Oogenesis in phthirapterans (Insecta: Phthiraptera). I. Morphological and histochemical characterization of the oocyte nucleus and its inclusions

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Abstract

We characterize morphological and histochemical changes occurring within the oocyte nucleus (germinal vesicle) during oogenesis in two phthirapteran species: the pig louse, *Haematopinus suis* (Anoplura) and the pigeon louse, *Columbicola columbae* (Mallophaga). In previtellogenic oocytes, within the oocyte nucleus the chromatin condenses and forms the karyosome. In contact with the karyosome numerous dense and highly heterogeneous nuclear bodies occur. We demonstrate that these nuclear inclusions have complex structure and contain RNA and argyrophilic proteins of nucleolar organizer (Ag-NOR proteins). Results of immunogold electron microscopy experiments are also presented. The obtained results suggest that the phthirapteran nuclear bodies are assemblages of ribonucleoproteins that are stored in the oocyte nucleus and might be utilized during early stages of embryonic development.

In the investigated species, the nuclear envelope of the germinal vesicle is equipped with characteristic protrusions. Ultrastructural analysis revealed striking similarity of these structures to the initial stages of the formation of accessory nuclei. Based on these results, we speculate on the possible evolutionary origin of the accessory nuclei in phthirapterans.
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1. Introduction

It is well established that the interphase cell nucleus contains distinct morphological and functional compartments: a nuclear envelope with nuclear pores, the nucleolus, the chromatin in a various degree of condensation and the interchromatin space (Spector, 1993, 2001; Driel van et al., 1995; Lamond and Earnshaw, 1998; Dundr and Misteli, 2001). The nucleolus is the most prominent nuclear substructure and probably the best characterized (Scheer and Hock, 1999; Carmo-Fonseca et al., 2000). The major function associated with this organelle is ribosome biogenesis, a process that entails transcribing the rDNA, processing of the pre-rRNA transcripts and assembling the rRNA with ribosomal proteins. The nucleolus is a highly dynamic domain and displays a great ultrastructural variability. To a large extent, the morphological organization of nucleoli reflects the degree of rRNA synthesis taking place in cells (reviewed by Olson et al., 2000).

The interchromatin space comprises characteristic structures: perichromatin fibrils and granules, interchromatin granule clusters and various nuclear bodies (NBs). The morphology and molecular composition of NBs are complex, and their functions are not fully understood (Brasch and Ochs, 1992; Roth, 1995; Matera, 1999). Immunoelectron and immunofluorescent studies have revealed a specific class of NBs containing factors involved in RNA splicing and processing (Gall et al., 1999; Gall, 2000). They have been referred to as coiled bodies in the nuclei of mammalian somatic cells, spheres or snurposomes in amphibian and Binnenkörper or endobodies in insect germinal vesicles. Recently, these structures have been renamed Cajal bodies (CB) in honor of their first investigator (Gall, 2000). CB contain, among others, splicing factors (snRNAs, Sm and SR proteins), basal transcription factors TFIIF, TFIIH, polymerases RNA I, II, III, stem loop binding protein 1 (SLBP1) and the survival motor neuron protein (SMN protein). Similarly to the nucleolus, CB stain positively with the Ag-NOR technique,

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and contain small nucleolar RNAs (snoRNAs) and nucleolar proteins: fibrillarin, B23, nucleolin, Nopp140, NAP57 (reviewed in Matera, 1999; Gall, 2000). The Cajal body-specific protein is p80-coilin (Gall, 2000; Meister et al., 2002; Massenet et al., 2002; Handwerger et al., 2003). Most recently, Darzacq et al. (2002) identified a new family of small RNAs, the small Cajal body-specific RNAs or scaRNAs that localize specifically to CB.

The function of CB is still debated. The most widely accepted model proposes that CB are an assembly site for factors involved in transcription and splicing, i.e. the small nuclear ribonucleoprotein particles (snRNPs) (Ogg and Lamond, 2002). Recently, it has been demonstrated that CB are involved in posttranscriptional modification of spliceosomal snRNAs (Darzacq et al., 2002). Mature snRNPs are transported from CB to the regions of splicing and/or processing of different types of RNA, i.e. to the nucleolus (snoRNPs) or to the speckles and sites of active transcription (snRNPs) (Matera, 1998, 1999; Carvalho et al., 1999; Carmo-Fonseca, 2002).

The oocyte nuclei (germinal vesicles) of many organisms are favorable material for morphological analyses because of their large size and a relatively high number of various nuclear structures. They often contain, in addition to the nucleoli, one or more CB. In *Xenopus* germinal vesicles, the latter nuclear domains are perfectly spherical and consist of three major parts: a spherical body (matrix), smaller spherical or nearly spherical granules on the surface (called B-snurposomes), and inclusions of various sizes that resemble the surface granules (B-like inclusions) (Callan, 1986; Gall, 1991; Gall et al., 1995). Based on EM and immunocytochemical studies it was proposed that *Xenopus* CB are the sites for the assembly of transcription complexes, so-called transcriptosomes, the unitary particles containing either polymerase RNA I, II or III and the appropriate factors involved in the synthesis and processing of RNA transcripts (i.e. snRNPs, SR proteins) (Gall et al., 1999; Gall, 2000). Transcriptosomes are subsequently exported from CB to the appropriate regions within the nucleus, e.g. to the nucleolus (pol I), to the speckles in somatic nuclei, or B-snurposomes in the oocyte nuclei, where they are stored and eventually transported to the sites of transcription (pol II and pol III) (Parfenov et al., 1996; Gall et al., 1999; Gall, 2000).

