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Egg shells of mallophagans and anoplurans (Insecta: Phthiraptera): morphogenesis of specialized regions and the relation to F-actin cytoskeleton of follicular cells

M. Zawadzka, W. Jankowska, S. M. Biliński

Abstract. The egg shells of investigated phthirapterans consist of three basic elements: an anterior operculum, a main egg shell and a posterior hydropyle. In some species these elements show further regional specializations: bristles and projections that facilitate attachment to feathers of the host, micropyles and aeropylar openings. All of the egg shell specializations are formed by distinct subpopulations of follicular cells. Staining with rhodamine-conjugated phalloidin has revealed that these subpopulations significantly differ in the distribution of microfilaments (F-actin). In this respect four morphological categories of the follicular cells have been distinguished: (1) cells devoid of processes and microvilli, with basal arrays of microfilaments, responsible for the secretion of a flat chorion; (2) cells devoid of processes and microvilli, separated by intercellular spaces, with basal arrays of microfilaments, responsible for the secretion of attachment structures; (3) cells equipped with actin-containing processes, responsible for the formation of micropyles or aeropyles, and (4) cells equipped with bundles of microvilli, responsible for the formation of hydropyles.

Keywords: Oogenesis, egg shells, chorion, follicular epithelium, microfilaments, Phthiraptera

Introduction

Insect egg shells are complex, extracellular structures, consisting of a large number of structural proteins that are synthesized and secreted by mesodermal follicular cells in an exact temporal and spatial order (reviewed by Margaritis, 1985; Regier & Kafatos, 1985). Generally, the egg shells are composed of two layers: an external chorion and internal vitelline envelope (vitelline membrane). In the majority of investigated insects the chorion consists of several sublayers and shows regional specializations, e.g. an operculum (the 'exit gate' for a larva), respiratory or attachment structures, and the micropylar apparatus which facilitates sperm entry during fertilization (Margaritis, 1985). Classic morphological and biochemical studies have shown that various egg shell regions are secreted by distinct groups of the follicular cells (Margaritis et al., 1980; Regier et al., 1980) and that in some model systems at least these groups synthesize different sets of the egg shell proteins (Mazur et al., 1980).

Most of the recent papers describing the morphogenesis of the egg shell specializations deal with the micropyles (e.g. Yamauchi & Yoshitake, 1984; Zarani & Margaritis, 1986, 1991a, b, c; Wenzel et al., 1990; Landim & Yabuki, 1995). These studies have revealed that the micropylar channels are molded around long cellular extensions of the follicular cells. The only exception to this rule has been recently described in the hymenopteran, *Eurytoma amygdali* (Zarani & Margaritis, 1994). Although the formation of other regional specializations of the chorion has not been analyzed

[1]Department of Systematic Zoology, Institute of Zoology, Jagiellonian University, R. Ingardena 6, PL 30-060 Kraków, Poland.

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Correspondence to: Szczepan M. Biliński. Tel: 004812 6336377 ext. 409; Fax: 004812 6343716; E-mail: sbili@ zuk.iz.uj.edu.pl

in detail, it has become evident that this process usually depends on various modifications of the apical surface of the follicular cells (Regier et al, 1980; Mouzaki et al., 1991).

The present paper reports the results of comparative studies on the morphogenesis of egg shell specializations in seven species of mallophagans (*Eomenacanthus stramineus, Uchida pallidulus, Menopon gallinae, Goniocotes gallinae, Lipeurus maculosus, Columbicola columbae, Trichodectes canis*) and one of anoplurans (*Haematopinus suis*). It is shown that the specializations are formed by distinct groups of follicular cells, which differ not only in morphology but also in the distribution of filamentous actin (F-actin).

Materials and methods

The specimens were collected from domestic pigs (*Haematopinus suis*), dogs (*Trichodectes canis*), hens (*Eomenacanthus stramineus, Uchida pallidulus, Menopon gallinae, Goniocotes gallinae*), pigeons (*Columbicola columbae*) and pheasants (*Lipeurus maculosus*).

Light (LM) and transmission electron microscopy (TEM)

Dissected ovaries were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, at room temperature, rinsed and postfixed in 1% osmium tetroxide in the same buffer. After dehydration in a series of ethanols and acetone, the material was embedded in Epon 812. Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined in a JEM 100SX electron microscope at 60 kV. Semithin (0.7 μm) sections were stained with 1% methylene blue in 1% borax and examined in a Jenalumar (Zeiss Jena) microscope.

Scanning electron microscopy (SEM)

Chorionated eggs were isolated, fixed (as described in the previous section), dehydrated and critical-point dried. The specimens were then mounted on holders covered with double-stick tape, coated with carbon and gold and examined in a JSM 5410 scanning electron microscope using an accelerating voltage of 25 kV.

