

## Light- and electron-microscopic observations on the relationship between prelampbrush oocytes and surrounding granulosa cells in the laying Japanese quail (*Coturnix coturnix japonica*)

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**Summary** — Transmission electronmicroscopic (TEM) observations demonstrated that the most superficial region of quail oocytes during the prelampbrush stage differs locally from the deeper ooplasm and is an active zone which forms exoplasmic cones, ridges or knob-like protrusions in the direction of/or in the granulosa cells. This exoplasm, in which no mitochondria were seen, is separated from the endoplasm, by a narrow interrupted filamentous layer. Using a lipid-preserving method of fixation, morphological evidence was found for the transport of lipid material from the granulosa cells into the exoplasm of the oocyte. Open intercellular bridges between exoplasm and granulosa cell cytoplasm were also seen. Differences between the electronmicroscopic aspect of clear and dark granulosa cells have been described.

**Japanese quail / prelampbrush oocyte / avian ovarian granulosa cell / ooplasm / lipid**

**Résumé** — Étude avec le microscope photonique et électronique des relations existant entre l'oocyte (avant le stade en écouvillon) de caille et les cellules de la granulosa qui l'entourent. À l'aide du microscope électronique, nous avons observé que l'ooplasm le plus superficiel a une structure qui diffère localement de l'ooplasm plus profond. L'ooplasm superficiel forme par endroits des cônes ou crêtes d'exoplasm dirigées vers ou dans les cellules de la granulosa. L'exoplasm ne contient pas de mitochondries et est séparé de l'endoplasm par une mince couche filamenteuse interrompue, visible après l'emploi d'un fixateur qui préserve bien les lipides. Nos observations suggèrent que du matériel lipidique provenant du cytoplasme des cellules de la granulosa est transporté vers l'exoplasm. De plus, l'exoplasm présente localement des ponts intercellulaires ouverts en communication directe avec le cytoplasme des cellules de la granulosa. Les différences d'ordre ultrastructural entre les cellules claires et foncées de la granulosa sont décrites.

**caille japonaise / oocyte prelampbrush / cellule de la granulosa ovarienne / ooplasm / lipide**

## INTRODUCTION

In vertebrates the function of the granulosa cells surrounding the oocyte is not completely understood and also differs widely according to the developmental stage of the follicle. Electronmicroscopic observations have demonstrated that in sauropsidian follicles the morphology of the granulosa cells is typical of that of biosynthetically active secretory cells owing to the presence of abundant rough endoplasmic reticulum, Golgi complexes and mitochondria, especially during the early stages of intrafollicular development (Bellairs, 1965; Wyburn *et al.*, 1966; Rahil and Narbaitz, 1973; Klosterman, 1987). The transfer of organelles (transosomes or lining bodies) from the granulosa cells into the oocyte has been observed both in birds (Press, 1964; Bellairs, 1965; Wyburn *et al.*, 1966) and in chelonians (Rahil and Narbaitz, 1973).

In the present work, transmission electron microscopy (TEM) was used to study the relationship between the quail prelampbrush oocyte and the surrounding granulosa cells. The term prelampbrush stage was used (Callebaut, 1973) and not Balbiani or dispersed Balbiani stage as used by Bellairs (1967) since we have shown by appropriate fixation methods (Callebaut, 1984) that part of the elements from the Balbiani complex persist in their paranuclear position for a much longer period than is usually assumed. Indeed, its presence near the germinal vesicle can still be demonstrated during at least the entire phase I of the ensuing lampbrush stage (in quail oocytes with a diameter of  $\approx 700 \mu\text{m}$ ).

In the present study we provide evidence that there exist important interactions between the superficial ooplasm (called exoplasm) and the granulosa cell cytoplasm.