During the past years, it has been demonstrated that NBs, morphologically and biochemically similar to CB, are present in germinal vesicles of many insects (*Acheta domesticus*, Gall et al., 1995; Tsvetkov et al., 1997; Filek et al., 2002; *Carabus violaceus*, Jaglarz, 2001; *Panorpa communis*, Batalova and Tsvetkov, 1998; *Tenebrio molitor*, Tsvetkov et al., 1997; Bogolyubov, 1998; Bogolyubov et al., 2000; *Anthonomus pomorum,* Świątek, 1999). Furthermore, in representatives of several insect groups, the oocytes are equipped with specific organelles termed accessory nuclei (AN) (King and Fordy, 1970; Cassidy and King, 1972; Meyer et al., 1979; Biliński, 1989, 1991a,b; Biliński et al.,

1993, 1995a,b). AN contain inclusions of nuclear origin (pseudonucleoli) embedded in a translucent matrix (Biliński et al., 1993). The immunocytochemical analysis of AN in hymenopterans demonstrated that pseudonucleoli are molecularly similar to *Xenopus* CB, which suggests that these structures may be homologous (Biliński and Kloc, 2002). Accessory nuclei and the structurally complex NBs have been also described in phthirapteran oocytes (Ries, 1932; Biliński, 1989).

Selected aspects of phthirapteran oogenesis were presented previously (Biliński and Jankowska, 1987; Zawadzka et al., 1997; Żelazowska and Biliński, 1999, 2001). Here, we focus on the ultrastructural, histochemical, and immunocytochemical analysis of the germinal vesicle and nuclear substructures in two species representing different subordinate phthirapteran groups: the pig louse, *Haematopinus suis* (Anoplura) and the pigeon louse, *Columbicola columbae* (Mallophaga). We present, for the first time, data on the nucleus structure in anoplurans. Based on the characteristic ultrastructural features of the nuclear envelope and its protrusions we speculate on the evolutionary origin of AN in phthirapterans.

2. Material and methods

The adult females of *Haematopinus suis* used in this study were collected from domestic pigs; the specimens of *Columbicola columbae* were collected from pigeons.

2.1. Histological and ultrastructural analyses

2.1.1. Whole mount preparations

The ovaries were dissected and fixed in 4% formaldehyde (freshly prepared from paraformaldehyde) in phosphate-buffered saline (PBS). The specimens were then rinsed in PBS, mounted on microscope slides and examined in a Jenalumar (Zeiss, Jena, Germany) microscope equipped with Nomarski interference contrast.

2.1.2. Light (LM) and transmission electron microscopy (TEM)

The ovaries were dissected and 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 (Fullam Inc., Latham, NY, USA). Semithin sections (0.5 μm) were stained with 1% methylene blue in 1% borax, and examined in a Jenalumar light microscope. Ultrathin sections (90 nm) were contrasted with uranyl acetate and lead citrate and examined in a Philips 300 or Jeol 100 SX TEM at 60 or 80 kV.

2.1.3. Immunoelectron microscopy

For immunogold labeling the ovaries were fixed in 2%

glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 20 min or in a mixture of 2% formaldehyde (freshly prepared from paraformaldehyde) with 1% glutaraldehyde in phosphate buffer (pH 7.4) for 20 min. They were processed as described for LM and TEM, except that they were not postfixed in osmium tetroxide and the blocks were polymerized at 50 °C. Ultrathin sections were collected on formvar coated nickel single slot grids and etched on droplets of 0.5% NaOH in ethanol followed by 5% H_2O_2 for 7 min, rinsed several times with distilled water, and blocked with 2% bovine serum albumin (BSA) or 2% fish gelatin in PBS for 20 min. Grids were incubated with primary antibody (for a list of antibodies see Table 1) diluted 1:50–1:800 in PBSB (PBS + 1%BSA + 0.1%NaN$_3$) or PBSF (PBS + 1% fish gelatin + 0.1%NaN$_3$) overnight at 4 °C. Subsequently, they were washed 5 times in PBS and incubated for 2 h at room temperature with the secondary antibody diluted 1:50 or 1:100 in PBSB or PBSF. The secondary antibodies were appropriate goat anti-mouse and anti-rabbit or donkey anti-goat IgG (whole molecule) conjugated to 10 or 18 nm gold particles (Sigma Chemical Co., St Louis, MO, USA). Following incubation, grids were washed 2 times in PBS and 3 times in distilled water. After drying, the sections were contrasted with uranyl acetate and lead citrate and examined in Jeol 100 SX electron microscope at 80 kV. Control sections were treated as described above, but the primary antibody was omitted.

2.1.4. Triton X-100 extraction

Dissected ovaries were Triton X-100 extracted (for a description of the method see Biliński et al., 1995a). In brief, isolated ovarioles were incubated in modified Hanks' buffer (0.05 M phosphate buffer pH 7.3, 2 mM EGTA, 50 mM MES, 12 mM MgCl$_2$) containing 1% Triton X-100. After extraction, the material was fixed in 2.5% glutaraldehyde in the same buffer at room temperature, rinsed and postfixed in 2% osmium tetroxide and 0.8% potassium ferricyanide in 0.1 M phosphate buffer pH 7.4 for 1 h. The ovarioles were rinsed in water and additionally fixed in 0.15% tannic acid and contrasted with uranyl acetate. After dehydration in a series of ethanols and acetone, the material

was embedded in Epon 812. Ultrathin sections were contrasted with lead citrate and examined in a Philips 300 TEM at 60 kV.

2.2. Histochemical analyses on semithin sections

The ovaries were dissected and fixed in 4% formaldehyde (freshly prepared from paraformaldehyde) in PBS for 40 min at room temperature. The material was then rinsed with PBS and after dehydration in a graded ethanol series, infiltrated and embedded in acrylic resin (Histocryl, Agar, Stansted, UK).

2.2.1. Nucleic acids (DNA, RNA) detection

Semithin sections (0.5 μm) were stained with propidium iodide (Sigma) in PBS (1:800) for 40 min, and subsequently with 0.3 μg/ml of diamidino-2-phenylindole dihydrochloride (DAPI, Sigma) in PBS for 30 min. All stainings were proceeded at room temperature in the darkness. The sections were then rinsed with PBS, mounted in glycerol in McIllvaine's buffer, pH 4.2 or in a medium containing 80% glycerol, 20% Tris buffer, pH 9 and 50% n-propylgalate, and examined in a Jenalumar (Zeiss) or Leica DMR epifluorescence microscope equipped with appropriate filters.

