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Polarized distribution of binding sites for concanavalin A and wheat-germ agglutinin in the zona pellucida of goodeid oocytes (teleostei)*

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Summary. Zonae pellucidae of the viviparous goodeid teleosts *Girardinichthys viviparus, Xenoophorus captivus,* and *Xenotoca eiseni* were investigated ultrastructurally, and binding sites for ConA and WGA were localized on cross-sections using a colloidal gold technique. In late stages of development, the oocytes are surrounded by a three-zonated acellular matrix multiply perforated by pore canals allowing long microvilli of the oocyte to penetrate interstices of the follicle epithelium. Together, the surface of the microvilli and zona pellucida is coated by a thin layer of homogeneous slightly electron-dense material. In early oogenesis, the thin acellular layer is entirely packed with binding sites for WGA, whereas those for ConA occur only sparsely. Three-zonated zonae pellucidae amply contain both WGA and ConA receptors. The asymmetric labelling pattern obtained with both lectin protein gold preparations indicates a polarized organization of the different glycoconjugates. WGA receptors are concentrated within the outer region of the zona pellucida. Labelling with ConA-HRP-Au complexes produced heavy deposits of marker beads within the inner two thirds of the zona pellucida and weak labelling of the superficial coat. After prolonged digestion with neuraminidase, WGA binding sites were no longer detectable.

Introduction

Teleost eggs, like secondary mammalian oocytes, are surrounded by highly specialized extracellular matrices. For piscine vitelline envelopes the term chorion is most widely used, among several other synonyms; yet, in accordance with recent literature, we will refer to it as the zona pellucida in order to avoid further confusion and to underline apparent morphological and refractive characteristics common to these structures in a great variety of organisms.

At first glance, reproductive patterns of viviparous teleosts and mammals seem to be completely unrelated. Anatomical dissimilarities are especially due to the absence of a uterus and implantation in fishes. Moreover, fertilization in goodeid teleosts is intrafollicular, but soon followed by evacuation of the zygotes or early cleavage stages into a preformed ovarian cavity where 'hatching' occurs and gestation continues to term in the absence of all egg envelopes (Mendoza 1940). Despite apparent differences in structure and sequence of reproductive events, it can, however, be noted that in both mammals and goodeids the span of time during which the zona pellucida is functionally active in relating the oocyte or developing embryo to its exterior environment covers a period including the late stages of oogenesis and postovulatory accommodation of the embryo within the female genital tract. Developmental correspondence at that stage should imply further analogies in the structural and functional versatility of the acellular egg envelopes in mammals and certain viviparous teleosts, despite being evolutionary highly disparate creatures.

Several zona functions documented in mammals such as spermatozoon recognition (Jones et al. 1988), induction of the zona reaction (Barros and Yanagimachi 1972), sperm receptor activity (Florman and Wassarman 1985), induction of the acrosome reaction (Bleil and Wassarman 1983), maintenance of the structural integrity of the matrix (Dunbar and Bundman 1987), and antigenicity (Shivers 1975) have been associated with the presence and distribution of various glycoproteins. Present knowledge about probable asymmetry in the organization of such compounds in zonae pellucidae derives mainly from binding studies of lectins with carbohydrate moieties in zona glycoconjugates (Nicolson et al. 1975; Dunbar et al. 1980; Ahuja and Bolwell 1983). The present study was designed to localize binding sites for concanavalin A (ConA) and wheat-germ agglutinin (WGA), two plant lectins commonly present in extracellular matrices, in three representatives of a family of viviparous teleosts undergoing gestation within the ovarian cavity.

Materials and methods

Ovaries of *Girardinichthys viviparus, Xenoophorus captivus,* and *Xenotoca eiseni* were fixed by immersion of 2–3 mm pieces in 2% paraformaldehyde + 2.5% glutaraldehyde in 0.1 *M* cacodylate buffer (pH 7.4) supplemented with 0.05% $CaCl_2$ for 2 h, and material for ultrastructural investigations was postfixed in 1% OsO_4 for 1 h. Embedding was either in a mixture of Epon and Araldite resins for ultrastructural morphology or in Lowicryl K4M (Lowi-Werke) for lectin histochemistry as described in Altman et al. (1984). In samples submitted to the latter procedure, accessible aldehyde groups were blocked after fixation by incubation in 0.1 *M* cacodylate buffer containing 0.1 *M* NH_4Cl. The colloidal gold suspension (mean particle diameter = 20 nm) was prepared following the method of Frens (1973). Coupling of horseradish peroxidase (HRP) (type VI, Sigma) and ovomucoid (type III, Sigma) was done as described in Geoghean and Ackerman (1977).