## MATERIALS AND METHODS

After decapitation of laying Japanese quail (*Coturnix coturnix japonica*) and opening of their abdomen, pieces of ovary were removed and fixed in 1% glutaraldehyde in 0.05 M sodium cacodylate buffer containing 0.01% malachite green, according to the procedure of Lawton (1989). After 2 h fixation the pieces were (without rinsing) either placed directly in 70% alcohol for 3 h (fixation method 1) or directly in 1% aqueous osmium tetroxide for 1 h (fixation method 2).

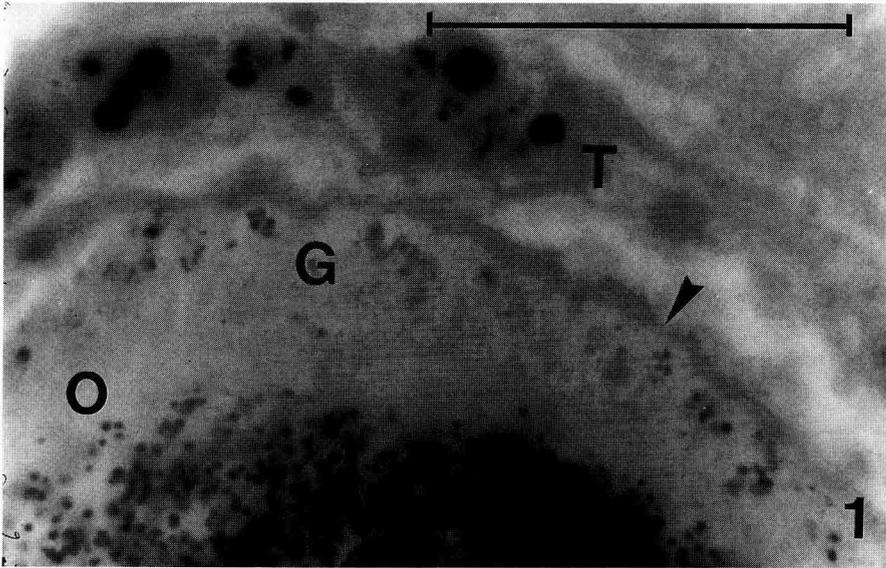
After fixation method 1, the pieces were passed through 50% alcohol (3 h), 30% alcohol (3 h), tap water during 1 night and then also postfixed in 1% aqueous osmium tetroxide for 1 h. After rinsing in tap water, the tissues (both from methods 1 and 2) were postfixed in 2% aqueous uranyl acetate (1 h), followed by rinsing in tap water. For light microscopic studies, the tissues were dehydrated in alcohol and embedded in paraffin or glycol methacrylate. Eight- $\mu\text{m}$  thick paraffin sections and 1- $\mu\text{m}$  thick glycol methacrylate sections were made. For electron microscopic studies, the tissues were dehydrated in alcohol and infiltration and embedding was performed *via* propylene oxide into LX-112 Resin (Ladd).

The ultrathin sections made with an LKB ultratome were stained with 10% uranyl acetate in methanol for 7 min, followed by staining with lead citrate (Reynolds, 1963). The ultrathin sections were then studied with a transmission electron microscope (Siemens Elmiscop 101).

## RESULTS

### *Light microscopic observations*

After fixation method 1, besides the intra-oocytal lipid spherules around the Balbiani complex and germinal vesicle, numerous groups of lipid spherules (of similar size) were seen in the granulosa layer (fig 1). However, on the paraffin sections it was not always clear whether the lipid spherules were localized in the granulosa cells



