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PHYSIOLOGICAL AND ULTRASTRUCTURAL ASPECTS
OF LATE VITELLOGENESIS AND OVULATION
IN PACIFIC HERRING (Clupea harengus pallasii)

by

Daniel J. Gillis

BSc. University of British Columbia, 1967

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

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of

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Physiological and ultrastructural aspects of late vitellogenesis

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Physiological and ultrastructural aspects of late vitellogenesis and ovulation in Pacific herring (Clupea harengus pallasii)

Abstract

Three stages of Pacific herring oocytes, late vitellogenic, ovulating and ovulated, were examined histologically and histochemically. Late vitellogenesis was documented by means of alkali-labile protein phosphorus (ALPP) assay of serum and liver tissue in males and females, and gonadal tissue in females. ALPP levels both in male and female livers remained constant over the study period but there were significant differences in values between males and females. ALPP values for male serum were constant, but those for females declined. Female gonad values declined (110 mg/l to 15 mg/l) during ovulation. Hepatosomatic indices for males were constant during the ovulation period, but those for females declined (1.6% to 0.9%).

Pacific herring eggs underwent two hydrations. During ovulation, Moisture values increased from 67% to 76% moisture. The second occurred following fertilization and during water-hardening with a further increase to 83% moisture. Unfertilized eggs dehydrated to 74% moisture when exposed to hydration water of the same salinity used for fertilized eggs.

PAS-staining localized glycogen in the vitellogenic egg cytoplasm and demonstrated the distribution of mucosaccharides in all stages. The thin adhesive epilayer found on the egg surface in ovulating and subsequent stages was PAS-negative, suggesting that this adhesive layer is mostly proteinaceous. Alkaline phosphatase was localized in follicular granulosa and thecal cells, but not in oocyte structures, suggesting that the former tissues constitute the locations of vitellogenin dephosphorylation and possibly cleavage into phosvitin and lipovitellin.

SEM and TEM studies demonstrated that egg membranes consist of five layers in late vitellogenic and transitional stages. These layers are the externus, distal interlayer,

internus, proximal interlayer and the sub-internus. The proximal and distal interlayers do not appear to have been described elsewhere. A thin, electron-dense layer was deposited on the external surface of the externus during ovulation.

A mechanical means of achieving egg membrane semipermeability during the first hydration by means of compression of the pores in the internus was suggested by TEM results. Both male and female liver tissues contained electron-dense and electron-lucent hepatocytes, however the latter were scarce in male livers but constituted over half of the female liver tissue. Male and female hepatocytes contained secretory vacuoles. In ovulating females, secretory vacuoles were larger and more abundant, and were associated with Golgi structures.

Dedication

This thesis is dedicated to my wife, Miki S. Maeba and children, Mieko, Kimi and John whose understanding and encouragement have made this work possible.

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Introduction

The processes of vitellogenesis and ovulation have been investigated and described for several teleosts. Much of the emphasis has been placed on economically important groups such as the salmonids, (e.g., Fluge, 1964a, 1964b; Hurley and Fisher, 1966; Beams and Kessel, 1973; Hara and Hiri, 1978; Nagahama et al., 1978; Stehr and Hawkes, 1979; Campbell and Idler, 1980; van Boheman et al., 1981; van Boheman et al., 1982; Weigand and Idler, 1984; Groot and Alderdice, 1985), and on species that are easily maintained under laboratory conditions, such as the killifish, Fundulus heteroclitus (e.g., Anderson, 1968; Kuchnow and Scott, 1977; Dumont and Brummet, 1980; Wallace and Selman, 1980; Selman and Wallace, 1983), the goldfish, Carassius auratus (e.g., Khoo, 1975; Nagahama et al., 1976; Khoo, 1979; Nagahama et al., 1983; Kagawa et al., 1984); the catfish, Heteropneustes fossilis (e.g., Nath and Sundararaj, 1981; Sundararaj and Nath, 1981), the Medaka, Oryzias latipes (e.g., Tesoriero, 1977a, 1977b; Iwamatsu, 1980; Iwamatsu and Ohta, 1981) and for several other species. In view of the ecological importance of clupeids to trophic dynamics in coastal ecosystems (Hourston and Haeghele, 1980), their phylogenetic position and long-standing economic importance (Whitehead, 1985), and recent efforts at domestication and artificial propagation (Morita, 1985), it is remarkable that vitellogenesis and ovulation have not been more thoroughly investigated for this group of teleosts. Among the difficulties associated with investigations of clupeids have been the cost associated with sampling marine populations and the difficulty of maintaining clupeids for long periods under laboratory conditions. Recently developed technology has facilitated marine impoundment of Pacific herring (Clupea harengus pallasii) (Gillis, 1979; Brett and Solmie, 1982; Gillis et al., 1982; Kreiberg et al., 1982). Herring impoundments permit reliable time-series sampling from discrete populations of sexually maturing herring.

The present study examines the process of late oocyte maturation and ovulation in Pacific herring. Oocyte provisioning, or exogenous vitellogenesis, maturation and ovulation

synchronization and ultrastructural changes have been examined.

DISTRIBUTION AND LIFE HISTORY

Pacific herring populations are distributed along the continental shelves and coastal waters from the north coast of Mexico, northward to the Beaufort sea, thence southward along the Asian and Korean coasts. They are also distributed along the Arctic coasts of Asia and North America (Blaxter, 1985; Hay, 1985). Their centre of abundance along the eastern Pacific rim lies along the British Columbia coast from Puget Sound in the south to Dixon Entrance in the north (Hourston and Haegele, 1980).

Adult Pacific herring feed in the summer months in shelf waters off the west coast of Vancouver Island and Hecate Strait. A few populations remain in larger inland bodies of water such as Georgia Strait and coastal fjords. Between October and December, herring form into large aggregations and begin their shoreward migrations to their near-shore spawning grounds. Upon reaching the vicinity of their spawning grounds, breeding populations inhabit deep channels. During the daylight hours they rise to the surface to feed on zooplankton populations at dusk (Blaxter, 1985). Feeding gradually slows and eventually stops prior to spawning. Following gamete maturation in over-wintering areas, Pacific herring enter the shallows where they spawn on marine macrophytes. Spawning is a mass event with all members of a spawning population depositing their gametes in from one to several days (Hourston and Haegele, 1980). Gamete maturation is synchronous among experienced spawners but secondary and tertiary spawning waves, separated by 10 to 15 days, may occur. Herring involved in the secondary waves are smaller, and presumably younger (Hay, 1985).

OOCYTE PROVISIONING

In most teleosts, the ovary is a hollow paired organ consisting of oogonia, oocytes and their surrounding follicle cells, supporting tissue or stroma and vascular and nervous tissue (Nagahama, 1983). Wallace and Selman (1981) summarize three ovarian types

classified according to the patterns of oocyte development. In fish with synchronous ovaries, all of the oocytes, once formed, mature and ovulate in unison. Synchronous ovaries are found in species that spawn once and then die, such as the anadromous Oncorhynchus species. Most teleosts have group-synchronous ovaries which contain at least two populations of oocytes at some time in their reproductive cycle. A fairly synchronous population of maturing oocytes (defined as a clutch) and a more heterogenous population of smaller oocytes from which the clutch is recruited are observable in such ovaries. Pacific herring belong to this group. In fish with asynchronous ovaries, oocytes in all stages of development are present at the same time.

During early development, oogonia proliferate by means of mitotic division and become primary oocytes when the chromosomes enter meiotic division. Division stops at the diplotene stage of the first meiotic prophase (Wallace and Selman, 1981). The most obvious feature of the remaining development is the accumulation of yolk material within the oocyte. The first yolk constituents to appear are the yolk vesicles fated to become the cortical alveoli. Anderson (1968) demonstrated the association of vesicle formation and the internal synthetic structures of the oocyte and concluded that glycoprotein synthesis and vesicle formation were endogenous (within the oocyte). Lipid accumulation commences after the start of yolk vesicle formation. Its appearance can be considered to mark the commencement of endogenous vitellogenesis (Shackley and King, 1977; Weigand, 1982). The yolk globules which ultimately expand to become the most abundant storage product in most teleost eggs form concurrently with, but after the start of the formation of yolk vesicles. Their endogenous origin from the fusion of numerous small coated vesicles which first appear peripherally in the ooplasm has been confirmed by several ultrastructural studies (e.g., Droller and Roth, 1966; Anderson, 1968; Shackley and King, 1977). Even though yolk globule precursors are synthesized endogenously, it is unclear if appreciable endogenous vitellogenesis occurs. Immediately after the formation of

precursor yolk globules, exogenous vitellogenesis commences (Wallace and Selman, 1981; Ng and Idler, 1983).

Vitellogenin is a female specific lipophosphoprotein. Its occurrence in the blood plasma coincides with the appearance of yolk globules in the oocyte (Aida et al, 1973, cited in Ng and Idler, 1983). Vitellogenin is comprised of a lipid-containing fraction, lipovitellin and a phosphorylated protein fraction, phosvitin. Mano and Lipman (1966a) observed that there are several forms of phosvitin which differ in their amount of phosphorylation as well as in other ways. At the start of exogenous vitellogenesis, non-phosphorylated phosvitin is incorporated by the oocytes. The degree of phosphorylation increases as vitellogenesis proceeds. The highest phosphorus levels are observed in the latter stages of development prior to ovulation (Mano, 1969, 1970). The hormonal control of hepatic vitellogenesis has recently been elucidated by several authors (e.g., Wallace and Selman, 1981; Kagawa et al, 1982; Nagahama et al, 1982; Nagahama, 1983).

In the present study, oocyte provisioning in the later stages of oogenesis was examined by means of alkali-labile protein phosphorus assay of female serum, gonad and liver. Serum and liver tissues of males served as controls. The site of re-manufacture of vitellogenin into phosvitin and lipovitellin was investigated by means of alkaline phosphatase localization. Mucosaccharide and glycogen localization within the developing oocyte were investigated by means of PAS staining.

Late development and ovulation synchronization are apparent in the results of Brett and Solmie (1982) and Kreiberg et al, (1982). They measured temporal changes in gonadosomatic indices for Pacific herring impounded under a variety of stocking densities and other factors. In the present study, gonadosomatic and hepatosomatic indices, and egg moisture content have been measured to assess the degree of maturation and ovulation synchronization.

HEPATIC AND OVARIAN ULTRASTRUCTURAL CHANGES DURING LATE VITELLOGENESIS AND OVULATION

Ultrastructural changes in female hepatocytes related to vitellogenesis have been described by Peute *et al.*, (1978) for the zebrafish, Brachydanio rerio, and by van Bohemen *et al.*, (1981) for rainbow trout, Salmo gairdneri. Ultrastructural changes associated with vitellogenesis for both species included the proliferation of rough endoplasmic reticulum, the enlargement of Golgi bodies and the development of electron-dense Golgi inclusions. For the zebrafish, electron-dense and electron-lucent hepatocytes were identified. Electron-lucent hepatocytes contained less dense cytoplasm and the rough endoplasmic reticulum appeared to consist of dilated saccules. In addition, there was a proliferation of nuclear pores in zebrafish hepatocytes. For both species, glycogen and lipid accumulation accompanied the onset of vitellogenesis, but these gradually disappeared, and were absent in hepatocytes of late vitellogenic females. Following ovulation and spawning in both species, hepatocyte structure regressed to pre-vitellogenic configurations. In the present study, hepatocytes sampled from late vitellogenic, ovulating and fully ovulated female livers are compared. In addition, female hepatocytes for each period are compared with those sampled from males during the same period.

Several authors have examined ultrastructural changes in oocytes at various stages of development. The structure and development of the egg membrane have been investigated in salmonids by Flugel, (1964a); Hurley and Fisher, (1966); Beams and Kressel, (1973); Stehr and Hawkes, (1979); and by Groot and Alderdice, (1985). Other authors have examined yolk formation in the guppy, Lipistes reticulatus, (Droller and Roth, 1966); a variety of oocyte developmental changes in Fundulus heteroclitus, (Anderson, 1968; Kuchnow and Scott, 1977; Dumont and Brummet, 1980; Wallace and Selman, 1980); and in other teleosts (e.g., Anderson, 1967; Livni, 1971; Wourms, 1976; Nagahama *et al.*, 1976; Flegler, 1977; Shackley and King, 1977; Tessoriero, 1977a; Hart and Yu, 1980; Iwamatsu

and Ohta, 1981). In the present study, ultrastructural changes in oocytes from late vitellogenic, ovulating and ovulated ovaries were examined by scanning and transmission electron microscopy. In addition, fertilized water-hardened eggs were examined by transmission electron microscopy.

Materials and Methods

FISH CAPTURE AND IMPOUNDMENT

Pacific herring were captured by purse-seine in the Gulf Islands south of Nanaimo, British Columbia in January and early February of 1984, and transported by tank barge to the Pacific Biological Station experimental fish farm in Departure Bay near Nanaimo. Several fishing and transfer events were required to secure about 50 tonnes of fish in a single master impoundment. Sub-samples were taken in 3 hours from the master impoundment on February 9, 1984, and transferred to two 3 meter cubic impoundments. To make the transfers, the web of the master impoundment was pulled from the water, concentrating the fish near the surface. Fish were randomly dip-netted in groups of ten to 50 fish and placed in the head of a sluice containing flowing water. This process was continued until approximately 1,500 fish had been sluiced to each of the small impoundments. Each small impoundment was covered with a protective net to prevent bird predation. A sample was taken from the impoundments on the following day to test sampling routines. The fish were allowed to recover for a period of one week prior to the commencement of sampling. Consistent with the behaviour of wild unimpounded fish, the impounded fish were not fed throughout the sampling period.

SAMPLING ROUTINE

Sampling was conducted on February 16 and 23; March 1, 5, 8, 9, 10, 12, 13, 14, 15, 16, 19, 22 and 29; and April 5 and 12. For each sampling day, the following routine was observed. The impoundment web was raised, concentrating the fish near the surface. Fish were dipped from the impoundment at random and placed in 25 l plastic pails containing sea water of the same salinity and temperature as that found in the impoundment. Sampling continued until 40 to 60 fish had been placed in each of two pails. The pails were then taken to laboratory facilities provided at the Pacific Biological Station. All fish were lethargic on arrival at the lab after the ten minute trip due to anoxic conditions during

transport. The fish in one pail were revived using compressed air. The unrevived pail of fish was emptied into a large sink where fish under 100 g, and injured or diseased fish were removed from the sample. Ten male and female fish were taken randomly from the remaining fish. For late vitellogenic and transitional fish, sex was determined by making a small incision in the lateral body wall and examining the gonads directly. Sex for mature fish was determined by the nature of the gametes spontaneously extruding from the urogenital papilla. The fish in the final sample were numbered from 1 to 10 for each sex for identification in subsequent steps. Following standard length measurements, excess slime and moisture were damp-towelled from each fish prior to weighing. Each fish was then thoroughly dried and the blood collected by amputation of the tail followed by draining the blood from the caudal vessels into 1.5 ml disposable polypropylene centrifuge vials. Blood serum was separated by centrifugation and stored on dry ice. Gonads were dissected from each fish and weighed. Ovarian sub-samples were taken from each female from the same position in each ovary and stored on dry ice. Livers for both males and females were carefully dissected, taking care to excise all of the tissue. Livers, including gall bladders, were weighed for each fish and stored on dry ice. All tissue samples except serum were taken in duplicate. One to two gram ovarian samples were taken from each female in triplicate and placed in pre-weighed plastic weighing boats and re-weighed for subsequent moisture determination.

For 5 sampling days starting on March 19, eggs were fertilized and water-hardened for four hours in sea water. Eggs were also exposed to sea water for four hours without fertilization. Percent moisture content for fertilized water-hardened and unfertilized water-hardened eggs were determined by the same method used for ovarian samples.

Ovarian samples were fixed for enzyme histochemistry in 80% ethanol. Routine perfusion fixation for scanning and transmission electron microscopy is presented under "Scanning Electron Microscopy", below.

ALKALI-LABILE PHOSPHORUS ASSAY

Species specific radioimmunoassay methods have been developed (eg. Idler *et al.*, 1979; Opresko and Wiley, 1984) for vitellogenin, however, the validity of using direct alkali-labile phosphorus determination in estimating female-specific phosphoprotein (vitellogenin) has been verified by Emersen and Petersen (1976) and more recently by Tinsley (1985). The method of direct determination of alkali-labile phosphorus was used in the present study because of the ease with which it could be modified to accommodate the processing of large numbers of samples of different tissues over a relatively short period of time. The method adopted was modified from those cited by Martin and Dotty (1949) and Wallace and Jared (1967).

