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Structure and development of the egg of the glossiphoniid leech *Theromyzon rude*: characterization of developmental stages and structure of the early uncleaved egg

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Summary

Some aspects of the reproductive biology of the glossiphoniid leech, *Theromyzon rude*, under laboratory conditions, and the staging and structure of its uncleaved egg were studied. Sexually mature animals form breeding communities and fertilization occurs in the ovisacs, presumably around the time of egg laying. Oviposition may be postponed for hours or days, but the eggs in the ovisacs remain blocked at first meiotic metaphase. Development of the uncleaved egg, from the time of oviposition to completion of the first cleavage division, has been subdivided into six stages. At 20 °C, the six developmental stages take 5–6 h. Characterization of the stages is based on observations of both live and fixed/cleared eggs. Discharge of the first pole cell, by the end of stage 1a, is associated with the movement of a ring of contraction between the equator and the animal pole. Discharge of the second pole cell, by the end of stage 1b, is accompanied by contraction of the animal hemisphere which becomes a cone-shaped structure. Polar rings and meridional bands of contraction make their appearance by stages 1c and 1d, respectively. Constriction of the polar rings and shortening of the meridional bands, during stage 1e, lead to accumulation of ooplasm at both egg poles. In this manner, the teloplasm or pole plasm forms. Completion of the first cleavage furrow, by the end of stage 1f, is preceded by dorsoventral flattening of the egg and rearrangement of its teloplasm and perinuclear plasm. Structure of the early uncleaved egg has been studied with transmission and scanning electron microscopy of intact or permeabilized preparations. The plasmalemma forms numerous long and some short microvilli evenly distributed across the egg surface. The ectoplasm includes many vesicles, mitochondria, granules and an elaborate network of filament bundles. Staining of this network with rhodamine-labelled phalloidin indicates that it contains F-actin. A less complex network of similar filaments is also present throughout the yolky endoplasm. The meiotic spindle lies close to the egg surface and not far from the presumptive animal pole ectoplasm. It comprises highly developed poles whose structure and relationships are described.

Key words: egg structure, egg development, leech, *Theromyzon rude*, cleavage.

Introduction

Much of the early and late literature on glossiphoniid leeches indicates that the embryos may be advantageously used for the study of developmental processes (Whitman, 1878; Schleip, 1936; Dawydoff, 1959; Fernández & Olea, 1982). Recent studies in developmental neurobiology have successfully utilized the leech embryo in the exploration of the role of cell lineage and cell–cell interactions in the development of the nerve cord (Weisblat, 1981; Stent & Weisblat, 1982, 1985; Weisblat & Stent, 1982; Weisblat, Kim & Stent, 1984).

Another interesting feature of the leech embryo is related to the possibility of tracing the cytoplasm of many of its cells to specific regions of the uncleaved egg. Thus, the poles of the egg include organelle-rich domains of ooplasm (called teloplasms) destined to be utilized in the formation of ectodermal and mesodermal cells. Hence, the animal and vegetal teloplasms may be repositories of certain morphogenetic determinants. Completion of meiosis and formation

of teloplasm are accompanied by prominent deformation movements of the uncleaved egg. Thus, the glossiphoniid leech egg represents a potentially valuable material for the study of the mechanism underlying both mosaic development and motility in nonmuscle cells. The manner of formation of ooplasmic domains in the egg of certain glossiphoniids, such as those of the genera *Glossiphonia* and *Theromyzon*, are only comparable to those presented by the egg of the oligochaete *Tubifex* (see Penners, 1922; Shimizu, 1982*a*, 1986). It is not surprising then that the study of their eggs attracted the attention of many early investigators of metazoan development such as Whitman (1878). His work deserves special mention because he presented a surprisingly detailed and accurate description of the structure and development of the uncleaved egg of some *Glossiphonia*. Some preliminary information on the structure and development of the uncleaved egg of the glossiphoniid *Theromyzon rude* is found in Fernández (1980) and in Fernández & Olea (1982).

This paper is the first of a series intended to analyse the structure and development of the uncleaved egg of *T. rude*. In this presentation we examine some aspects of the reproductive biology of the leech under laboratory conditions and also provide a description of the structure and of the stages of development of the uncleaved egg.

Materials and methods

Adult specimens of the leech *Theromyzon rude* were collected in ponds of the Golden Gate Park (San Francisco, California) and around Calgary (Canada), during June and July. Animals were found under rocks or hard objects, either alone or in groups. They were placed in large plastic jars containing artificial spring water (see Fernández & Olea, 1982). Breeding was performed in a thermoregulated chamber at 14°C in complete darkness. If breeding needs to be accelerated, a group of animals may be removed and placed at higher temperature. In this manner, the availability of mature eggs was controlled. The characteristic social behaviour of leeches during the breeding season is also seen under laboratory conditions. Thus, solitary and grouped animals are found in the breeding jars. The former are unfed, have recently fed or have already laid eggs. Grouped leeches constitute breeding communities of variable size. Animals enter the community as males, with guts full of blood, and leave as females, with empty guts and highly developed ovisacs. At 14°C, gravid leeches usually leave the breeding community 1–2 days before oviposition.

Eggs are considered ready to be laid when their colour turns brown and when they become clustered within the ovisacs. Each cluster presumably comprises the set of oocytes destined to be housed within a cocoon. Animals lay four to six cocoons per clutch, with each cocoon including 15–30 eggs. Accordingly, one clutch includes 60–180 eggs.

In order to postpone egg laying, gravid females may be continuously agitated in a shaker or their suckers can be rendered useless by piercing them with minute insect pins. The latter procedure was used when mechanical agitation failed to block egg laying. Uncleaved eggs may be obtained from laid cocoons or directly from the ovisacs. Since development of the egg starts as soon as it leaves the ovisacs and takes a couple of hours to complete oviposition, eggs within a single cocoon develop asynchronously when laid naturally (for more details see Fernández, 1980; Fernández & Olea, 1982). Removal of ripe eggs from the ovisacs has the advantage that all of them will develop synchronously. For this purpose, unanaesthetized leeches were cut and pinned open in a Sylgard-filled Petri dish. The paired ovisacs were removed and opened in artificial spring water, which is a convenient culture medium for uncleaved eggs.

Development of the uncleaved egg may be studied at temperatures ranging from about 8 to 25°C. Development seems to be reversibly blocked at approximately 3°C. Thus, eggs may be stored at that temperature for a couple of hours if needed. A total of approximately 2000 eggs, coming from 50 pregnant leeches, was used in this study.

Examination of live and cleared eggs under the dissecting microscope

Development of the uncleaved egg may be conveniently studied by combining the observation of live with fixed/cleared eggs. Eggs were fixed in ALFAC (95% ethyl alcohol, 85 ml; 40% formaldehyde, 10 ml; glacial acetic acid, 5 ml) for 12–24 h at room temperature. After dehydration in absolute ethanol (two changes of 1 h each), eggs were cleared and stored in methyl benzoate. Live and cleared unmounted whole eggs were examined against a dark background using reflected cold light.

Light and transmission electron microscopy

Eggs were fixed 1·5–2 h in 50% Karnovsky solution (see Fernández & Stent, 1980) or in 3% glutaraldehyde in 0·1 M-phosphate buffer pH 7·4, containing 0·15% of tannic acid. The latter fixation, performed at room temperature, gave reasonably good preservation of microtubules and microfilaments. After rinsing in buffer at room temperature, eggs were postfixed in 1% OsO_4 for 1 h at room temperature in the dark, dehydrated in graded ethanol and slowly embedded under vacuum in Epon 812. For light microscopy, 1 μm sections of the embedded egg were stained with 1% toluidine blue in 1% sodium borate. For electron microscopy, thin sections of the embedded egg were placed on collodion-coated copper grids and double stained with alcoholic uranyl acetate and lead citrate. Sections were viewed in a Philips EM 300 electron microscope.

Scanning electron microscopy

Eggs were fixed in 2·5% glutaraldehyde in 0·1 M-cacodylate buffer pH 7·4 for 2 h at 4°C. After rinsing in the same buffer, eggs were dechorionated (removal of the perivitelline envelope) with fine tweezers and postfixed in 1% OsO_4 at room temperature and darkness for 2–3 h. Tissues dehydrated in graded acetone were critical-point dried from CO_2, mounted and then coated with a layer of gold approximately 30 nm thick. For this purpose, a Polaron

E 5000 sputter apparatus was used. Samples were examined in a Philips EM 300 electron microscope, equipped with a scanning device.