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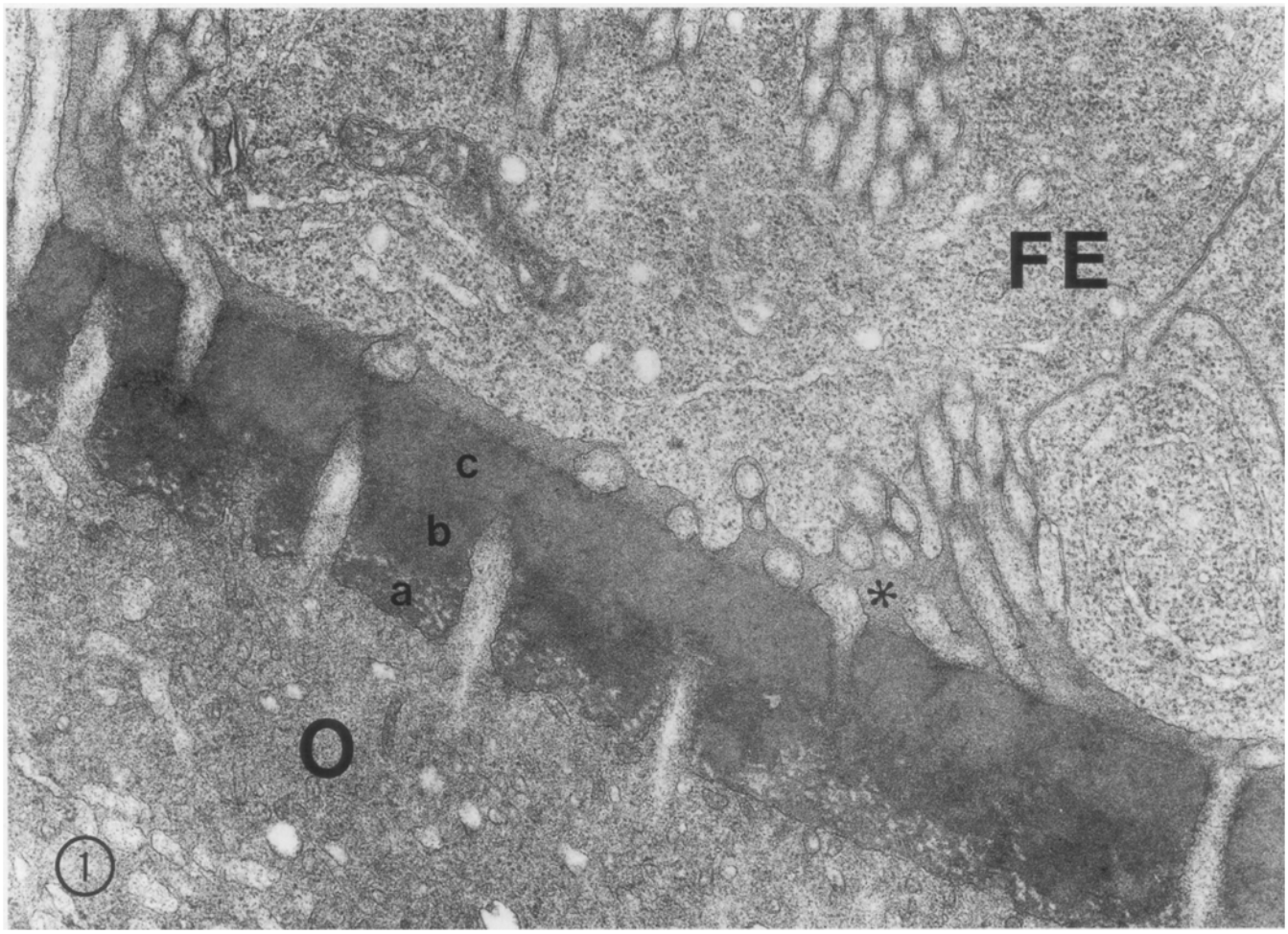


