Oogenesis of the Mayfly Habrophlebia eldae: Synthesis of Vitelline and Chorionic Envelopes

Massimo Mazzini and Elda Gaino

Istituto di Difesa delle Piante (M.M.), Università della Tuscia, Viterbo and the Istituto di Zoologia (E.G.), Università di Genova, Genova, Italy

The ultrastructure of developing ovarian follicles inside the panoistic ovarioles of Habrophlebia eldae were examined to observe the events occurring during egg maturation up to the full formation of the chorionic envelopes. The early vitellogenic follicles are coupled by gap junctions and are extensively interlocked with the oocyte plasma membrane via microvilli. With the onset of vitellogenesis, coated pits and coated vesicles are precursors to yolk deposition and are visible at the follicle cell-oocyte interface. Postvitellogenic development entails the deposition of the egg envelopes. The vitelline envelope arises from the coalescence of rectangular plaques whose precursors are visible in Golgi complexes as heterogeneous electron-opaque granules. A chorionic pattern of ridges on the egg surface characterizes the shell of H. eldae. The fully developed chorion shows three distinct regions with differently organized patterns. A fine layer of fibrous material (a secretion of the follicle cells, Ephemeroptera devoid of accessory glands) adheres to the egg chorion and is probably involved in attachment to the substrate.

Key words: oocyte growth, Ephemeroptera, egg shell, electron microscopy

INTRODUCTION

Numerous scanning electron microscope (SEM) studies have been performed on ephemeropteran eggs [Alba-Tercedor and Sowa, 1987; Flowers, 1980, 1986; Kopelke, 1980; Kopelke and Müller-Liebenau, 1981a, b, 1982; Malzacher, 1982, 1986; Pescador and Peters, 1982; Gaino and Mazzini, 1984, 1987; Mazzini and Gaino, 1985; Sowa and Soldàn, 1986]. These studies have mainly revealed the characteristics of chorion sculpture, the micropyle, and the attachment structures, which can be particularly useful in constructing ootaxonomic keys.

There have been no recent publications on Ephemeroptera reproduction. The anatomical and histological features of the female reproductive system was examined in six European species [Soldàn, 1979]. This study, using classical cytological methods, describes the structure of the ovary in different developmental stages. No
ultrastructural information on the oogenesis of mayflies has yet been published, with the exception of some data on the mature egg shell of *Habrophlebia eldae* [Jacob and Sartori, 1984], the revised Italian *H. fusca*. The present paper is the first report on the fine structure of Ephemeroptera oogenesis. It describes the morphological events during oocyte development and the follicular cells during the oogenesis of *H. eldae*, as observed by transmission (TEM) and scanning electron microscopy.

**MATERIALS AND METHODS**

Ovarian follicles were obtained by dissection of larvae and young nymphs of *H. eldae* collected in the Erro Stream (Piemonte). The eggs were taken from mature nymphs and imagos.

**Scanning Electron Microscopy Analysis**

Eggs stored in 80% ethanol or fixed for 2 hr in 5% glutaraldehyde and 4% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.2, at 4°C were dried by the critical-point method using liquid CO₂ in a Bomar apparatus. They were attached to specimen holders with silver conducting paint, coated with gold in a Balzers Union evaporator, and observed with a Philips EM 505 or a Coates & Welter Cwiskan 106A field emission scanning electron microscope. Some eggs were observed without critical-point treatment.

**Thin-Section Analysis**

Ovarian follicles at different developmental stages were fixed for 1 hr in 5% glutaraldehyde and 4% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.2, at 4°C placed for 12 hr in cacodylate buffer, postfixed in 1% OsO₄ for 1 hr, dehydrated in a graded ethanol series, and embedded in Epon 812 and Epon-Araldite. Sections were cut with either an LKB III or a Reichert OM U2 ultratome, mounted on grids, stained with uranyl acetate and lead citrate, and observed with a Philips EM 300 at the Institute of Zoology of the University of Siena.