To explore the organization of the ectoplasmic cytoskeleton, eggs were treated with extraction buffer containing the nonionic detergent Triton X-100. Successful preparations of whole-mounted cytoskeletons of early uncleaved eggs were obtained with the extraction buffer designed by Schliwa (1980, see also Schliwa & van Blerkom, 1981). This buffer includes Mg^{2+}, Pipes, Hepes, EGTA and 0·15 % Triton X-100 at pH 7. Dechorionated eggs were briefly washed in extraction buffer without detergent and then treated for 5–10 min in the same buffer with detergent at room temperature. After a brief rinse in buffer without detergent, eggs were fixed in 3 % glutaraldehyde in 0·1 M-cacodylate buffer for 1 h at room temperature. After rinsing in cacodylate buffer for 1 h, eggs were postfixed in 1 % OsO_4 for another hour at room temperature. Tissues were then dehydrated in graded acetone, critical-point dried from CO_2 and covered with gold.

Staining of isolated cortices and of whole-mounted permeabilized eggs with rhodamine-labelled phalloidin (RLP)

Eggs treated with the Schliwa extraction buffer were briefly rinsed and then fixed in 4 % paraformaldehyde in 0·1 M-phosphate-buffered saline (PBS) pH 7·4, for 15 min at room temperature. After rinsing in PBS another 15 min, permeabilized eggs were incubated in a solution of approximately 1 $\mu g\,ml^{-1}$ of RLP (kindly provided by Dr Schliwa) in PBS for 20 min at room temperature. Eggs were again rinsed in PBS for about 30 min and finally mounted under coverslip in glycerol–PBS (9:1) containing 0·1 M-n-propyl gallate. To avoid compression of eggs, the corners of the coverslip were provided with small plasticine stoppers. The samples were examined in a Zeiss microscope equipped with epifluorescence illumination and utilizing the rhodamine excitation filter.

To determine the distribution of actin filaments across the endoplasm and their connections with the ectoplasmic actin network, stained eggs were either fractured or slightly compressed under the coverslip.

To prepare isolated egg cortices, dechorionated eggs were placed on coverslips coated with 0·1 % L-polysine (Sigma). After breaking the egg with tweezers, jets of extraction buffer with detergent were directed against the preparation. The extraction continued for 5–10 min. After buffer rinsing, fixation and staining with RLP, egg peels were similarly mounted under coverslips without stoppers and viewed under epifluorescence optics.

Results

Observations on the behaviour of *T. rude* breeding in the laboratory, and also in the field, indicate that copulation probably takes place during the period of several weeks in which animals remain together in breeding communities. Copulation in *T. rude* is known to last for several days (Wilkialis & Davies, 1980). Since at 14 °C animals leave the community a few days before egg laying, the final steps of egg maturation and perhaps fertilization take place in solitary animals. Data presented under Materials and methods strongly suggest that fertilization in *T. rude* occurs in the ovisacs. This conclusion can also be extended to other glossiphoniids because fertilized eggs may be obtained directly from the ovisacs of several different species used in our laboratory (unpublished observations). Sometimes eggs removed from the ovisacs appear to lack a fertilization membrane, the perivitelline chamber is absent and development fails to start. These features probably indicate that the eggs were not fertilized and therefore the cortical reaction has not yet taken place. This result strongly suggests that fertilization may occur a few hours before the initiation of egg laying.

When pregnant leeches are subjected to continuous agitation in a shaker or their suckers are immobilized, egg laying may be postponed for hours or days. Egg laying may also be interrupted and resumed later if during delivery the mother is disturbed. Examination of freshly laid eggs and of eggs that have remained in the ovisacs for varying lengths of time reveals that development of their meiotic nuclei has not progressed beyond metaphase I. Thus, it is clear that intraovarian development of the fertilized egg is subjected to meiotic arrest.

Staging of the development of the uncleaved egg

Stage 1 of development has been subdivided into six substages designated 1a–1f. Each stage is defined as that moment in which the process diagnostic for that stage has been completed. A diagrammatic representation of the development of the uncleaved egg is shown in Fig. 1. The following characterization of the developmental stages of the uncleaved egg is based on observations of both live (Fig. 2) and fixed/cleared (Fig. 3) eggs developing at 20 °C.

Stage 1a. Emission of the first pole cell (0.00–2.00 h)

Fertilized eggs removed from the ovisacs or spontaneously laid by the mother have a perivitelline space filled with fluid. This fluid includes tiny particles and becomes opalescent when the eggs are properly illuminated. Fresh eggs are spherical and yellow and measure 650–700 μm in diameter (Figs 2A, 3A). The first sign that the meiotic arrest has been released and that development is under way is the appearance of a shallow circular depression, 25–50 μm in diameter, at

the egg surface. This region, called the grey spot, corresponds to the outer pole of the meiotic spindle. It marks the site of release of the pole cells and accordingly the animal pole of the egg. Between 1.15 and 1.45 h, the egg develops two lobes as an equatorial or slightly supraequatorial ring of contraction appears (Fig. 2B). As this ring of contraction moves towards the animal pole, the first pole cell emerges at the centre of the grey spot (Fig. 2C). This is a transparent cell, 25–50 μm in diameter, that remains connected to the surface of the grey spot until discharge of the second pole cell.

Stage 1b. Emission of the second pole cell (2.00–3.00 h)

Deformation of the animal hemisphere of the egg often ceases shortly after emergence of the first pole cell. Although the egg resumes its spherical shape (Fig. 2C), it soon enters into a new period of deformation. Flattening of both hemispheres, along the

