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Ultrastructure and Histochemistry of the Periplasm in Oocytes of Sturgeons during Egg Envelope Formation*

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In the cytoplasm of oocytes (ooplasm) located in ovarian follicles with diameters 2000 μm and 2150 μm in *Acipenser gueldenstaedtii*, and 2000 μm and 2350 μm in *A. baerii*, periplasm containing a basophilic compartment and endoplasm containing reserve materials was formed. Vesicles involved in polyspermy blocking and in the formation of the embryo were located in the periplasm. These included compact (cCGs), low-electron-dense cortical granules (lCGs), and lamellar bodies. The cCGs were bounded by a membrane, comprised fibrillar material, fibrils and rod-shaped components. The lCGs were membrane-bounded and contained fibrillar material and granular inclusions. Endoplasmic reticulum (ER) and Golgi complexes were involved in the formation of cCG and lCG. The basophilic compartment, ER and Golgi vesicles participated in the formation of lamellar bodies. They comprised numerous membranes and fibrillar material. It is assumed that they transfer membranes and their precursors to the growing furrow during cleavage and release their content to organize the extracellular matrix. The location of compounds in the developing egg envelope of *A. gueldenstaedtii* was presented and discussed. Ovaries of both investigated species represented the first pubertal stages of development. Such fish should not be used for reproduction.

Key words: Oogenesis, cortical granules, Acipenseriformes, Chondrostei.

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Oocytes of sturgeons are equipped with cortical granules that function during entry of a spermatozoon into the egg and during subsequent fusion of gametes. In response to fertilisation they exocytose their contents from the oocyte surface to the perivitelline space (GINSBURG 1961; CHERR & CLARK 1985; DETTLAFF *et al*. 1993; PSENICKA *et al*. 2010). In teleost fish they are also termed cortical alveoli or cortical vesicles, and are produced by endoplasmic reticulum (ER) and Golgi complexes (SELMAN & WALLACE 1989; HART 1990; KUNZ 2004). They are membrane-bound organelles containing numerous spherical or irregular inclusions named spherical bodies and nucleoids (IWAMATSU & OHTA 1976; BEGOVAC & WALLACE 1988; MOTTA *et al*. 2005a, b). They fill most of the oocyte cytoplasm (ooplasm) or locate in the peripheries, close to the plasma membrane (oolemma) (IWAMATSU & OHTA 1976; BEGOVAC & WALLACE 1988; SELMAN & WALLACE 1989; HART 1990; TSAO *et al*. 1999; MOTTA *et al*. 2005a, b). The colloid that they release contains a metalloproteinase (alveolin), sialoglycoproteins and high-molecular-mass polysialoglycoproteins (hyosophorins) (HART 1990; KUDO & TESHIMA 1998; SHIBATA *et al*. 2012). In *Cyprinus carpio*, two types of distinctive cortical granules were observed (LINHART *et al*. 1995). In *Carassius auratus gibelio* they contain calcium-dependent, C-type lectins that play a role in polyspermy blocking (DONG *et al*. 2004). In *Thunnus thynnus* they are probably not involved in this process (ABASCAL & MEDINA 2005). In sturgeons, the content of cortical granules holds protein-bound acid mucopolysaccharides and granules were designated as lysosome-like organelles (DETLAFF *et al*. 1993).

Eggs of sturgeons including the investigated species, *Acipenser gueldenstaedtii* and *Acipenser baerii*, are equipped with multiple micropyles lo-

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cated at the animal region that allow entry of numerous spermatozoa (CHERR & CLARK 1982; DEBUS *et al*. 2008; PSENICKA *et al*. 2010; ŻELAZOWSKA 2010). Observation of egg surfaces in *A. transmontanus* and in *A. baerii* during gamete fusion revealed that after incorporation of a spermatozoon, the contents of cortical granules was released into the space between the oocyte and egg envelope. This caused the agglutination of spermatozoa in the micropylar canals, thus preventing polyspermy (CHERR & CLARK 1985; PSENICKA *et al*. 2010). These eggs were deprived of their most external layer of egg envelope, the so-called jelly envelope. In specimens that spawn spontaneously in moving freshwater, the jelly envelope is sometimes washed out by the water current, and it is accepted that the presence of a jelly envelope is not a prerequisite for fertilisation (CHERR & CLARK 1985). In oocytes of the investigated species, the layer of egg envelope located most externally to the oolemma slightly thickens. In *A. baerii* this layer is called the adhesive layer or jelly coat (DEBUS *et al*. 2008; PSENICKA *et al*. 2010). In *A. gueldenstaedtii* this layer is referred to as the extrachorion (ŻELAZOWSKA 2010). It is assumed that these layers of egg envelope are necessary not only to anchor the eggs to the river bottom, but also to increase mobility of spermatozoa.