Samples of liver or gonad tissue (0.2 to 0.3 g) were weighed into 150 by 15 mm culture tubes and homogenized in sufficient glass distilled water to bring the volume to 5 ml. Serum samples (0.05 to 0.25 ml) were similarly diluted to 5 ml. Five ml of cold (1 to 3°C) 10% trichloroacetic acid (TCA) was added. The tubes were vortexed and the mixture was held at 4°C overnight. The mixture was incubated in a water bath at 90°C for 30 min, centrifuged at 3000 g for 10 min, and decanted, retaining the precipitate in the original tube. The precipitate was vortexed with 10 ml of ethanol, held at 90°C for 5 min, centrifuged at 3000 g, and the supernate decanted, followed by the same treatment with mixed solvents (ethanol : ether : acetone : chloroform, 2 : 2 : 2 : 1) without heating. The precipitate was then dissolved and the alkali-labile phosphorus liberated by incubation at 100°C for 15 min in 1 ml of 2N NaOH. The solution was cooled in an ice bath and neutralized by adding 1 ml of 2N HCl. Five ml of an equal volume mixture of isobutyl alcohol and toluene was added, followed by 2 ml of a freshly prepared equal volume mixture of molybdate and silico-tungstate (Martin and Dotty, 1949) reagents. Alkali-labile phosphorus was extracted by vigorously inverting each tube 15 times followed by centrifugation at 3000 g for 5 minutes to separate aqueous and organic phases.

The remainder of this spectrophotometric determination was conducted according to the method of Martin and Dotty (1949). Prepared standards ranged from 1.1 to 36 mg/l phosphorus. Standards and reagent blanks were run with each set of samples. The method was tested on composite tissue samples from Pacific herring and rainbow trout prior to its application to study samples.

EGG MOISTURE DETERMINATION

Pre-weighed ovarian, fertilized water-hardened, and unfertilized water-hardened samples were dried in a glass dessicator under vacuum over Drierite (anhydrous calcium sulfate) overnight. Dried samples were weighed and the percent moisture determined. Hydration water salinities were determined by argentometric methodology (Standard Methods, 1975).

ALKALINE PHOSPHATASE LOCALIZATION

Eighty percent ethanol-fixed ovarian samples were ethanol-dehydrated then infiltrated and embedded in paraffin (56°C) within 24 hours of collection, and stored at 4°C. Trimmed tissue blocks were soaked in distilled water overnight to facilitate 10 μ m sectioning. A modified Gomori method was used as follows. Sections were deparaffinized in chloroform and hydrated in graded ethanol solutions followed by a 45 min incubation at 37°C in a solution consisting of 30 ml of solution A, (1.2 g CaCl_2 ; 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 6.1 g hydroxymethylaminomethane; distilled water to make 1 l) and 50 ml of solution B (1% solution of sodium beta-glycerophosphate) with the pH adjusted to 9.8 with 0.1N HCl. The sections were then washed in distilled water; 2% cobalt nitrate for 5 min; washed in distilled water; 2% yellow ammonium sulfide for 2 min; washed in distilled water; counterstained with eosin; dehydrated in graded ethanols, cleared in xylene and mounted. Control sections were dry-heated to 100°C prior to processing to destroy enzyme activity.

CARBOHYDRATE AND MUCOSACCHARIDE LOCALIZATION

Fixation and sectioning was achieved as for alkaline phosphatase localization. Deparaffinized sections were treated with alcoholic periodic acid (1 g periodic acid

dissolved in 100 ml of 90% ethanol) for 2 hours followed by a 5 min wash in 90% ethanol and a quick hydration to distilled water. Sections were then treated with Schiff's reagent for 10 min. Sections were transferred through three 1-min changes of sulfite solution (5 ml 10% $K_2S_2O_5$, 5 ml 1N HCl, water to make 100 ml), washed in running water for 5 min, and counterstained in fast green (1% fast green in 2% acetic acid, diluted 1:10 with distilled water for use), dehydrated in graded ethanols, cleared and mounted. Control sections were treated with fresh human salivary amylase. One control and one experimental slide were not counterstained for comparison purposes. Sections for both alkaline phosphatase and PAS methods were viewed and photographed using a Carl Zeiss Photomicroscope. Exposures were made on Kodak 35 mm type 5060 Panatomic-X film developed in Kodak HC-110 developer. Color exposures were made on Kodak type 5018 Ektachrome film with E-6 processing.

SCANNING ELECTRON MICROSCOPY

Tissue fixation for both transmission and scanning microscopy was achieved by perfusion fixation according to the method of Hinton (1975) modified as follows to accommodate Pacific herring. The osmolarity of herring plasma was measured and the results used to adjust the osmolarity of the perfusate. The perfusate solution was prepared by dissolving 4.28 g sodium cacodylate and 25 ml of 8% glutaraldehyde to a total volume of 95 ml in distilled water. The pH was adjusted to 7.4 with 0.1N HCl. Distilled water was added to bring the total volume to 100 ml. The osmolarity was adjusted to 440 mOs with NaCl (about 0.25 g).

Fish to be perfused were taken from the revived pail of fish and killed by a series of blows to the head without rupturing the vascular system, especially the gill tissue. The fish were placed in supine positions in V-shaped grooves carved in styrofoam blocks. The styrofoam supports provided convenient pinning substrate during subsequent operations. The perfusion apparatus consisted of a 50 ml reservoir connected to 1.5 m of polyethylene

tubing (1.22 mm O.D. and 0.76 mm I.D.) heat flared at the free end. The reservoir was suspended 1 metre above the fish.

The heart and ventral aorta were exposed by an incision in the mid-ventral body wall. a moistened white cotton thread was passed beneath the bulbus and a loose thumb knot applied. The flared free end of the cannula was inserted through an incision in the ventricle, into the bulbus. The cannula was secured in the bulbus with the cotton thread. Perfusion was started immediately and continued for 10 to 15 min. After perfusion had ceased, the sutures and cannula were removed from the fish. Yellowish coloration appearing on the white ventral body surface indicated successful perfusion. Perfused fish were wrapped in wet paper towelling and allowed to fix for an additional hour.

For scanning electron microscopy, ovarian samples were dissected from the fish and stored in the perfusate fixative for 30 min to 1 hour. Samples were then rinsed in 2 changes for 10 min each of rinsing buffer consisting of 2.14 g sodium cacodylate dissolved in 90 ml of distilled water with the pH adjusted to 7.4 with 0.1N HCl and the osmolarity adjusted to 440 mOs with NaCl (about 1.6 g). The rinsing buffer was replaced with OsO_4 fixative for 1 hour. Part A of the fixative consisted of 1 g of OsO_4 dissolved in 50 ml of distilled water while part B consisted of 8.56 g sodium cacodylate dissolved in 90 ml of water followed by pH adjustment to 7.4, addition of 2 g of sucrose and dilution to 100 ml with distilled water. One part of each solution was mixed to produce the final 1% OsO_4 secondary fixative. Following fixation, tissues were rinsed in 2 changes of rinsing buffer, dehydrated in graded ethanols and stored in stoppered vials in 100% ethanol.

Ovarian samples were dried by replacing the 100% ethanol with amyl acetate in a graded series and drying the samples in a critical point apparatus which uses CO_2 replacement of amyl acetate. Mounted eggs and ovarian tissue were gold coated prior to viewing in an ETEC Autoscan scanning electron microscope with the accelerating potential set to 20 kV. Exposures were made on Ilford FP4 120 rollfilm, developed in Kodak HC110 developer and

printed on Ilford variable contrast paper.

TRANSMISSION ELECTRON MICROSCOPY

Sample tissues were dissected from perfused fish and all except ovarian tissues were minced under perfusate fixative then rinsed in buffer, post-fixed in OsO_4 , rinsed again and dehydrated in graded ethanols. For soft tissues the ethanol was replaced with propylene oxide in 3 changes of 100% and infiltrated for 1 hour each in 20:1, 10:1, 5:1 and 1:1 mixtures of propylene oxide in Spurr (1969) medium hardness epoxy embedding resin. Tissues were allowed to infiltrate overnight in the 1:1 mixture with the stopper removed from the vial to permit evaporation of the propylene oxide. Infiltrated tissues were oriented in fresh medium and cured at 70°C overnight in a vacuum oven with a very low vacuum applied. Kidney, optic nerve, retina, white and dark skeletal muscle, intestine, liver and ovarian tissues were thus processed to verify the quality of preservation of this vascular perfusion technique. Liver tissues from both males and females, and ovarian tissues were subsequently taken as experimental tissues.

Cured and trimmed blocks were 'silver' sectioned (65 to 85 nm) with glass knives, stained with alkaline lead citrate stain formulated as follows. Lead citrate (1 g) was suspended in distilled water to make 100 ml. To make the final stain, 1 ml of 1N NaOH and 9 ml of water was combined with 10 ml of the suspension and mixed in a CO_2 free environment. Sections were stained for 10 min followed by rinsing in distilled water and viewing in a Phillips EM 300 transmission electron microscope with the accelerating potential set to 80 kV. Eggs from ovarian tissue were treated identically except that the time in each infiltration was extended to 1 day and Spurr's hard formulation was used. Photographic exposures were made on Kodak type 4489 electron microscope film, developed in Kodak D-19 developer and printed on Ilford variable contrast paper.

CALCULATION AND STATISTICS

Computer programs were written to facilitate the calculation of alkali-labile phosphorus,

egg moistures and gonadosomatic and hepatosomatic indices. Correlation, ANOVA and Student's-t test programs were written and applied to various data. Conventional algorithms were used in statistical programs (Scalzo and Hughes, 1975; Coulombe, 1983). Arcsine transformation was applied to ratio data prior to its processing in statistical tests (Zar, 1974). Statistical significance was accepted at the highly significant (99%) level.

Results

FISHING AND IMPOUNDMENT

Mortality related to the transfer operation was estimated at 5% based on a count of dead fish removed from the impoundments one week after transfer. Most of the initial mortalities suffered predator or net damage prior to capture. Open wounds became infected resulting in the death of the fish. Subsequent mortality was estimated at less than 1% per week. There was no evidence of predators (otters and mink) having entered the impoundments during the sampling period.

The impoundment stocking density was estimated at approximately 1 kg/m^3 . This density was well below 20 kg/m^3 density for short-term confinement and below 10 kg/m^3 (Gillis 1979) and 8 kg/m^3 (Kreiberg et al., 1982) suggested for long-term confinement. The behaviour of the impounded herring was typical of that previously described for low density stocking (Gillis, 1979; Brett and Solmie, 1982; Gillis et al., 1982). Pacific herring normally cease feeding during the final stages of maturation and spawning (Hourston and Haegele, 1980). The impounded experimental fish did not appear to feed. However, when copepods and other zooplankton were evident in the impoundment on April 5 and April 12, The impounded herring resumed feeding even though the females were still ripe.

GONADOSOMATIC AND HEPATOSOMATIC INDICES (GSI; HSI)

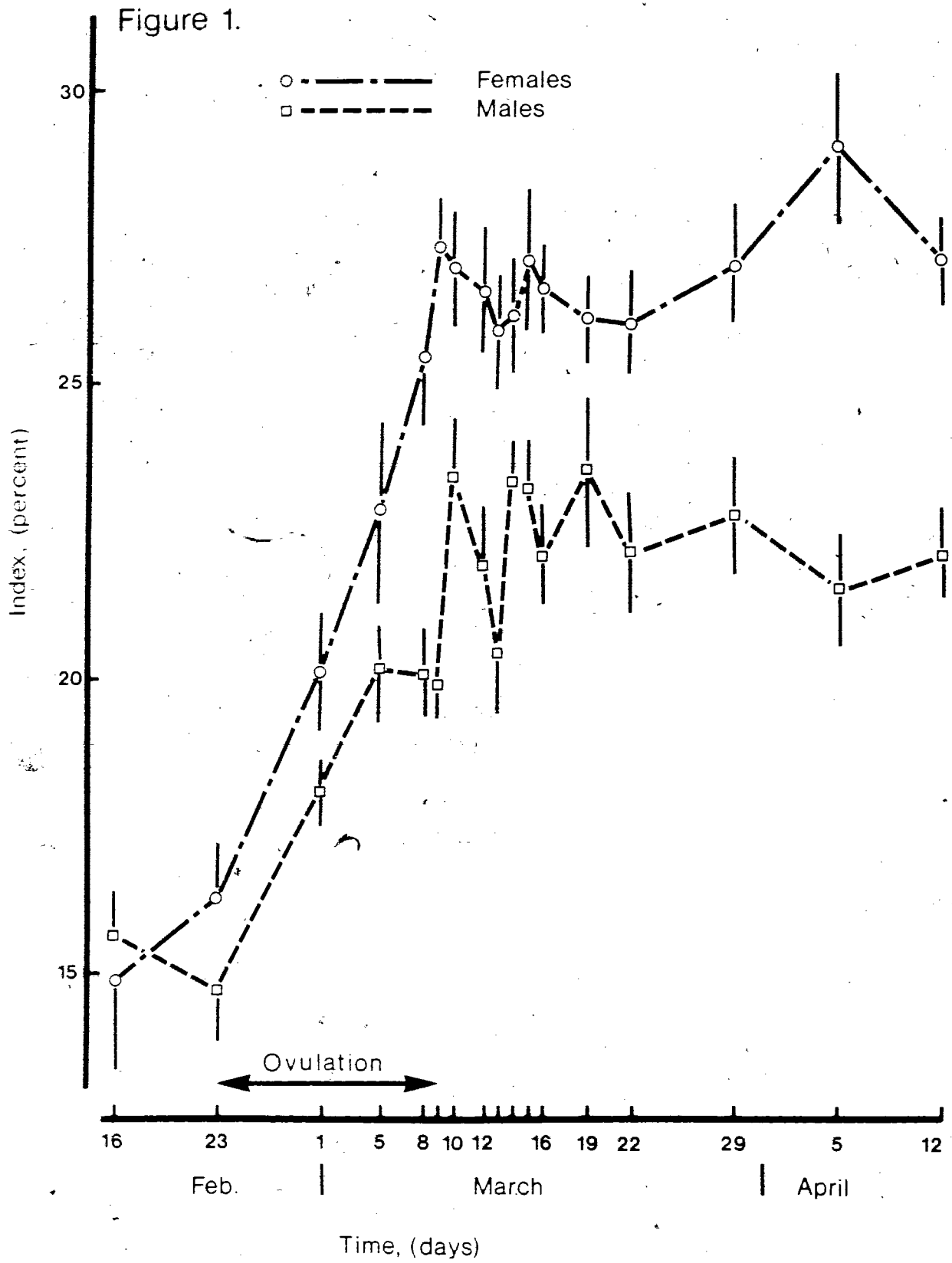
The GSI for females rose from 15% at the beginning of the study period to approximately 27% after ovulation (Figure 1). The GSI for males rose from 15% to 23% during the same period. Both for males and females, the pre-ovulatory rise in GSI was significant and the post-ovulatory variability in GSI values for both males and females was not significant. The mean GSI values for males were always lower than those for females, except for the first sampling day.

At the beginning of the sample period female mean HSI values were twice as high as mean

Figure 1.

Gonadosomatic indices for male and female Pacific herring.

The gonadosomatic index is the percentage of total somatic weight (including gonads) comprised of gonad weight. Each plotted point represents the mean for ten fish. The vertical bars are standard errors of the mean values.



values for males (Figure 2). Mean values for females were always higher than those for males. Mean HSI values for males remained relatively constant over the sampling period. There was no statistical significance in the variability of male values. The precipitous decline in values for females over the pre-ovulatory period (Feb. 16 to March 9) was significant but the variability following ovulation up to March 29 was not significant.

ALKALI-LABILE PHOSPHORUS

A final test of the modified method using a composite sample of herring ovaries confirmed the suitability of the method ($n=10$; range, 37.4 to 42.8 mg/l; standard deviation, 1.47 mg/l; mean, 40.0 mg/l; Standard error of the mean, 0.47 mg/l). The presumptive late vitellogenic increase in female gonad ALPP was not significant but the decline during population ovulation was significant (Figure 3). The decline in values for female serum and liver over the total sample period were both significant. During the late vitellogenic and ovulation periods, mean values for female serum were always higher than those for female liver and both male tissues; mean values for female liver were always higher than those for male liver; mean values for male serum were always higher than those for male livers.

EGG MOISTURE DETERMINATION

Herring eggs underwent two distinct hydrations (Figure 4). The first occurred during ovulation. Percent moisture increased from 66% to 76% and remained at the latter level until the eggs were spawned and fertilized. Immediately following fertilization the eggs hydrated to approximately 83% moisture. Spawned but unfertilized eggs dehydrated to approximately 74% moisture. The difference between ovulated, fertilized water-hardened, and unfertilized water-hardened moisture was significant. The amount of water taken up during the second hydration was not correlated with the salinity of the hydration water ($r = 0.3$; $P > 0.05$) over the limited range of salinities tested (23.00 to 28.29 parts per thousand). The amount of water lost by unfertilized eggs was also not correlated with salinity ($r = 0.41$; $P > 0.05$).

Figure 2.

Hepatosomatic indices for male and female Pacific herring.

The hepatosomatic index is the percentage of total somatic weight (including liver and gonads) that is comprised of liver tissue. Liver weights included the weight of the gall bladder which was invariably full. Each plotted point represents the mean for ten fish.

The vertical bars are standard errors of the mean values.

Figure 2.

