thin electron-dense chorion (about 0.1  $\mu\text{m}$  thick) (Fig. 2G), which, at higher magnification, is seen to consist of two layers (endochorion and exochorion) separated by a darker line (Fig. 2H). The exochorion is enriched with irregularly flocculated material (Fig. 2H).

Ovulated eggs, observed under SEM, have an irregular surface because of the reduced thickness of the chorion, which tends to pucker over the vitelline envelope (Fig. 2I).

## Discussion

The present investigation confirms that meroistic telotrophic ovarioles, characterized by nurse cells that supply the oocyte with molecules and organelles during its previtellogenic growth, represent the typical model of Ephemeroptera



**Fig. 2**—Ultrastructural view of some phases of the eggshell (vitelline and chorionic envelopes) synthesis in *Cloeon dipterum*. —**A**. Sketch of a meroistic telotrophic ovariole showing the presence of a single maturing oocyte (O) per ovariole and the trophic cord (TC) connecting it to the nurse cell (NC) located in the tropharium (T). —**B**. Section showing the extension of the trophic cord (TC) that connects the growing oocyte (O) to the nurse cell (NC) of the tropharium. Follicle cells (FC) bound the oocyte except for the region crossed by the cord. N, nucleus of the oocyte. —**C**. Elongated follicle cells (FC) interlock apically with the plasma membrane of the oocyte (O) via a few microvilli (arrows). N, nucleus of the oocyte. —**D**. Electron-dense granules (arrows) accumulate at the oocyte (O)/follicle cell (FC) interface. —**E**. Tropharium (T) whose morphology is consistent with degenerative processes. O, oocyte. —**F**. Hardly discernible vitelline envelope (VE) interposed between follicle cells (FC) and oocyte (O). —**G**. An ovulated egg showing its thin chorionic envelope in the form of a continuous electron-dense coat (arrows). —**H**. Detail of the chorion revealing its organization in endochorion (EnC) and exochorion (ExC) separated by a darker line. Note the flocculated material adherent to the exochorion (arrows) and the granular appearance of the vitelline envelope (VE). O, oocyte. —**I**. SEM view of a region of the ovulated egg surface. Note the puckered appearance of the chorion as a result of its reduced thickness.

ovaries, as proposed by Gottanka and Büning (1993). The finding of a nutritive cord, connecting each egg to its nurse cells, resolves the doubt of Ikigame *et al.* (2000) about the ovaries of *C. dipterum* belonging to the meroistic telotrophic model.

In Ephemeroptera, owing to the short life of the adults, numerous eggs complete their development in the mature nymphs before the nymphs emerge from the water (Koss 1968). In these insects, follicle-cell activity is particularly intense owing to the complexity of the exochorion, which is usually decorated with a variety of sculptures (Koss and Edmunds 1974; Gaino and Mazzini 1988, 1989; Gaino and Flannagan 1995).

Mayflies are typically oviparous and *B. rhodani* shows the classical mayfly ovary model. In each ovariole the oocytes are arranged in a linear sequence and their growth proceeds from the distal region towards the basal one, in such a way that already in the nymphs with dark wing-pads, which are about to emerge, the eggs closer to the oviduct are ovulated. This process continues in the subimago.

*Cloeon dipterum* stands out among Ephemeroptera because of its ovoviviparity, a condition that leads to a drastic reduction in the number of growing eggs per ovariole. In this species, egg maturation lasts longer because the vitelline envelope becomes evident in the subimago and the chorion in the imago. Therefore, in agreement with Ikigame *et al.* (2000) vitelline and chorionic envelopes are completed in the imaginal stage. The maturation of a single egg per ovariole in *C. dipterum* is synchronized with the achievement of the adult stage.

The comparison between the oviparous *B. rhodani*, and the ovoviviparous *C. dipterum*, both belonging to the same family Baetidae, showed that the morphology and thickness of the egg envelopes reflect the biology of the species. Indeed, the thick egg envelopes of *B. rhodani* typically meets the requirement for laid eggs to survive in the environment up to the final development of the larvae, while eggs developing inside the mother can do this without such a protection.

Also, follicle cells behave differently in *B. rhodani* and *C. dipterum*. In the former, they are cube-shaped in the early stages of vitellogenesis and only after the synthesis of the vitelline envelope do they gradually extend to follow the oocyte growth. In the latter, the egg growth expands the follicle-cell epithelium already before the onset of eggshell elaboration, here including the synthesis of the vitelline envelope precursors.

In *B. rhodani*, the endochorion undergoes progressive changes, as usually occurs for the more complex egg layers in many insects (see review in Margaritis 1985; Rościszewska 1995). It is worth stressing that a fibrous exochorion is quite uncommon in Ephemeroptera because in these insects the exochorion has a more solid organization (Gaino and Mazzini 1988, 1989; Gaino *et al.* 2001). Nevertheless, this solidity could be assured by the chorionic pillars. They cross,

at intervals, the exochorion and connect its ‘honey-comb roof’ to the alveolate endochorion, thereby preventing the collapse of this layer. The presence of holes, pillars and a ‘roof network’ in the chorion of *B. rhodani* has been frequently observed in the eggs of other insects, regardless of their taxonomic position (see review in Margaritis 1985).

In *C. dipterum* the chorion is very thin and the flocculated material adherent to the exochorion could be responsible for the firm adhesion of the laid eggs to the substratum before the moving out of the larvae, as suggested by Degrange (1960). Indeed, at contact with water, the eggs hatch almost immediately because the larvae, the heads of which are provided with a special ‘ruptor ovi’, open the chorion along a polar region of weakness that facilitates their escape (Degrange 1959, 1960).

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