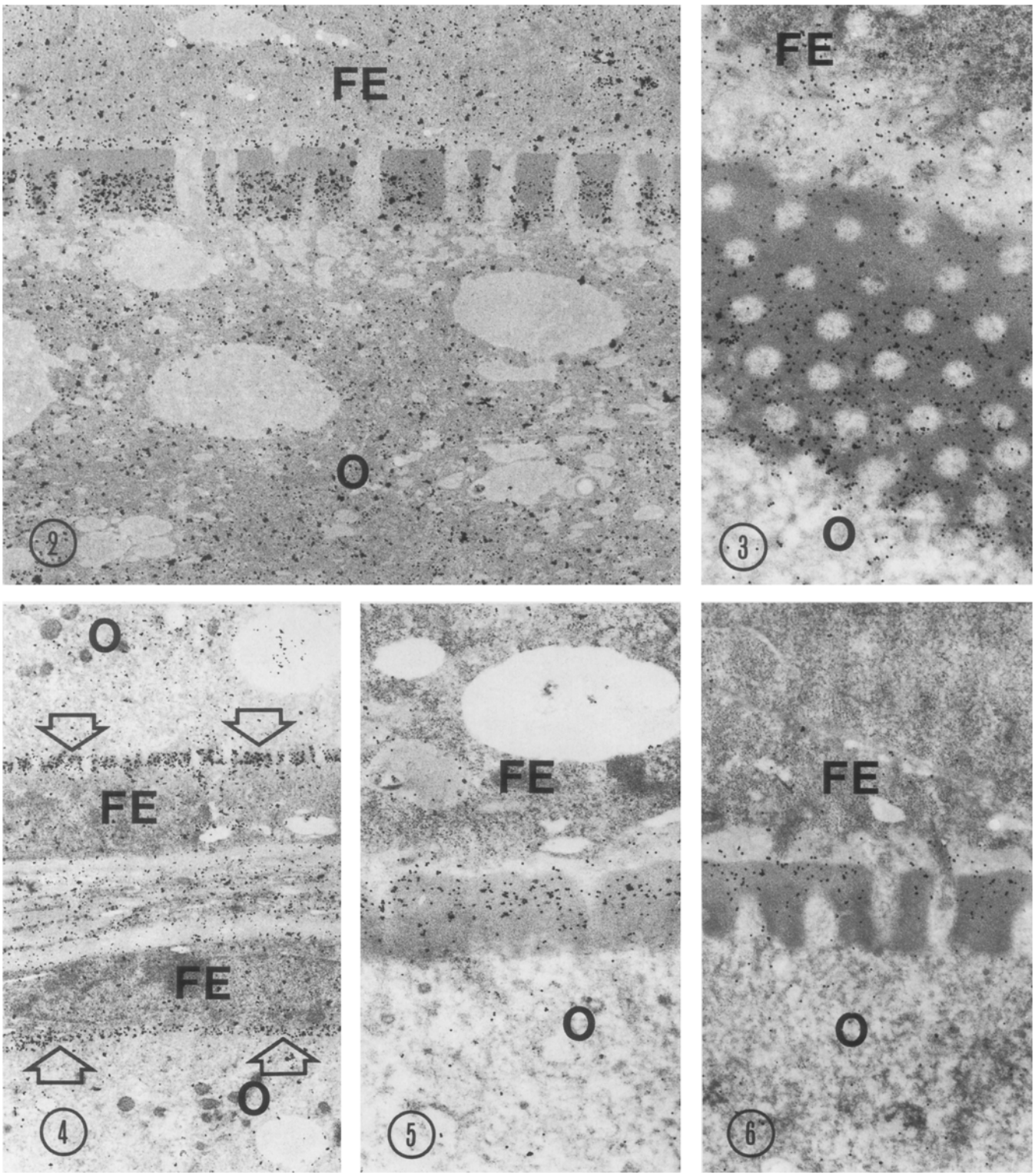
Fig. 1. Zona pellucida in *Xenoophorus captivus*. It is composed of three zonulae (*a–c*) and contains pore canals through which pass microvilli of the oocyte (*O*). An external coat (indicated by the *asterisk*) separates the follicle epithelium (*FE*) from the surface of the microvilli and the zona pellucida. ×28800