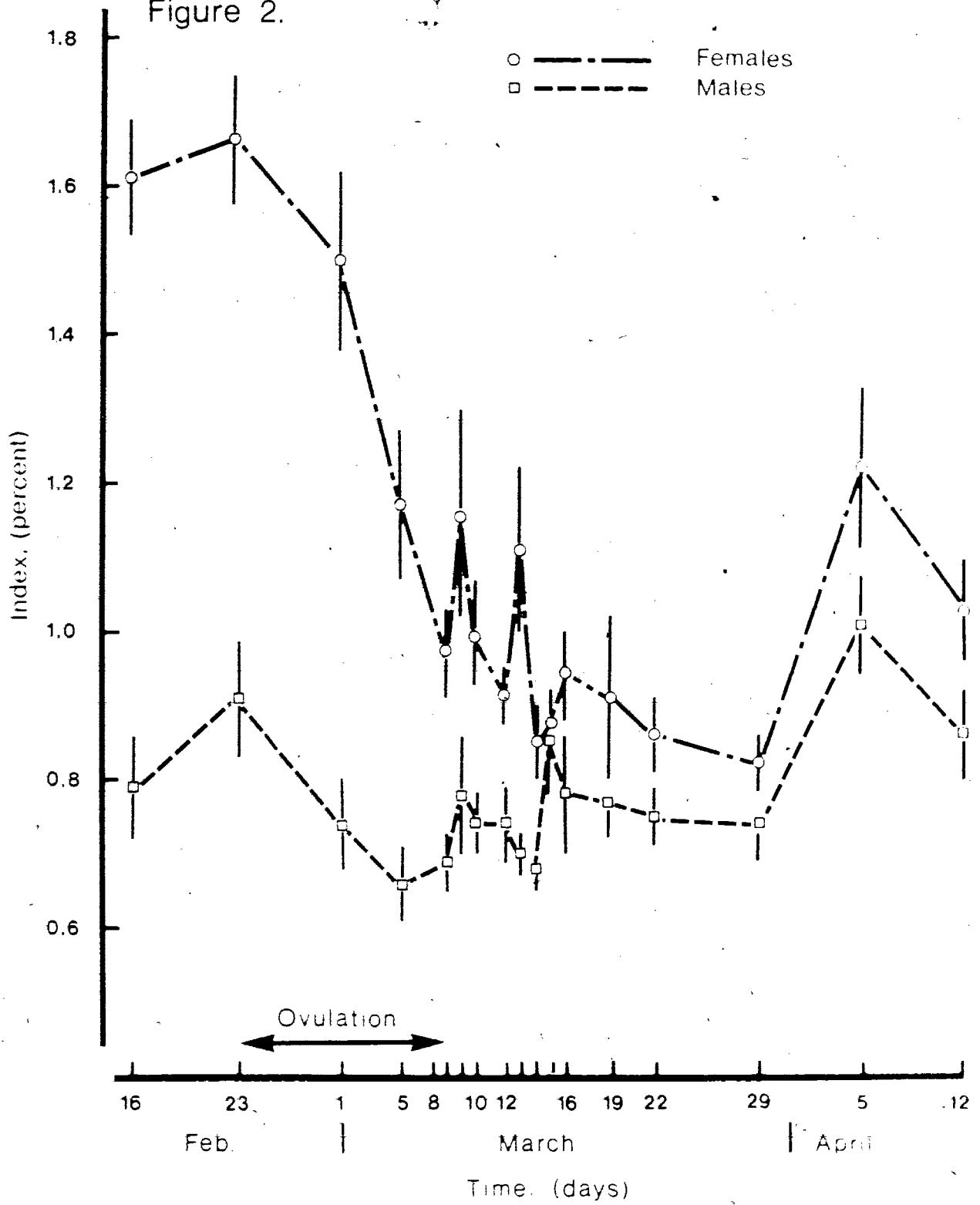


Figure 3.

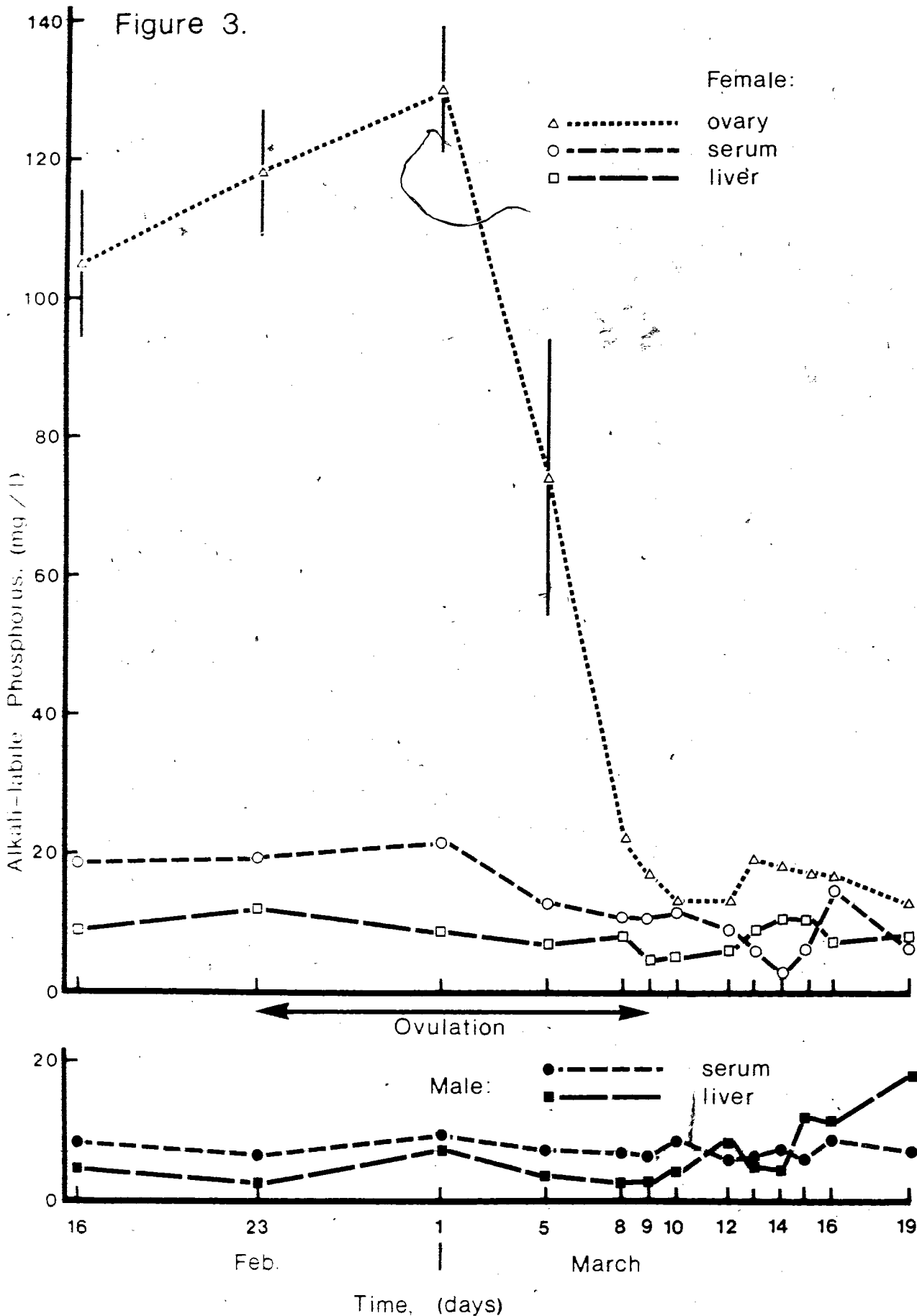
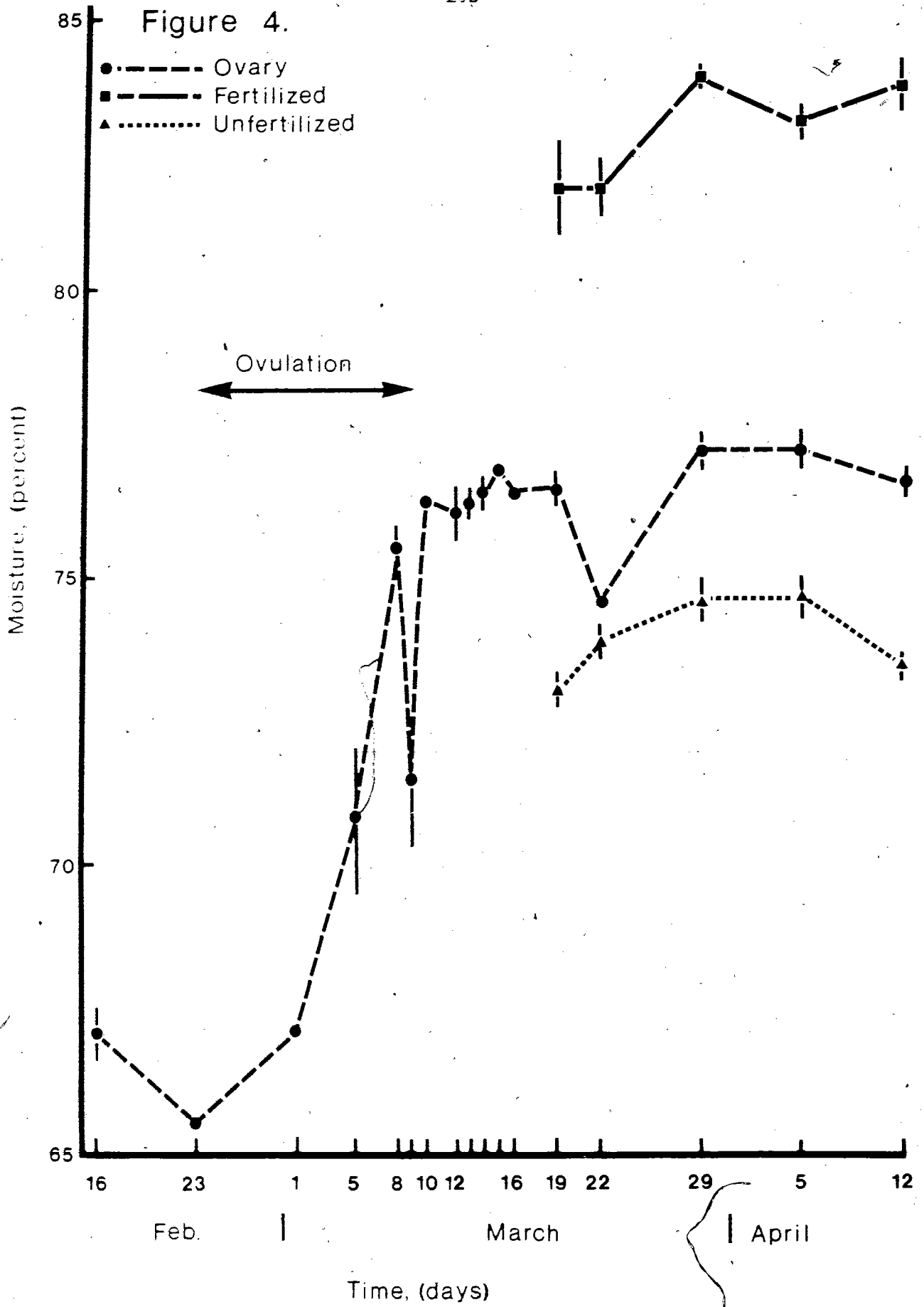


Figure 4.

Percent moisture content for ovaries, and fertilized and unfertilized, water-hardened eggs of Pacific herring.

Each plotted point represents the mean for 10 fish. The value for each fish is the mean of triplicate determinations. Each point therefore represents 30 determinations.

Figure 4.



ALKALINE PHOSPHATASE LOCALIZATION

Sectioning was greatly facilitated by soaking trimmed blocks in distilled water overnight. Block softening did not interfere with alkaline phosphatase or PAS reactions. Chloroform was used as the transitional vehicle during infiltration. Ovulated eggs did not infiltrate as expected. The chloroform diffused freely from the egg but the paraffin failed to infiltrate, resulting in shrunken and deformed sections of ovulated eggs. Late vitellogenic and ovulating eggs infiltrated normally. Alkaline phosphatase activity was localized in granulosa and thecal cells (Figure 5). Alkaline phosphatase activity was further localized in the margins of the granulosa cells where they touch the externus of the egg membrane (Figure 6). The granules in the granulosa cells, and the thecal cells appeared heavily stained. Alkaline phosphatase was absent from oocyte microvilli and other oocyte structures. Alkaline phosphatase was not localized in sections of ovulated eggs.

CARBOHYDRATE AND MUCOSACCHARIDE LOCALIZATION

Glycogen was localized in the ooplasm between yolk globules (Figures 7 and 8). Glycogen deposition was particularly heavy in the vicinity of the germinal vesicle (Figure 7b). Mucosaccharide was absent from the yolk globules, the externus and the distal interlayer, but was present in the cortical granules, the granulosa layer and the proximal interlayer (Figure 8). The granules in the granulosa cells were not stained with either Schiff's reagent or fast green. The cytoplasm immediately surrounding the granules stained strongly PAS-positive. The internus and sub-internus stained weakly PAS-positive. The adhesive layer between two adherent eggs stained PAS-negative (Figure 9). Glycogen localization was confirmed by TEM results (Figure 10).

ELECTRON MICROSCOPY

Ultrastructural examination of kidney, optic nerve, retina, white and dark skeletal muscle, intestine, liver and ovarian tissue revealed that the whole fish vascular perfusion method

Figure 5.

Alkaline phosphatase localization, (Modified Gomori and eosin).

5a. A late vitellogenic oocyte which was heat treated prior to staining to destroy enzyme activity. The egg membrane (**Em**), germinal vesicle (**gv**) and unstained granulosa (**g**) are visible. (70X)

5b. A late vitellogenic oocyte which was stained without prior heat treatment. Granulosa cells (**g**) stain black, showing the location of alkaline phosphatase activity. (70X)

5c. An ovulating oocyte which was heat treated prior to staining to destroy enzyme activity. Granulosa cells (**g**) are unstained. The germinal vesicle (**gv**) is completing its meiotic divisions in this section. (70X)

5d. A section of the same oocyte as 5c which was not heat treated prior to staining. Granulosa cells (**g**) stain black, showing the location of alkaline phosphatase activity. The dividing germinal vesicle (**gv**) is also apparent in this section. (70X)

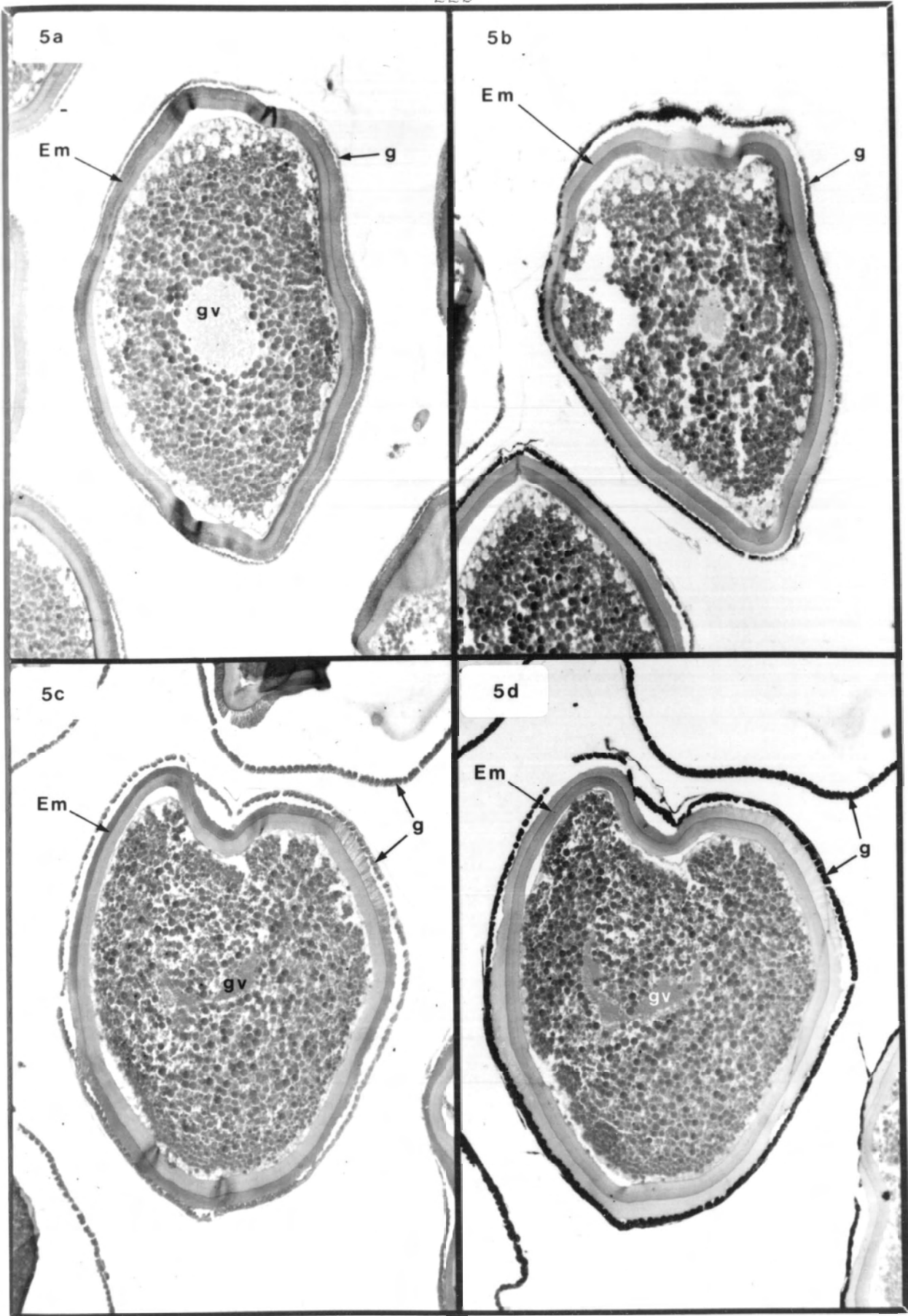
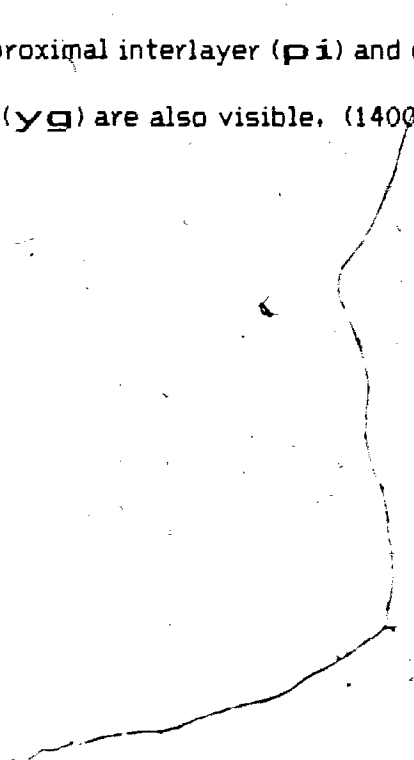


Figure 6.