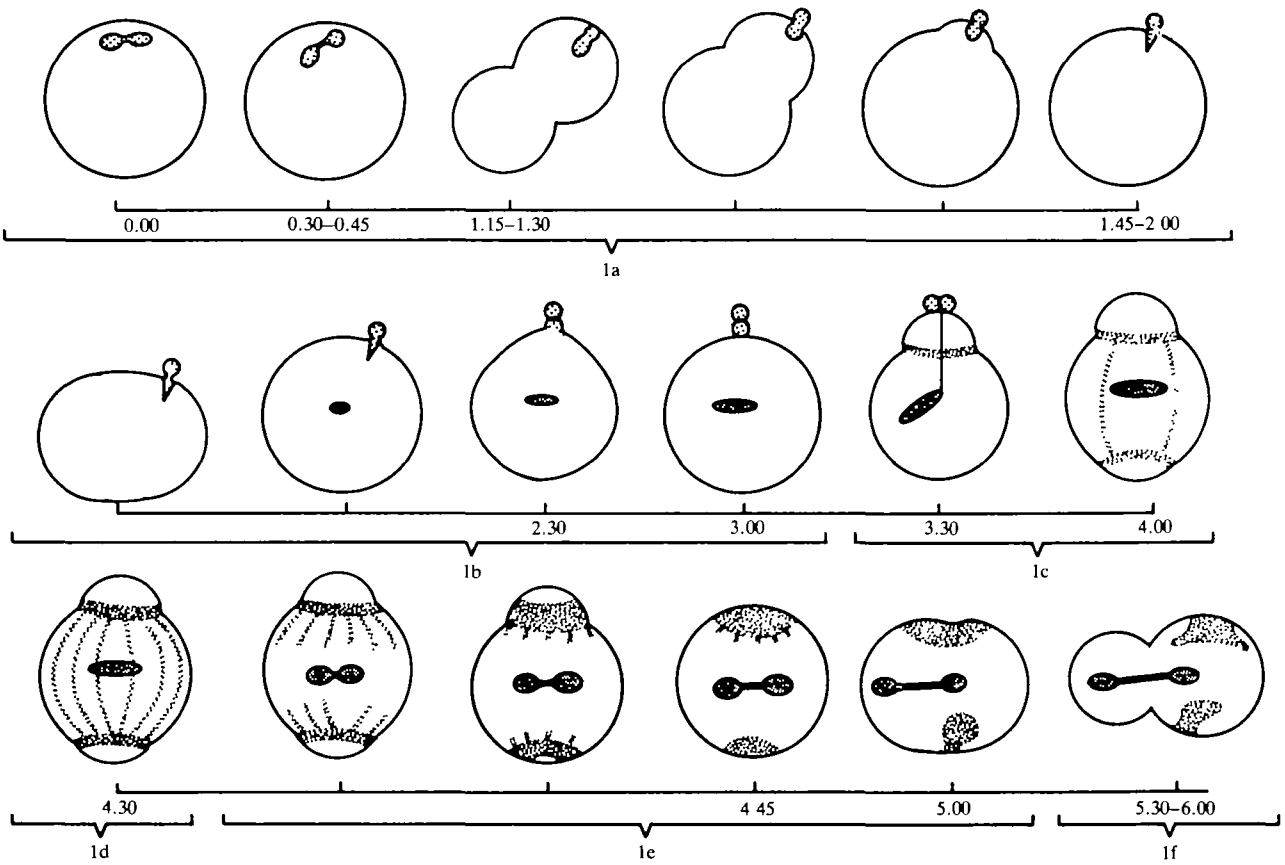


Fig. 1. Diagrams that illustrate the development of the uncleaved egg of *T. rude* at 20°C. Drawings are side view representations based on the observation of both live and cleared eggs. The time of development (0–6.00 h) and the staging of the uncleaved egg (1a–1f) are indicated. The figure-eight structure of stage 1a eggs and the conic structure of stage 1b eggs correspond to the meiotic nucleus I and II, respectively. Ripe eggs, removed from the ovisacs or laid spontaneously (0 h eggs), are blocked at metaphase I. During stage 1a, the first meiotic division is completed. Notice that emission of the first pole cell is preceded by the appearance of a ring of contraction that moves from the equator toward the animal pole. During stage 1b, the second meiotic division is completed and formation of the perinuclear plasm is initiated at the centre of the egg. Emission of the second pole cell is also preceded by a deformation movement of the animal hemisphere of the egg. At about the same time a deformation of the vegetal hemisphere causes the establishment of the vegetal protuberance. During stage 1c, the egg becomes engaged into a series of deformation movements leading to redistribution of its ectoplasm. In this manner, the polar rings and the first meridians are formed. By this time, the perinuclear plasm has extended into a prominent disk-shaped structure. Completion of the system of meridians is reached by the end of stage 1d. During stage 1e, and as a result of constriction of the polar rings and shortening of the meridians, ectoplasm is accumulated at the egg poles to constitute the animal and vegetal teloplasms. Meanwhile, the perinuclear plasm has turned into a figure-eight shape and then a dumb-bell-shaped structure. Dorsoventral flattening of the egg is initiated at stage 1e. Completion of the first cleavage division takes place by the end of stage 1f. Notice that during this stage the teloplasm and the perinuclear plasm have moved in opposite directions. Since the first cleavage furrow bisects the perinuclear plasm, it passes beside the teloplasm. In this manner, the egg divides unequally yielding two blastomeres of different size and structure. For more details see the text.

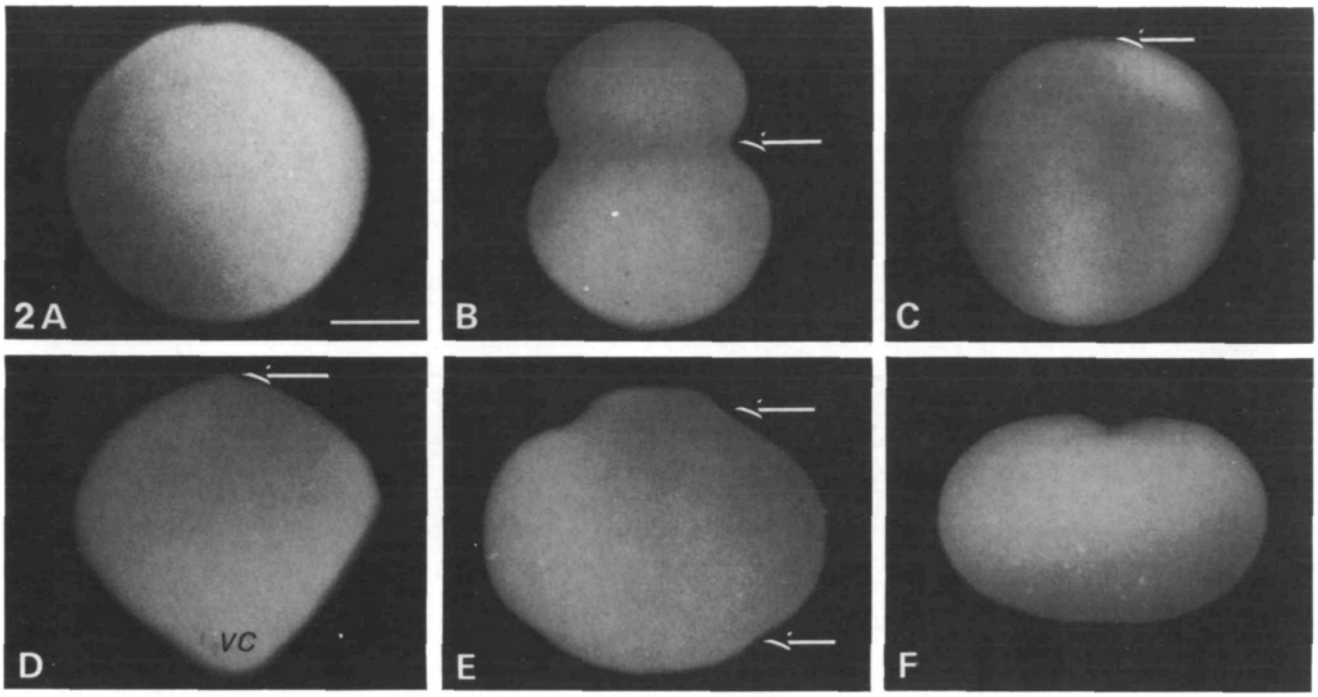


Fig. 2. Side view of live eggs photographed under dark-field illumination. (A) early stage 1a egg; (B) late stage 1a egg showing the ring of contraction (arrow) associated with release of the first pole cell; (C) early stage 1b egg in which the first pole cell (arrow) appears at the centre of the flattened animal pole; (D) late stage 1b egg showing the deformation movements associated with release of the second pole cell (arrow) and with formation of the vegetal protuberance (*vc*); (E) stage 1c egg viewed at the time of establishment of the two polar rings (arrows); (F) stage 1f egg viewed shortly before appearance of the first cleavage furrow. Magnification bar, 200 μm.

animal–vegetal axis, results in the egg resembling a biconvex lens. After about 30 min, the egg again recovers its spherical shape. At 2.30–2.45 h the animal hemisphere of the egg is deformed into a cone-shaped structure which persists for about 15 min (Fig. 2D). During this time the second pole cell emerges from the animal pole immediately below the first pole cell. These two cells are similar in size and appearance and lie one on the top of the other. As release of the second pole cell is completed, the vegetal hemisphere deforms into a cone-shaped structure similar to that produced shortly before in the animal hemisphere. This deformation lasts for approximately 15 min and is followed by the protrusion of the vegetal pole. This constitutes the vegetal protuberance (Fig. 2D).

Inspection of cleared eggs at late stage 1b shows that a mass of opaque ooplasm appears at the centre of the egg. Moreover, after singamy is completed this central mass of ooplasm is destined to house the cleavage nucleus. For that reason, the central domain of ooplasm is designated as perinuclear plasm (Fig. 3B).

Stage 1c. Formation of the polar rings (3.00–4.00 h)

After termination of the animal and vegetal hemisphere deformation, many eggs suffer a brief dorsoventral flattening of their animal hemisphere. Meanwhile, the first pole cell comes down to the egg surface. Division of the first pole cell does not seem to occur.

Another episode of deformation movements takes place at both hemispheres of the egg 30–45 min later. First, a ring of contraction appears at the upper third of the animal hemisphere which bulges out in the form of a dome-shaped structure about 100 μm high (Fig. 2E). By this time, the animal pole is marked by a shallow depression that may include the two degenerating pole cells. The ring of contraction of both live and fixed/cleared eggs becomes increasingly conspicuous as whitish or opaque ooplasm gradually accumulates at its walls. The animal polar ring, thus formed, is about 50 μm thick and 250–500 μm in diameter (Fig. 3C). The vegetal polar ring forms similarly, but later, at the other hemisphere of the egg. The vegetal polar ring is often smaller (150–400 μm in diameter) and less marked on the egg surface (Fig. 2E).