RNA staining

The ovaries were fixed in 8% formaldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h, at room temperature. The material was then infiltrated and embedded in JB-4 (Polysciences, Warrington, PA, USA; for details see manufacturers' instructions). Sections (4–6 μm) were stained with 1% toluidine blue, pH 4.8.

Fluorescence microscopy

The ovaries were dissected in 4% formaldehyde, freshly prepared from paraformaldehyde in PBS for 40 min at room temperature. The specimens were then rinsed in PBS and stained with rhodamine-labeled phalloidin (Sigma Chemical, St Louis, MO, USA) in PBS (1 μg/ml) for 30 min at room temperature in the dark. Labeled ovaries were mounted on microscopic slides and examined using a Jenalumar epifluorescence microscope (Zeiss Jena) equipped with appropriate filters.

Results

Gross morphology of the ovary

The ovaries of investigated species are of the polytrophic-meroistic type (for classification and characterization of insect ovaries see King & Büning, 1985; Štys & Biliński, 1990; Büning, 1994). They are composed of five loosely arranged ovarioles. Within each ovariole a terminal filament, germarium, vitellarium and short pedicle can be distinguished. The vitellarium comprises 3–4 ovarian follicles (egg chambers) in a linear arrangement; each egg chamber consists of an oocyte and seven polyploid nurse cells, and is surrounded by a mesodermal follicular epithelium. During previtellogenesis, follicular cells become binuclear and gradually diversify into four subpopulations:

(1) cells covering the nurse cells;
(2) cells migrating between the nurse cells and the anterior pole of the oocyte;
(3) columnar follicular cells covering lateral aspects of the oocyte, and
(4) cells surrounding the posterior pole of the oocyte.

Subpopulations 2, 3 and 4 are responsible for the formation of three basic elements of the egg shell (see below). For further descriptions of phthirapteran oogenesis see Ries (1932), Biliński & Jankowska (1987) and Biliński (1989).

Architecture of egg shells

The egg shells of all investigated species consist of three basic elements: (1) an anteriorly located operculum; (2) a main egg shell covering lateral aspects of the oocyte, and (3) a posterior hydropyle (Fig. 1). In some species these elements show further regional specializations: micropyles, aeropylar openings and bristles or projections which facilitate attachment to feathers of the host (Figs 1, 2a, d, and e). A list of regional specializations of the egg shells in investigated species is presented in Table 1.

As in other insects, the egg shells of phthirapterans are composed of an outer chorion and an inner vitelline envelope, adjacent to the plasma membrane. The chorion, in turn, consists of a thin trabecular layer and an outermost, homogeneous chorionic layer (Fig. 2j). In the pig louse, *H. suis* the latter part comprises a perforated roof and a solid floor (see below). All surface specializations of the egg shells (bristles, micropyles, hydropyles) are built of the homogeneous chorionic layer only. The follicular cell imprints have never been observed on any of the investigated eggs.

Aeropyles

The egg shells of all the investigated mallophagans are solid and devoid of openings (except for micropyles; see below). In contrast, the outermost chorionic layer of the pig louse,

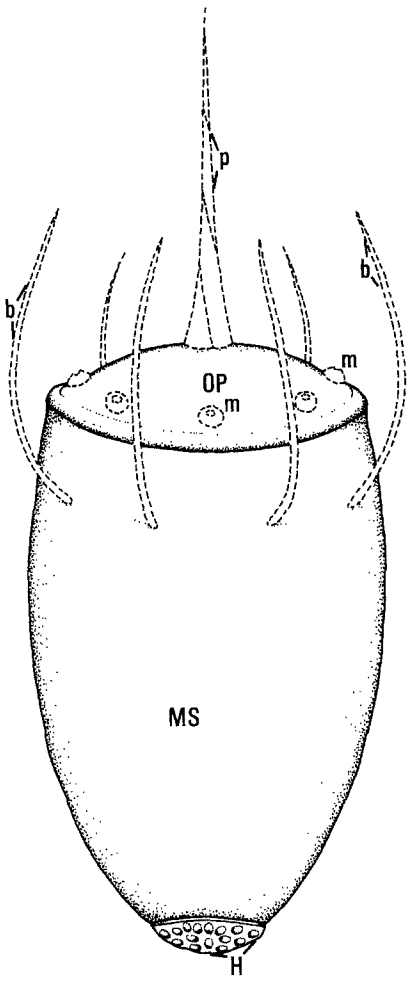

Fig. 1 Schematic representation of a generalized phthirapteran egg shell; basic elements (H, hydropyle; MS, main egg shell; OP, operculum) and regional specializations (b, bristles; m, micropyle; p, opercular projection).