**Freeze-Fracture Analysis**

Ovarian follicles were fixed in glutaraldehyde-formaldehyde as above and gradually infiltrated in glycerol to a final concentration of 30%. They were then rapidly frozen in freon 22 cooled to the temperature of liquid nitrogen. Fracture and platinum carbon coating were carried out in a Balzers BAF 301 freeze etching apparatus set at −115°C. Replicas were eventually digested with Clorox, picked up with formvar-coated copper grids, and examined with a Philips EM 301 electron microscope.

**RESULTS**

The ovary of *H. eldae* consists of a parallel array of panoistic ovarioles. As observed by Soldàn [1979] in the Ephemeroptera female reproductive system, each ovariole includes a number of follicles that follow one another in a linear sequence of increasing size. The most anterior part of the ovariole includes the terminal filament (TF), which attaches the ovariole to the inner body wall. The germarium is located under the TF, and its apical portion contains a cluster of oogonia among the differentiating oocytes and peripherally placed prefollicular cells. The last follicle in the
ovariole is always in vitellogenic growth, whereas the preceding one is in previtellogenesis or early vitellogenesis. Ovulation of the terminal follicle in the ovariole seems to trigger inception of vitellogenesis in the penultimate one.

Early in ovarian development, the oocytes at the posterior of the germarium become associated with the columnar follicular epithelium made up of tightly packed cells (Fig. 1). These cells are anchored basally to the tunica propria and apically interlocked with the oocyte plasma membrane via a few microvilli (Fig. 2). No prominent intercellular spaces can be observed between adjacent follicle cells (FC) or at the follicle cell-oocyte (FC-OC) interface (Fig. 2). In early vitellogenic follicles, microvilli emerging from the oocyte surface extensively interlock with the follicle cells. Thin sections at the FC-OC interface reveal numerous coated pits having the characteristic clathrate pattern, and the cortical ooplasm appears to contain many coated vesicles and yolk spheres (Fig. 3). The yolk spheres that lie close to the oolemma are formed by the coalescence of small vesicles into larger aggregates. When vitellogenesis commences, electron-dense amorphous material begins to be irregularly positioned at the FC-OC interface (Fig. 4). The follicular epithelium consists of densely packed cells containing many mitochondria and ribosomes; the latter are free or associated with the endoplasmic reticulum. Most of the cell volume is occupied by a large nucleus with uniformly dispersed chromatin (Fig. 5). At this stage the follicle cells are closely apposed to one another via macular-type gap junctions (Fig. 6). Their contact surface lies almost perpendicular to the egg surface (Fig. 5).

Later the FC flatten to less than 3 μm (Figs. 7 and 8) and are joined by desmosomes and septate junctions located near the FC-OC interface (Fig. 8). The contact surfaces between follicle cells have increased and are oriented at more oblique angles to the longitudinal axis of the egg (Fig. 8). The elongate nucleus has its major axis parallel to the oocyte surface, is characterized by one or sometimes two prominent nucleoli, and occupies a major portion of the cell volume (Figs. 7 and 8). Rough endoplasmic reticulum is very abundant as vitelline envelope deposition proceeds. Golgi complexes are more numerous during this and subsequent egg shell deposition phases than during the earlier stages of vitellogenesis.

Vitelline membrane deposition is indicated by the presence of electron-dense material on the surface of the oocyte (Figs. 7–10). Vitelline envelope precursors are visible in the Golgi complexes (Fig. 9). The material is located upon the oocyte surface in electron-dense ellipsoidal granules of about 0.3 μm (Fig. 7), which subsequently fuse (Fig. 9) into rectangular plaquets that form a coat about 0.4 μm thick (Fig. 8). Freeze-fracture analysis showed that the fully formed vitelline envelope arises from the coalescence of the rectangular plaquets, which are composed of heterogeneous particles (Fig. 10).