Alkaline phosphatase localization, magnified view, (Modified Gomori and eosin).

This section of a late vitellogenic oocyte shows alkaline phosphatase activity in the granulosa (**g**) and thecal (**th**) cells. Enzyme activity is absent from oocyte microvilli located within the egg membrane pores. Also apparent in this section are the three principal layers of the egg membrane, the externus (**E**), internus (**I**) and sub-internus (**SI**), as well as two additional layers, the proximal interlayer (**pi**) and distal interlayer (**di**). Yolk vesicles (**yv**) and yolk globules (**yg**) are also visible. (1400X)



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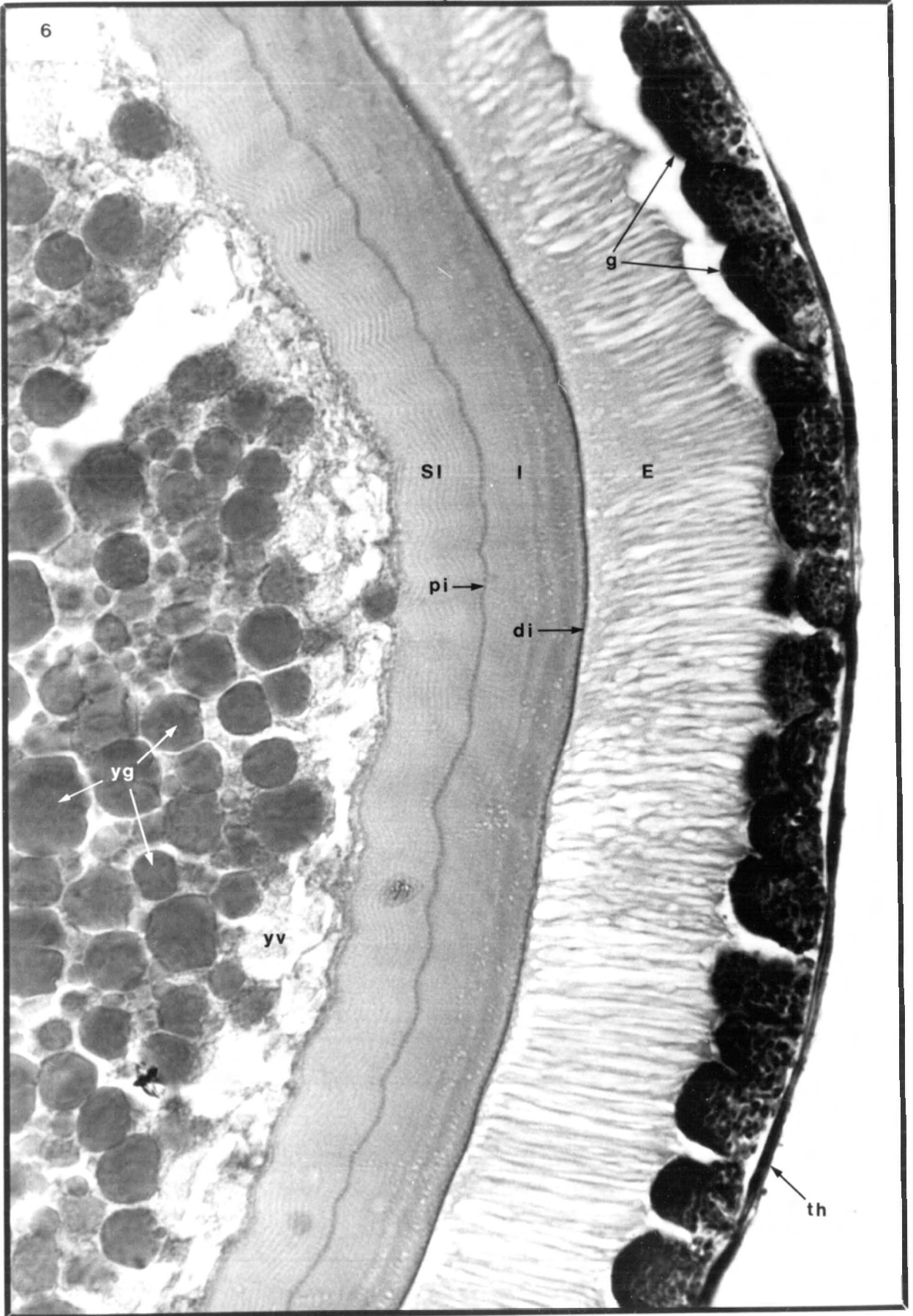


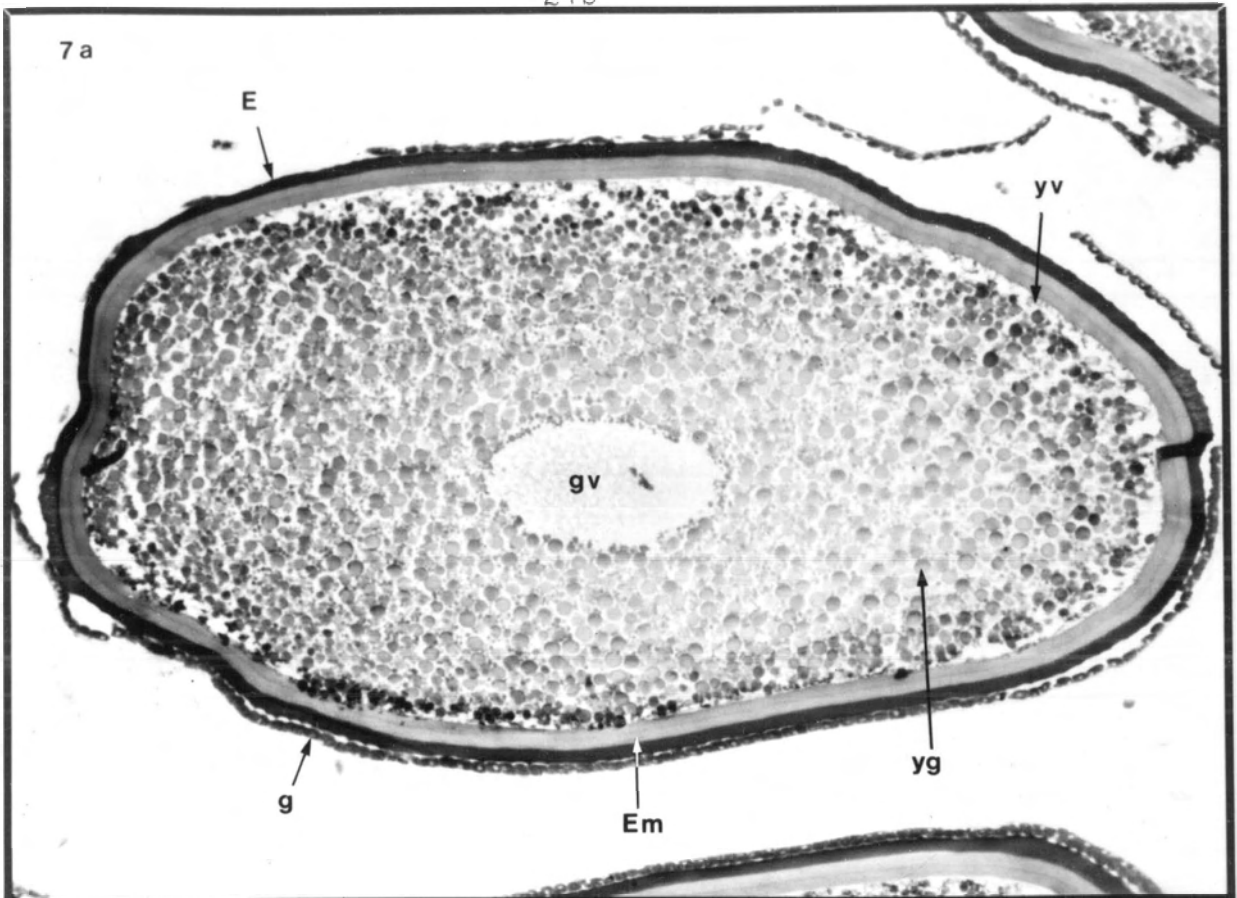
Figure 7.

Mucosaccharide and glycogen localization, (PAS and fast green).

7a. This late vitellogenic section was treated with fresh human salivary amylase prior to PAS-staining. PAS-reactivity was determined from color micrographs of this section. The germinal vesicle (**gv**) and yolk globules (**yg**) were PAS-negative while yolk vesicles (**yv**) were strongly PAS-positive. The egg membrane (**Em**) and granulosa cells (**g**) were weakly positive. The externus (**E**) of the egg membrane and the granulosa cells appear dark in this section due to their strong affinity for the fast green counterstain. (160X)

7b. A section of a late vitellogenic oocyte which was not treated with salivary amylase prior to PAS-staining. Numerous glycogen granules (**gl**) are apparent in the cytoplasm between the yolk globules. Glycogen deposition is particularly heavy in the vicinity of the germinal vesicle. (160X)

7a



7b

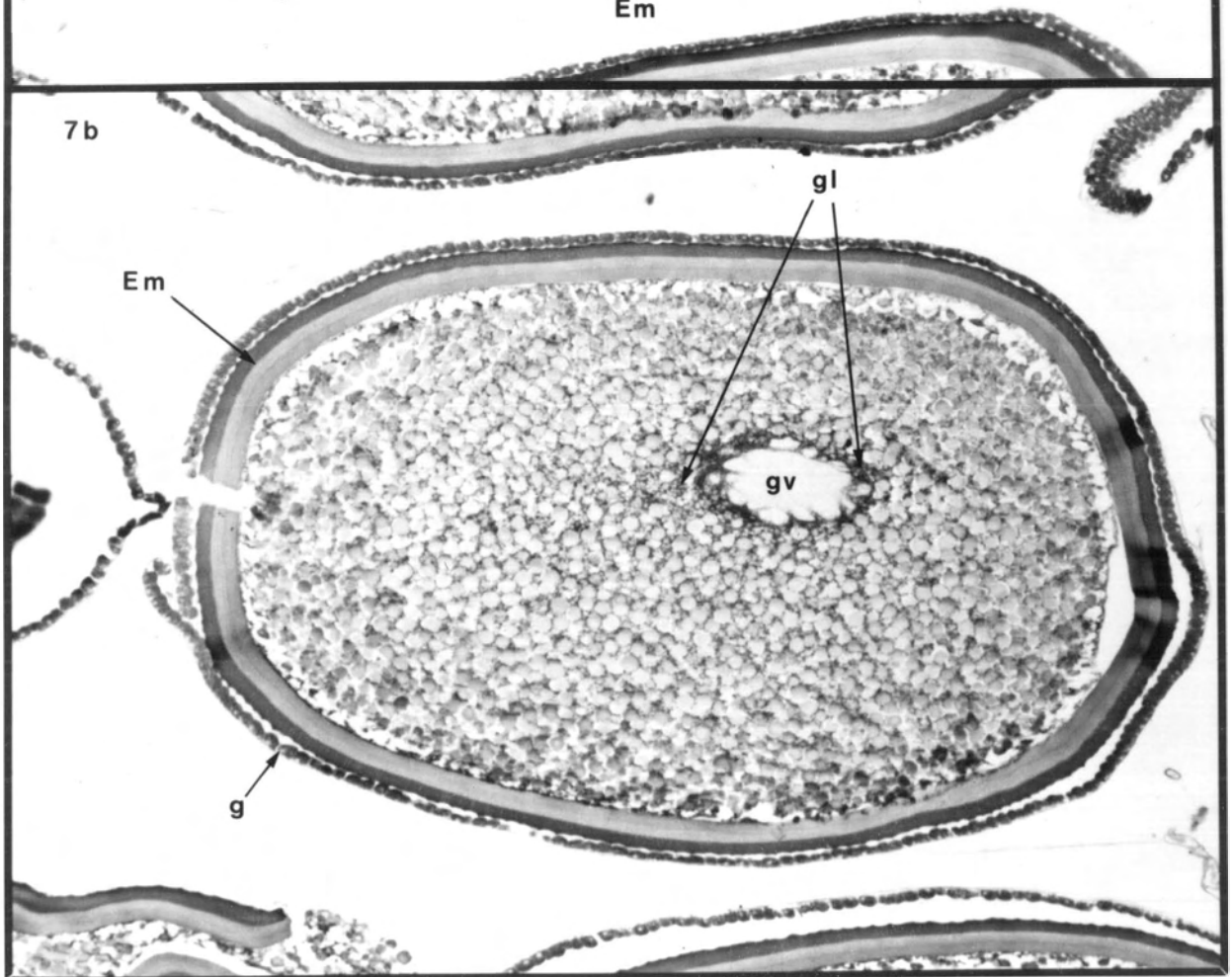


Figure 8

Mucosaccharide localization, enlarged view, (PAS and fast green).

This section of a late vitellogenic oocyte was treated with fresh human salivary amylase prior to staining. Yolk globules (**yg**) and granulosa cell granules (**gr**) stain PAS-negative. Yolk vesicles (**yv**), the proximal interlayer and the cytoplasm immediately surrounding the granulosa granules are PAS-positive. The remaining egg membrane structures; the externus (**E**), internus (**I**), the sub-internus (**SI**) and the distal interlayer (**di**) stain weakly PAS-positive. (700X)

Figure 9.

Adherent eggs, (PAS and fast green).