H. suis is perforated by numerous canals (Figs 2a and b) that facilitate gas exchange between the environment and the developing embryo (Hinton, 1981). In accordance with the term used by Hinton (1981) these structures will be referred to as aeropyles (APLs). Analysis of semithin sections has revealed that the APLs of the operculum are markedly wider (Fig. 2b) and remain separated (Fig. 2j), while those penetrating the main egg shell fuse laterally, forming a broad, air-filled (Hinton, 1981) space. This space divides the outermost chorionic layer into a porous 'roof' and a solid 'floor' (Fig. 2k). As opposed to other insects (e.g. dipterans, Margaritis et al., 1980; Mouzaki & Margaritis, 1991; Mouzaki et al., 1991) these layers are not connected by vertical 'pillars'.

The APLs are formed around broad cellular processes of the follicular cells (Figs 2j and k). Processes penetrating the operculum are much larger, more voluminous, and as a rule, contain nuclei; they are connected with the main body of the cell by means of narrow stalks (Fig. 2j). The chorion *in statu nascendi* consists of numerous, intermingled fibers that surround the processes of the follicular cells (Figs 2h and i).

Micropyles

Micropyles (MPLs) have been found only in some species (*E. stramineus*, *U. pallidulus*, *M. gallinae*, *L. maculosus* and *T. canis*). They have the appearance of low hemispherical or conical protuberances, and are located next to the rim of the operculum, forming a characteristic 'micropylar ring' (Figs 2d and e). Each MPL contains a single, narrow (0.5–4 μm in diameter) micropylar canal (Figs 2e, 3c). The canals span the outermost chorionic layer reaching the trabecular layer of the chorion. In the dog louse, *T. canis* all MPLs are combined into a fence-like structure that is perforated by several, relatively broad micropylar canals (report in preparation).

In all the species MPLs are molded around cellular processes of the follicular cells (Fig. 3c).

Attachment structures

Egg shells of the representatives of the family Menoponidae (*E. stramineus*, *U. pallidulus* and *M. gallinae*) are equipped with variously shaped attachment structures: bristles covering the anterior part of the main egg shell and opercular projections (Figs 2d, 3a). These structures are formed within intercellular spaces which develop between the follicular cells at the onset of choriogenesis (Figs 3a and b). The follicular cells responsible for the formation of the bristles are always larger than those secreting the remaining (flat) regions of the main egg shell. Since their baso-apical axes are tangential to the oocyte surface, resulting bristles are oriented towards the anterior pole of the shell (Fig. 3a, arrows).

Table 1 Specializations of basic egg shell elements (operculum, main egg shell, hydropyle) in investigated phthirapterans

Species	Length of egg (μm)	Operculum	Main egg shell	Hydropyle
Anoplurans				
Haematopinus suis	1500	Aeropyles	Aeropyles	—
Mallophagans				
Eomenacanthus stramineus	800	Micropyles and a projection	Bristles	—
Uchida pallidulus	500	Micropyles and a projection	Bristles	—
Menopon gallinae	500	Micropyles and a projection	Bristles	—
Goniocotes gallinae	350	—	—	—
Columbicola columbae	750	—	—	Surrounded by a collar
Lipeurus maculosus	450	Micropyles	—	—
Trichodectes canis	300	Micropyles	—	—