2.2.2. Ag-NOR technique

The Ag-NOR staining of 0.5 μm thick sections was performed according to the method of Howell and Black (1980) modified by Biliński and Bilińska (1996). Briefly, sections were stained in a 1:2 mixture of 2% gelatin in 1% formic acid and 50% silver nitrate (Sigma). The staining was carried out in a moist chamber at 37 °C for 9 min. After rinsing the slides were counterstained with DAPI (0.3 μg/ml) in PBS for 20 min at room temperature in the dark, rinsed in PBS and mounted in glycerol in McIllvaine's buffer, pH 4.2. The slides were examined in a Jenalumar microscope.

Table 1
List of antibodies used in immunoEM analyses

Antibodies		Antigens revealed
Mouse monoclonal	(1) K-121	2,2,7-Trimethylguanosine cap of snRNAs
	(2) 8WG16	Carboxyl-terminal domain of polymerase II RNA
	(3) Coilin	p80-Coilin
	(4) αSC-35	SR protein, non-snRNA splicing factor of pre-mRNA
Goat polyclonal serum	(5) A-16	Fibrillarin
Rabbit polyclonal sera	(6) C23 (H250)	Nucleolin
	(7) H195	SMN protein

(1) Calbiochem, Oncogene Research Products, Cambridge, MA, USA; (2) Abcam, USA; (3) Zymed, San Francisco, California, USA; (4) Accurate Chemical and Scientific Corp., Westbury, NY, USA; (5), (6), (7), Santa Cruz Biotechnology, USA.
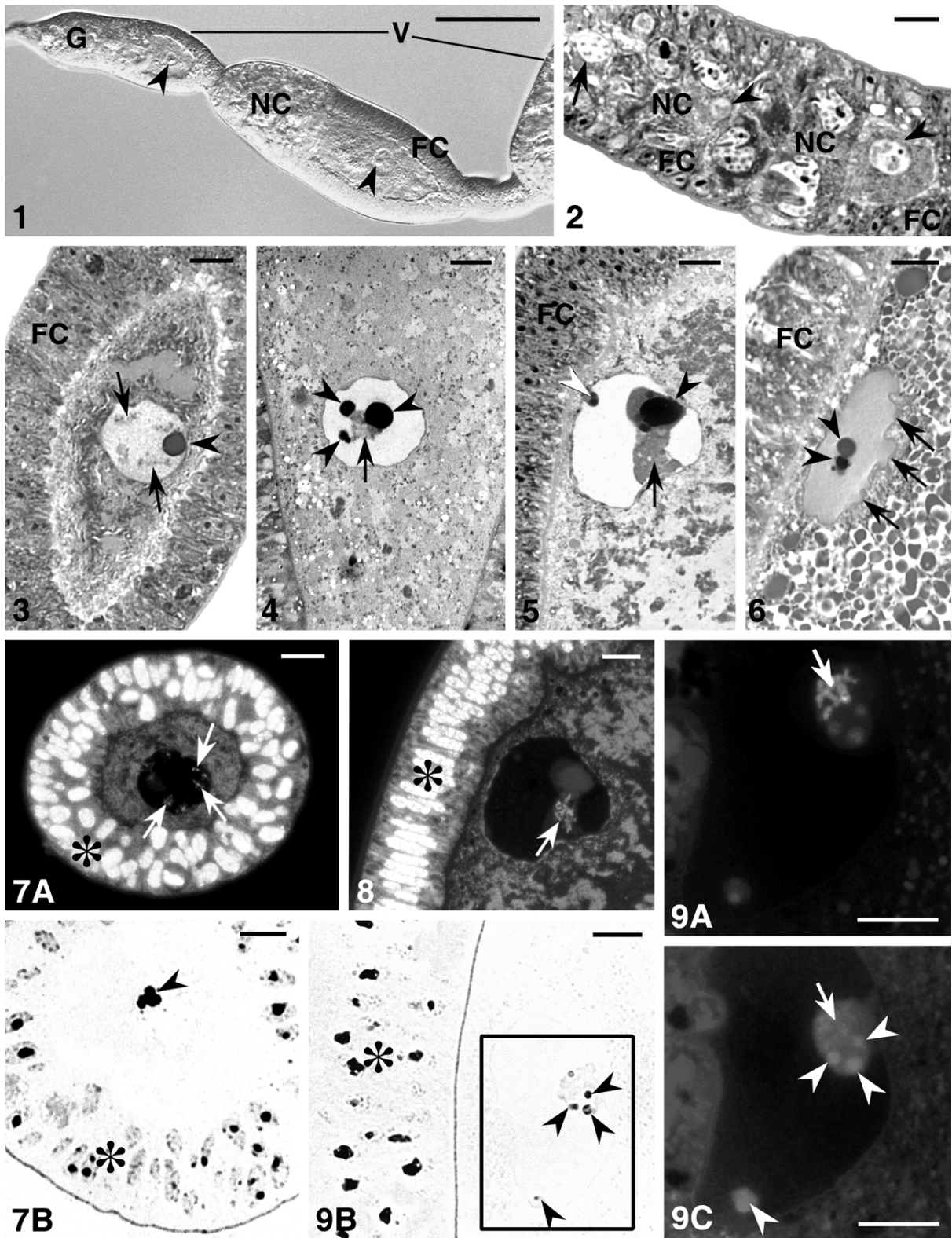
3. Results

3.1. Gross architecture of the female reproductive system

The female reproductive system of the studied species is composed of paired ovaries, short lateral oviducts, and a common oviduct that leads into a vagina. Also, a pair of accessory glands that secrete the cement used for attaching the eggs to substratum and spermatheca are present. Within female reproductive organs specialized cells, the mycetocytes, housed by symbiotic microorganisms are localized (for a detailed description of ovarian mycetocytes and microorganisms see Żelazowska and Biliński, 1999).

The ovaries are composed of five loosely arranged ovarioles of the polytrophic-meroistic type (for the description and classification of insect ovaries see Büning, 1994; Biliński, 1998). Each ovariole is composed of three parts: the terminal filament, germarium, and vitellarium (Fig. 1). The terminal filaments join up with each other and form the ligament that attaches the gonad to the body wall. The germaria are short and contain a few clusters of oogonial cells. It appears that within the clusters, only one cell, the future oocyte enters the prophase of the first meiotic division (Fig. 2, arrow). The basal part of the germarium is occupied by early previtellogenic ovarian follicles which are surrounded by somatic prefollicular cells (Fig. 2). The boundary between the germarium and the vitellarium is not distinct (Figs. 1 and 2). In the vitellarium, there are 3–8 linearly arranged ovarian follicles, in subsequent stages of oogenesis (Fig. 1). Each follicle is composed of an oocyte and 7 nurse cells and surrounded by follicular cells (Figs. 1, 2 and 15).