The aim of this study was to determine the structure and composition of the peripheral ooplasm during formation of egg envelopes in oocytes of *A. gueldenstaedtii* and *A. baerii*. The peripheral ooplasm is referred to as the periplasm, and was not separated by a distinct cytoskeletal barrier from the endoplasm that contained large yolk platelets and lipid droplets. The periplasm comprised cortical granules, free ribosomes and numerous vesicles (ER and Golgi, vitelline envelope precursors, lamellar bodies, granules of pigment, yolk precursors). A distinct basophilic compartment (irregularly-shaped zones devoid of organelles, surrounded by vesicles of Golgi and ER) was also discerned in the periplasm. In *A. baerii* oocytes, a region of greater periplasm thickness was distinguished. In this region an aggregation of mitochondria immersed in a high-electron-dense granular material was located. In developing embryos of *A. baerii*, this aggregation of mitochondria was discerned as the mitochondrial cloud and nuage (SAITO *et al*. 2014). Near the oolemma, in both investigated species, filaments organized in bundles and oriented parallel to the surface of oocytes were located. They separated the oocyte cortex from the periplasm. In cortex only cortical granules and coated vesicles occurred. These granules were previously incorrectly referred to as yolk platelets (ŻELAZOWSKA 2010).

Examination of the process of oogenesis and embryo development in old fish lineages such as sturgeons are useful for the resolution of controversies over classification and relationships, and also for drawing conclusions on phylogeny (PSENICKA *et al*. 2010; SAITO *et al*. 2014). Natural populations of the investigated species have declined but specimens are reared in aquaculture. Information on gonadal development and puberty of cultured fish is incomplete and further investigations are necessary (HURVITZ *et al*. 2007). This information can be used to develop methods that allow to determine ovarian developmental stages with minimal damage or stress to cultured fish and to improve commercial farming.

Materials and Methods

Ovarian follicles were isolated from ovaries dissected from two specimens of *A. gueldenstaedtii* (sexually mature, body length 1.5 m) and two specimens of *A. baerii* (sexually mature, body length 1.5 m). Specimens were fed nutritive fodder with low content of lipids and proteins and with natural food. They were raised in artificial ponds near Kraków in the south of Poland.

Light (LM) and transmission electron microscopy (TEM)

Samples were fixed in ice-cold 2.5% glutaraldehyde (Sigma, U.S.A.) in 0.1 M phosphate buffer (pH 7.4). After fixation for 4 hours, they were rinsed and post-fixed in 1% osmium tetroxide in the same buffer containing saccharose, dehydrated in a graded series of ethanol and acetone and embedded in epoxy resin Agar 100 (Agar, U.K.). Semithin sections (0.7 μm) were stained with 1% methylene blue in 1% borax and photographed under a Jenalumar (Carl Zeiss, Germany) light microscope. Ultrathin sections (90 nm) were contrasted with uranyl acetate and lead citrate and examined in a JEOL JEM 100SX (JEOL, Tokyo, Japan) transmission electron microscope at 80 kV.

Histochemical and fluorescence analyses on semithin sections

Samples were fixed in 4% formaldehyde in phosphate-buffered saline (PBS) for 4 hours, rinsed with PBS, dehydrated in a graded series of ethanol, then infiltrated and embedded in acrylic resin (Histocryl, Agar). Oocytes were examined using at least 18-24 sections for each staining. Treatment of semithin sections (0.7 μm) with 2% periodic acid for 30 min. and staining with Janus green B revealed that all ovarian follicles were alive until tissue preparation. Janus green B adsorbed and-or formed insoluble complexes with proteins (LAZAROW & COOPERSTEIN 1953). Some

sections were stained with 1% bromophenol blue on a hotplate to detect structural proteins, and with solution containing fuchsin, 1 N hydrochloric acid and potassium pyrosulphite at room temperature for 60 min. to detect neutral polysaccharides. To detect lipids that were not extracted during the embedding procedure, sections were stained in a solution containing fuchsin, 1 N hydrochloric acid and potassium pyrosulphite, rinsed and then tinted with 1:1 mixture of 1% Lyon blue in 99.8% ethanol and 1% acetic acid. Detected lipids were grey. Glycoproteins were analyzed by staining in 0.1% amido black 10B in a 1:7:2 mixture containing glacial acetic acid, methanol and water. Acidic mucopolysaccharides were analyzed by staining on a hotplate in 0.1% alcian blue (Sigma) in water acidified with 1% acetic acid. Glycogen was analysed by staining in carmine Besta. Calcium was detected by staining in 0.1% alizarin S for 60 min, followed by rinsing with water and staining with 1% methylene blue in 1% borax. The detected calcium was purple.