Stage 1d. Establishment of the system of meridians (4.00–4.30 h)

Meridional bands of contraction are initiated at the equator of the egg in the form of short and shallow grooves first seen by midstage 1c. Grooves become

prominent as they grow in length and depth and whitish ooplasm accumulates at their walls (Fig. 3C). Since the majority of the grooves reach both polar rings, a system of meridians forms. By the end of the stage 1d, ten to fifteen such meridians (20–60 μm thick) have formed and the egg now has a pumpkin-like appearance. Meanwhile, fixed/cleared eggs show that the perinuclear plasm has grown into a disk-shaped structure which reaches 150–300 μm in diameter (Fig. 3C).

Stage 1e. Formation of the teloplasm (4.30–5.00 h) The grooves diminish first at the equator and then progressively toward the poles of the egg. This causes a shortening of the meridians towards the poles and concentration of ooplasm in the vicinity of the polar rings. Concomitantly, increasing constriction of the polar rings leads to gradual accumulation of their ooplasm at both poles of the egg (Fig. 3D). Formation of the teloplasm terminates when the system of meridians disappears from the egg surface. Since

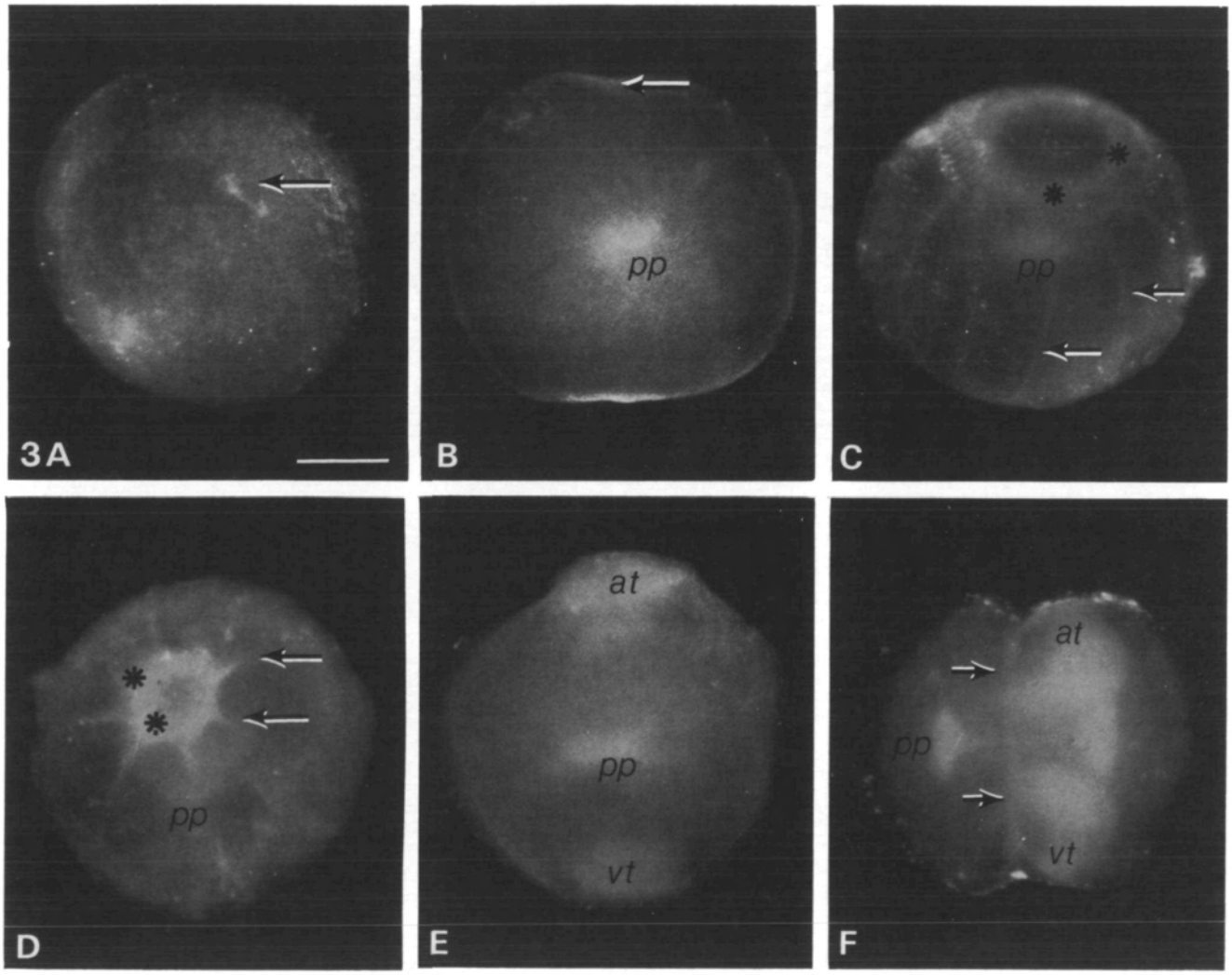


Fig. 3. Cleared eggs photographed under dark-field illumination. (A) early stage 1a egg, viewed from the animal pole, showing the opaque figure-eight meiotic nucleus (arrow); (B) side view of a midstage 1b egg showing the nascent perinuclear plasm (*pp*). The meiotic nucleus lies at the top of the egg (arrow); (C) dorsolateral view of a late stage 1c egg showing the animal polar ring (asterisks), part of the meridians (arrows) and the disk-shaped perinuclear plasm (*pp*); (D) midstage 1e egg, viewed from the animal pole. The constricted animal polar ring (asterisks), shortened meridians (arrows) and the perinuclear plasm (*pp*) are shown; (E) side view of a late stage 1e egg, in which formation of the teloplasm has been completed. The animal (*at*) and vegetal (*vt*) teloplasms appear as opaque masses of ooplasm accumulated at the corresponding egg poles. The perinuclear plasm (*pp*) is converted into a flattened figure-eight structure; (F) side view of a late stage 1f egg showing how the trajectory of the first cleavage furrow (arrows) determines the formation of two unequal blastomeres. Cell CD is larger than cell AB and includes most of the animal (*at*) and vegetal (*vt*) teloplasms. The cleavage furrow has subdivided the perinuclear plasm (*pp*) into two symmetrical halves. Magnification bar, 200 μm.

this event first happens in the vegetal hemisphere of the egg, the vegetal teloplasm forms first and the animal teloplasm forms last (Fig. 3E).

Stage 1f. Completion of the first cleavage furrow (5.00–6.00 h)

Another change in the overall shape of the egg, from spherical to biconcave, is initiated during stage 1e and is completed during stage 1f (Fig. 2F). This conformational change is brought about by dorsoventral flattening of the egg and deepening of its poles. Fixed/cleared eggs show that during this process the teloplasm undergoes two important topographical changes. First, it is displaced at that side of the egg from which the blastomere CD will originate. Second, the teloplasm begins to be internalized and thus less and less of it remains adjacent to the surface. Simultaneously, the elongated dumb-bell-shaped perinuclear plasm moves toward the region of the egg destined to form the AB blastomere. The first cleavage furrow appears at the dorsal surface of the egg, just beside the animal teloplasm. In this manner, the deepening cleavage furrow subdivides the perinuclear plasm into two symmetrical halves (Fig. 3F). Moreover, the CD blastomere encloses most of the egg teloplasm and thus is larger than the AB blastomere.

Structure of the early uncleaved egg

The preceding description of developmental stages of *T. rude* uncleaved eggs reveals that this cell is engaged in a stereotyped sequence of peculiar deformation movements leading to completion of meiosis and ooplasmic segregation. As a first step towards the understanding of these processes, our attention was directed to the study of the structure of the early stage 1a egg.

Examination of whole-mounted fixed/cleared eggs with the dissecting microscope revealed that most of the egg cytoplasm corresponds to yolk. The figure-eight-shaped opaque body, visualized at one pole of the egg, corresponds to the meiotic female nucleus (Fig. 3A). Further details on the structure of the egg were obtained by examination of sectioned material under the light and electron microscopes. These studies show that the early uncleaved egg consists of endoplasm, a layer of ectoplasm and the meiotic female nucleus (Fig. 4). Since examined eggs were all fertilized, they must also include the sperm nucleus. Unfortunately it has not yet been possible to detect it in early uncleaved eggs.