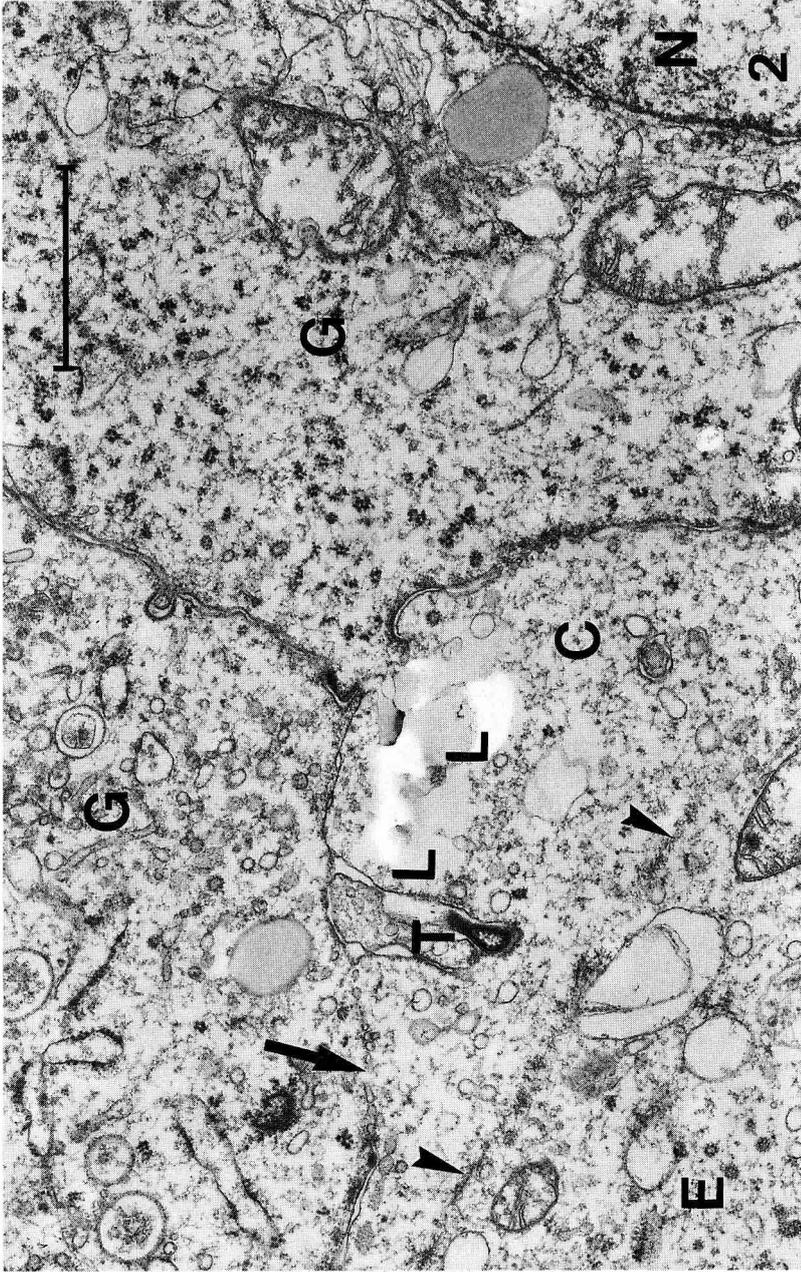
**Fig 1.** Micrograph of undeparaffinized section through quail follicle containing an oocyte in prelampbrush stage; G: granulosa layer containing numerous groups of lipid granules with the same diameter as those seen in the ooplasm (O); T: theca with large lipid inclusions; arrowhead indicates basement membrane; fixation: glutaraldehyde malachite green -70% alcohol- osmium tetroxide. Scale bar: 50  $\mu$ m.

or between them. After glycol methacrylate embedding, no lipid inclusions were visible in the granulosa layer, indicating that they had been solubilized by the histological procedure.