Some semithin sections were subjected to silver staining in a 1:2 mixture of 1% gelatin (Sigma) in 1% formic acid and 50% silver nitrate (Sigma) according to a procedure described in BILIŃSKI & BILIŃSKA (1996). Some sections were stained with a mixture of 1% azure II in water, with 1% methylene blue in 1% borax and with 1% toluidine blue in water for examination by light microscopy.

Nucleic acids (DNA and RNA) were detected in sections stained with DAPI (Sigma) for 30 min in the dark and with propidium iodide (Sigma) for 50 min. Lectins were detected by incubation of sections for 25 min at room temperature in a moist chamber in the dark in a solution of stock fluorescein isothiocyanate (FITC)-labeled concanavalin A (ConA) from *Canavalia ensiformis* (Sigma) diluted in 0.3 M methyl-α-D-mannopyranoside (Sigma) in PBS. Stock solution of ConA contained 2 mg of ConA dissolved in 2 ml 0.9% NaCl. Non-specific staining was not blocked (haemoglobin was not added). Dark (unstained) areas represented organelles and zones in the periplasm that were inhibited by methyl-α-D-mannopyranoside from a solution (comprised lectins). In sections incubated in a solution of stock of ConA-FITC in PBS without addition of methyl-α-D-mannopyranoside FITC-labeled ConA was localized at residues of D-mannose. Images were acquired in a Leica DMR (Germany) light and epifluorescence (FLM) microscope equipped with appropriate filters. Contrast of some sections was enhanced by Nomarski differential interference.

Results

In fragments of ovary of *A. gueldenstaedtii* two consecutive stages of ovarian follicles containing oocytes surrounded by an egg envelope were distinguished according to diameters. These were: follicles with a diameter of 2000 μm (stage 5 of egg envelope formation according to ŻELAZOWSKA 2010) (Fig. 1A) and those with a diameter of 2150 μm (stage 6 of egg envelope formation, not shown). In fragments of ovary of *A. baerii* ovarian follicles with diameters of 2000 μm and 2350 μm were observed (not shown). Each ovarian follicle consisted of a spherical, late vitellogenic oocyte surrounded by follicular cells situated on a basal lamina (Figs 2E, J & 3A-C). The ovarian follicles were enclosed in a layer of thecal cells. The egg envelope consisted of a vitelline envelope composed of four sublayers (a filamentous layer most proximal to the oocyte, a trabecular layer t2, a homogeneous layer and a trabecular layer t1), the chorion and the extrachorion nearest to the apical surfaces of follicular cells (i.e. located most externally to the oolemma) (Figs 1A, 2A-J & 3A-D).

A thin layer of periplasm and an extensive and abundant endoplasm was distinguished in the ooplasm (Figs 1A-D, 2A-I & 3A-D). Periplasm was located near the oolemma and contained cortical granules, lamellar bodies, granules of pigment, vesicles of rough ER (rER), mitochondria, and free ribosomes (Figs 1A-D, 2A-I & 3A-D). Periplasm was devoid of large yolk platelets (Figs 1A-D, 2A-I & 3A-D). No glycogen granules were detected in the endoplasm or periplasm (not shown).

Two types of cortical granules were distinguished: compact (cCGs) and low-electron-dense (lCGs) (Fig. 1A, B, D). Medium and high electron dense granules surrounded by a membrane represented cCGs. They were ellipsoid or irregular (Fig. 1B, D). Their content formed rod-shaped components (Fig. 1B, arrows, D) and a fibrillar material attached to numerous regularly-arranged fibrils (Fig. 1D). In the initial stages of growth of cCGs most fibrils were loosely packed in the granule (Fig. 1D, arrow), while the number of rod-shaped components and the degree of compaction of fibrils increased in the fully grown granules (Fig. 1B). Initial stage and fully grown cCGs were present in the ooplasm in the stages examined. Initial stage cCGs were always in close proximity to Golgi complexes (not shown). Fully grown cCGs were distributed randomly (Fig. 1B). Spherical or oval vesicles represented lCGs (Fig. 1B). They were surrounded by a membrane and contained coarse granular inclusions and irregularly arranged fibrillar material (Fig. 1B). They arose by fusion of rER and Golgi vesicles in the periplasm (Fig. 1B). Some lCGs fused with lamellar bodies. Lamellar bodies contained several concentrically arranged membranes and fibrillar material identical to that in cCGs, lCGs and rER (Fig. 1A, B).