The zone of adhesion is shown by the heavy arrow between the two opposed externi (**E**). This zone stains PAS-negative, but the externus in the immediate vicinity of the adhesion zone stains weakly PAS-positive. The dark material between the yolk globules (**yg**), is PAS-positive glycogen in this amylase-untreated section. (400X)

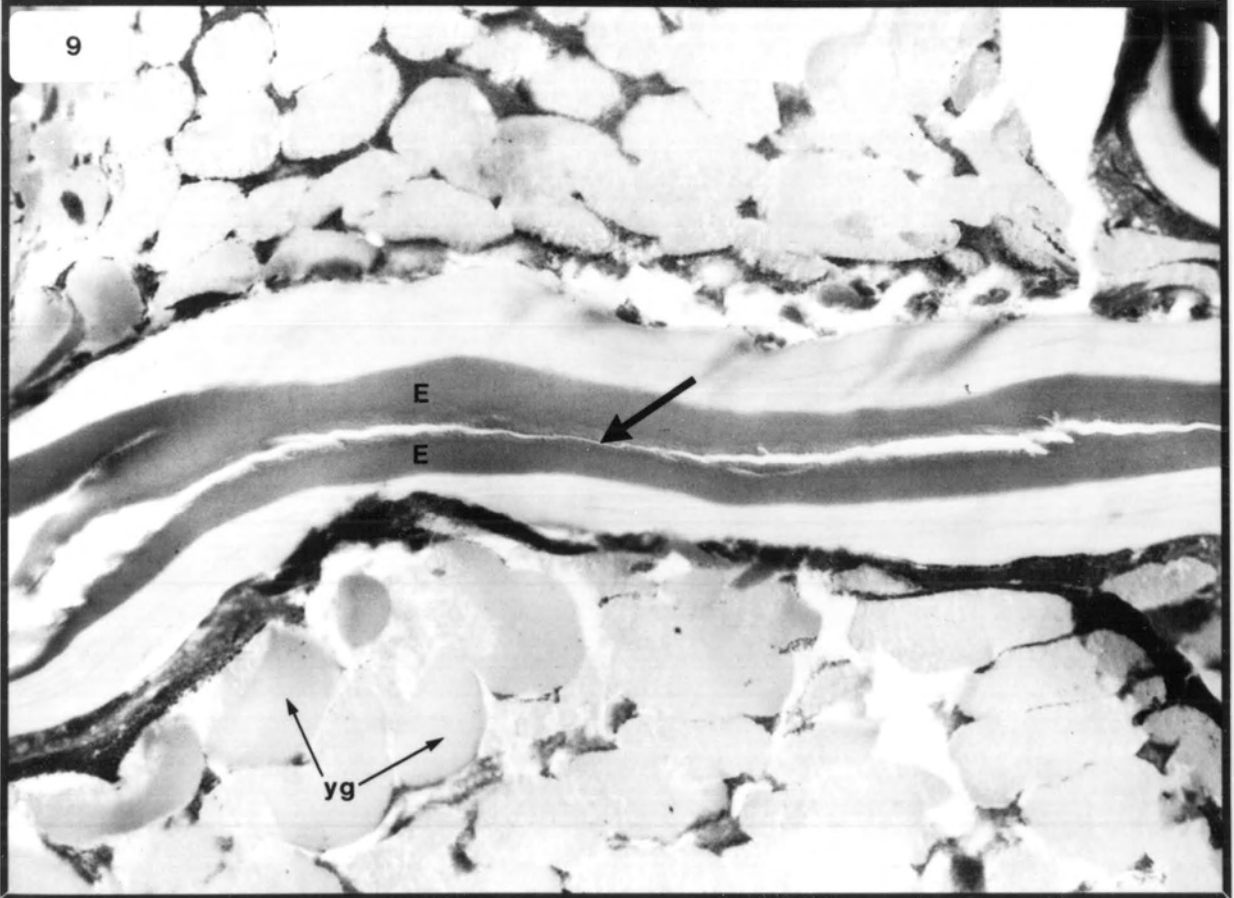
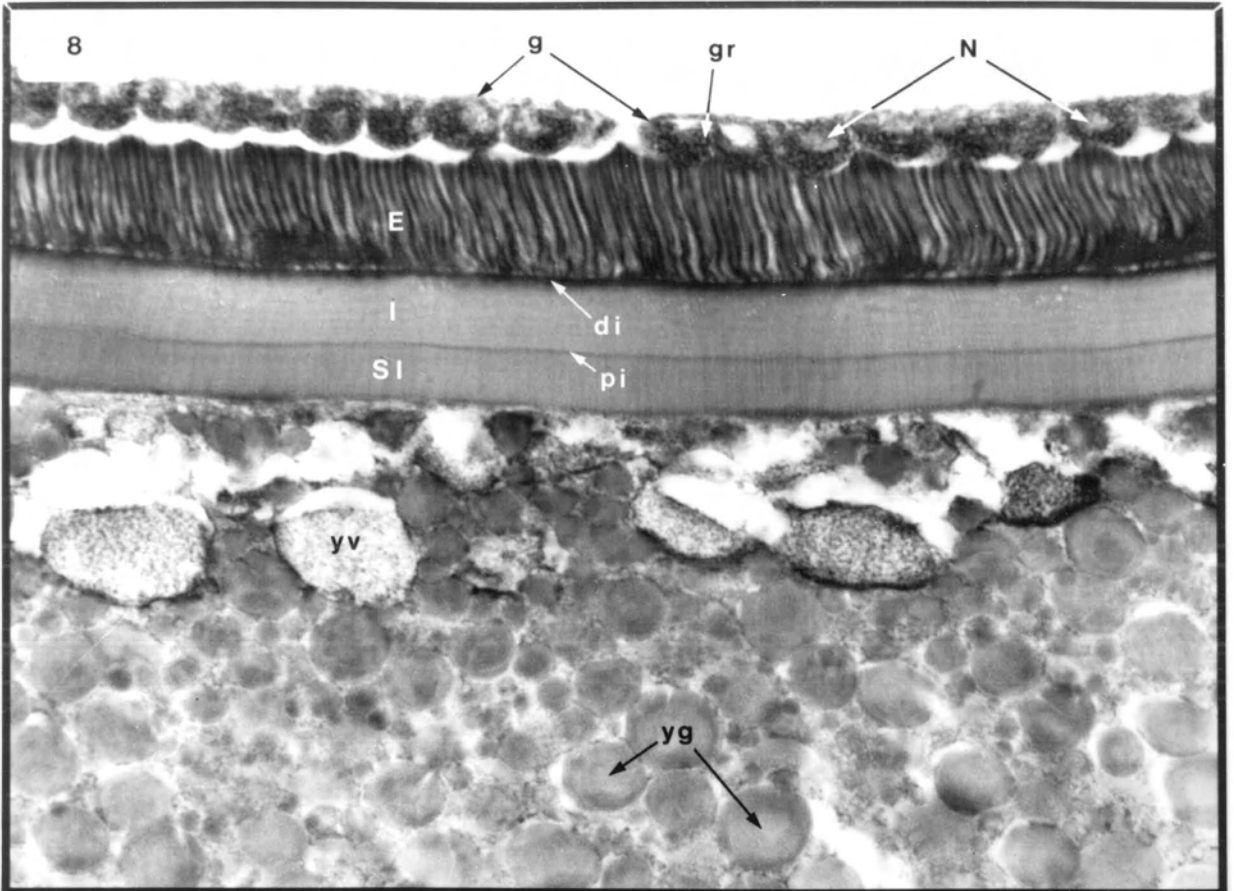
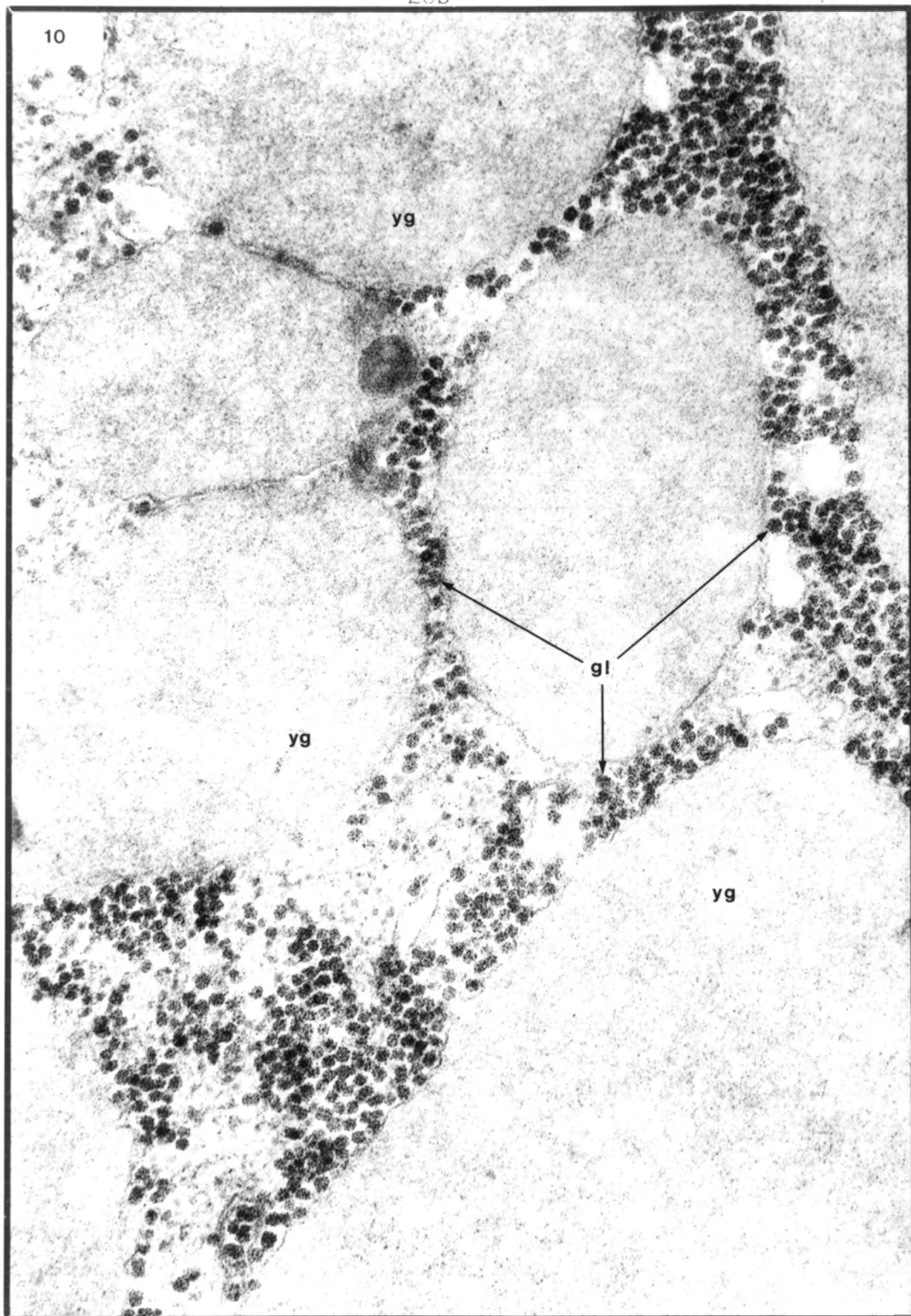


Figure 10.

Transmission electron micrograph of yolk globules and glycogen granules.

Glycogen granules (**gl**) are located in the cytoplasm between membrane bound yolk globules (**yg**). This micrograph confirms glycogen as the PAS-positive staining granules seen in Figure 7b. (83,000X)



used produced excellent microstructural preservation. The perfusate did not initiate blood clotting, eliminating any requirement for prior vascular system flushing or the use of anti-clotting agents.

Epoxy resin infiltration of late vitellogenic and ovulating eggs occurred as expected, but infiltration of ovulated eggs did not. The propylene oxide transition vehicle diffused readily from the eggs, but the resin infiltrated less readily. Ovulated eggs invariably shrank and became distorted. Shrunken eggs popped audibly during infiltration. Popped eggs assumed their original shapes and floated. Longer residency times in each graded mixture did not achieve satisfactory infiltration. Reasonable infiltration was achieved by puncturing fixed eggs prior to their introduction to the first transition solution. Internal egg morphology was disrupted in the vicinity of the puncture, but satisfactory sections were taken from positions distant from the puncture.

LATE VITELLOGENIC MORPHOLOGY

Late vitellogenic oocytes were enclosed within follicles which were attached to abundant ovarian stroma (Figure 11a). Oocyte vascularization was evidenced by raised ridges on the surface of the follicle (Figure 11b). The follicle was comprised of two layers, the outer theca and the inner granulosa layer (Figures 11c and 11d).

Atretic oocytes were present in late vitellogenic ovaries (Figure 11a) but not in ovulating or ovulated ovaries. Atretic oocytes were seldom larger than 100 μ m (Figure 12). Atretic oocytes were also observed in paraffin sections prepared for light microscopy (not illustrated). Atretic oocytes were completely covered with thecal, but not granulosa cells. Atretic oocyte thecal cells were individually distinguishable (Figure 12b). PAS staining characteristics of the atretic oocyte membrane were identical to those of the internus and sub-internus of late vitellogenic oocytes.

STRUCTURAL CHANGES DURING OVULATION

Several structural changes accompanied ovulation, but the definitive event was the

Figure 11.

Scanning electron micrographs of late vitellogenic oocytes.

11a. Several oocytes are shown. All have intact thecal and granulosa layers. Abundant ovarian stroma (**OS**) and atretic oocytes (**ao**) are visible.

11b. A single vitellogenic oocyte is shown with its associated stroma and atretic oocytes. A capillary network (**C**) is evident beneath the thecal layer.

11c. A sectioned vitellogenic oocyte is shown. Visible are the germinal vesicle (**gv**), yolk (**Y**), egg membrane (**Em**), granulosa cells (**g**) and thecal layer (**th**).

11d. An enlarged view of the egg membrane is shown. The externus (**E**), internus (**I**) and sub-internus (**SI**) are visible. Membrane pores (**mp**) are visible in the externus.