Endoplasm or vitelloplasm

The endoplasm consists of numerous darkly stained yolk platelets scattered across a lightly stained matrix (Fig. 4). Yolk platelets are about 0·1–15 μm in diameter, lack a limiting membrane and contain a regular

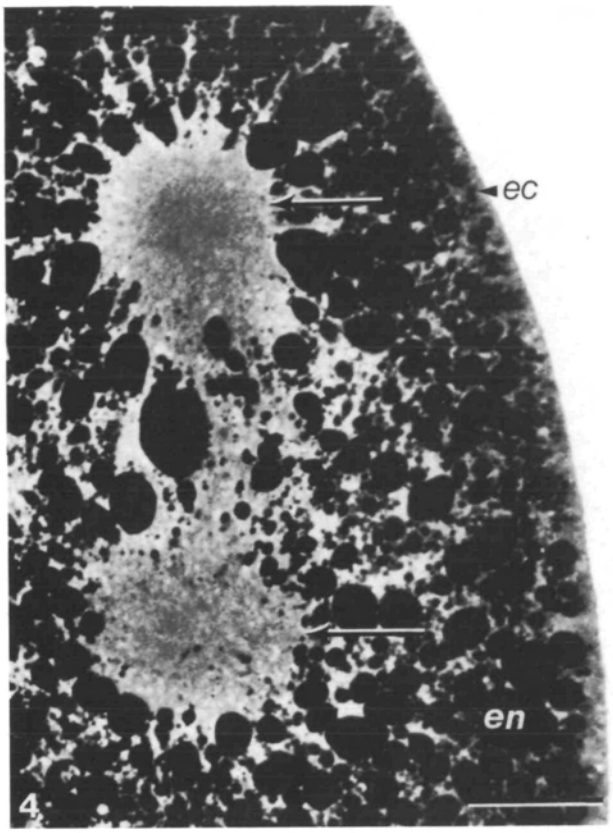
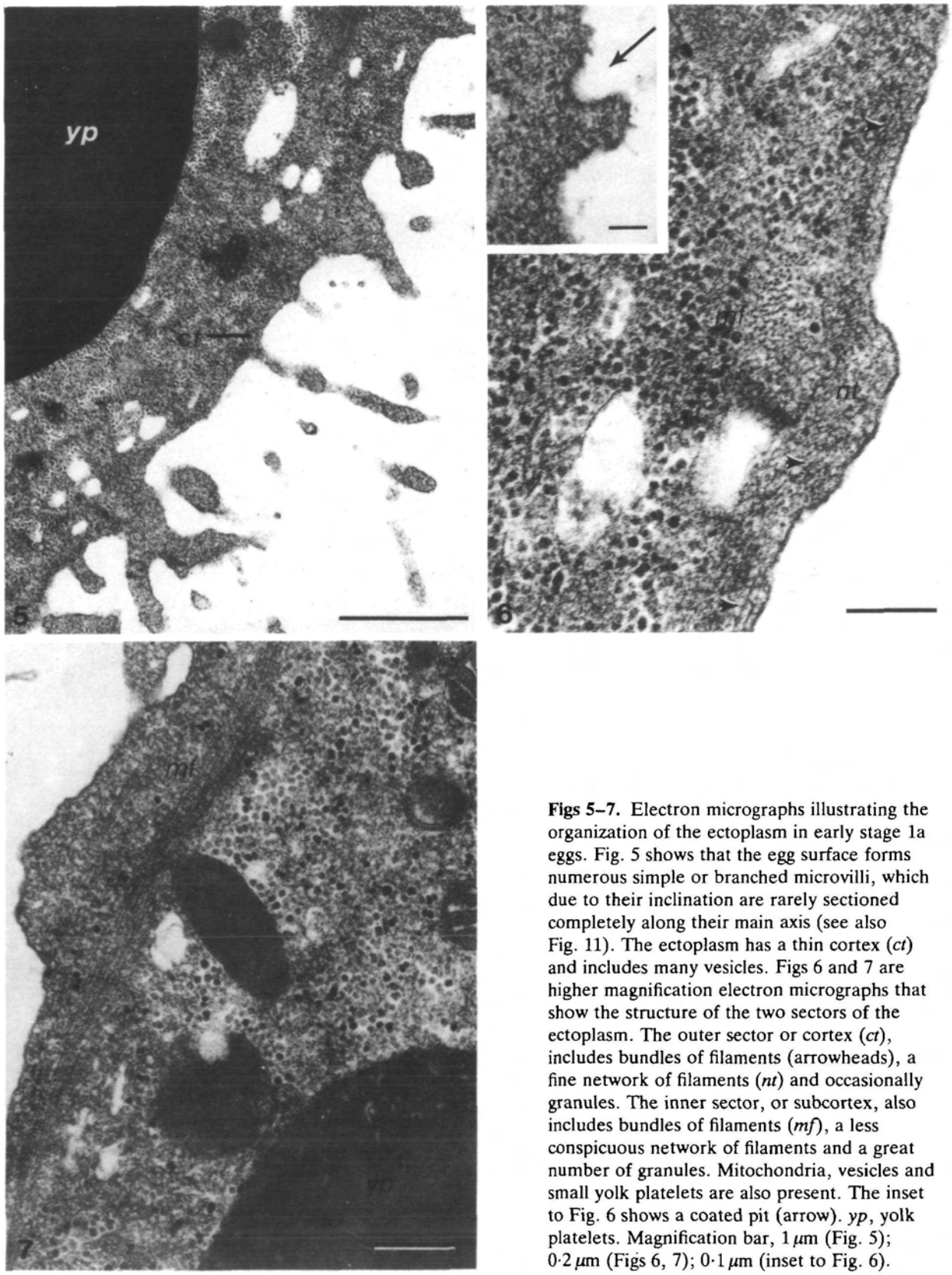


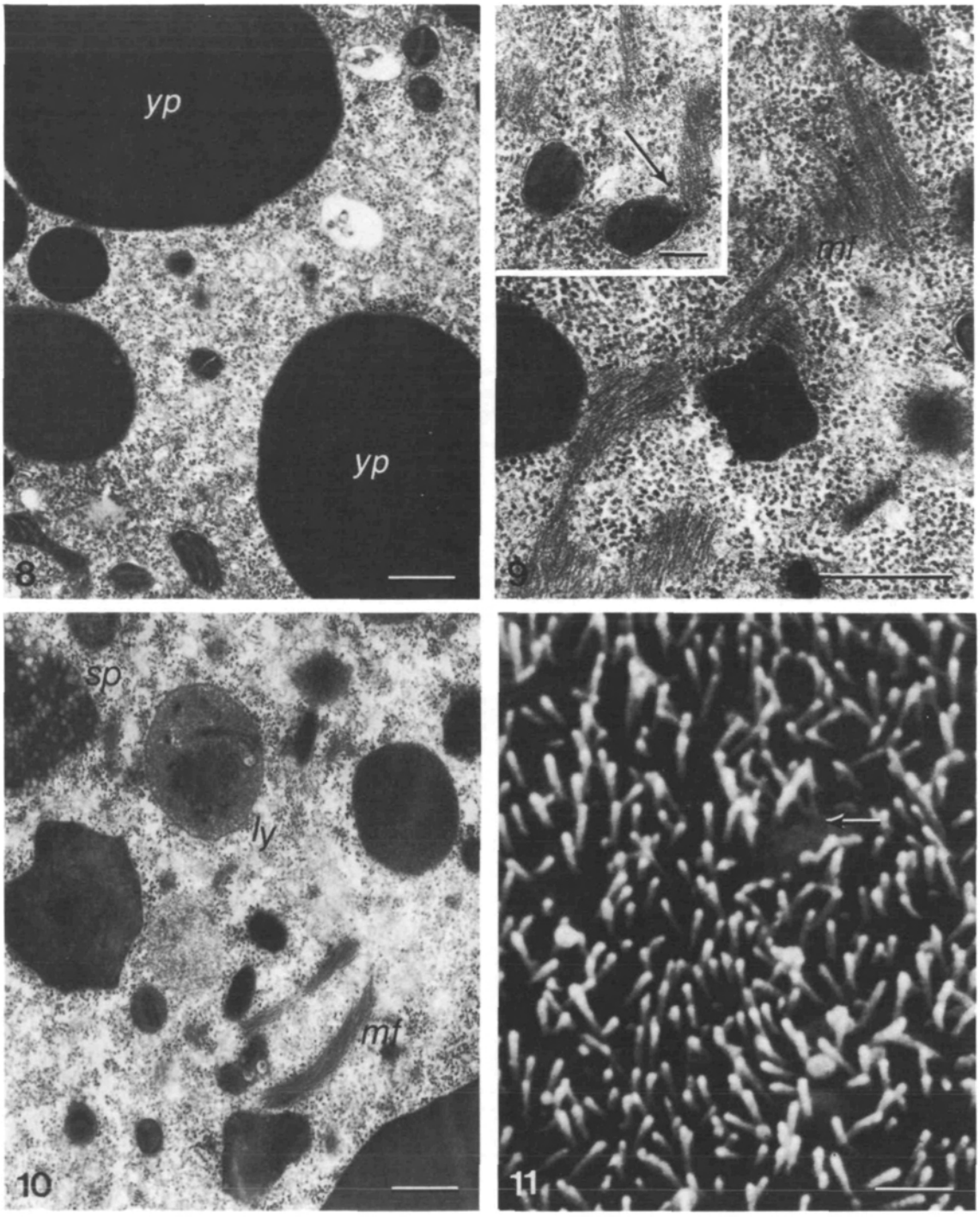
Fig. 4. Epon-embedded section stained with toluidine blue showing the main structural components of an early stage 1a egg. The ectoplasm (*ec*) forms an irregular, lightly stained layer at the egg periphery. The endoplasm (*en*) stains darker due to heavy accumulation of yolk platelets. The meiotic nucleus consists of two prominent poles (arrows) linked to one another by a bridge of ooplasm. The latter includes the spindle and the chromosomes, not shown in the micrograph. Magnification bar, 40 μm.