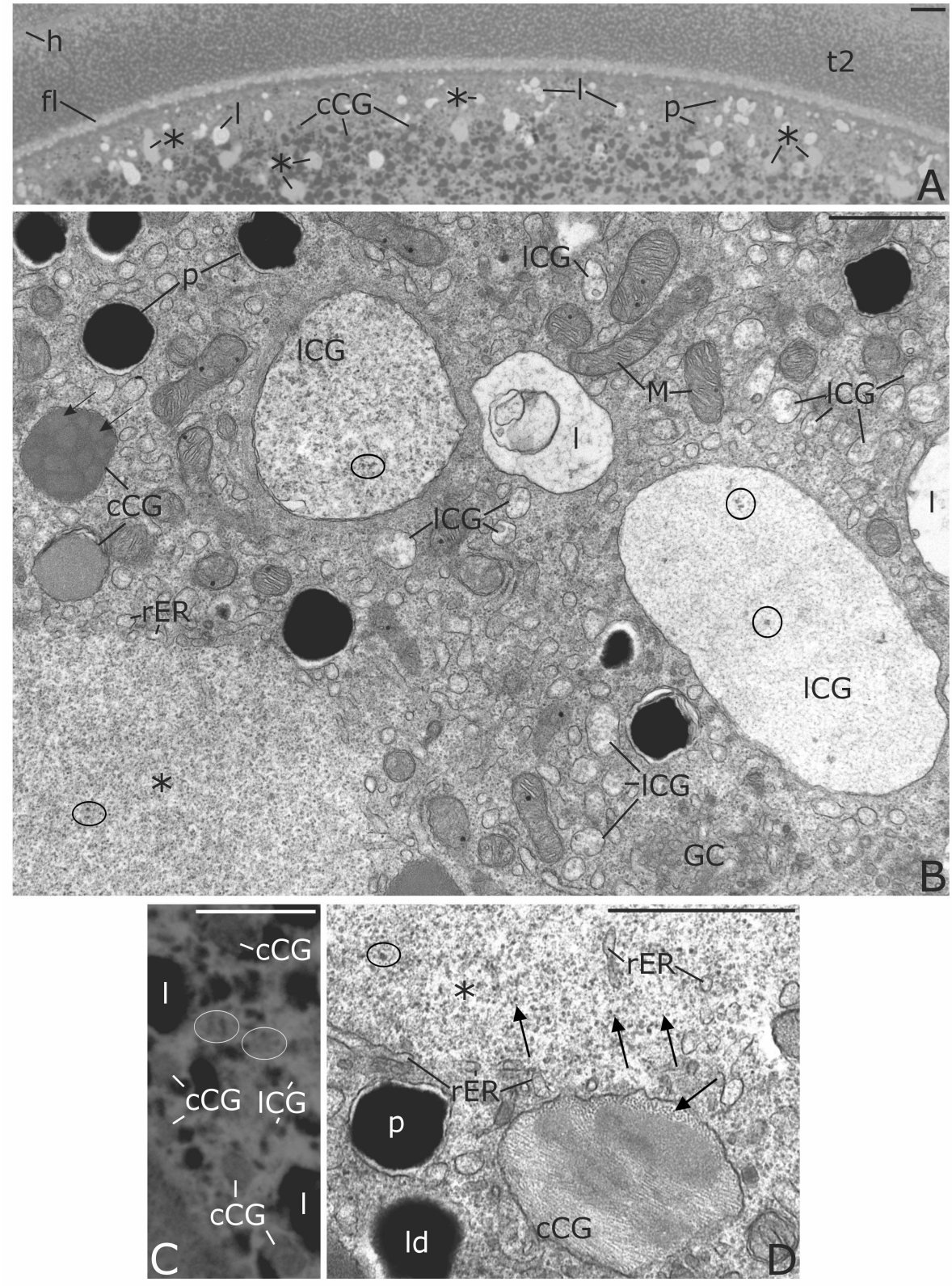

Fig. 1. *A. gueldenstaedtii*. Periplasm of oocytes at stage 5 of egg envelope formation. cCG – compact cortical granules, lCG – low-electron-dense cortical granules containing fibrillar material, black oval lines – granular inclusions. (A) Tangential section, * – basophilic compartment. LM. (B) Arrows indicate cross-sectioned rod-shaped components of fully grown cCG. TEM. (C) Lectins gave no fluorescence in cCGs, lCGs, l and in basophilic compartment (encircled with white oval lines). FLM. (D) Fragment of basophilic compartment and initial-stage cCG, arrows indicates cross sectioned fibrils. Note the presence of fibrillar material in cCG and in the basophilic compartment. TEM. Bars (A), (C) = 1000 μm (B), (D) = 1 μm. Abbreviations (refer also to Figs 2 & 3): fl – filamentous layer in the vitelline envelope, t1– trabecular layer t1, h – homogeneous layer, t2 – trabecular layer t2, ch – chorion, ex – extrachorion, l – lamellar bodies containing fibrillar material and membranes, GC – Golgi complex, rER – rough ER, M – mitochondria, ld – lipid droplets, p – pigment granules, Y – yolk platelets, FC – follicular cells.