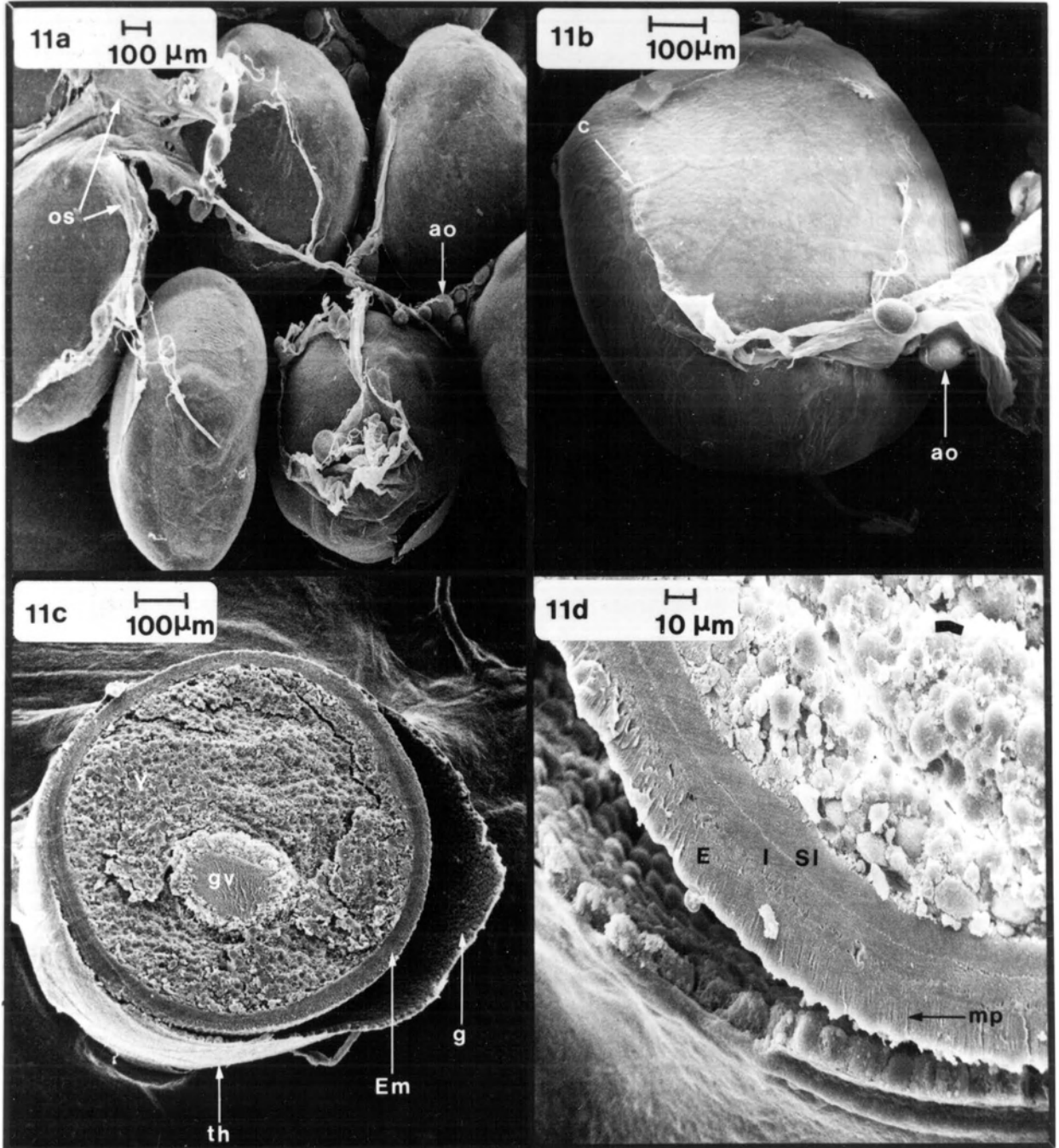


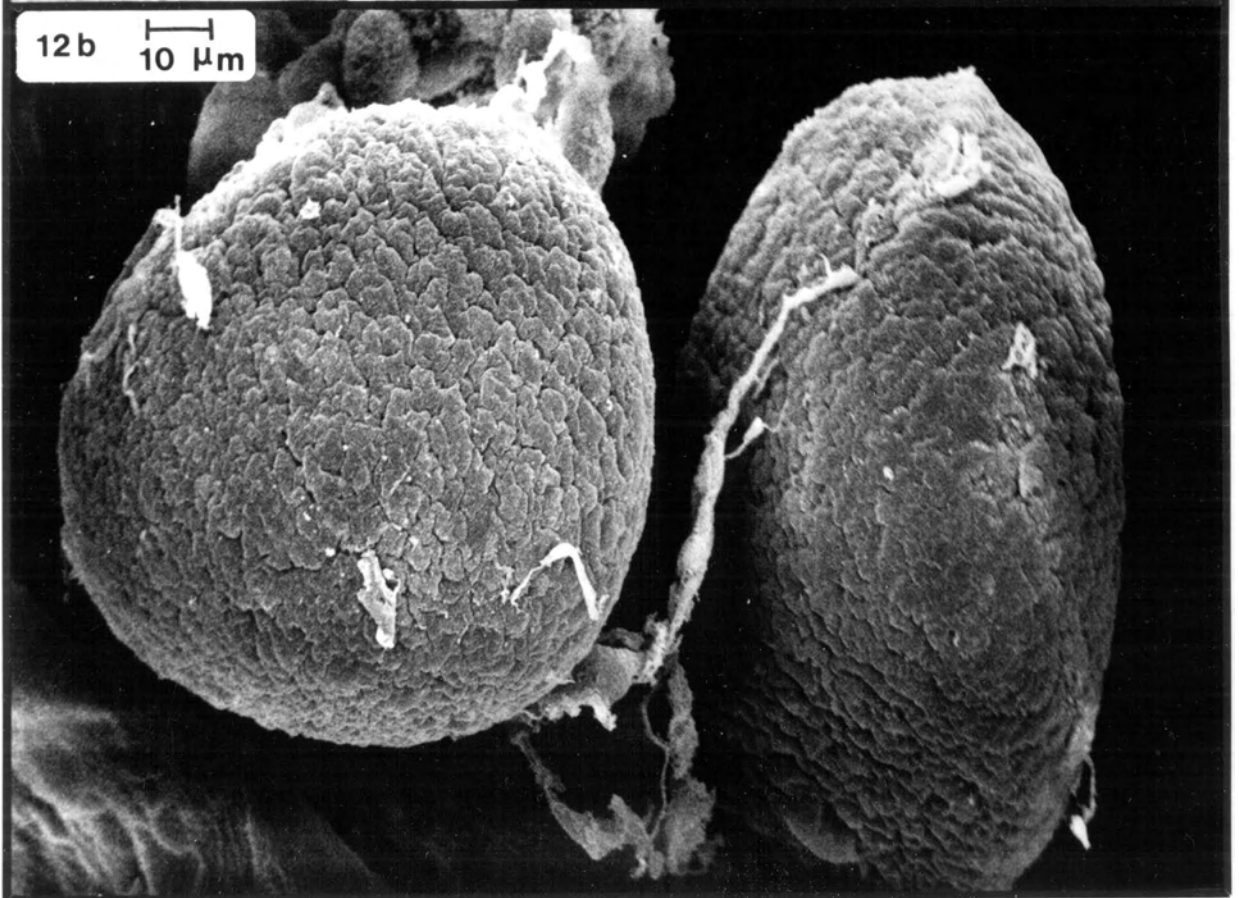
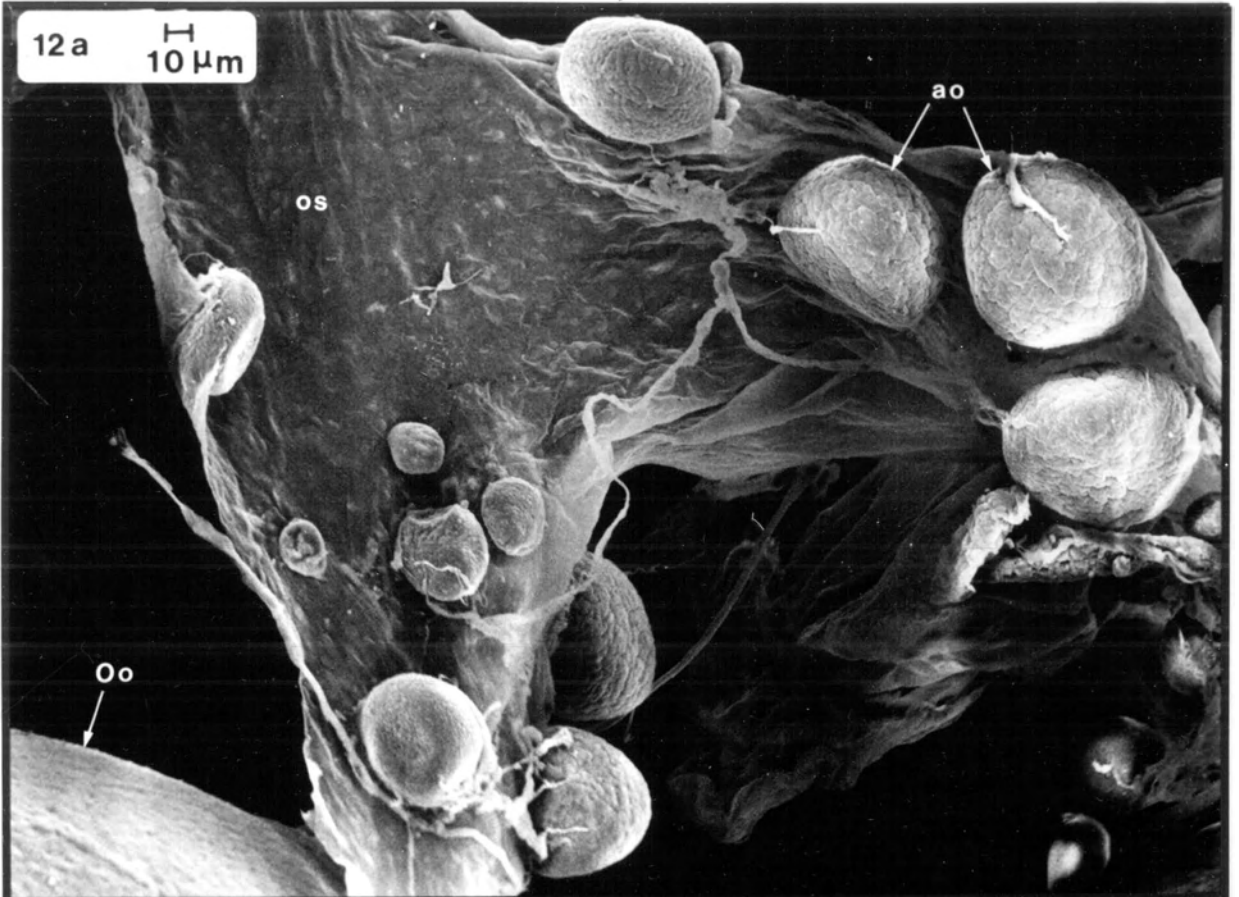
Figure 12.

Scanning electron micrographs of atretic oocytes from a late vitellogenic ovary.

12a. This view shows several atretic oocytes (ao), and their association with the ovarian stroma (os). The atretic oocytes vary in size from 20 to 90 μ m in this field. The tneca of a single oocyte (oo) is visible in the lower right corner.

12b. Two atretic oocytes are shown in this view. Paraffin sections for light microscopy revealed that these oocytes are covered with thecal but not granulosa cells. The viewer of these micrographs is seeing the external surface of the thecal cell layer. They are attached to the ovarian stroma.

29b



expulsion of the oocyte from the follicle to become a fertile egg. During the early stages of ovulation, the germinal vesicle completed its meiotic divisions and the vesicle dissociated (Figures 5c and 5d). Atretic oocytes were not seen attached to ovarian stroma. The ethanol in which samples of ovulating ovary were fixed was turbid following fixation. This turbidity was not caused by dissociation of the follicular or stromal tissue as this tissue was still intact. The turbid particles consisted of detached atretic oocytes and minor amounts of unidentifiable debris. Rents and tears appeared in the follicular and stromal tissue (Figure 13a) and the oocytes were expelled from the follicle (Figure 13b). The margin of the follicle constricted the emerging oocyte. The surface texture of the emerging oocyte was deeply indented (Figure 13c) and each indentation (formerly occupied by a single granulosa cell) contained numerous irregularly distributed pores. The first hydration occurred following emergence from the follicle. The eggs apparently swelled, since much of the external and internal texture of the egg membrane was smooth (Figure 13d). The egg assumed a more spherical appearance (Figure 14a).

Micropyles were easily observed in ovulated eggs (Figure 14b). Externally, the micropyle was located at the center of a 50 μm diameter crater-like depression (Figure 15a). The external diameter of the opening averaged 1.2 μm (Figure 15b). Internally, the micropylar opening was located at the apex of a conical mound with a base diameter of 25 μm (Figure 15c). The diameter of the internal opening averaged 0.5 μm (Figure 15d). The micropylar canal was lined with annular ridges (Figure 15b).

A fine fibrillar network and patches of egg membrane material were present on the proximal surfaces of the cells (Figure 16a). During ovulation the fibrillar network and the proximal membrane of the cell were the first to degrade (Figure 16b). Internally, late vitellogenic granulosa cells were filled with granules (Figure 17a). RER was located in the cytoplasm lining the margins of the granules. During ovulation, granulosa granules were less abundant, and the RER assumed stacked profiles along the distal margins of the cells

Figure 13.

Scanning electron micrographs of ovulating oocytes.

13a. Several oocytes (Oo) are shown enclosed within folds of ovarian stroma (os). Several rents are visible in the stroma and thecal layers. The oocyte identified by the heavy arrow has partly emerged from the degenerating membranous tissue. Atretic oocytes are absent.

13b. The emerging oocyte identified in 12a is enlarged in this view. The oocyte appears constricted by the thecal tissue (th) in the equatorial region, suggesting that the theca may contain some contractile tissue.

13c. This view shows an enlargement of the exterior surface of the externus. Numerous depressions are apparent. Each depression was formerly occupied by a single granulosa cell. Membrane pores (mp) are irregularly distributed within each depression.

13d. A section of transitional oocyte is shown. Yolk has been removed. Visible are the externus (E), internus (I) and sub-internus (SI). The laminar nature of the internus and sub-internus are clearly visible in this fortuitous section. Membrane pores (mp) are visible in all three layers. The interior surface of the sub-internus is also shown with regularly arranged internal pore openings. A single microvillus (mv) is seen emerging from the sub-internus.

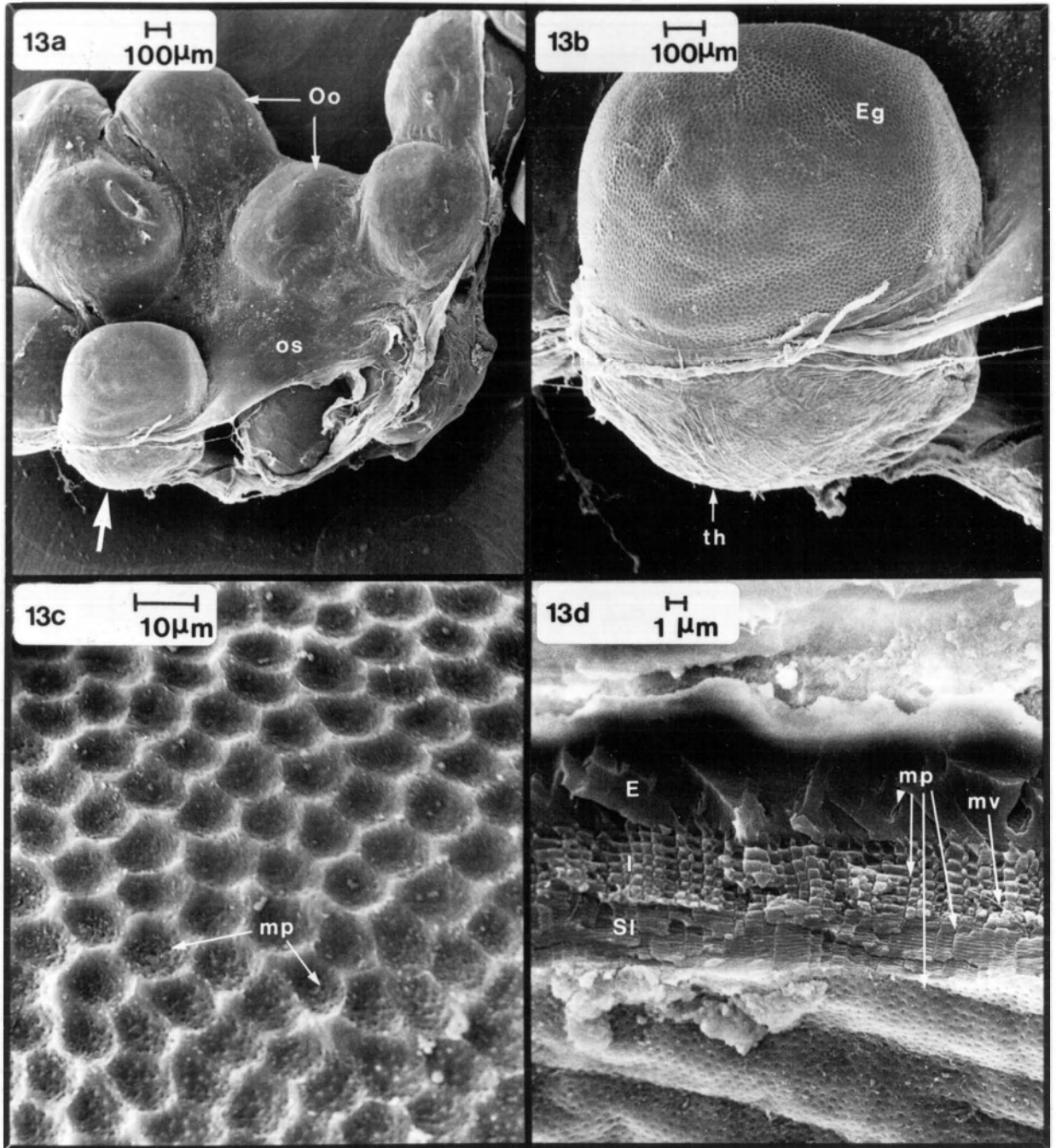


Figure 14.

Scanning electron micrographs of ovulated eggs.

14a. The egg shown (**Eg**) is fully ovulated and has completed its first hydration. The egg has taken on a more spherical appearance. The position of the micropyle (**mi**) is shown within a shallow crater. Note the light circular area on the surface of the externus surrounding the micropyle crater.

14b. The micropyle region is shown in magnified view. The depressions in the surface of the externus are less apparent than the same structures in transitional eggs, but are still visible. External openings of egg membrane pores can also be seen.

14c. A fractured section of the egg membrane is shown. Visible are the externus (**E**), distal interlayer (**di**), internus (**I**), proximal interlayer (**pi**) and the sub-internus (**SI**). Membrane pores (**mp**) can be seen in the externus. The laminar nature of the internus and sub-internus are also visible.

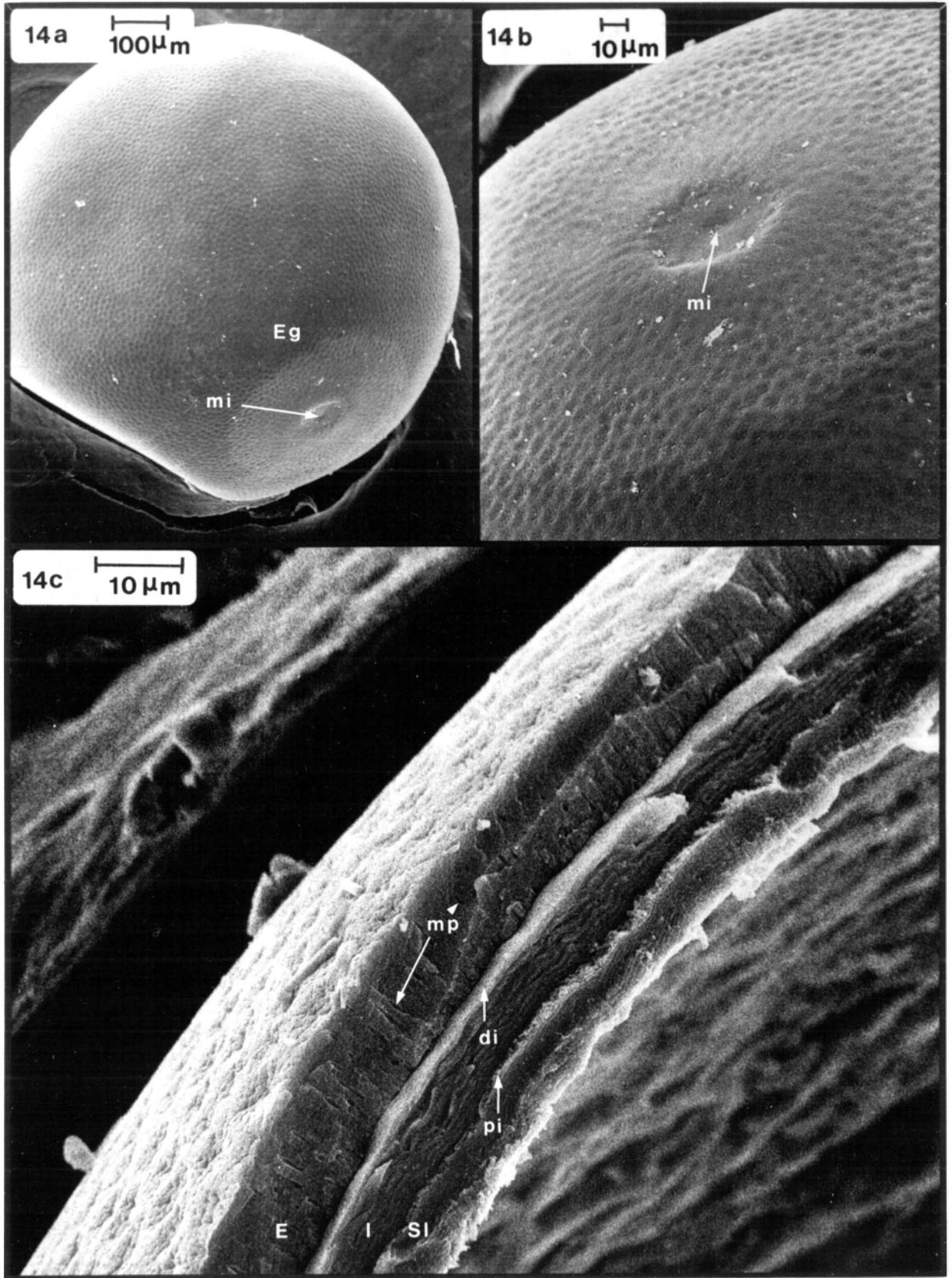


Figure 15.