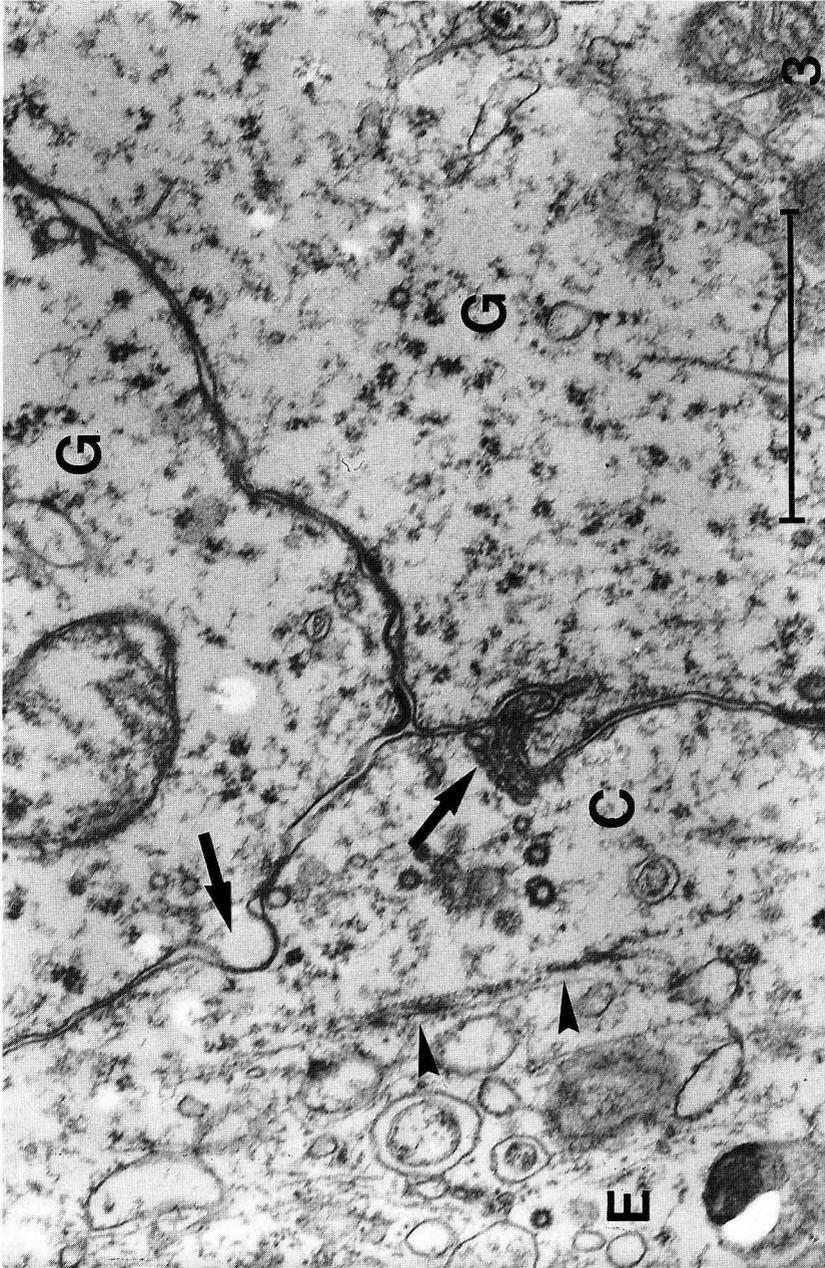
#### ***Transmission electron microscopic observations***

With fixation method 1 the lipids were well conserved, also at the electron microscopic level. The superficial ooplasm was seen to form different kinds of exoplasmic extensions in the direction of/ or in the granulosa cells. The first type was seen to point to the junction of 2 granulosa cells and often had the form of a cone or ridge with a

broad base (fig 2). The exoplasmic areas were incompletely separated from the remainder of the ooplasm by a nearly plane region of filaments (probably actin bundles). By contrast to the underlying endoplasm, no mitochondria were seen in the exoplasmic cones (fig 2). The latter contain groups of lipid elements localized below intercellular communications between a granulosa cell and the exoplasm. Belairs (1967) described them on a drawing as pinocytotic-like vesicles, since by her fixation and embedding method the lipid material was probably solubilized. Sometimes globular material seems to be in the process of expulsion by the granulosa cell cytoplasm in the direction of the exoplasm (fig 3).