3.2. Oocyte nucleus (germinal vesicle)

The process of oogenesis in phthirapterans has been previously divided into five consecutive stages: previtellogenesis (stages 1–2), vitellogenesis (stages 3–4) and choriogenesis (stage 5) (Biliński and Jankowska, 1987). In previtellogenic oocytes, the germinal vesicle is large, round

and centrally located (Figs. 1 and 2-arrowheads and Fig. 3). This localization is maintained until early vitellogenesis (stage 3) (compare Figs. 1–4 and 5 and 6). As first yolk spheres and lipid droplets begin to accumulate within the oocyte cytoplasm, the spherical germinal vesicle shifts to a mid-region of the cortical ooplasm (Fig. 5). At the next stage (late vitellogenesis) the oocyte significantly increases its volume due to accumulation of reserve materials. The germinal vesicle remains near the surface of the oocyte but its shape changes from spherical to oval (Fig. 6). The ultrastructure of germinal vesicles in the pig louse, *Haematopinus suis* and the pigeon louse, *Columbicola columbae* differs and will be presented in separate sections.

3.2.1. Haematopinus suis

In the germinal vesicle of *H. suis*, besides chromatin, characteristic heterogeneous dense structures (2–4 μm in diameter), which will be referred to as nuclear bodies (NBs), occur. Their morphology and localization change during the course of oogenesis.

During early previtellogenesis (stage 1), the germinal vesicle comprises aggregations of chromatin, lying in contact with the nuclear envelope, and a few NBs variably distributed within nucleoplasm (Figs. 2–3). EM analysis has revealed that during this stage NBs are composed of material of different electron density and structure (Fig. 12). Five types of this material can be distinguished:

(1) coarse-granular material of high electron density,
(2) fine-granular material of high electron density,
(3) fine-granular material of medium electron density,
(4) fine-granular material of low electron density,
(5) homogeneous material of high electron density.

The spatial relationship between different types of NBs components appears rather variable. However, the homogeneous material (type 5) is consistently found in contact with the fine-granular material of high electron density (type 2) throughout the entire oogenesis (Figs. 12–14 B-arrows).

Figs. 1–9. *Haematopinus suis*: Fig. 1: Apical part of an ovariole with the germarium (G) and the vitellarium (V) comprising ovarian follicles in subsequent stages of oogenesis; germinal vesicles are indicated by arrowheads; nurse cells (NC), follicular cells (FC). Whole mount preparation, Nomarski interference contrast, scale bar = 100 μm. Fig. 2: Apical region of an ovariole with oogonial cells in prophase of the first meiotic division (pachytene) (arrow). Two, early-previtellogenic ovarian follicles differentiated into an oocyte, a group of nurse cells (NC), and surrounded by follicular cells (FC). In germinal vesicles (arrowheads) the aggregations of chromatin and NBs are visible. LM, methylene blue, scale bar = 10 μm. Fig. 3: Early previtellogenic oocyte (stage 1). The germinal vesicle contains chromosomes (arrows) and nuclear body (arrowhead); follicular cells (FC). LM, methylene blue, scale bar = 10 μm. Fig. 4: Late previtellogenic oocyte (stage 2). Condensed chromatin forms the karyosome (arrow), NBs—arrowheads. LM, methylene blue, scale bar = 10 μm. Fig. 5: Early vitellogenic oocyte (stage 3). NBs (arrowhead) are in contact with the karyosome (arrow). Note also the peripheral dense body (white arrowhead) localized in the vicinity of the nuclear envelope; follicular cells (FC). LM, methylene blue, scale bar = 10 μm. Fig. 6: Germinal vesicle within the cortical ooplasm (late vitellogenic oocyte, stage 4); the karyosome and all NBs (arrowheads) are localized in the center of the nucleoplasm. Note folds (arrows) of the nuclear envelope; follicular cells (FC). LM, methylene blue, scale bar = 10 μm. Fig. 7: Early previtellogenic oocyte (stage 1). Histocryl sections stained with DAPI (A) and Ag-NOR technique (B). Note the condensed chromatin (A, arrows), and Ag-NOR-positive NBs (B, arrowhead), nuclei of follicular cells (asterisk) A, FLM, scale bar = 10 μm. B, LM, scale bar = 10 μm. Fig. 8: Early vitellogenic oocyte (stage 3). Histocryl section stained with DAPI. The chromatin forms the karyosome (arrow), nuclei of follicular cells (asterisk). FLM, scale bar = 10 μm. Fig. 9: Vitellogenic oocyte. Histocryl sections stained with DAPI (A), Ag-NOR technique (B), and propidium iodide (C). The region shown in A and C corresponds to the boxed area in B. Karyosome (white arrow); NBs in contact with the karyosome, and a peripheral dense body (arrowheads) are Ag-NOR-positive (B) and RNA-positive (C); nuclei of follicular cells (asterisk). A, FLM, scale bar = 10 μm. B, LM, scale bar = 10 μm. C, FLM, scale bar = 10 μm.
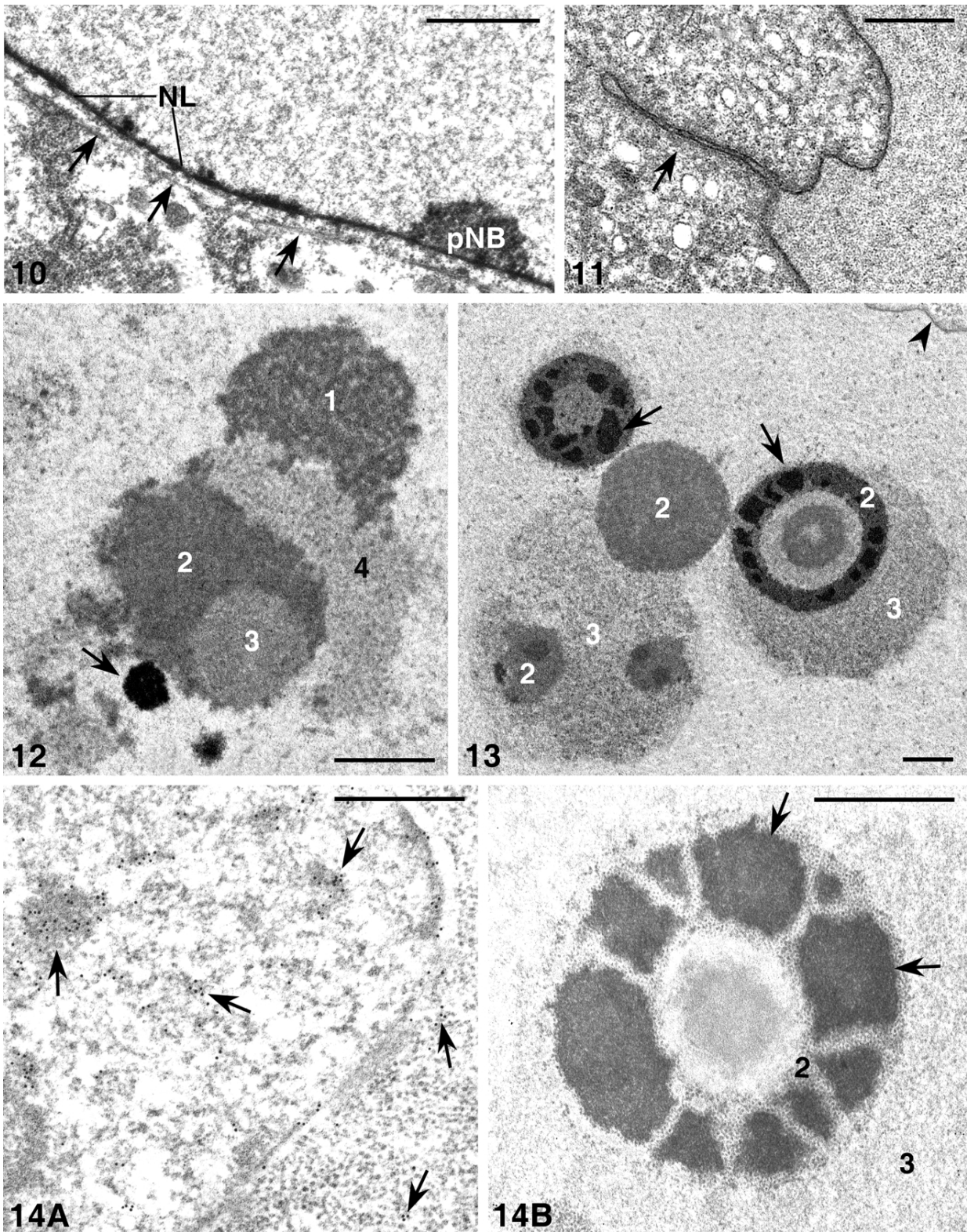
Table 2
The results of histochemical analyses. All stainings were performed on semithin Histocryl sections