array of highly osmiophilic granules. Some platelets have outer and inner regions that stain differently. The outer region stains darker because its granules are more closely packed (Fig. 8). However, very small patches of less-densely packed granules may be found across the outer region (Fig. 7). Yolk platelets are not segregated by size and their distribution is generally random. Alignment of yolk platelets occurs around the poles of the meiotic spindle (Fig. 16). The vitelloplasmic matrix includes moderate numbers of small mitochondria, an assortment of dense bodies and numerous granules about 25 nm in diameter (Figs 8–10). Some dense bodies have limiting membranes and may include vesicles and clumps of electron-dense material. These bodies are 1–2 μm in diameter and may correspond to lysosomes (Fig. 10). Other dense bodies exhibit a spongy structure due to the presence of numerous vesicular profiles. The nature of these spongy bodies is unknown, but they

may correspond to transformed yolk platelets (Fig. 10). Finally, some dense bodies have very irregular outlines and resemble lipid droplets. Granules of 25 nm are also abundant in other regions of the egg and may correspond to glycogen particles (our unpublished observations). Many bundles of thin filaments, 7–8 nm in diameter, have been found in lacunae-type expansions of the matrix containing



Figs 5–7. Electron micrographs illustrating the organization of the ectoplasm in early stage 1a eggs. Fig. 5 shows that the egg surface forms numerous simple or branched microvilli, which due to their inclination are rarely sectioned completely along their main axis (see also Fig. 11). The ectoplasm has a thin cortex (*ct*) and includes many vesicles. Figs 6 and 7 are higher magnification electron micrographs that show the structure of the two sectors of the ectoplasm. The outer sector or cortex (*ct*), includes bundles of filaments (arrowheads), a fine network of filaments (*nt*) and occasionally granules. The inner sector, or subcortex, also includes bundles of filaments (*mf*), a less conspicuous network of filaments and a great number of granules. Mitochondria, vesicles and small yolk platelets are also present. The inset to Fig. 6 shows a coated pit (arrow). *yp*, yolk platelets. Magnification bar, 1 μm (Fig. 5); 0·2 μm (Figs 6, 7); 0·1 μm (inset to Fig. 6).



Figs 8–10. Electron micrographs illustrating the organization of the endoplasm in early stage 1a eggs. Fig. 8 shows the structure of the endoplasm in regions where yolk platelets (*yp*) are relatively more abundant than the surrounding matrix. The latter has some mitochondria and numerous granules scattered across an apparently empty space. It is thought that this space results from the combination of unsatisfactory preservation of the cytoplasm and reduced development of a cytoskeleton. Figs 9 and 10 show the structure of regions of the endoplasm where dense bodies, mitochondria and small yolk platelets are accumulated. In these regions the matrix is relatively more abundant and one also finds numerous bundles of thin filaments (*mf*). Spongy bodies (*sp*) and structures interpreted as lysosomes (*ly*) are indicated. The inset to Fig. 9 shows a bundle of thin filaments that appears to make contact with the surface of a mitochondrion (arrow). Magnification bar, 0·5 μm (Figs 8–10); 0·2 μm (inset to Fig. 9).

Fig. 11. Scanning electron micrograph showing the surface organization of an early stage 1a egg. Microvilli are rather straight but point in different directions. A small number of short or stubby microvilli is detected elsewhere. The arrow indicates a branched microvillus. Magnification bar, 2 μm.

accumulations of dense bodies, small yolk platelets and mitochondria (Figs 9, 10). Interestingly these filament bundles are often seen making close contacts with mitochondria and dense bodies (Fig. 9).

Ectoplasm

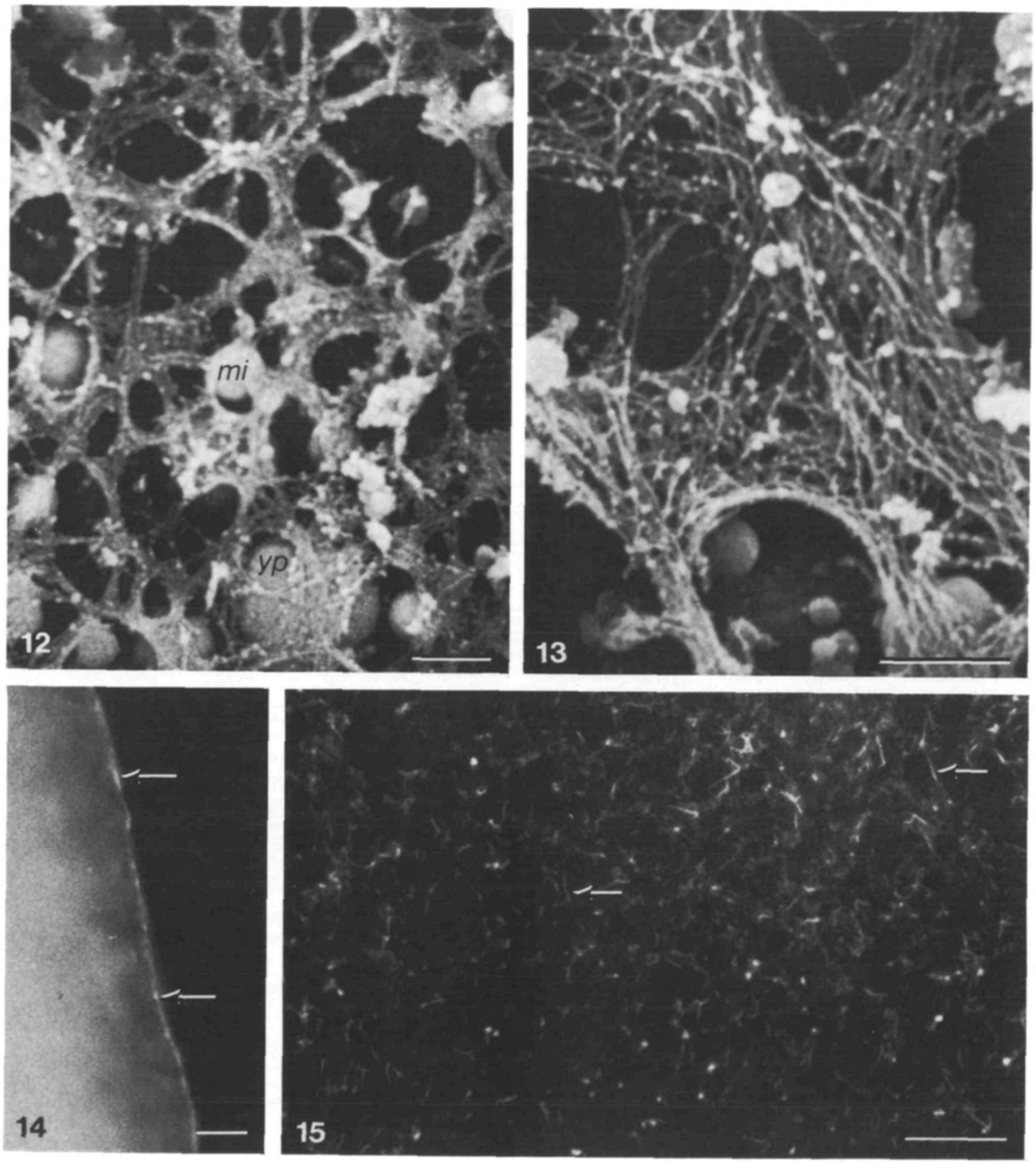
An irregular layer of ooplasm (2–10 μm thick) that has few small yolk platelets is seen around the egg surface. Although this layer is continuous with the endoplasmic matrix, it differs markedly from it. The main difference is concerned with the accumulation of vesicular and fibrillar material in the ectoplasm (Figs 5–7). The layer of ectoplasm may be subdivided into two sectors. The thin outer sector, which corresponds to the egg cortex, includes few granules and numerous 7–8 nm thick filaments. Some of these filaments form a subplasmalemmal fine network (Figs 6, 7) and the rest is arranged in bundles running more or less parallel to the surface. The thick inner sector, which corresponds to the subcortical layer, is more granular than fibrillar and includes numerous vesicles and various organelles. A fine network of filaments is much less evident, but bundles of filaments are seen travelling in various planes. Obliquely oriented bundles are sometimes seen passing into the outer layer, whereas perpendicularly oriented bundles may be seen entering microvilli. Therefore, bundles of filaments seem to form a coarse network of filaments which extends throughout the entire ectoplasm. Rounded or oval vesicles appear scattered across the inner sector of the ectoplasm. These vesicles sometimes enclose granular or membranous material. The plasmalemma of the early uncleaved egg forms numerous long (1–2 μm) and some short (approximately 0·5 μm) microvilli. These two types of microvilli intermingle and their distribution is largely isomorphic. Microvilli are found on the egg surface singly or in groups. In the latter case, two or more microvilli are seen to originate from a short and broad common stalk (Figs 5, 11). A bundle of thin filaments extends from the tip of the microvillus to the inner or outer sector of the layer of ectoplasm. A fine network of filaments may be found in some microvilli, but granules are rarely seen. The plasmalemma between microvilli generally displays irregular outlines and may form coated or uncoated pits (Fig. 6). In some cases vesicles are seen clustered at the neighbourhood of such pits.