**Fig 2.** TEM micrograph of section through exooplasmic cone (C) of prelambrush oocyte pointing to the junction of 2 neighbouring granulosa cells (G); N: nucleus of one of the granulosa cells containing many ribosomes; at the top of the exooplasmic cone an interruption in the membranes is seen, below which a group of lipid granules (L) has accumulated; the arrow indicates the area where the membranes between exoplasm and granulosa cell cytoplasm are absent; T: transosome; E: endoplasm containing mitochondria, separated from the exooplasmic cone by an interrupted plane region of filaments (arrowheads); fixation method 1. Scale bar: 1  $\mu$ m; x 28 000.



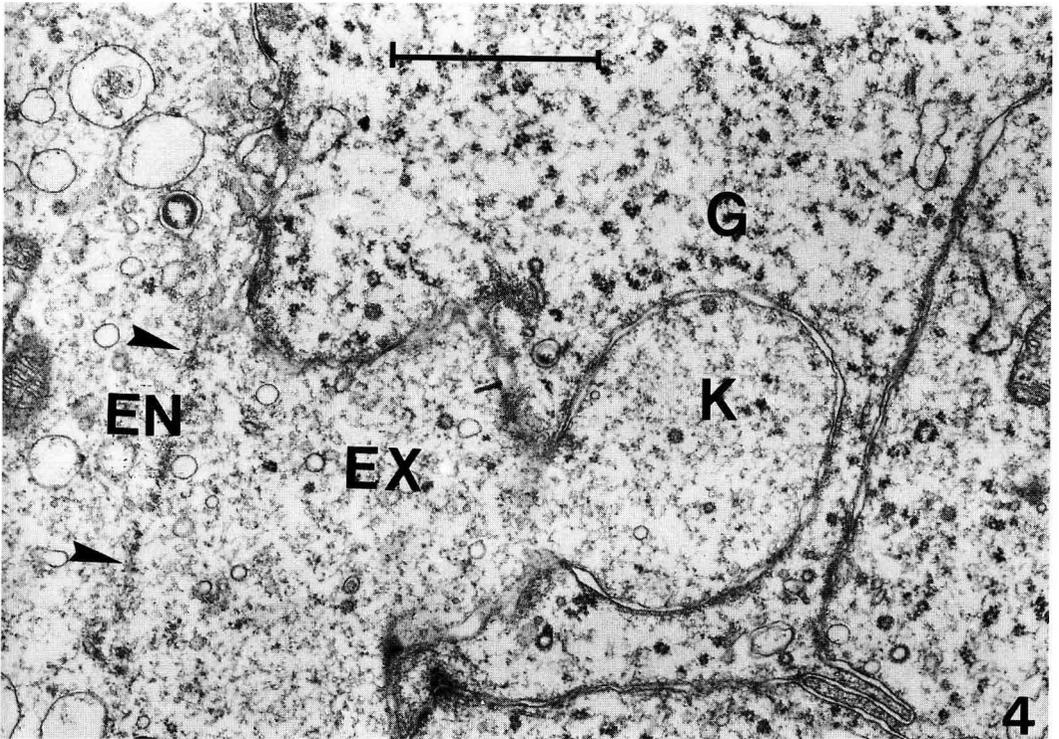
**Fig 3.** TEM micrograph of section through exoplasmic cone (C) pointing to the junction of 2 granulosa cells (G). Note globular extension (indicated by upper arrow) of the granulosa cell cytoplasm into the exoplasm of the oocyte; E: endoplasm of prelambrush oocyte, incompletely separated from exoplasmic cone by interrupted narrow layer of filaments (arrowheads); lower arrow indicates exoplasmic protuberance; fixation method 1. Scale bar: 1  $\mu\text{m}$ ; x 40 000.

In several places of the exoplasmic cones direct continuity between the granulosa cell cytoplasm and the exoplasm seemed to exist thus forming true intercellular communications (fig 2).