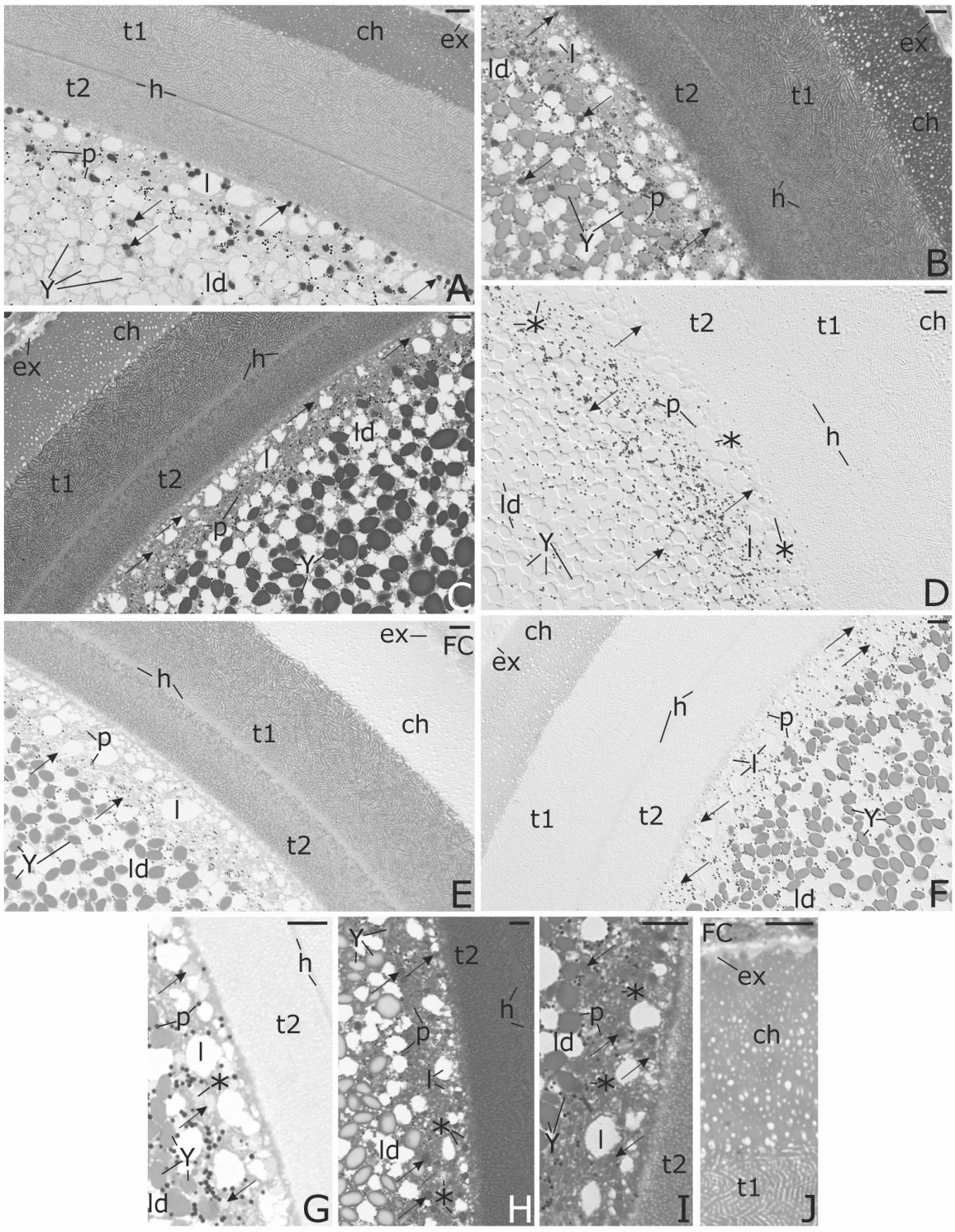

Fig. 2. *A. gueldenstaedtii*. Oocytes at stage 5 of egg envelope formation, arrows indicates cCG, * – basophilic compartment of the periplasm. (A) Janus green B – insoluble complexes with proteins. (B) Polysaccharides. (C) Glycoproteins. (D) Acidic mucopolysaccharides in cCGs and in the basophilic compartment. (E) Structural proteins in the t1 and t2 of the vitelline envelope. (F) Silver impregnation to pigment granules. Acidic proteins in yolk platelets and in the chorion. (G) Toluidine blue staining. (H) Lipids (in membranes) and polysaccharides in cCGs. (I)-(J) Calcium in cCGs, in the basophilic compartment and in ER, in the chorion and in the extrachorion. LM. Bars = 1000 μm.