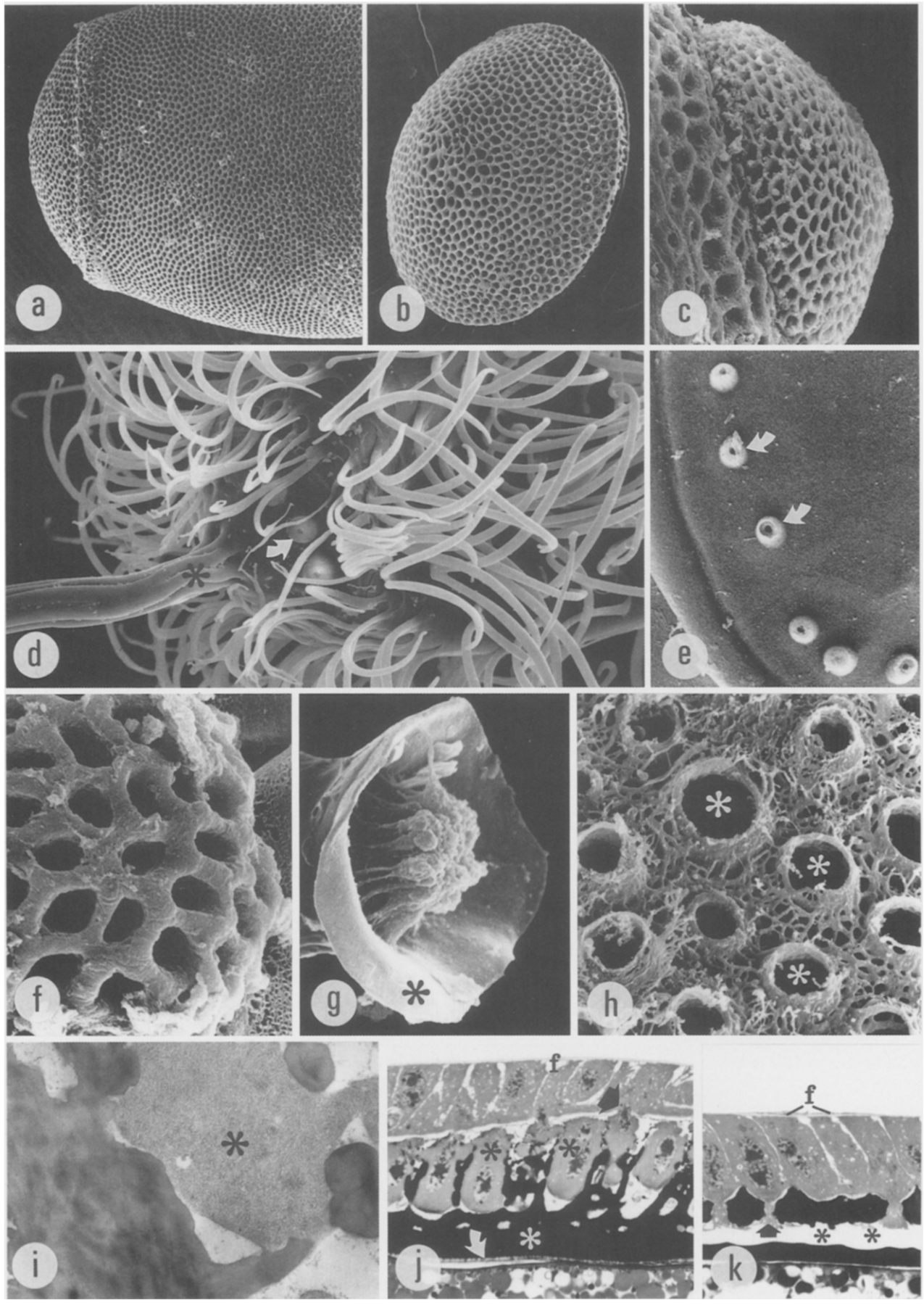
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Fig.2 **a**, *Haematopinus suis*. Operculum and anterior part of the main egg shell; note numerous aeropyles perforating both parts of the shell. Scanning electron microscope (SEM) (× 250). **b**, *Haematopinus suis*. Isolated operculum. SEM (× 300). **c**, *Haematopinus suis*. Hydropyle. SEM (× 1500). **d**. *Eomenacanthus stramineus*. Anterior part of the egg shell; note bristles, opercular projection (asterisk) and micropyles (arrow). SEM (× 500). **e**, *Lipeurus maculosus*. Fragment of the operculum with micropyles (arrows). SEM (× 2000). **f**, *Lipeurus maculosus*. Hydropyle. SEM (× 5400). **g**, *Columbicola columbae*. Hydropyle; note the collar-like structure (asterisk). SEM (× 3000). **h**, *Haematopinus suis*. Detail of developing chorion; follicular epithelium has been stripped away in distilled water; note chorionic fibers and aeropylar openings (asterisks). SEM (× 4000). **i**, *Haematopinus suis*. Follicular cell process (asterisk) surrounded by chorionic fibers. TEM (× 9500). **j**, *Haematopinus suis*. Developing operculum; note broad projections of follicular cells (black asterisks) that contain nuclei and trabecular layer of the chorion (white arrow); black arrow indicates a stalk connecting the projection with the main body of the follicular cell. f, follicular epithelium; white asterisk, homogeneous chorionic layer. LM, semithin section, methylene blue (× 700). **k**, *Haematopinus suis*. Developing main egg shell; note broad space (asterisks) separating a perforated roof from the solid floor of the homogeneous chorionic layer. f, follicular epithelium; arrow, projection of the follicular cell. LM, semithin section, methylene blue (× 700).

Hydropyles

According to Hinton (1981), hydropyles (HPLs) of phthirapterans are responsible for the uptake of water. In investigated species HPLs are slightly convex or hemispherical and comprise numerous, slender (2–3 μm in diameter) canals (Figs 2c, f and g). The canals are arranged parallel to each other and penetrate the whole thickness of the chorion (Fig. 3d). They are molded around bundles of long microvilli that protrude from the apical surfaces of follicular cells covering the posterior oocyte pole (Fig. 3e). In pigeon louse, *C. columbae*, the HPL is connected with the main egg shell by a short pedicle and encircled by a collar-like structure (Fig. 2g).