Further details on the organization of filaments in the early uncleaved egg have been obtained by examination, under the scanning electron microscope, of whole-mounted permeabilized eggs. Low-magnification electron micrographs (Fig. 12) show filaments arranged into strands or bundles that constitute a three-dimensional network throughout the ectoplasm. The strands differ in length and thickness, and the spaces between them are thus of different sizes and shapes. The spaces of the network probably contained small yolk platelets, dense bodies, vacuoles or mitochondria removed during the extraction procedure. High-magnification electron micrographs (Fig. 13) show that filaments frequently change direction as they pass from one strand into another. In this manner, a much finer network with tiny spaces is formed. These spaces probably contained smaller structures such as vesicles or granules, extracted from the ectoplasm during permeabilization.

Staining of permeabilized eggs with the actin probe, rhodamine-labelled phalloidin, demonstrates that the early uncleaved egg contains a large amount of F-actin. As shown in Figs 14 and 15, the ectoplasm includes an actin network that probably corresponds to the network of filaments seen under the transmission and scanning electron microscopes. As expected, changes in the geometry of the network generate variations in its staining with the actin probe (see Fig. 15). Another interesting feature of the ectoplasmic F-actin network is that it extends into the endoplasm, where it forms a tenuous network around the yolk platelets.

Meiotic nucleus

The main axis of the meiotic nucleus of the early uncleaved egg lies parallel or slightly oblique to the egg surface. It consists of two voluminous and symmetrical poles linked to one another by a strand of ooplasm (about 60 μm long), containing the spindle and the chromosomes (Figs 3A, 4). The latter are very small and display a metaphase arrangement. Such arrangement of the chromosomes has also been detected in ripe eggs forced to remain in the ovisacs for varying lengths of time. The anaphase movement of the chromosomes, however, is initiated soon after the eggs abandon the ovisacs. Therefore, it is quite clear that eggs are subjected to meiosis I metaphase-arrest. Changes in the orientation of the meiotic spindle take place during anaphase I (our unpublished observations), presumably as a result of changes in the organization of the spindle poles. Hence, it was of interest to determine the structure and relationships of the poles of the early meiotic spindle. Toluidine-blue-stained sections show that the poles of the meiotic spindle consist of centrosome, centrosphere and astrosphere (Fig. 16). The centrosome appears as a rounded unstained central region of the pole enclosing a pair of darkly stained granules, about 1 μm thick, interpreted to be the centrioles. The centrosphere may be subdivided into inner and outer sectors that stain differently. The inner sector is 10–20 μm in diameter and includes particles that stain with toluidine blue. The outer sector is 40–50 μm in diameter and stains much



Figs 12–13. Scanning electron micrographs of permeabilized whole-mounted early stage 1a eggs, illustrating the organization of filaments across the ectoplasm. Fig. 12 shows how bundles of filaments form a coarse network that surrounds structures identified as mitochondria (*mi*) and yolk platelets (*yp*). Spaces in the network were probably occupied by similar structures removed during the extraction procedure. Fig. 13 reveals two additional features of the coarse network. First, that the network is clearly three-dimensional and second, that filaments in the bundles form an additional smaller network. The tiny spaces of the latter network are probably occupied by granules removed during permeabilization. Magnification bar, 2 μm (Fig. 12); 1 μm (Fig. 13).

Figs 14–15. Fluorescence micrographs of permeabilized whole-mounted early stage 1a eggs, stained with rhodamine-labelled phalloidin illustrating the organization of F-actin throughout the ectoplasm. Fig. 14 is a micrograph of an egg in focus at its edge and reveals the highly fluorescent layer of ectoplasm (arrows). This result has been interpreted as due to heavy concentration of F-actin in the ectoplasm. Fig. 15 corresponds to a top view of the animal hemisphere of the egg. F-actin is seen to form a network that in many places consists of strongly fluorescent rods (arrows) interpreted as being thick bundles of filaments. Magnification bar, 10 μm (Fig. 14); 30 μm (Fig. 15).

lighter. The astrosphere may reach 250 μm in diameter and is made of numerous straight or curved fibres that may be more than 100 μm long. The astral fibres of the two spindle poles extend both into the endoplasm and ectoplasm. The electron microscope shows that the inner centrosphere includes granules and numerous microtubules, which are seen to travel away from the centrosome periphery in all directions (Fig. 17). In the outer centrosphere, microtubules gather in bundles that constitute radial fibres