Periplasm contained a basophilic compartment (asterisks in Figs 1A-B, D & 2D, G-I; white oval lines in Fig. 1C) comprising irregularly arranged fibrillar material (Fig. 1B, D), as well as fibrils morphologically identical to those in the cCGs (arrows in Fig. 1D) and granules identical to those in lCGs (Fig. 1B, D, encircled). Some rER and Golgi vesicles disposed of their contents in the baso-

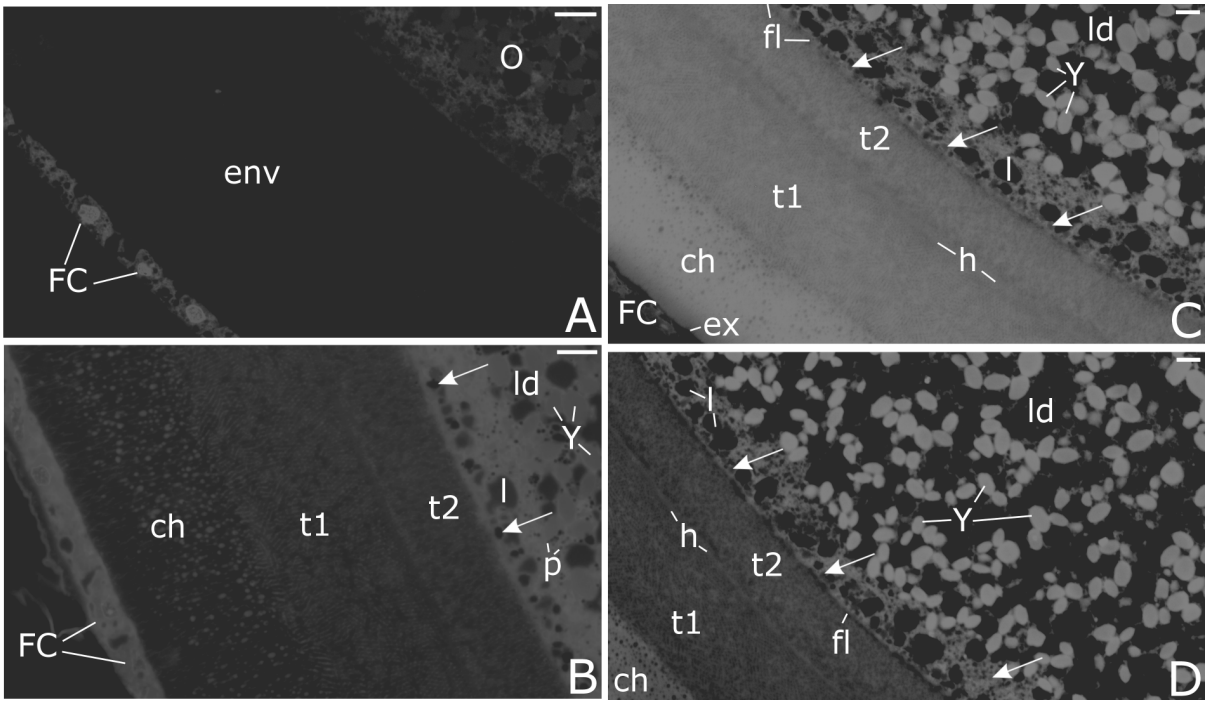

Fig. 3. *A. gueldenstaedtii.* Ovarian follicles at stage 5 of egg envelope formation, arrows indicate cCG. (A) DNA in the mitochondria gave bright fluorescence, O – oocyte, env – egg envelope. (B) DNA in mitochondria and RNA in free ribosomes gave bright fluorescence. Fluorescence in the egg envelope came from free ribosomes in the oocyte microvilli and processes of FCs. (C) D-mannose residues gave bright fluorescence. (D) Lectins, ld – lipid droplets were washed out during fixation. FLM. Bars = 1000 μm.

philic compartment, where it was subsequently enclosed by fusing membranes from these emptied vesicles (Fig. 1B, D). These membranes formed a barrier between the basophilic compartment and the periplasm. Some rER and Golgi vesicles were located within this compartment (Fig. 1D).