Distribution of actin filaments in the follicular cells

At the onset of choriogenesis the follicular cells are already diversified into four subpopulations (see above). Staining with rhodamine-conjugated phalloidin has revealed that these subpopulations differ in the distribution of microfilaments.

The cells covering the lateral aspects of the oocyte (i.e. those responsible for the formation of the main egg shell and bristles) comprise basally located arrays of parallel microfilaments (Fig. 4a). Microfilaments of the neighboring cells are aligned and oriented perpendicular to the long axis of the oocyte. The apical parts of these cells are devoid of distinct microfilaments (not shown). The only exception to this rule has been found in the pig louse, *H. suis*. Here, in the apices of the follicular cells large accumulations of microfilaments have been identified (Fig. 4). Further analysis has shown that these microfilaments are localized within the processes of the follicular cells (see above) and gather mainly in the vicinity of their membranes (Fig. 4d).

In the basal parts of the follicular cells surrounding the anterior pole (i.e. those responsible for the formation of the operculum and MPLs), individual microfilaments occur (Fig. 4b); their orientation is random and not related to the oocyte axes. The cellular processes that serve as templates for micropylar canals comprise distinct accumulations of filamentous actin (Fig. 4c).

The follicular cells covering the posterior oocyte pole (i.e. those responsible for the formation of the HPL) are devoid of basal arrays of microfilaments. The microvilli of these cells contain numerous, parallel actin filaments (Figs 4e and f).

After chorion deposition, and before the passage of the oocyte from the ovarian follicle to the oviduct, a major reorganization of F-actin cytoskeleton takes place in the main and anterior follicular cells. Within each cell the microfilaments gather and form thick bundles (stress fibers) which are arranged parallel to each other and span the whole diameter of the cell (Fig. 4g). The orientation of the stress fibers in the neighboring cells is random and not related to the oocyte axes (Fig. 4g).

Discussion

Although the architecture of the egg shell has been studied in numerous representatives of several insect orders (see e.g. Scali & Mazzini, 1981; Ogorzałek, 1987; Mazzini & Gaino, 1990; Rościszewska, 1991; Kambysellis, 1993; Mazzini et al., 1993; Simiczyjew, 1994), its morphogenesis has been reported only for the dipterans, i.e. *Drosophila melanogaster* (Margaritis et al., 1980; Margaritis, 1986), *Dacus oleae* (Mouzaki et al., 1991), *Rhagoletis cerasi* (Mouzaki & Margaritis, 1991), *Ceratitis capitata* (Zarani & Margaritis, 1991b), and *Bradysia tritici* (Wenzel et al, 1990); the lepidopterans, i.e. *Bombyx mori* (Yamauchi & Yoshitake, 1984), *Antheraea polyphemus* (Regier et al., 1980; Mazur et al, 1980), *Lymantria dispar* (Leclerc & Regier, 1993); the plecopterans, i.e. *Perla marginata* and *P. pallida* (Rościszewska, 1995) and the hymenopteran, *Eurytoma amygdali* (Mouzaki & Margaritis, 1994; Zarani & Margaritis, 1994). These analyses have indicated that during oogenesis of 'higher' (holometabolous) insects the follicular epithelium gradually diversifies into morphologically distinct subpopulations which, in turn, are involved in the formation of certain regional specializations of the egg shell. In the most thoroughly investigated species, the fruit fly, *Drosophila melanogaster*, as many as 10 subpopulations have been distinguished (Margaritis et al., 1980).

Preliminary histological studies (Biliński & Jankowska, 1987) have shown that in the bird louse, *E. stramineus*, the follicular epithelium differentiates into four basic subpopulations. Three of them, the anterior cells, the main body cells and the posterior cells, contribute to choriogenesis and secrete the operculum, the main egg shell and the hydropyle, respectively (Biliński & Jankowska, 1987). Our present investigations have indicated that at least in some phthirapteran species the basic subpopulations consist of morphologically distinct groups of cells. These groups significantly differ in