Ultrathin sections were cut with glass knives and placed on nickel grids. The sections were incubated at 20° C with either 0.1 mg/ml ConA (Sigma) or 0.1 mg/ml WGA in phosphate-buffered saline (PBS) for 10 min, rinsed with PBS (3 × 30 s) and then floated for 15 min on drops containing HRP-Au and Ovo-Au, respectively. After washing, the sections were stained with uranyl acetate. Specificity of binding was tested with 0.2 *M* methyl-α-D-mannopyranoside (Sigma) for ConA and with 0.2 *M* *N*-acetyl-D-glucosamine (Sigma) for WGA. Several sections were incubated with 0.5 *U* neuraminidase (type V, Sigma)/ml 0.05 *M* PBS in a wet chamber at 37° C for 16.5 h. Afterwards, the sections were washed in PBS and incubated first with WGA and then with Ovo-Au. In control preparations the enzyme was omitted.

Results

The ultrastructural and histochemical data noted in this study were obtained from experiments involving the germinal tissue of ovaries in the goodeid teleosts *Girardinichthys viviparus, Xenoophorus captivus,* and *Xenotoca eiseni.* Goodeid ovaries normally contain follicles in all stages of development. As the cells become slightly columnar during maturation the height of the simple follicle epithelium increases. A micropyle, characteristic of oviparous teleosts, was not observed and species-specific variabilities of ultrastructure or histochemical reactivity worth mentioning have not been ascertained.

At an early stage of development, the squamous follicle epithelium in goodeid ovaries is closely apposed to the oocyte. During maturation, as irregular ooplasmic projections grow out, an interstitial gap opens between the oolemma and the plasmalemmas of follicle cells. Gradually, long microvilli form on the oocyte and become covered with an extracellular coat; further acellular material is deposited between the bases of the microvilli. Toward the end of oogenesis, the zonae pellucidae of goodeid ova reach 1–1.5 μm in thickness, showing a tripartite structure due to concentric zonulae (Fig. 1) that differ in their width, texture, and stainability. It is multiply perforated by pore canals allowing oocyte microvilli to penetrate the follicle epithelium. The oolemma borders on the bottom layer (*a* in Fig. 1) of the extracellular matrix without a perivitelline space, and electron micrographs provide ample evidence of vesicular transport of macromolecules on the oocyte surface. The apposed cytoplasm of the follicle epithelium appears exocytotically quiescent. The loosely aggregated material in *a* becomes the compacted electron-opaque substance of zonula *b*. The transition to the less electron-dense top layer (*c* in Fig. 1) is not well defined, but the outer demarcation of the zona



Figs. 2–6. Portions of oocytes (*O*), follicle epithelium (*FE*), and zonae pellucidae of follicles in *Xenoophorus captivus* (**Fig. 2**), *Xenotoca eiseni* (**Figs. 3–5**), and *Girardinichthys viviparus* (**Fig. 6**)

Fig. 2. binding of ConA is densest on the lower two thirds of the mature zona pellucida. ×10800

Fig. 3. Oblique section through the zona pellucida showing densely spaced pore canals. ConA binding sites predominate within the innter regions of the zona pellucida. ×21600

Fig. 4. Portions of two follicles at an early stage of development. WGA binding sites (indicated by the *arrows*) cover the entire zonae pellucidae. ×9185

Figs. 5 and 6. WGA binding sites within the outer regions of a zona pellucida. **Fig. 5** ×18380, **Fig. 6** ×18750

pellucida is clear-cut. A moderately electron-dense, homogeneous coat covers the exterior of the whole structure including the microvilli of the oocyte.

Thin immature zonae pellucidae reacted weakly to tests for ConA receptors, whereas the broader ones in follicles of advanced maturity bound high quantities of this lectin, which revealed a markedly polarized distribution (Figs. 2 and 3). The labelling pattern was characterized by dense binding of marker beads to the inner two thirds of the extracellular matrix roughly corresponding to zonulae *a* and *b*, whereas the outer region *c* of the zona pellucida was virtually unlabelled. Receptors for WGA could be localized on zonae pellucidae of goodeid eggs at all stages of development. Initially, the marker beads were uniformly distributed (Fig. 4) but with increasing thickness of the matrix, WGA binding sites were shifted into a near-surface position predominantly occupying zonula *c* in mature follicles (Figs. 5 and 6). The external coat on the zonae pellucidae and the oocyte microvilli was moderately labelled in response to both ConA-HRP-Au and WGA-Ovo-Au procedures. Lectin-protein-gold labelling was specific, since inhibitory saccharides effectively blocked labelling. Treatment of ultrathin sections with neuraminidase resulted in negative responses to subsequent tests for WGA-binding sites on zonae pellucidae. Control preparations without the enzyme, however, reacted positively.