	Stage of oogenesis	Chromatin/karyosome	Nuclear bodies (NBs)	Peripheral dense bodies
DNA	1	+	−	−
	2	+	−	−
	3, 4	+	−	−
DNA and RNA	1	+	+	+
	2	+	+	+
	3, 4	+	+	+
Argyrophilic proteins of nucleolar organizer	1	−	+	+
	2	−	+	+
	3, 4	−	+	+

Abbreviations: 1—early previtellogenesis (stage 1); 2—late previtellogenesis (stage 2); 3, 4—vitellogenesis (stages 3 and 4).

During the successive stages of oogenesis (late previtellogenesis, stage 2 and early vitellogenesis, stage 3) chromatin within the oocyte nucleus condenses and forms the karyosome (Fig. 4). The latter structure stains with propidium iodide and is DAPI-positive (Figs. 7(A), 8 and 9(A) and (C)). In the end of previtellogenesis, NBs increase in number and translocate towards the karyosome (Figs. 4 and 5). In early vitellogenic oocytes (stage 3), within the germinal vesicle distinct small aggregations of electron-dense, fine-granular material are observed. These aggregations are initially associated with the nuclear envelope and will be referred to as peripheral dense bodies (Figs. 5, 9(B) and (C) and 10).

During late vitellogenesis (stage 4), the peripheral dense bodies move towards the center of the germinal vesicle and together with the karyosome and NBs form an irregular, polymorphic structure (Fig. 6). At this stage, the NBs consist of concentric layers of high (type 2) and medium (type 3) electron dense, fine-granular material (Figs. 13 and 14(B)). In addition, the type 2-material is interspersed with aggregates of dense homogenous material (type 5) (Figs. 13 and 14(B)).

Histochemical analysis showed that both the NBs and the peripheral dense bodies stain positively with the Ag-NOR method, which indicates that they contain argyrophilic proteins of nucleolar organizer (Ag-NOR proteins) (Figs. 7(B) and 9(B)). Double staining of semi-thin sections with the silver salt and DAPI confirmed a direct contact of the karyosome and NBs (Figs. 9(A) and (B)). All NBs appear RNA-positive, as they stain positively with propidium iodide, and do not stain with DAPI (compare Figs. 9(A) and (C)). The summarized results of histochemical stainings are presented in Table 2.

To gain insight into the molecular composition of NBs in *H. suis* immunogold labeling with antibodies against snRNAs and various nuclear proteins was performed (for the list of antibodies used see Table 1). In the investigated species, none of the germinal vesicle NBs have been labeled (see Fig. 14(B)). The comparison of the immunolocalization of snRNPs particles (K-121 antibody) in the oocyte with that in the follicular cells shows that although labeling of the cytoplasm in both cell types is similar, i.e. gold particles form small aggregates dispersed within cytoplasm (Fig. 14(A)), there is a significant difference in staining of the nucleoplasm (Figs. 14(A) and (B)). The K-121 antibody labels many small irregular foci within the follicular cell nuclei (Fig. 14(A)) while, no structure is labeled within the germinal vesicle (Fig. 14(B)).