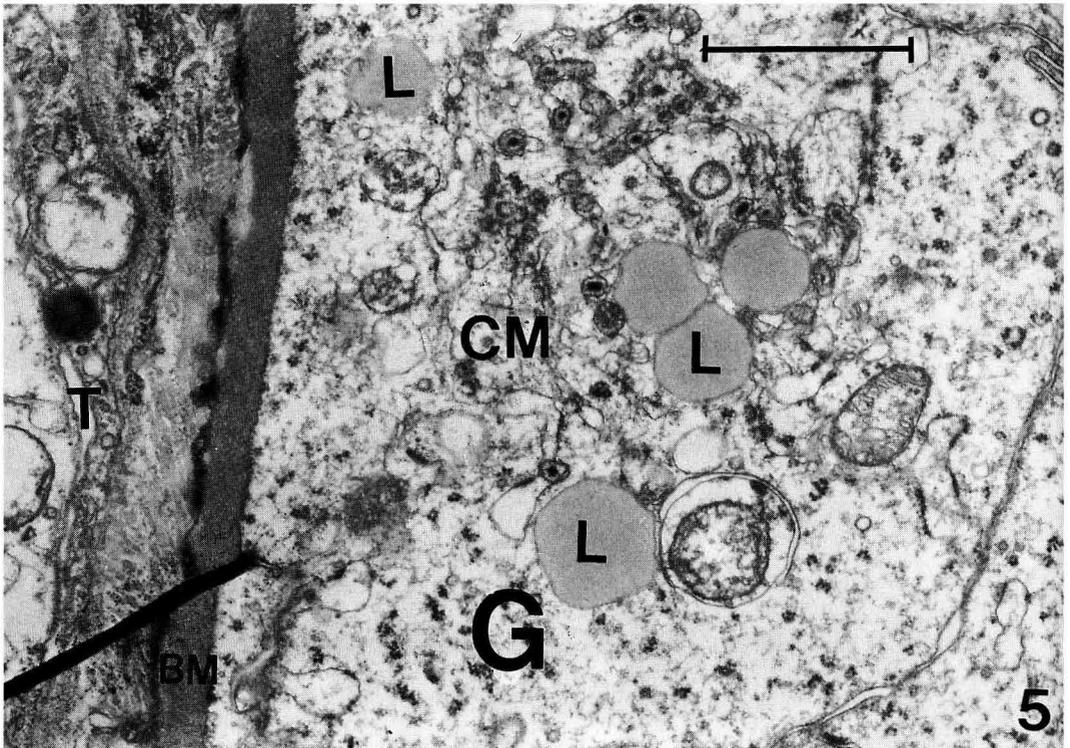
The granulosa cells were also seen to give off transosomes in the exoplasmic cones (fig 2). The second type of exoplasmic extension was seen to form a large knobby protrusion (sometimes starting from an exoplasmic cone) into a granulosa cell (fig 4). A third smaller type, the so-called "protuberance" (probably corresponding to the description of Bellairs, 1965) also often originating from the exo-

plasmic cones was seen to invaginate into the neighbouring granulosa cell (fig 3). In or around the "complex mass" (Bellairs' denomination: 1965) of the granulosa cells, numerous fat spherules were occasionally seen (fig 5). With fixation method 1 the complex masses were sometimes seen to contain elements resembling nucleated red blood cells and with the size range of viral particles. No lipid material could be observed between the granulosa cells or between the granulosa cells and the basement membrane.

Dark granulosa cells could be detected locally after fixation method 2 (fig 6).



**Fig 4.** TEM micrograph of section through a large knobby protrusion (K) coming from the exoplasm (EX) and deeply indented in the neighbouring granulosa cell (G); EN: endoplasm of preampbrush oocyte; arrowheads indicate interrupted plane region of filaments; fixation method 1. Scale bar: 1  $\mu\text{m}$ ; x 28 000.