Scanning electron micrographs of external and internal micropyle openings of ovulated eggs.

15a. This view shows the external micropyle opening (mi) located at the center of a shallow, 50 μ m diameter crater-like depression. Egg membrane pores (mp) are visible in this field.

15b. An enlarged view of the external opening of the micropyle is shown. The diameter of the opening is 1.2 μ m.

15c. The internal configuration of the micropyle is shown. The micropyle is located at the apex of a conical mound which has a diameter of 25 μ m at its base. The wrinkled appearance of the internal surface of the sub-internus can be seen in this field.

15d. An enlarged view of the internal opening of the micropyle is shown. The opening is approximately 0.5 μ m in diameter.

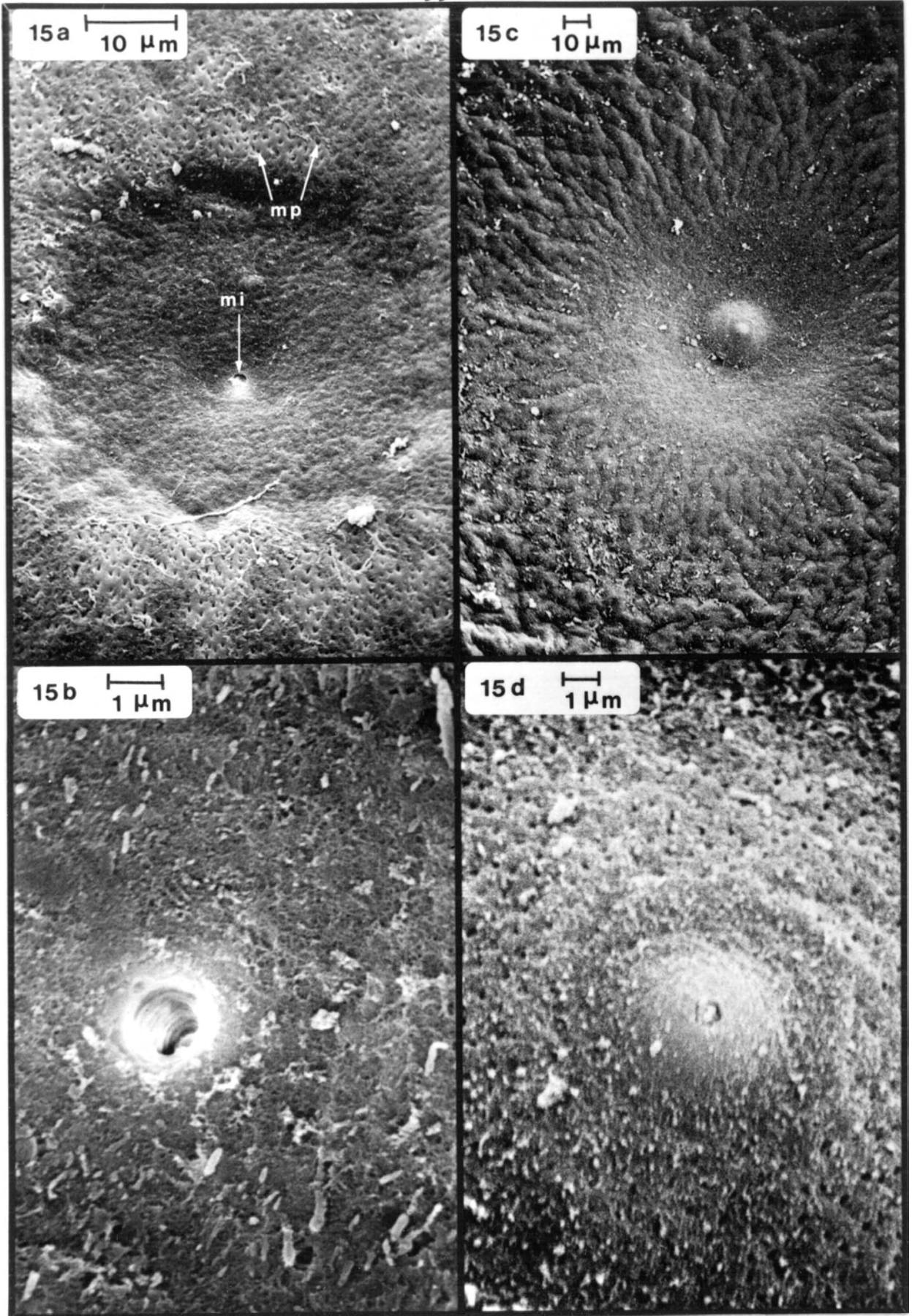


Figure 16.

Scanning electron micrographs of granulosa cells.

16a. This layer of granulosa cells was peeled from a late vitellogenic oocyte. The under-surface of the granulosa cells is shown. An extensive fibrillar network (fn) is seen on the under-surface of the cells and in the spaces between cells.

16b. This layer of granulosa cells was peeled from an ovulating oocyte. The under-surface of the granulosa cells is shown. The membranes and fibrillar network have disappeared revealing the granulosa granules (gr). Many of the granules are dished or indented on one side.

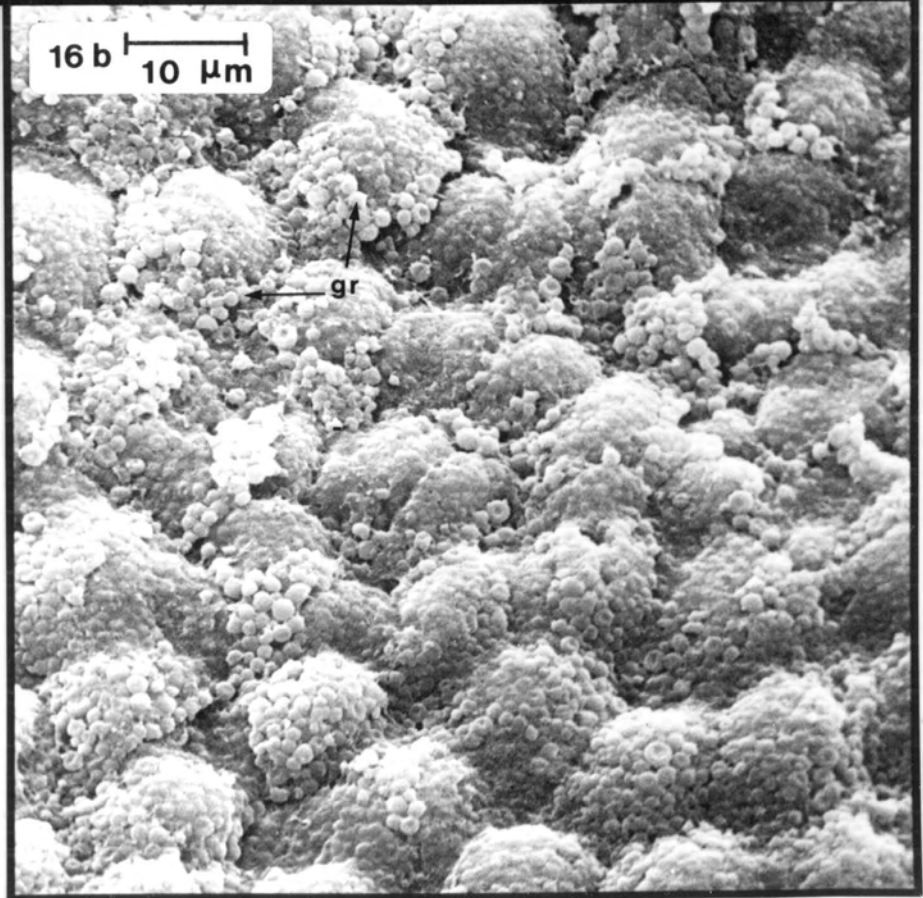
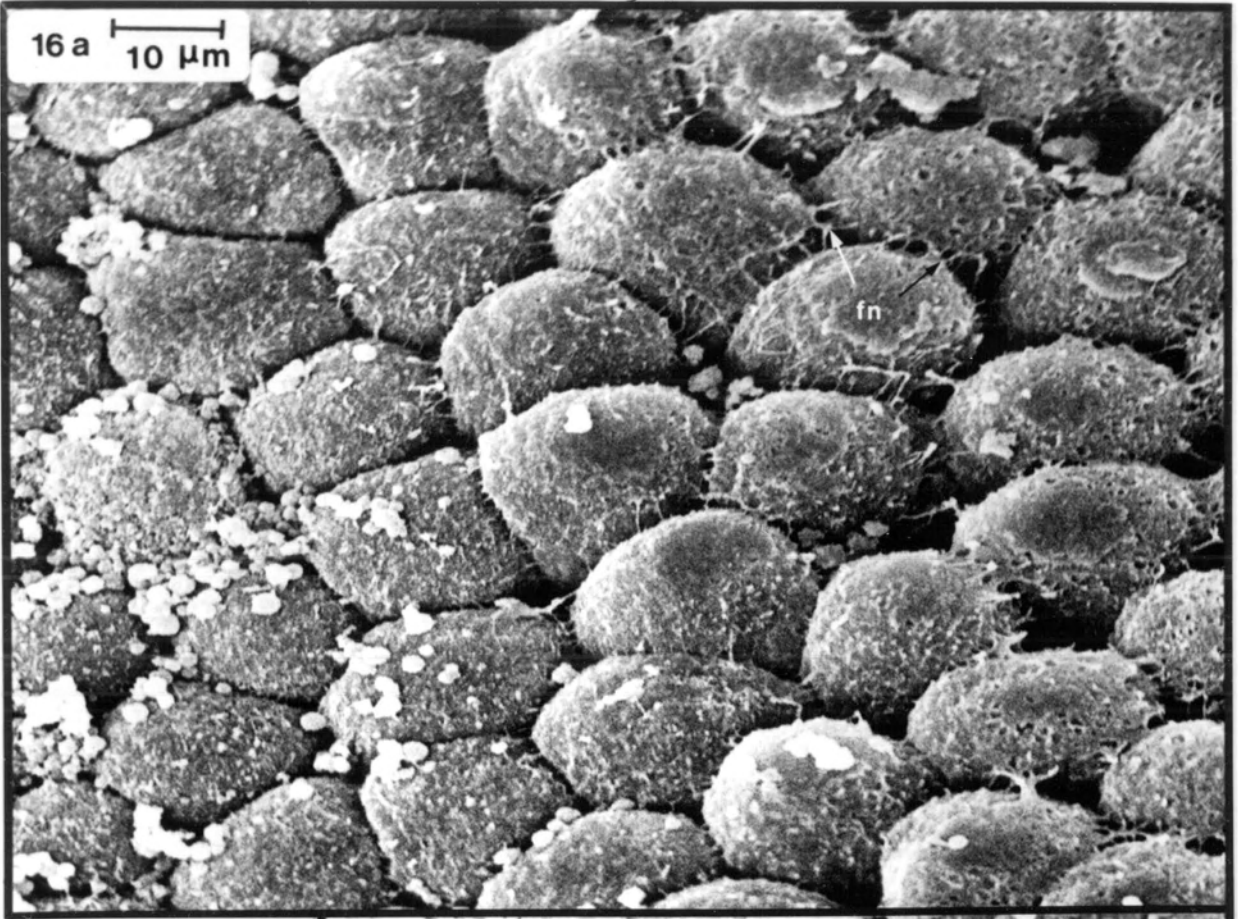
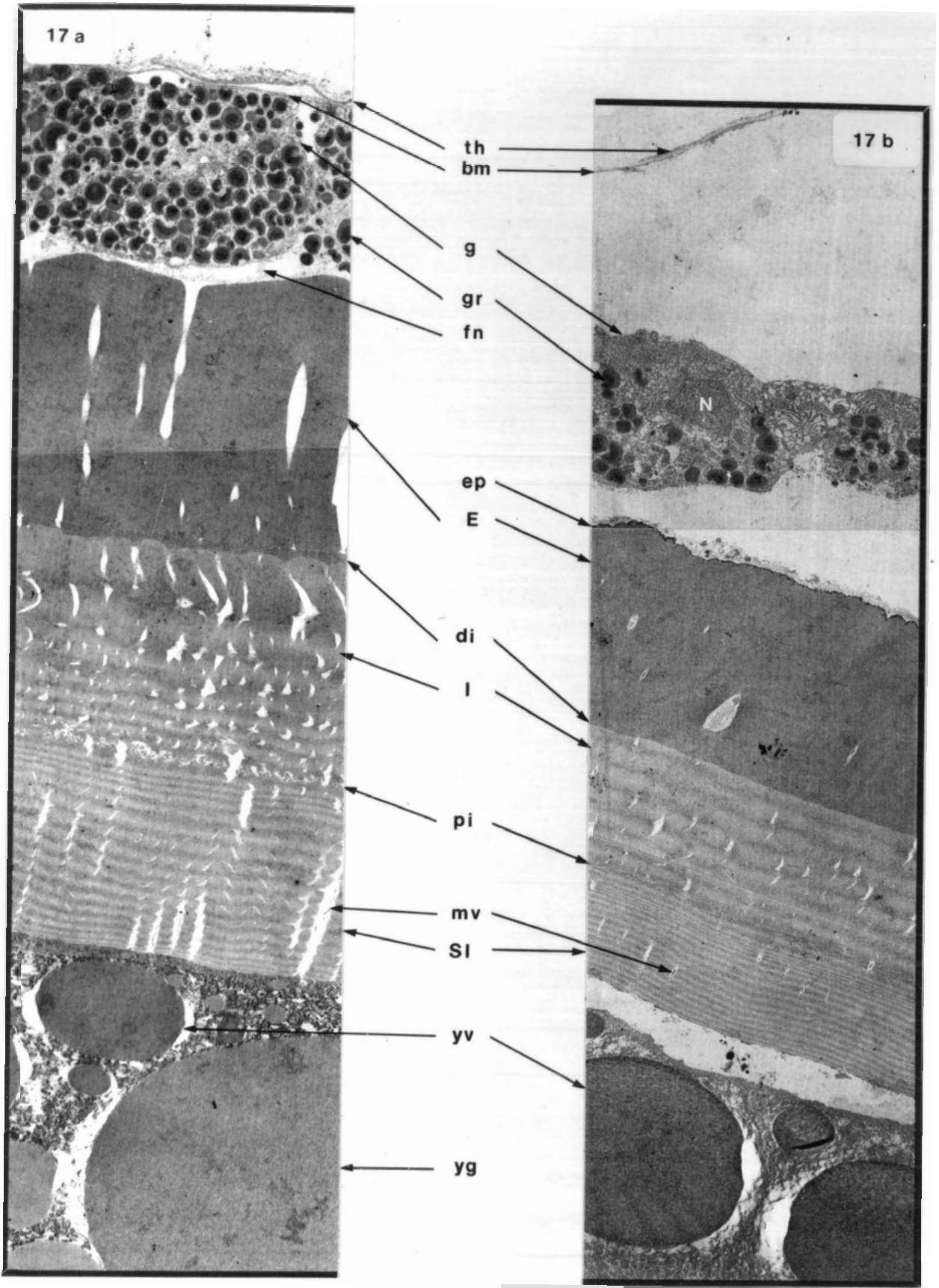


Figure 17.

Transmission electron micrographs of late vitellogenic and ovulating oocytes. (3,000X)

Sections of late vitellogenic and ovulating oocytes are shown in 17a and 17b respectively. The observable structures are the thecal cells (th), basement membrane (bm), granulosa cells (g), granulosa cell granules (gr), fibrillar network (fn) in the vitellogenic egg, the epilayer (ep) in the ovulating egg, externus (E), distal interlayer (di), internus (I), proximal interlayer (pi), microvilli (mv) with membrane pores, sub-internus (SI), yolk vesicles (yv) and yolk globules (yg). Membrane pores are seen in all three major membrane layers in both sections. In 17b, the epilayer is visible on the external surface of the externus and the pores of the internus are open. The space between the sub-internus and the yolk cytoplasm in 17b is artifactual.



(Figure 17b).

The membrane of Pacific herring late vitellogenic oocytes consists of five layers. (Figures 17a, 13d and 14c). The sub-internus was located next to the ooplasm and was separated from it by the plasma membrane. The sub-internus was laminar in structure. The laminations were compacted when compared with those of the internus. Pores in the sub-internus ran relatively straight, taking the shortest route from the ooplasm to the next layer. Microvilli, originating from the oocyte were observable in the pores. The structure of the sub-internus did not change appreciably during or after ovulation (Figures 17b and 18a).

The sub-internus was separated from the internus by a thin layer of loosely packed fibrous material (Figure 19b). This layer was described as the proximal interlayer.