3.3. Nuclear envelope

In *H. suis,* the oocyte nucleus is surrounded by a typical envelope, composed of inner and outer membranes pierced by numerous pores. The inner membrane is lined by the nuclear lamina (Fig. 10). Triton X-100 extraction visualized a single layer of microtubules within the perinuclear ooplasm (Fig. 10, arrows).

During vitellogenesis (stages 3–4) and choriogenesis (stage 5), the nuclear boundary appears regular when viewed in a light microscope (Figs. 5 and 6). However, EM reveals that in some areas the nuclear envelope forms long, finger-shaped protrusions (Fig. 11, arrow). Their apical part is dilated or club-shaped while the basal part is constricted, generating a kind of stem that connects these structures with the nuclear envelope (Fig. 11). The detailed

Figs. 10–14. *Haematopinus suis*: Fig. 10: Ultrastructure of Triton X-100 extracted oocyte. Microtubules in perinuclear ooplasm (arrows), peripheral dense body (pNB), nuclear lamina (NL). TEM, scale bar = 1 μm. Fig. 11: Fragment of the germinal vesicle in the vitellogenic oocyte. The protrusion of the nuclear envelope (arrow) contains no electron-dense material. TEM, scale bar = 1 μm. Fig. 12: Ultrastructure of the early previtellogenic nucleus (stage 1). NBs consist of morphologically distinct components (1–4, marked respectively; 1, 2, 3, 4, for detailed description see Results). Note also the aggregation of homogeneous electron-dense material (type 5) (arrow). TEM, scale bar = 1 μm. Fig. 13: In the vitellogenic oocyte nucleus, NBs are composed of concentrically arranged layers of fine-granular material of high (2) and medium (3) electron density; homogeneous electron-dense material (arrows). Arrowhead points at the germinal vesicle envelope. TEM, scale bar = 1 μm. Fig. 14: Vitellogenic oocyte. Immunogold staining with K-121 monoclonal antibody. A, fragment of follicular cell and B, a nuclear body within the germinal vesicle. A, Irregular foci, both in the nucleus and cytoplasm, labeled with K-121 antibody are indicated by arrows, scale bar = 500 nm. B, Note lack of labeling over the nuclear body, homogeneous electron-dense material (arrows), digits indicate NB material type 2 and type 3 scale bar = 1 μm.
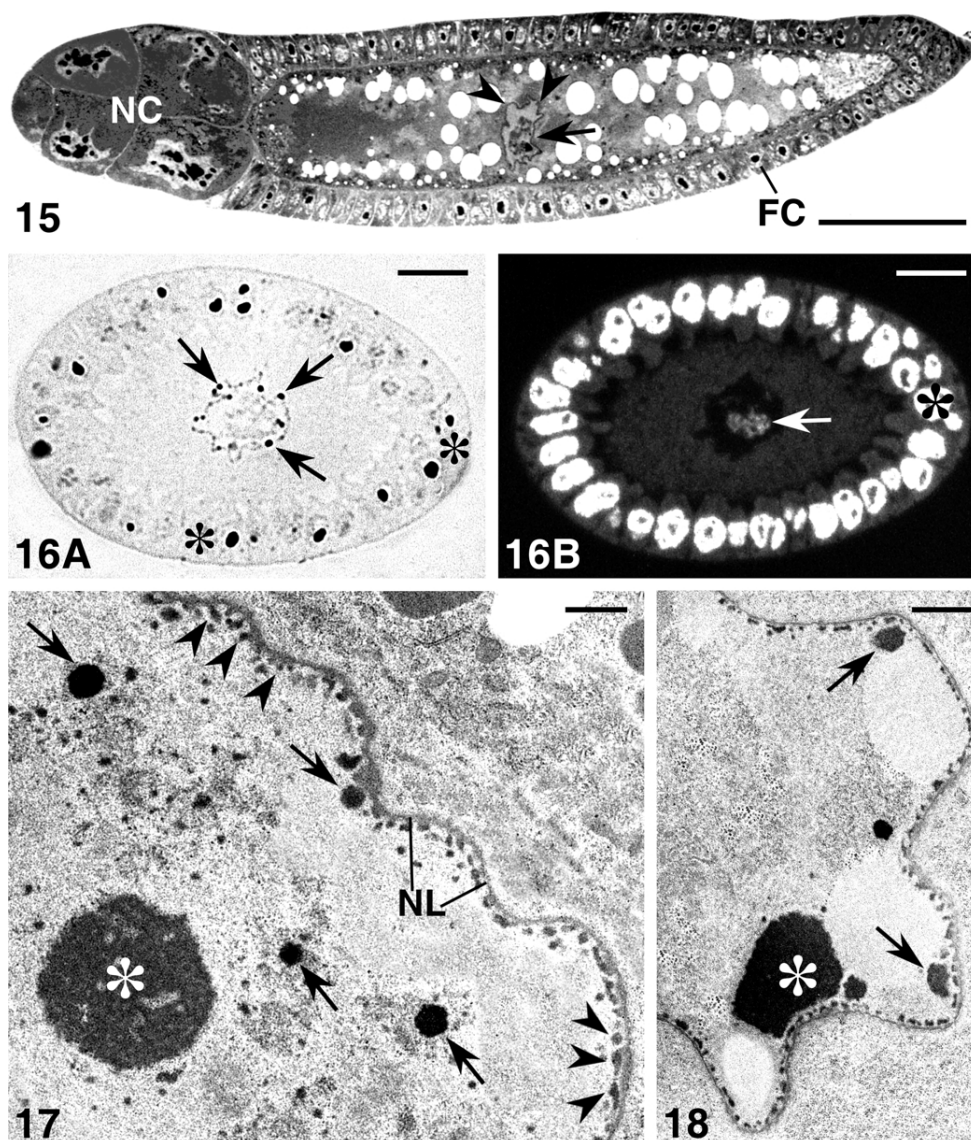
Figs. 15–18. *Columbicola columbae*: Fig. 15: Early vitellogenic ovarian follicle (stage 3). Note protrusions of nuclear envelope (arrowheads) and karyosome (arrow) within the germinal vesicle; nurse cells (NC), follicular cells (FC). LM, methylene blue, scale bar = 50 μm. Fig. 16: Early vitellogenic oocyte (stage 3) stained with Ag-NOR technique (A) and DAPI (B). A, NBs within the protrusions of nuclear envelope are Ag-NOR-positive (arrows); B, within the germinal vesicle only the karyosome (arrow) is DAPI-positive, asterisks-nuclei of follicular cells. A, LM, scale bar = 10 μm. B, FLM, scale bar = 10 μm. Fig. 17: Ultrastructure of the early vitellogenic germinal vesicle (stage 3). Aggregation of fibro-granular (asterisk) and homogeneous high electron-dense material (arrows) within the nucleoplasm. Note a thick layer of the nuclear lamina (NL) underlined by regularly distributed small aggregations of fibro-granular material (arrowheads). TEM, scale bar = 1 μm. Fig. 18: Ultrastructure of the early vitellogenic (stage 3) germinal vesicle. The aggregations of fibro-granular (asterisk) and homogeneous (arrows) material are localized within the protrusions of the nuclear envelope. TEM, scale bar = 1 μm.