Discussion

Zonae pellucidae in viviparous goodeids are moderately thick and lack the complexity of zonation found in most oviparous teleosts (Götting 1967; Manner et al. 1977; Dumont and Brummett 1980). Nevertheless, the hyaline homogeneous structure observed by light microscopy can ultrastructurally be resolved into layers of different compactness and electron density. Heterogeneity in its physicochemical composition is indicated by the lectin-binding procedures, which reveal asymmetry in the distribution of carbohydrate moieties, but at the same time show evidence that the entire zona pellucida in goodeid ovarian follicles contains glycoconjugates. This is not generally observed in teleosts, since histochemical tests have demonstrated that the zona material is highly variable and polysaccharides may be missing either totally or in parts (reviewed in Guraya 1986). Zonae pellucidae of mammals are matrices primarily of glycoprotein but often include considerable amounts of acid and neutral glycosaminoglycans (Dietl 1986a, b; Guraya 1985).

Asymmetry in the chemical structure of mammalian zonae pellucidae has previously been documented by applying lectin histochemistry to intact eggs or isolated zonae (Nicolson et al. 1975). In the present study, the method used involves incubations of ultrathin tissue sections and thus avoids the disadvantages due to penetration hindrances. The results show that unlike the patterns noted in some rodents, ConA binding sites in goodeid zonae pellucidae are not randomly distributed but concentrated within the deeper regions of the matrix. Receptors for WGA are usually localized to the outer surface of zonae pellucidae (Nicolson et al. 1975), which is also reflected in the finding that the presence of this lectin can block sperm binding to the zona (Oikawa et al. 1973, 1974). Other data indicate that anionic groups are located within the outermost layers, investing the whole structure with an acidic and strongly hydrated sphere (Yanagimachi et al. 1973; Dietl et al. 1983; Shimizu and Yamada 1986). Dietl and co-workers (1983) analysed zona glycoproteins and identified the spectrum of carbohydrate residues. These included neuraminic acid; moreover, Shimizu and Yamada (1986) using the digestion technique with neuraminidase showed that sialic acids account for the acidic nature of the zona surface. The WGA-binding sites in zonae pellucidae of goodeid oocytes are mostly located within the exterior regions and are susceptible to neuraminidase digestion. It seems likely, therefore, that interrelational stages between oocyte and spermatozoon in the course of both mammalian postovulatory and teleost preovulatory fertilization involve surface phenomena such as cell-to-cell recognition and binding, which function on a similar physicochemical basis.

The question concerning the origin of the zona material has not yet been solved conclusively, although the present opinion is that it is produced and secreted by the oocyte itself (Haddad and Nagai 1977; Tesoriero 1977; Bleil and Wassarman 1980; Dietl 1986a, b). The present study provides some circumstantial evidence in support of this view. Firstly, morphological observations show that the oocyte surface is active in exocytotic and/or endocytotic processes, whereas the follicle cells are not. Secondly, the thin zona pellucida at an early stage of development strongly binds WGA, but its affinity to ConA is much less pronounced. The latter reactivity increases at the inner portions of the zona as it broadens, apparently due to deposition of additional material from within.

References

Ahuja KK, Bolwell GP (1983) Probable asymmetry in the organization of components of hamster zona pellucida. J Reprod Fertil 69:49–55

Altman LG, Schneider BG, Papermaster DS (1984) Rapid embedding of tissues in Lowicryl K4M for immunoelectron microscopy. J Histochem Cytochem 32:1217–1223

Barros C, Yanagimachi R (1972) Polyspermy preventing mechanisms in the golden hamster egg. J Exp Zool 180:251–266

Bleil JD, Wassarman PM (1980) Synthesis of zona pellucida proteins by denuded and follicle-enclosed mouse oocytes during culture in vitro. Proc Natl Acad Sci USA 77:1029–1033

Bleil JD, Wassarman PM (1983) Sperm-egg interactions in the mouse: sequence of events and induction of the acrosome reaction by a zona pellucida glycoprotein. Dev Biol 95:317–324