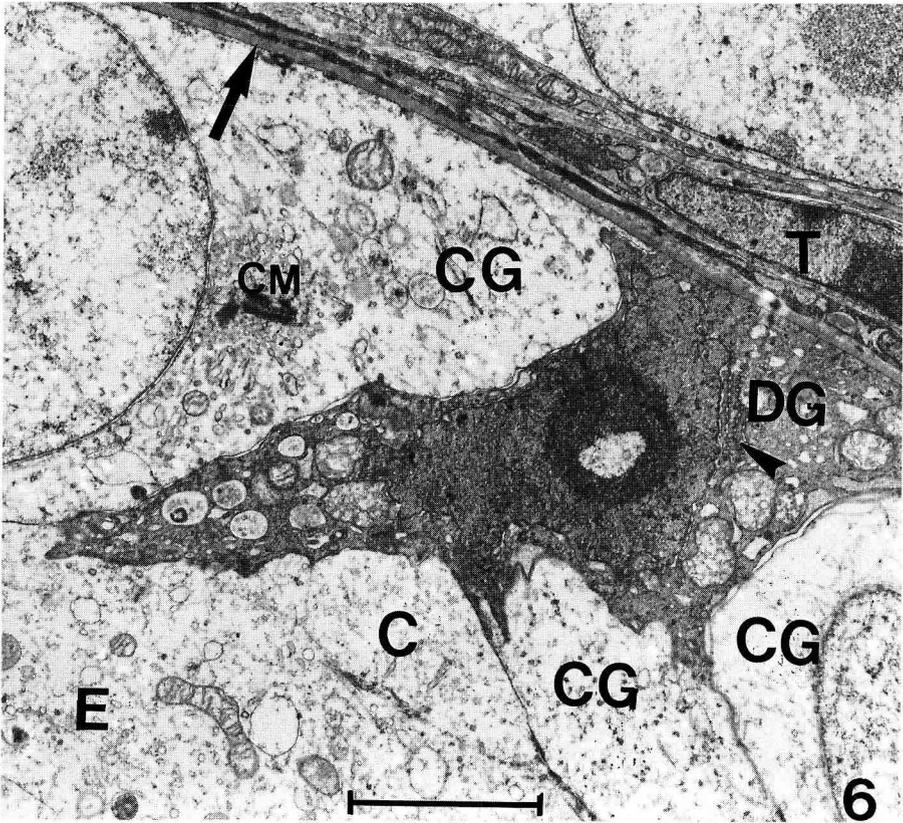
**Fig 5.** TEM micrograph of section through complex mass (CM) of granulosa cell (G) at the surface of prelampbrush oocyte; L: lipid granules; T: theca; BM: basement membrane; fixation method 1. Scale bar:  $1 \mu\text{m} \times 28\ 000$ .

These dark cells have a rather angular aspect with numerous tentacle-like extensions near the basement membrane, between the clear rounded granulosa cells and also extend to the exoplasmic cones of the oocyte.

Although the dark granulosa cells have a prominent nucleolus, their nucleus is smaller than that in the clear granulosa cells. The nuclear wall has an irregular outline and the nucleoplasm is as densely stained as the cytoplasm between the numerous vacuoles. The boundary between nucleus and cytoplasm is therefore not

easily visible. This gives the cell a mouldy wood aspect. Sometimes the ooplasm of the prelampbrush oocyte was not wholly covered by granulosa cells and even extended to the basement membrane. After fixation method 2, the lipids were less well conserved.

In the ooplasm of some of the prelampbrush oocytes a voluminous body (partially surrounded by a cell membrane: trilaminar at high magnification) as large as a whole granulosa cell (and probably representing an engulfed granulosa cell) was seen. It seemed to be composed of remnants of



**Fig 6.** TEM micrograph of a section through the follicle wall of a prelampbrush oocyte after fixation method 2; the very dark granulosa cell (DG) pressed between 3 clear granulosa cells (CG) can be clearly seen. Note the angular aspect of the dark cell with tentacle-like extensions near the basement membrane, between the other granulosa cells and the exoplasmic cone (C) of the prelampbrush oocyte; E: oocyte endoplasm containing mitochondria. The nucleoplasm of the dark cell is very dense and the nuclear membrane has an irregular form (arrowhead indicates annulate lamellae close to the nuclear membrane); T: theca; arrow indicates basement membrane; CM: complex mass with paired centrioles and numerous small vesicles. Scale bar: 2  $\mu$ m; x 12 000.

cell organelles forming vacuoles and irregular clumps with variable aspect and diameter. A central part containing dense granular material and resembling a nucleolar area could sometimes be observed.