Granules of pigment in the periplasm were surrounded by a membrane and composed of an electron-dense material (Fig. 1B, D). In the most voluminous oocytes, all pigment granules were porous and composed of a core and dense cortex (not shown).

The endoplasm occupied the central region of the oocyte and contained reserve materials such as yolk platelets and lipid droplets (Figs 2A-I & 3B-D) and numerous organelles (mitochondria – Fig. 3A-B, free ribosomes – Fig. 3B, granules of pigment, rER, Golgi complexes – not shown). In the external region of the endoplasm, pigment granules and cCGs (Fig. 2A-I), as well as oval or irregular compartments that were the same as the basophilic compartment in the periplasm and contained acidic mucopolysaccharides were present (asterisk with two lines in Fig. 2D).

The rER vesicles comprised proteins and calcium-protein compound (Fig. 2C, G, I). The cCGs contained polysaccharides (Fig. 2B, H), glycoproteins (Fig. 2C) and calcium compound (Fig. 2I). Acidic mucopolysaccharides were located in cCGs, lCGs and in the basophilic compartment (Figs 1B, D & 2D). Lectins were present in cCGs (Fig. 1C & Fig. 3D, arrows) and in the basophilic compartment (white oval lines in Fig. 1C) as well as in the lCGs (Fig. 1C). They were represented by fibrillar material observed in ultrathin sections (Fig. 1B, D). Fibrillar material was also observed in lCGs (Fig. 1B). Incubation of sections with ConA showed the absence of D-mannose in cCGs, lCGs and in the basophilic compartment (Fig. 3C). Mitochondria and free ribosomes were detected in the periplasm (Fig. 1B, D & Fig. 3A, B).

Histochemistry of the developing egg envelope in *A. gueldenstaedtii*

All layers of the vitelline envelope were composed of structural proteins (Fig. 2E) and comprised glycoproteins (Fig. 2C), residues of D-mannose (Fig. 3C) and lectins (Fig. 3D). The chorion and extrachorion were comprised of neutral polysaccharides (Fig. 2B), glycoproteins (Fig. 2C), calcium (Fig. 2J) and residues of D-mannose (Fig. 3C). Proteins in the chorion and extrachorion stained with Janus green B (Fig. 2A) and impregnated with silver nitrate (Fig. 2F). Lectins were not present in chorion and extrachorion (Fig. 3D).

Discussion

Periplasm and centrally located endoplasm were distinguished during the formation of the egg envelope in the oocytes of the investigated species. Periplasm contained cortical granules (cCG and lCG), lamellar bodies and a basophilic compartment. These present results support previous observations in *A. baerii* showing that the content of cortical granules was released from oocytes by exocytosis during fertilisation and prevented the access of supernumerary spermatozoa to the ooplasm (PSENICKA *et al*. 2010). This is most probably due to the action of lectins released from cCGs. Calcium, crucial during fusion of a spermatozoon with an egg, is stored in ER and in cortical granules. Added to water, calcium fosters motility of spermatozoa, the acrosomal reaction and hence promotes fertilisation in sturgeons (CHERR & CLARK 1985; PSENICKA *et al*. 2010). During fertilisation it is released from vesicles of ER and induces the cortical reaction (HART 1990; KINSEY *et al*. 2007). The function of cCGs in sturgeons is probably not limited to blocking spermatozoa. TEM observation of sections and incubation with ConA indicated that they are derivatives of ER and Golgi complexes, and should be considered rather as secretory vesicles than strictly lysosome-like organelles. Some lysosome-like properties may be ascertained to the basophilic compartment of the periplasm. It was surrounded by vesicles of Golgi and ER that sometimes fused and formed a double membrane.