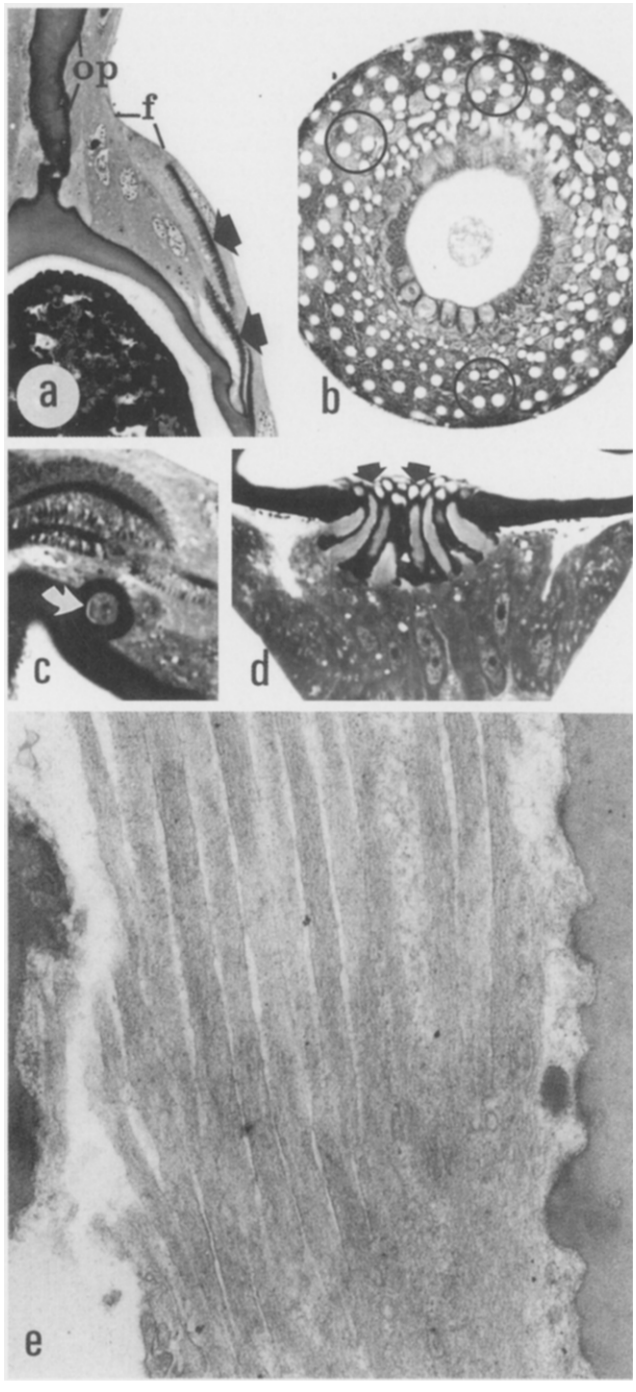

Fig. 3 **a**. *Uchida pallidulus*. Anterior part of a choriogenic ovarian follicle (longitudinal section). f, follicular epithelium; op, opercular projection; arrows, bristles. LM, semithin section, methylene blue (× 500). **b**. *Eomenacanthus stramineus*. Anterior part of a choriogenic ovarian follicle (cross section); bristles develop within intercellular spaces (encircled). LM, semithin section, RNA staining (× 850). **c**, *Menopon gallinae*. Anterior part of a choriogenic ovarian follicle (tangential section). Note micropyle and its canal (arrow). LM, semithin section, methylene blue (× 900). **d**, *Goniocotes gallinae*. Posterior part of a choriogenic ovarian follicle (longitudinal section); note developing hydropyle and its canals (arrows). LM, semithin section, methylene blue (× 900). **e**, *Goniocotes gallinae*. Bundle of microvilli within the hydropylar canal. TEM (× 31 000).

the distribution of the microfilaments, and most importantly participate in the formation of various egg shell specializations. On these grounds we distinguished four morphological categories of the follicular cells.

1. Cells devoid of cellular processes and microvilli, not separated by intercellular spaces, with basal arrays of microfilaments, and responsible for the secretion of the 'flat' chorion (Fig. 5A).
2. Cells devoid of cellular processes and microvilli, separated by intercellular spaces, with basal arrays of microfilaments, and responsible for the secretion of attachment structures (Fig. 5B).
3. Cells equipped with actin containing processes, responsible for the formation of MPLs and APLs (Fig. 5C).
4. Cells equipped with bundles of microvilli, and responsible for the formation of HPLs (Fig. 5D).

In studied phthirapterans, the above categories of follicular cells occur in various combinations which result in a rather high diversity of egg shells. Obviously, the more the categories differentiate during oogenesis, the more complicated the shell that is formed. Figure 6 shows diversification of the follicular epithelium into subsequent subpopulations and categories in the pig louse, *H. suis*, and representatives of the family Menoponidae, characterized by the simplest and most complex egg shells, respectively.

Openings perforating insect egg shells are, as a rule, formed around processes of specialized follicular cells (micropylar canals of dipterans and lepidopterans; Yamauchi & Yoshitake, 1984; Wenzel et al., 1990; Zarani & Margaritis, 1986, 1991a, b, c) or elongated microvilli present at three-cell junctions (hydropylar canals of silkmoths; Regier et al., 1980). We may add to this list, bundles of microvilli that serve as templates for the formation of hydropylar canals in phthirapterans. Ultrastructural as well as immunohistochemical studies on various dipteran and lepidopteran species have shown numerous microtubules and microfilaments inside the processes of the follicular cells (Margaritis, 1984; Yamauchi & Yoshitake, 1984; Wenzel et al., 1990; Zarani & Margaritis, 1991a, c). These cytoskeletal elements are believed to participate in the elongation and/or stabilization of the processes. In contrast, analogous structures of mallophagans and anoplurans do not contain detectable amounts of tubulin (unpublished results) and are filled with microfilaments only. This may be related to the shape of the processes, which in phthirapterans are short and broad while in holometabolous insects they are slender and usually longer.