After vitelline envelope deposition, we observe its detachment from the follicle cells to form a sharp intercellular space. During this sequence of events, in which first the VE and then the chorionic envelope are formed, multiple Golgi complexes are evident inside the follicle cells, which are also seen to contain many mitochondria and abundant rough endoplasmic reticulum organized in parallel sinuous rows (Fig. 11). The morphological events leading to chorionic formation trigger inception by the deposition of electron-dense bodies (Fig. 11) released at the apical part of the FC where they coalesce to form the inner chorion layer. The latter first consists of discontinuous material apposed to the internal face of the FC (Fig. 12). This material
then becomes organized, first as a continuous sheet with two distinct zones: an inner homogeneous paler zone and an outer irregular darker zone (Figs. 12, 13). As inner chorion layer deposition proceeds, a sort of periodic sequence of membrane-like units becomes a characteristic feature of this sheet.

As a consequence, the inner layer averages 0.3 μm in thickness when completely formed, with a periodic distribution of electron-dense lamellae more or less perpendicularly oriented with respect to the egg surface (Fig. 14). Each lamella, about 17 nm thick, is separated from the others by a distance of 10 nm.

At SEM level, the eggs of *H. eldae* are characterized by longitudinal costae arising from the chorionic surface and separated from each other by intercostal areas (Fig. 15). Adhesive material is often present on the egg chorion (Fig. 16). The fully formed chorion has a different costal and intercostal organization. The portion adjacent to the inner layer forms the intermediate chorionic layer and consists of loosely structured fibrillar material sometimes organized in an irregular lattice (Figs. 17 and 18). In the costal areas, the intermediate layer is particularly wide and constitutes the core of columnar projections arising from the egg chorion and separated from each other by chambers (Fig. 17). This intermediate coat supports the outermost layer of the chorion, 0.3 μm thick, which in both costal and intercostal areas is highly electron-opaque and consists of two laminae separated by a sinuous electron-transparent stratum. The outermost lamina has an irregular surface (Fig. 18), which imparts a granular appearance to the chorion coat and forms small protrusions.

**DISCUSSION**

This is the first time that the pattern of cell differentiation of the oocyte and ovarian follicle cells has been documented in Ephemeroptera. Our aim was to gain knowledge of the regulation of vitellogenesis in the panoistic-type ovary by describing the previtellogenic and vitellogenic stages in the synthesis of the vitelline and chorionic envelopes.

Our findings show that the follicular epithelium develops from prefollicular cells of the upper germaria region. During the early stages of vitellogenesis, the FC are coupled by macular-type gap junctions and extensively interlocked with microvilli that protrude from the oocyte surface. As the ovarian follicle undergoes rapid vitellogenic growth, the FC flatten. The decrease in height of the epithelium during oogenesis is accompanied by a simultaneous increase in egg envelope thickness. The FC cytoplasm is seen to contain abundant rough endoplasmic reticulum and numerous

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Fig. 1. Low power micrograph of the germarium region showing an oocyte (oc) encircled by follicle cells (fc). ×3,400.

Fig. 2. Enlargement of the previous micrograph showing portion of the follicle cell-oocyte interface (fc-oc) with a few microvilli (mv) and basement lamina (bl). ×12,000.

Fig. 3. Follicle cell-oocyte interface (fc-oc) from an early vitellogenic follicle with many microvilli (mv), coated pits (cp), a coated vesicle (cv), yolk spheres (y), and a desmosome (d). ×48,000.

Fig. 4. Electron-dense amorphous material (arrows) evident at the follicle cell-oocyte interface (fc-oc); follicle cell nucleus (n), mitochondria (m), yolk sphere (y). ×48,000.
Fig. 5, 6.
Figs. 7 and 8. An alleged sequence of vitelline envelope (ve) formation showing the coalescence of the electron-dense material upon the oocyte surface; follicle cell (fc), mitochondria (m), follicle cell nucleus (n), septate junctions (sj), yolk spheres (y). Both ×13,000.

Figs. 9 and 10. Vitelline envelope (ve) formation observed in a fixed oocyte (Fig. 9) and a freeze fracture replica (Fig. 10). Rectangular plaquets composed of particles are visible upon the follicle cell (fc); Golgi apparatus (g). Both ×32,000.