## DISCUSSION AND CONCLUSION

The present TEM study offers a clear view of granulosa cell and oocytic membranes and retains the lipids (after fixation method

1: Callebaut, 1990). This can be explained by: i), the presence in the glutaraldehyde fixative of malachite green which retains phospholipids in the tissues (Teichmann *et al*, 1972; Lawton, 1989); and ii), the treatment with 70% alcohol immediately after the primary fixation which not only prevents solubilisation of some fixed proteins (Silverton and Anderson, 1961) but also of some lipids: cholesterol and triglycerides (Callebaut *et al*, 1991).

Since De Somer's report (1905) it has been known that the smallest follicles in the ovary of the adult chicken contain numerous lipid spheres grouped round the Balbiani complex. The origin of these lipid spheres is not known, but the following hypotheses may be considered: i), they may be synthesized by the oocyte (so-called endogenous yolk formation); ii), they may be formed in the granulosa cells and then transported into the ooplasm; iii), they may be formed from precursor material in the theca and pass between the granulosa cells; iv), they may come from plasma lipids which penetrate through the follicle wall.

In the present study morphological evidence was found for the second possibility, but the other hypotheses cannot be excluded. Earlier studies seemed to indicate that some components, such as Golgi bodies (Brambell, 1926), were transported from the granulosa cells to the ooplasm before the onset of the rapid oocytal growth period. In a recent study we also found electron microscopic evidence for the delivery of lipids by the granulosa cells to the superficial avian ooplasm in the largest follicles during their final yellow yolk assemblage (Callebaut, 1991a). According to Kemp (1958), intercellular bridges exist between the avian granulosa and the oocyte. By contrast, Press (1959) and Bellairs (1965) found no evidence for such intercellular bridges. In the present study howev-

er, using a different method of fixation intercellular bridges were also seen.

Among vertebrates, true intercellular communications between granulosa cells and oocyte have been described by TEM only in squamate reptiles (lizards and snakes) (Ghiara and Filosa, 1966; Hubert, 1971; Neaves, 1971; Taddei, 1972; Bou-Resli, 1974). These communications originate through a secondary fusion of the oocyte with the granulosa cells before they begin to differentiate into pyriform cells (Andreuccetti *et al*, 1978). Let us mention that the class Aves also belongs to a similar genome lineage as do the reptilian suborder of the Squamata, suggesting a common phylogenetic past (Ohno, 1970).

Dark granulosa cells have been described at some periods (different according to the fixative used) of avian follicular development (Holl, 1890; Brambell, 1926; Marza and Marza, 1935; Press, 1964). They have been considered as degenerating cells (Brambell, 1926; Bellairs, 1965; Chalana and Guraya, 1980; Guraya, 1989). However, the present study suggests that these angular cells with long tentacular processes between the neighbouring granulosa cells and extending from the basement membrane to the oocyte have a holding or supporting function, as has already been suggested by Holl (1890). We have also described them at more advanced stages of follicular development (Callebaut, 1991b). As was the case in the lampbrush and beginning post-lampbrush stages (Callebaut *et al*, 1981), during the prelampbrush stage of the quail oocyte we could also discern 3 major ooplasmic zones by the trypan blue-induced fluorescence method (Callebaut and Sijens, 1985). The exoplasmic zone described here where uptake of material from the granulosa cells seems to occur probably corresponds to the narrow most superficial unlabelled ooplasmic zone seen

after trypan blue administration. It probably functions as an oocyte transit compartment and also exists during the lampbrush stage (Caliebaut, 1991b).

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