After the completion of choriogenesis, thick stress fibers appear within the follicular cells of investigated phthirapterans. Similar fibers ('bundles') have also been identified in the follicular cells of the German cockroach, *Blattella germanica* (Zhang & Kunkel, 1992). In accordance with Zhang and Kunkel (1992) we suggest that these fibers are responsible for ovulation, i.e. the passage of the developed, chorionated egg to the oviduct.

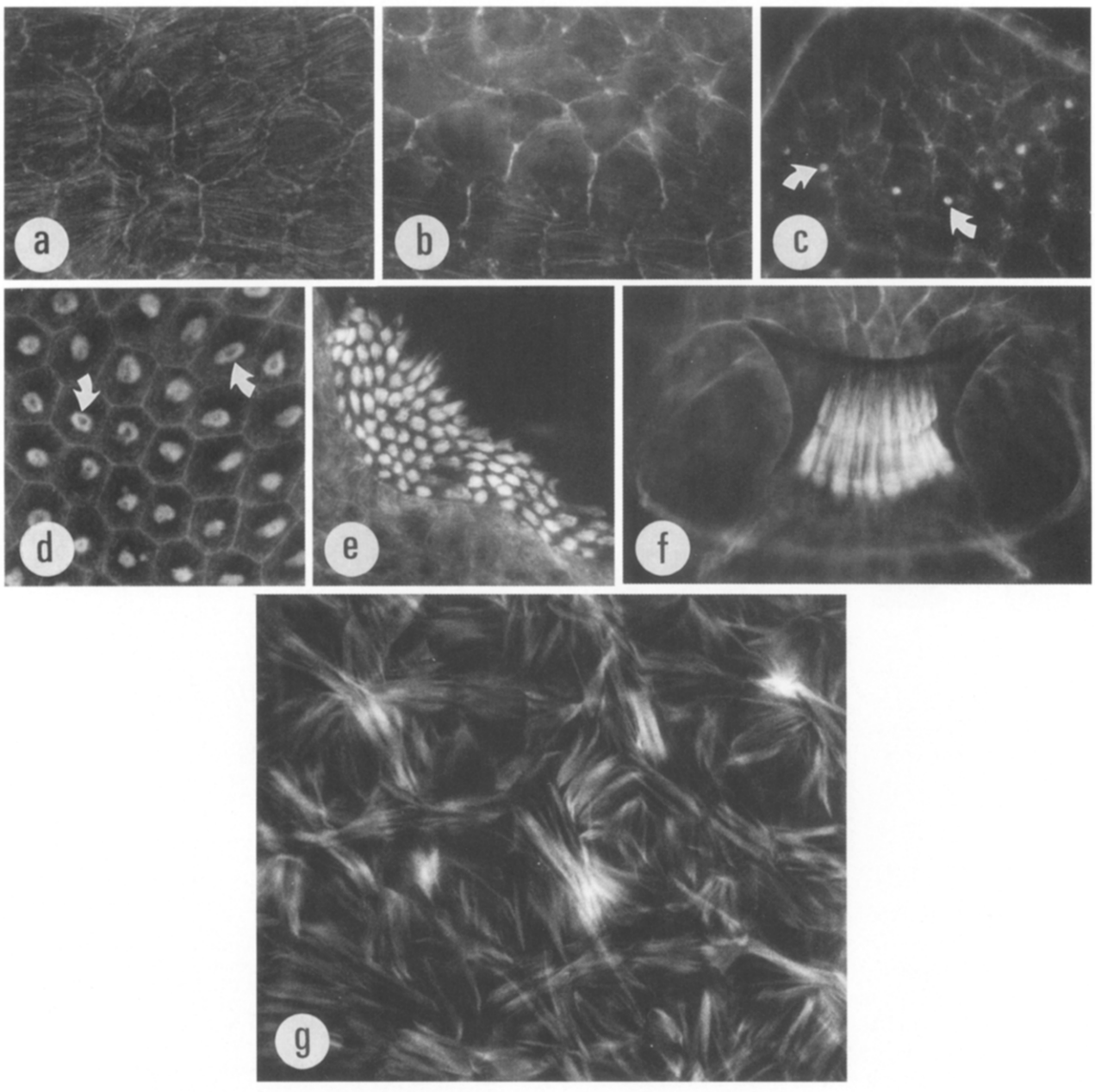

Fig. 4 **a**, *Columbicola columbae*. Basal arrays of microfilaments in follicular cells covering lateral aspects of the oocyte. Rhodamine–phalloidin (× 500). **b**, *Columbicola columbae*. Transition zone between anterior and lateral follicular cells; note individual microfilaments in the former subpopulation. Rhodamine–phalloidin (× 500). **c**, *Lipeurus maculosus*. Anterior follicular cells; note accumulations of microfilaments (arrows). Rhodamine–phalloidin (× 500). **d**, *Haematopinus suis*. Apical accumulations of microfilaments in follicular cells covering lateral aspects of the oocyte; the shape of some accumulations (arrows) suggests that microfilaments gather predominantly in the vicinity