Dietl J (1986a) Struktur und Funktion der Zona pellucida. F Enke, Stuttgart

Dietl J (1986b) Die Zona pellucida des Säugetiereies: Eine außergewöhnliche extrazelluläre Matrix. Naturwissenschaften 73:89–94

Dietl J, Czuppon A, Mettler L (1983) Characterization of porcine zona pellucida antigens by immunoaffinity chromatography and by high-pressure liquid chromatography. Am J Reprod Immunol 4:116–121

Dumont JM, Brummett AR (1980) The vitelline envelope, chorion, and micropyle of *Fundulus heteroclitus* eggs. Gamete Res 3:25–44

Dunbar BS, Bundman DS (1987) Evidence for a role of the major glycoprotein in the structural maintenance of the pig zona pellucida. J Reprod Fertil 81:363–376

Dunbar BS, Wardrip NJ, Hedrick JC (1980) Isolation, physicochemical properties, and macromolecular composition of zona pellucida from porcine oocytes. Biochemistry 19:356–365

Florman HM, Wassarman PM (1985) O-linked oligosaccharides of mouse egg ZP3 account for its sperm receptor activity. Cell 41:313–324

Frens G (1973) Controlled nucleation for the regulation of the

particle size in monodisperse gold suspensions. Nature (Phys Sci) 241:20–22

Geoghean WD, Ackerman GA (1977) Adsorption of horseradish peroxidase, ovomucoid and anti-immunoglobulin to colloidal gold for the indirect detection of concanavalin A, wheat germ agglutinin and goat anti-human immunoglobulin G on cell surfaces at the electron microscopic level. J Histochem Cytochem 25:1187–1200

Götting K-J (1967) Der Follikel und die peripheren Strukturen der Oocyten der Teleostier und Amphibien. Eine vergleichende Betrachtung auf der Grundlage elektronenmikroskopischer Untersuchungen. Z Zellforsch 79:481–491

Guraya SS (1985) Biology of ovarian follicles in mammals. Springer, Berlin Heidelberg New York

Guraya SS (1986) The cell and molecular biology of fish oogenesis. In: Sauer HW (ed) Monographs in developmental biology, vol 18. S Karger, Basel New York

Haddad A, Nagai MET (1977) Radioautographic study of glycoprotein biosynthesis and renewal in the ovarian follicles of mice and the origin of the zona pellucida. Cell Tissue Res 177:347–369

Jones R, Brown CR, Lancaster RT (1988) Carbohydrate-binding properties of boar sperm proacrosin and assessment of its role in sperm-egg recognition and adhesion during fertilization. Development 102:781–792

Manner HW, Vancura M, Muehleman C (1977) The ultrastructure of the chorion of the fathead minnow, *Pimephales promelas*. Trans Am Fish Soc 106:110–114

Mendoza G (1940) The reproductive cycle of the viviparous teleost, *Neotoca bilineata,* a member of the family Goodeidae. II. The cyclic changes in the ovarian soma during gestation. Biol Bull 78:349–365

Nicolson GL, Yanagimachi R, Yanagimachi H (1975) Ultrastructural localization of lectin-binding sites on the zonae pellucidae and plasma membranes of mammalian eggs. J Cell Biol 66:263–274

Oikawa T, Yanagimachi R, Nicolson GL (1973) Wheat germ agglutinin blocks mammalian fertilization. Nature 241:256–259

Oikawa T, Nicolson GL, Yanagimachi R (1974) Inhibition of hamster fertilization by phytoagglutinins. Exp Cell Res 83:239–246

Shimizu S, Yamada K (1986) The cytochemistry of glycoconjugates in the zona pellucida of murine ovarian oocytes and two-cell embryos. Histochem J 18:357–363

Shivers CA (1975) Immunological interference with fertilization. Acta Endocrinol (Suppl) 194:223–244

Tesoriero JV (1977) Formation of the chorion (zona pellucida) in the teleost, *Oryzias latipes*. I. Morphology of early oogenesis. J Ultrastruct Res 59:282–291

Yanagimachi R, Nicolson GL, Noda YD, Fujimoto M (1973) Electron microscopic observations of the distribution of acidic anionic residues on hamster spermatozoa and eggs before and during fertilization. J Ultrastruct Res 43:344–353