Fig. 5. Low-magnification micrograph showing the follicle cell-oocyte interface (fc-oc) in an early vitellogenic follicle; basement lamina (bl), mitochondria (m), follicle cell nucleus (n), yolk sphere (y). ×19,000.

Fig. 6. Freeze-fracture replica of the follicle cell-oocyte (fc/oc) interface in an early vitellogenic follicle; gap junctions (gj), microvilli (mv). ×31,000.
Golgi complexes. During this stage of synthetic activity, we observed electron dense granular material that was subsequently released to form the egg envelopes.

The vitelline envelope progresses through morphological changes that result in the formation of a thick coat. The peculiar inner region of the chorion is characterized by a periodic distribution of electron-dense lamellae perpendicularly disposed to the oocyte. Norton and Vinson [1982] observed a similar type of periodicity in the inner chorionic layer of the ichneumonid parasitoid Campoletis sonorensis, and Furneaux and Mackay [1972] reported the presence of polycrystalline proteinaceous material in several insect orders, excluding Ephemeroptera.

The presence of many coated pits and vesicles at the follicle cell-oocyte interface suggests that yolk deposition in H. eldae is accomplished by endocytic sequestration of material elaborated in a tissue other than the ovary, namely the fat body in insects [Engelmann, 1979] and the liver in nonmammalian vertebrates [Clemens, 1974]. The formation of yolk spheres can be seen as the progressive coalescence of smaller vesicles into larger aggregates.

It is interesting to observe that while the FC flatten, the septate junctions joining them increase in surface adhesion. This is principally because of their orientation, which changes from perpendicular to almost parallel, forming an oblique angle to the egg surface.

As in many other genera of mayflies [Degrange, 1960; Kopelke and Müller-Liebenau, 1981a,b, 1982; Koss, 1968, 1970; Koss and Edmunds, 1974; Soldàn, 1979], the material adhering to the chorionic surface in H. eldae may be due to the activity of the follicle cells. In fact, the absence of accessory reproductive glands [Brinck, 1957] or secretory activity in the epithelial cells of the oviduct [Soldàn, 1979] tends to confirm that the adhesive layers are secreted by the follicular cells at the end of their cycle of activity. The suprachorionic layer seems to be involved in adhesion of the cell to the substrate when the eggs are laid. SEM analysis of the other species of Ephemeroptera reveals a chorionic pattern of knob-terminated coiled threads for adhesion to the substrate [Gaino and Mazzini, 1987; Gaino et al., 1987]. The lack of specialized attachment structures in the egg of H. eldae suggests that this is achieved by the adhesive material.

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Figs. 11 and 12. Sequence showing the activity of the follicle cell (fc) during the formation of the inner chorion layer (icl); basement lamina (bl), rough endoplasmic reticulum (rer), septate junctions (sj). Figure 11, ×20,000; Figure 12, ×80,000.

Figs. 13 and 14. The inner chorion layer (icl) is first organized as a continuous sheet (Fig. 13) and then as a periodic sequence of electron-dense lamellae (Fig. 14) disposed perpendicularly to the egg surface. Figure 13, ×120,000; Figure 14, ×80,000.
Fig. 15–18.
REFERENCES


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Fig. 15. Whole egg of *H. eldae* observed with a scanning electron microscope (SEM). ×500.

Fig. 16. SEM view of the external surface of the egg with clear costal (c) and intercostal (ic) areas and traces of adhesive material (arrows). ×3,000.

Fig. 17. Organization of the chorion in the costal area. From the inside: inner chorion layer (icl), intermediate chorion layer (imcl), chambers (ch), outer chorion layer (ocl). ×20,000.

Fig. 18. Organization of the chorion in the intercostal area. Note the inner chorion layer (icl), the thin intermediate chorion layer (imcl), and the bilayered outer chorion layer (ocl). ×38,000.