of membranes of follicular cell projections. Rhodamine–phalloidin (× 650). **e**, *Haematopinus suis*. Posterior pole of a choriogenic ovarian follicle; note bundles of microvilli (cross 'optical' section). Rhodamine–phalloidin (× 500). **f**, *Columbicola columbae*. Posterior pole of a choriogenic ovarian follicle; note bundles of microvilli (longitudinal 'optical' section). Rhodamine–phalloidin (× 500). **g**, *Haematopinus suis*. Stress fibers in postchoriogenic follicular cells. Rhodamine–phalloidin (× 900).

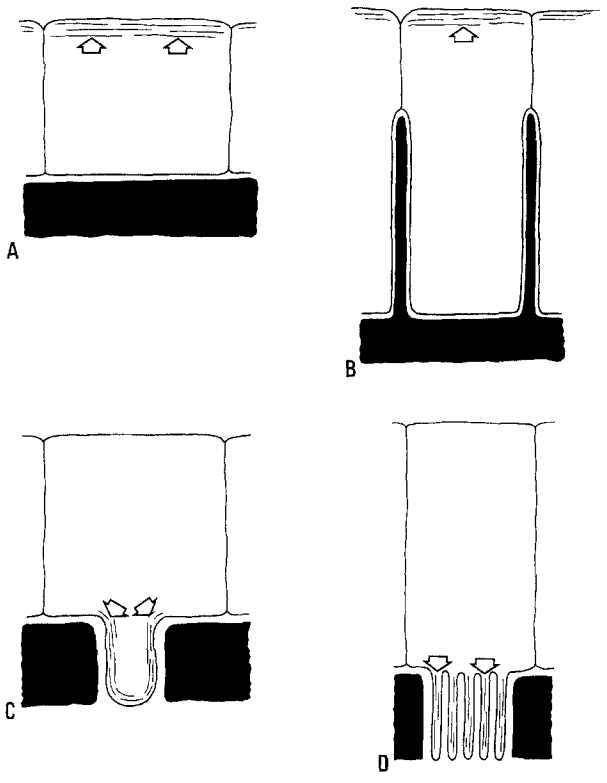

Fig. 5 Schematic representations of 4 described categories of follicular cells (**A, B, C, D**). Arrows indicate microfilaments; see Discussion for further description.

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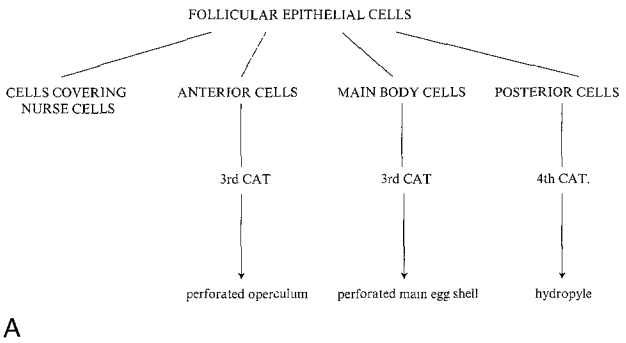

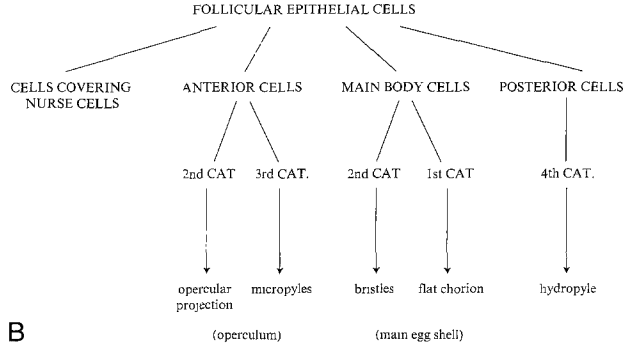

Fig. 6 Diversification of follicular cells into subpopulations and categories in **A,** the pig louse, *Haematopinus suis*, and **B**, representatives of the family Menoponidae. Arrows indicate types of chorionic structures produced; see Discussion for further description.

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