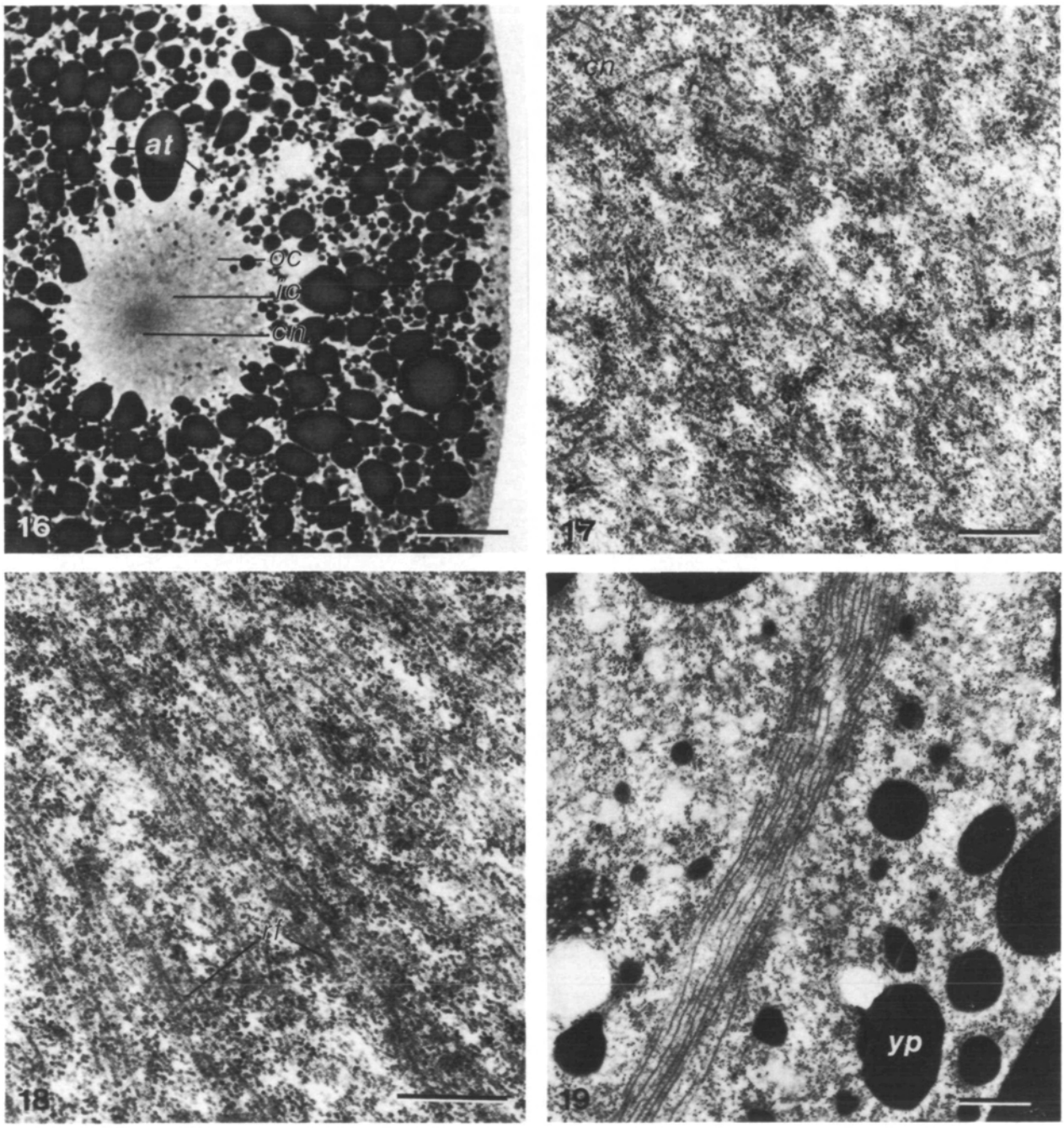


Fig. 16. Epon-embedded section of the inner pole of the meiotic spindle of an early stage 1a egg, stained with toluidine blue. The pole consists of the following components: centrosome (*cn*) enclosing the paired centrioles; inner centrosphere (*ic*); outer centrosphere (*oc*) and astrosphere (*at*). Magnification bar, 20 μm.

Figs 17–19. Electron micrographs illustrating the structure of different components of the meiotic spindle pole of Fig. 16. In the inner centrosphere (Fig. 17), bundles of microtubules intersect as they radiate from the centrosome periphery (*cn*). In the outer centrosphere (Fig. 18), bundles of microtubules constitute radial fibres (*rf*). Fig. 19 shows the structure of an astral fibre. It consists of an undulating bundle of microtubules coursing across the endoplasmic matrix. *yp*, yolk platelet. Magnification bar, 0·5 μm (Figs 17, 18); 1 μm (Fig. 19).

(Fig. 18). The cytoplasm between these fibres contains fewer granules, mitochondria and small yolk platelets. As radial fibres leave the centrosphere, they are funnelled between the yolk platelets to constitute the astral fibres (Fig. 19). The centrosome has not yet been examined under the electron microscope.

Examination of numerous thin sections reveals that the majority, if not all, of the microtubules of the early uncleaved egg are part of the female meiotic nucleus; microtubules are absent across most of the ectoplasm and endoplasm.

Discussion

Results presented in this paper indicate that the uncleaved egg of *T. rude* is very convenient material for the study of cytoplasmic movements associated with the expulsion of the pole cells and with ooplasmic segregation. Deformation movements are prominent and stereotyped. Since the ooplasm has a distinct whitish appearance and its redistribution mostly takes place across the egg surface, ooplasmic segregation may be studied in live eggs. Furthermore, very simple clearing techniques allow detailed observations of both deep and superficial translocation of ooplasm during teloplasm and perinuclear plasm formation.

Development of the uncleaved egg of *T. rude* may be subdivided into two periods. The early period (stages 1a and 1b) deals with completion of meiosis and initiation of the perinuclear plasm formation. The late period (stages 1c to 1f) deals with formation of the teloplasm and completion of the first cleavage division. A similar developmental sequence is seen in *Tubifex* eggs, which also form prominent accumulations of ooplasm (pole plasms) at both egg poles (Shimizu, 1982*a*). Moreover, the egg of this oligochaete includes a prominent actin lattice which seems to generate the motive force for the ooplasmic segregation (Shimizu, 1982*b*, 1984). Recent observations by the same investigator (Shimizu, 1986) indicate that accumulation and displacement of superficial ooplasm in the form of rings and meridians also seem to occur in *Tubifex* eggs. However, the eggs of *Tubifex* and *T. rude* differ in several respects including the types of deformation movements accompanying release of the pole cells. Thus, *Tubifex* eggs form meridional bands of contraction during release of the two pole cells. *T. rude* eggs, on the other hand, form rings of contraction during the same process. Finally, *Tubifex* eggs differ from *T. rude* eggs in that the former do not form a vegetal protuberance.

Meiotic arrest may be produced by the presence in the oocyte cytoplasm of a substance that blocks meiosis beyond metaphase I. Such a substance may be a cytostatic factor, perhaps similar to that partially purified from unfertilized *Xenopus* eggs by Meyerhof & Masui (1979*a*,*b*) and Masui, Meyerhof & Miller (1980). The factor might be quickly inactivated when *T. rude* eggs are exposed to the cocoon fluid or culture medium.

There are two reasons why it was of interest to study the structure of the ectoplasm. First, because the force-generating elements triggering the deformation movements of the egg probably reside at the cell surface. Second, because it is known that after fertilization the egg surface is subjected to profound remodelling. An extensive framework of actin filaments seems to be present throughout the ectoplasm of the early uncleaved egg. Since microtubules are not found across most of the ectoplasm and endoplasm, the cytoskeleton of the early uncleaved egg may largely consist of actin filaments. Of course the presence of intermediate filaments remains to be investigated. As it occurs in the egg of sea urchins (Burgess & Schroeder, 1977; Begg & Rebhun, 1979; Mabuchi, Hosoya & Sakai, 1980; Carron & Longo, 1982) and amphibia (Charbonneau & Picheral, 1983), assembly of cortical actin filaments and elongation of microvilli in *T. rude* eggs may also be induced at the time of fertilization. The fine network component of the ectoplasmic cytoskeleton may be considered to represent a sort of three-dimensional actin gel, that perhaps contributes to the mechanical properties of the egg surface (Kane, 1983). The coarse network component of the ectoplasmic cytoskeleton, on the other hand, would not only provide rigidity to the egg surface but also probably represents the potential contractile machinery responsible for the deformation movements of the egg. The bundle of filaments present in the microvilli is considered to be part of the coarse actin network and is probably engaged in shortening of microvilli taking place during stage 1a (our unpublished observations). Arrangement of filaments in fine or coarse networks may depend on the type of protein or factor associated with F-actin (see Kane, 1982, 1983; Schliwa, 1981). Moreover, it is quite possible that the two types of networks are interconvertible.

The available evidence indicates that the endoplasmic actin cytoskeleton seems to be composed of fewer elements and this situation might be related to the greater fluidity of the vitelloplasm in these eggs. However, there are regions of the endoplasm where large bundles of filaments appear to build up a complex cytoskeleton closely related to the surface of mitochondria as well as other structures. This observation suggests that an actin-based system for the translocation of organelles may be present across the endoplasm. Such systems may be responsible, for

example, for the concentration of ooplasm at the egg centre during perinuclear plasm formation.

Presence of pits along the plasmalemma and of many vesicles across the cortical ooplasm of leech early uncleaved eggs suggests that membrane may be flowing into and out of the egg surface. This flow may be related to membrane retrieval provoked by microvilli resorption. Presence of some short microvilli across the surface of 0 h eggs indicates that such processes may have already started in the intraovarian oocyte. Membrane retrieval is known to take place in other invertebrate eggs after cortical granule exocytosis which has expanded the area of the plasmalemma (Fisher & Rebhun, 1983). Thus, vesicles scattered throughout the ectoplasm would be formed by endocytosed plasmalemma.

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