analysis has not revealed any presence of protrusions completely detached from the nuclear envelope. Within the protrusions, accumulations of any type of the materials found within NBs have never been observed.

3.4. Columbicola columbae

In the other studied species, the pigeon louse, *C. columbae,* the germinal vesicle chromatin also condenses and forms the karyosome that is DAPI-positive (Fig. 16(B)).

The ultrastructural analysis of the germinal vesicle in this species showed that aggregations of fibro-granular material (Fig. 17, asterisks) and homogeneous high electron-dense material (Fig. 17, arrows) occur in the nucleoplasm. The inner membrane of the nuclear envelope is lined by the nuclear lamina (Fig. 17). In the nucleoplasm, beneath the lamina regularly distributed, small aggregations of fibro-granular material are found (Fig. 17, arrowheads). During vitellogenesis (stages 3–4) the nuclear envelope undergoes intensive folding along the entire perimeter (Figs. 15 and

18). The nuclear envelope folds are broad, dome-shaped (Fig. 18) or rarely long and finger-shaped (not shown). The bases of the folds do not constrict and never detach from the nucleus. Within both types of folds round areas of low electron-density and either one large or few small accumulations of fibro-granular material are observed (Fig. 18). The large accumulations stain positively with the Ag-NOR method (Fig. 16(A)) and are DAPI-negative (Fig. 16(B)).

4. Discussion

4.1. The karyosome and nuclear bodies in the germinal vesicle

There are two basic ovary/ovariole types in insects: panoistic and meroistic. In the panoistic ovaries all oogonia differentiate into the oocytes and the growing oocytes are accompanied only by somatic follicular cells. During previtellogenesis the oocyte nucleus engages in massive synthesis of various types of RNAs (primarily mRNAs and rRNAs) (reviewed in Büning, 1994). Thus, the entire pool of RNAs deposited in the ooplasm is synthesized by the oocyte itself. In the panoistic ovarioles of several insect groups (e.g. Orthoptera: Gryllidae, Mecoptera: Boreidae and Siphonaptera), within the germinal vesicle a selective amplification of the ribosomal genes (rDNA) takes place (Trendelenburg et al., 1973; Büning and Sohst, 1989; Biliński and Büning, 1998). As a result, a characteristic compact body, termed the extrachromosomal DNA-body, arises in the nucleoplasm. As oogenesis progresses, the extrachromosomal DNA-body fragments and disperses within the nucleoplasm which is accompanied by the formation of the multiple nucleoli (Trendelenburg et al., 1973; Büning and Sohst, 1989; Biliński and Büning, 1998).

In the meroistic ovaries, the nurse cells (or trophocytes) accompany the oocytes and support them with vast amounts of RNA (mostly rRNA, mRNA) and organelles (mitochondria, cisternae of endoplasmic reticulum, Golgi complexes). The nutritive role of the nurse cells is facilitated by the presence of stable cytoplasmic canals (intercellular bridges in the polytrophic ovarioles or nutritive cords in telotrophic ones) that link the nurse cells and the oocyte and serve as a transport route (for a review see Büning, 1994; Cooley and Theurkauf, 1994; Biliński, 1998). The presence of nurse cells relieves the oocyte from the necessity of massive syntheses. For this reason the germinal vesicle in the meroistic ovariole usually shows low transcriptional activity and its chromatin turns into the karyosome. The latter structure has been demonstrated in oocyte nuclei of dipterans, hymenopterans, neuropterans, coleopterans and mecopterans (for a review see Gruzova and Parfenov, 1993). Only in a few meroistic groups (including neuropterans, coleopterans and lower dipterans) some rRNA is synthesized by the oocyte itself (Lima de Faria

and Moses, 1966; Gruzova et al., 1972; Matuszewski and Kloc, 1976; Kloc et al., 1995; Kubrakiewicz and Biliński, 1995; Rübsam and Büning, 2001; Kubrakiewicz, 2002). As in the panoistic ovaries, the selective amplification of ribosomal genes in the germinal vesicles leads to the formation of the extrachromosomal DNA-body and ultimately to the appearance of the multiple nucleoli (Matuszewski and Hoser, 1975; Kubrakiewicz and Biliński, 1995; Kubrakiewicz, 2002; Mazurkiewicz and Kubrakiewicz, 2002). The amplified ribosomal genes contribute together with the nurse cells to a massive increase in biogenesis of ribosomes that results in an accelerated rate of oogenesis.

In the germinal vesicles of the investigated phthirapteran species, the chromatin condenses into the karyosome and neither the extrachromosomal DNA-body, nor the multiple nucleoli have been found. In addition to the karyosome, the oocyte nuclei of *H. suis* contain heterogenous NBs. They have rather complex structure and are composed of 5 types of material. We have demonstrated that these nuclear inclusions contain argyrophilic proteins of nucleolar organizer (Ag-NOR proteins), and are RNA-positive and DNA-negative. As oogenesis progresses, NBs as well as the peripheral dense bodies, which initially contact the nuclear envelope, translocate and ultimately associate with the karyosome. Similar movements of NBs within the germinal vesicle have been described in several coleopteran families (Carabidae, Curculionidae, Tenebrionidae) and are believed to reflect dynamic properties of these bodies and their involvement in the intensive nucleo-cytoplasmic exchange (Gruzova and Parfenov, 1993; Świątek, 1999; Jaglarz, 2001).