Cortical granules in teleost fish are composed of glycoproteins (hyosophorins) with properties similar to those of acidic mucopolysaccharides (SELMAN & WALLACE 1989; TSAO *et al*. 1999; SHIBATA *et al*. 2012). These glycoproteins bind calcium and form reservoirs of calcium located near the surface of the oocyte (TSAO *et al*. 1999). During fertilisation, hyosophorins in the perivitelline space are cleaved by a protease that coexists in cortical granules to form low-molecular-weight polypeptides. This results in the release of calcium, and the attraction of water by osmosis (HART 1990; TSAO *et al*. 1999; SHIBATA *et al*. 2012). Cortical granules also contain a glycosylated protein involved in the hardening of the egg envelope that takes place during fertilisation. This protein is an oocyte-specific proenzyme, the latent pro-alveolin (SHIBATA *et al*. 2012). During fertilisation, this inactive pro-form is released and activated by the removal of the pro-domain by a processing serine protease, which coexists in the cortical granules in a dormant form. Once activated, alveolin (a member of the astacin proteinase family) is released into the perivitelline space where it hydrolyzes glycoproteins in the egg envelope and allows their cross-linkage, with subsequent hardening of the egg envelope (MODIG *et al*. 2007; SHIBATA *et al*. 2012). The sialoglycoprotein of *Tribolodon hakonensis* exhibited serine protease activity and together with calcium was necessary to assemble vitelline envelope components into the fertilisation envelope *in vitro*. The assembly was analogous to blood coagulation (KUDO & TESHIMA 1998). In *Oncorhynchus mykiss*, a sialyltransferase that catalyses sialylation of polysialoglycoprotein expressed a developmental profile similar to that of STL2, a lectin, and was quite different from that of polysialoglycoprotein which was expressed during the yolk vesicle stage and later was down-regulated. The mRNA for this enzyme was expressed throughout oogenesis, down-regulated at late yolk vesicle stage and then up-regulated during vitellogenesis (ASAHINA *et al*. 2004). It is commonly accepted that endogenous lectins probably participate in transport of polysaccharides and/or polysaccharide components of glycoproteins from Golgi complexes (NOSEK 1984; HART 1990; DONG *et al*. 2004). These lectins are C-type, which are homologs of the type II antifreeze proteins that inhibit ice-crystal growth in cells of fish that can survive low temperatures (DONG *et al*. 2004). They have carbohydrate-recognition domains that bind D-mannose, and a calcium-binding site. They strongly agglutinated rabbit erythrocytes and are inhibited by D-mannose. The agglutination activity of lectins increased with increasing calcium concentration. Released from cortical granules during fertilisation, they are dephosphorylated and return to the original state during early development of the embryos (DONG *et al*. 2004).

It is assumed that in the investigated species lectins enclosed in the lCGs were transported to lamellar bodies where they might persist until cleavage of a fertilised egg. The common explanation for the presence of lamellar bodies in teleost fish oocytes was atresia or artifactual formation occurring during fixation (MIRANDA *et al*. 1999; SANTOS *et al*. 2008). In first-cleavage zygotes of the frog *Xenopus laevis*, three types of these bodies were discerned (SINGAL 1975). The first type is derived from Golgi complexes and is involved in the synthesis of plasma membrane during cleavage. These bodies were ascribed a role of transferring membrane precursors to the growing cleavage furrow (SINGAL 1975). The myelin-like figures and membrane whorls (second and third types of lamellar bodies in *X. laevis* cleaving zygotes according to SINGAL 1975) were observed in the oocytes of sturgeon. They were present only in previtellogenic oocytes, not during egg envelope formation (unpublished). In oocytes of the neotenic cave salamander *Proteus anguinus anguinus* they were involved in the formation of nascent endoplasmic membranes (BIZJAK MALI & BULOG 2010).

TEM observation of lamellar bodies revealed membranes and a fibrillar material identical to that observed in the lCGs. This supports the conclusion that lamellar bodies could play a role after fertilisation. Most probably, the fibrillar material contained lectins that, together with membranes, are transported in these bodies to the cleavage furrow. They are externalized and organize the extracellular matrix in developing embryos. In blastulae of *X. laevis*, lectins were found in extracellular matrix (ROBERSON & BARRONDES 1983).

Glycoproteins in the egg envelope of the teleost *Oryzias latipes* facilitated sperm guidance into the micropyle (IWAMATSU *et al*. 1997). A similar role can be ascribed to glycoproteins in the chorion and extrachorion in the egg envelope of *A. gueldenstaedtii*. The eggs of the sturgeon *A. transmontanus* required calcium to release jelly coats (CHERR & CLARK 1985). It is possible that in *A. gueldenstaedtii*, extracellular calcium stored in the chorion is necessary during the release of this most external investment of the egg. Results of this study confirmed the association of lectins with the vitelline envelope before fertilisation in lower vertebrates (ROBERSON & BARRONDES 1983).

Examination of ovaries in *A. gueldenstaedtii* and *A.baerii* showed that they represented the first pubertal stages of development. Most oocytes in gonads were in previtellogenic stages of growth and only a few ovarian follicles formed egg envelopes and gathered yolk (ŻELAZOWSKA *et al*. 2007; ŻELAZOWSKA & KILARSKI 2009; ŻELAZOWSKA 2010). Such fish should not be used for reproduction.

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