Recent analyses of insect NBs have shown that these structures, despite their variability in size and morphology, contain small nuclear RNAs, Sm proteins and coilin and are therefore homologous to CB (Gall et al., 1995; Batalova and Tsvetkov, 1998; Bogolyubov, 1998; Bogolyubov et al., 2000; Jaglarz, 2001). Surprisingly, the presence of the mentioned Cajal body markers in the lice NBs has not been confirmed by our immunoelectron studies. It might be speculated, in the light of such unexpected results, that the antibodies used in our experiments do not cross-react with the phthirapteran epitopes (except the K-121 antibody, see below). In this context, it is worth mentioning that recent molecular analyses have shown that lice are characterized by elevated levels of substitution in various mitochondrial genes (Shao et al., 2001; Page et al., 2002; Johnson et al., 2003). In insects, mtDNA usually evolves 10–20 times faster than nuclear DNA, whereas in lice this difference is 200–300 fold. It should be stressed, that such high relative rates are unknown in other organisms. Although at present there is no evidence that the louse nuclear genome and consequently proteome evolve at a substantially higher rate than in other insects, this possibility cannot be ruled out (Cruickshank et al., 2001; Page et al., 2002; Johnson et al., 2003).

In contrast to other antibodies used in our experiments,

the K-121 antibody, which recognizes the unique 2,2,7-trimethylguanosine (TMG) cap of small nuclear RNAs, labels irregular foci in the ooplasm as well as in the nucleoplasm and cytoplasm of the follicular cells, in the exact pattern demonstrated in other animal systems (Gall et al., 1995; Tsvetkov et al., 1997; Jaglarz, 2001; Biliński and Kloc, 2002). Unexpectedly, this antibody does not stain the oocyte NBs suggesting the absence of snRNAs in these structures. Alternatively, the TMG cap of snRNAs although present in the NBs may be (1) 'masked' by specific proteins that bind to this cap prior to transport of snRNPs from the cytoplasm into the nucleoplasm or (2) 'masked' as a result of very tight packaging of molecules within the NBs. The latter alternative is supported by exceptionally high electron density of some NB compartments.

Since the precise molecular composition of *H. suis* NBs remains elusive we can only speculate about their function. We suggest that they represent assemblages of ribonucleo-proteins stored in the germinal vesicle throughout advanced oogenesis and utilized during initial stages of embryogenesis. It is worth mentioning here, that similar function has been ascribed to CB in several insect and amphibian systems (Parfenov et al., 1996; Bogolyubov et al., 2000; Jaglarz, 2001; Biliński and Kloc, 2002).

4.2. Nuclear envelope features

Our ultrastructural studies have revealed that the nuclear envelope in the vitellogenic oocytes of *H. suis* is equipped with long, narrow, club-shaped protrusions. Similar projections have been described in the germinal vesicles of the apple blossom weevil, *Anthonomus pomorum* (Świątek, 1999). In the latter species, the protrusions of the germinal vesicle contain nuclear bodies and are likely to take part in the exchange of macromolecules between the nucleus and cytoplasm (Świątek, 1999).

In *H. suis*, the nuclear envelope projections are formed after the translocation of the peripheral dense bodies towards the karyosome, and do not contain the material found within NBs. However, these nuclear outgrowths strikingly resemble the initial stages of the formation of accessory nuclei (AN).

The AN have been demonstrated in the oocytes of several insect groups including hymenopterans, dipterans, mecopterans, and phthirapterans (mallophagans and in some anoplurans) (Ries, 1932; Hopkins, 1964; King and Fordy, 1970; Cassidy and King, 1972; Meyer et al., 1979; Zissler and Sander, 1982; Biliński and Jankowska, 1987; Biliński, 1989; Biliński, 1991a,b; Szklarzewicz et al., 1993; Biliński et al., 1993; Biliński et al., 1995a,b). EM studies revealed that AN arise from the nuclear envelope by budding or folding and are surrounded by an envelope identical to that of the oocyte nucleus (King and Fordy, 1970; Meyer et al., 1979; Biliński, 1989, 1991a). Ultrastructural studies suggest that both microtubules and the nuclear lamina are involved in the formation and/or stabilization of AN (Biliński, 1989,

1991a). In most of the cases, AN contain electron-dense inclusions termed pseudonucleoli (King and Fordy, 1970; Meyer et al., 1979; Biliński et al., 1993).

The localization of AN in insect oocytes differs. In hymenopterans, newly formed AN are transported from the perinuclear cytoplasm, towards the oocyte periphery (Hopkins, 1964; King and Fordy, 1970; Cassidy and King, 1972; Biliński et al., 1993). Their localization in the cortical ooplasm is a microtubule-dependent process (Biliński et al., 1995a). In mallophagans, anoplurans and dipterans they are gathered around the oocyte nucleus and some of them maintain the contact with the nuclear envelope by tapered stems (Meyer et al., 1979; Biliński and Jankowska, 1987; Biliński, 1989).

In both studied phthirapterans, 'canonical' AN are absent, however the nuclear envelope of their germinal vesicles proliferates and generates folds or protrusions. In *C. columbae,* the folds comprise accumulations of fibro-granular, AgNOR-positive material and do not detach from the nucleus throughout the entire course of oogenesis. In *H. suis,* the nuclear protrusions also remain attached to the nuclear envelope but they do not contain any inclusions. In the investigated species, the extensive folding of the nuclear envelope may be associated with the high rate of nucleo-cytoplasmic transport. However, since described protrusions/folds bear striking morphological resemblance to the initial stages of AN formation in *Eomenacanthus stramineus* (Biliński, 1989), we favor the idea that they may represent an early step in the path leading to the origin of AN during evolution of Phthiraptera.

In summary, the germinal vesicles of the studied species are characterized by: (1) the condensation of chromatin into the karyosome early during oogenesis, (2) the presence of numerous morphologically complex NBs, containing RNA and argyrophilic proteins of nucleolar organizer (Ag-NOR proteins), (3) the occurrence of nuclear folds and/or protrusions that resemble initial stages of AN formation.

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