A third layer was observed external to the proximal interlayer (Figure 17a). This layer (the internus) was similar in staining characteristics to the sub-internus (Figures 6 and 8). It was also laminar in nature. The internus was slightly thicker than the sub-internus in all sections examined, but the laminations were more widely-spaced and fewer in number. Pores within the internus took indirect, possibly helical paths (Figure 17a) in late vitellogenic oocytes. During the early stages of ovulation, the pore structure was unchanged (Figure 17b). However, during the first hydration which occurred after the expulsion of the oocyte, the pore structure collapsed. Internus pores were not observed in fully ovulated or fertilized egg sections (Figure 18a and 18b). In ovulated and fertilized egg sections, the internus was the same thickness or slightly thinner than the sub-internus.

An additional thin interlayer was observed between the internus and externus (Figures 17a and 17b). This layer (distal interlayer) was approximately twice as thick as the proximal interlayer and consisted of granular material (Figure 19a). There were no changes observed in this layer during ovulation.

Figure 18.

Transmission electron micrographs of ovulated and fertilized, water-hardened eggs.

(3,000X)

Sections of ovulated and fertilized, water-hardened eggs are shown in 18a and 18b, respectively. Observable structures include the epilayer (**ep**), externus (**E**), distal interlayer (**di**), internus (**I**), Proximal interlayer (**pi**) and sub-internus (**SI**). The externus in the ovulated egg has taken on a 'spongy' appearance. Membrane pores are visible in the externus and sub-internus of both sections. Note however that the pores are not observable in the internus in either sections.

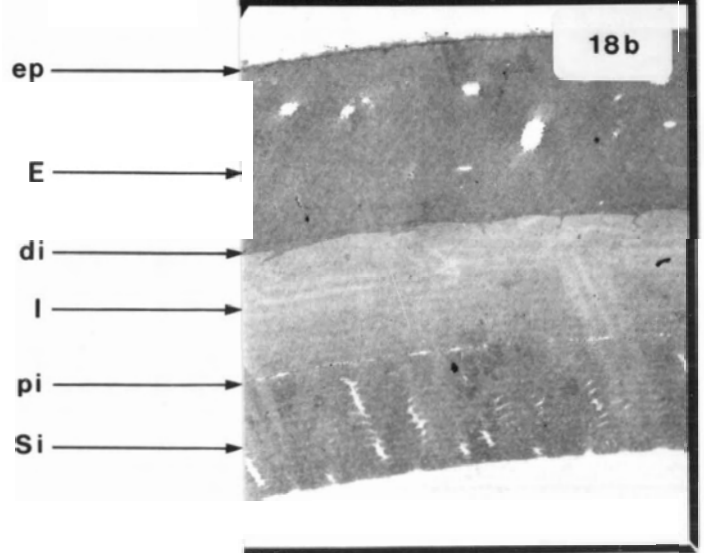
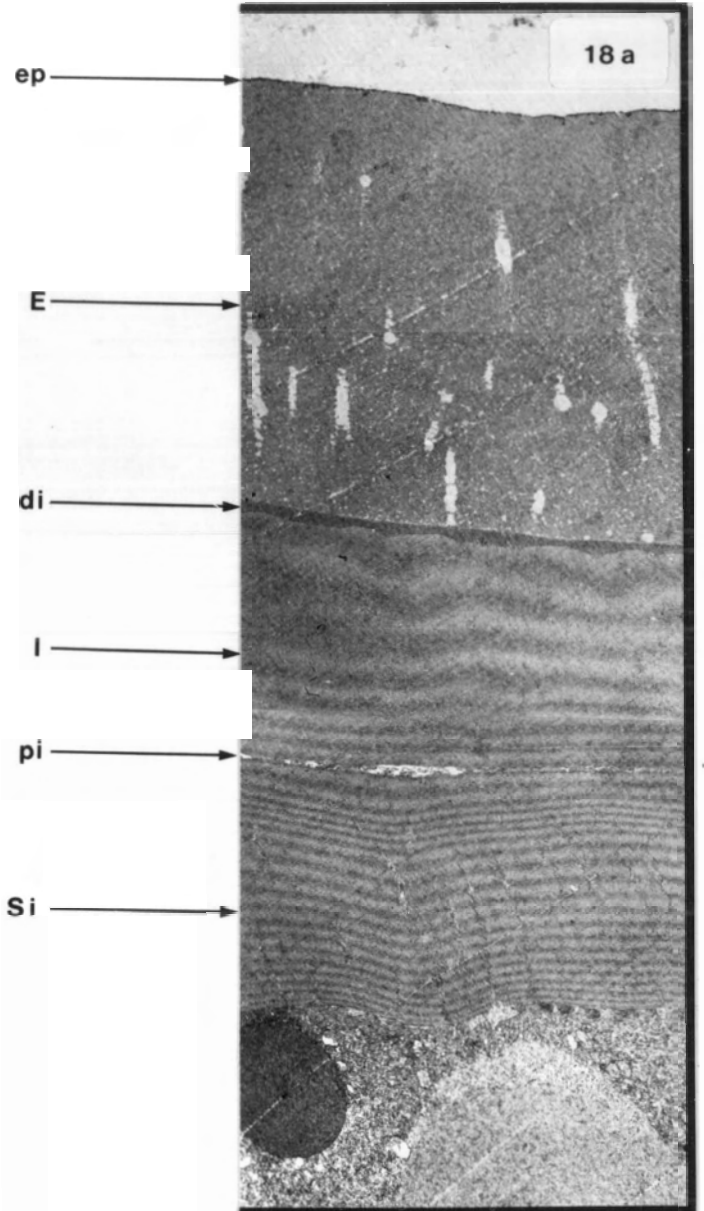


Figure 19.

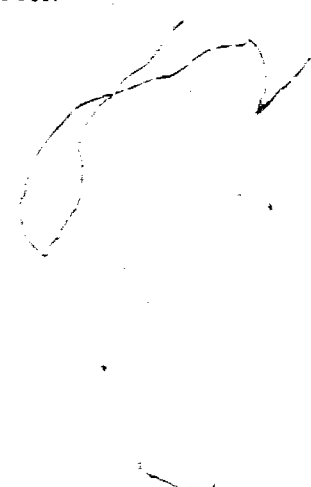
Transmission electron micrographs of proximal and distal interlayers of ovulating oocytes, and epilayers of ovulated and fertilized water-hardened eggs.

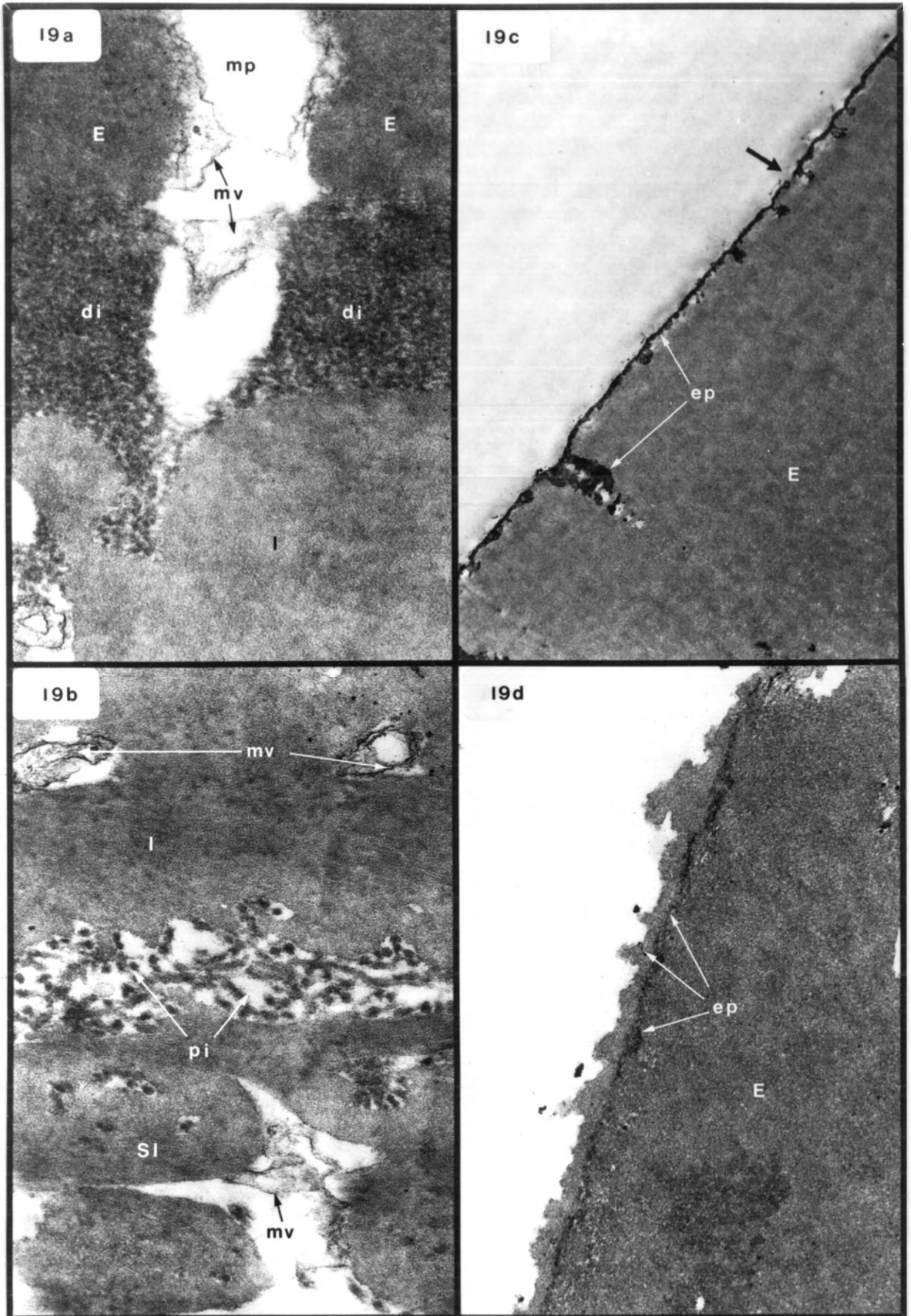
19a. The distal interlayer (**di**) is shown at high magnification to consist of numerous small granules. Also visible in this field are the externus (**E**), internus (**I**), remnants of microvilli (**mv**) and an egg membrane pore (**mp**). (45,000X)

19b. The proximal interlayer (**pi**) is shown in high magnification to consist of fibrous material. Also visible in this section are the internus, sub-internus and microvilli. (45,000X)

19c. This magnified view of the surface of the externus (**E**) shows the epilayer (**ep**) in an ovulated egg. This epilayer is responsible for the formation of the substance that renders spawned herring eggs strongly adhesive. (13,900X)

19d. The epilayer in this section of a fertilized, water-hardened egg has been activated, rendering the egg strongly adhesive. (21,000X)





A fifth layer was the outer-most layer in late vitellogenic oocytes (Figure 17a). Pores in this layer were straight and larger in diameter than those in either the internus or sub-internus. Staining characteristics of this layer differed markedly from those of all other layers. This layer was not laminated and appeared homogenous in structure. The externus appeared unchanged in the ovulating oocyte sections but appeared spongy in sections of fully ovulated eggs (Figure 18a). The spongy texture developed during the latter stages of ovulation. In sections of fertilized eggs (Figure 18b) the spongy texture was not observed.

During the early stages of ovulation, a sixth layer was deposited onto the external surface of the egg (Figure 17b). This epilayer (Figure 19c) appeared flat and compressed in ovulated eggs.

The terms 'sub-internus', 'internus' and 'externus' appear elsewhere in the literature (Groot and Alderdice, 1985), and are appropriately applied to the observed layers in Pacific herring. The terms 'proximal interlayer', 'distal interlayer' and 'epilayer' are used here in their descriptive context and are not considered names for the various layers.

Yolk vesicles were prominent in the peripheral ooplasm in all stages examined except fertilized eggs. Yolk vesicles in ovulated eggs are called cortical alveoli.

STRUCTURAL CHANGES DURING FERTILIZATION

The egg expanded after fertilization on contact with sea water, smoothing both external and internal membrane texture (Figure 20a and 20d). The epilayer was activated (Figure 19d) and the eggs became strongly adhesive. Debris accumulated on the surface of the eggs (Figure 20b) and this accumulated material consisted mostly of sperm cells (Figure 20c). Equatorial sperm diameters were approximately 0.8 μ m.

INTERNUS AND SUB-INTERNUS LAMINATIONS

The number of internus laminations increased from a mean of 7 in late vitellogenic oocytes to 8 in ovulated stages. The number of laminations in the sub-internus increased from a

Figure 20.

Scanning electron micrographs of fertilized, water-hardened eggs.

20a. Three adherent eggs are shown in this view.

20b. An enlargement of the joint between two adherent eggs is shown. Debris (d) is shown adhering to the exterior surface of the externus. There is a particularly heavy accumulation near the joint. These eggs have completed their second hydration. The egg surface is smooth and featureless.

20c. An enlargement of the adherent debris reveals that it consists mostly of sperm cells. The head of each sperm is about 1.2μ in length and 0.8μ in diameter.

20d. A sectional view of a joint between two adherent eggs is shown. The three major layers of the egg membranes are not apparent in this view.

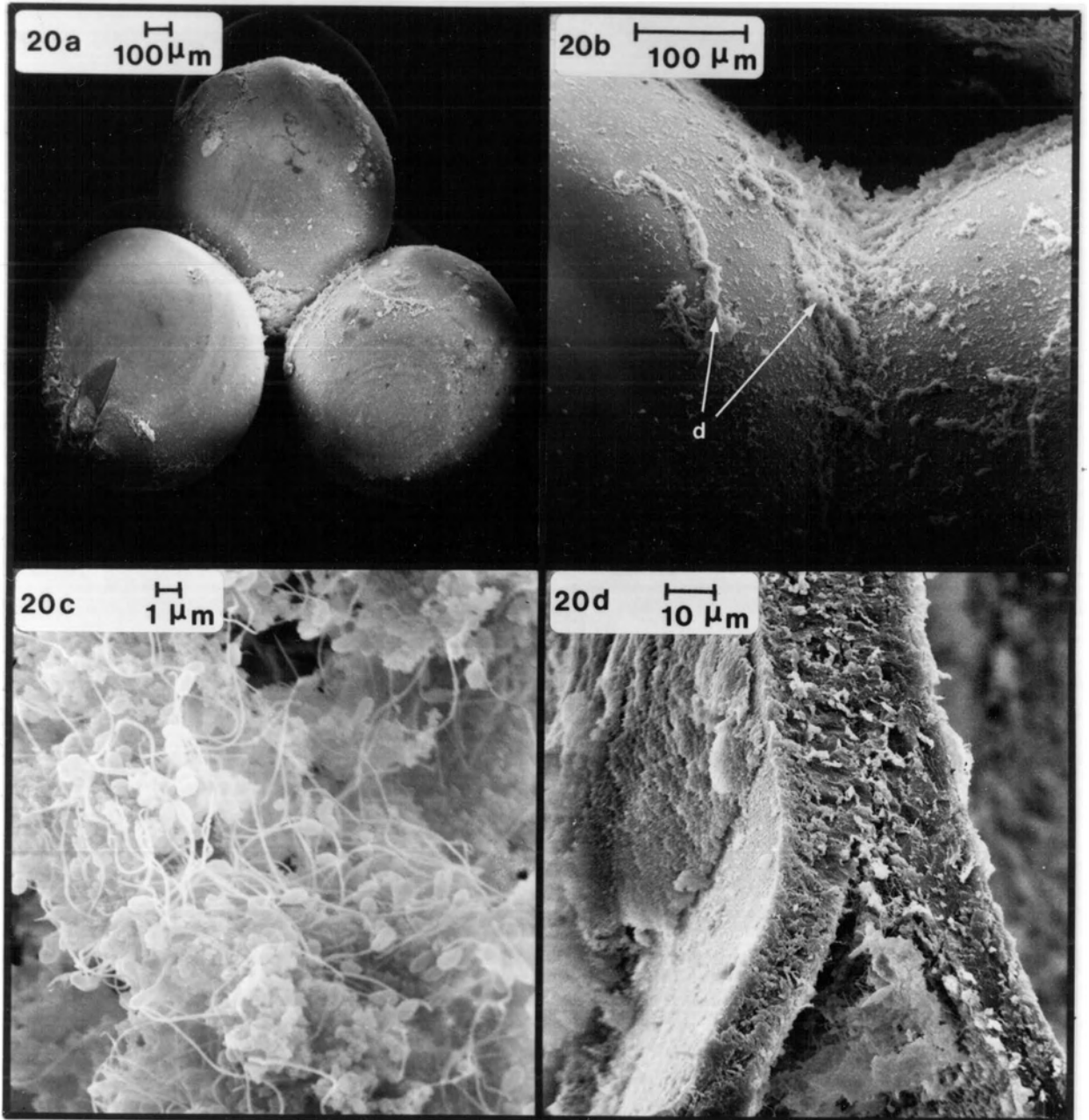


Figure 21.

Transmission electron micrographs of livers from males and females for late vitellogenic, ovulating and ovulated stages. (4,700X)

Female liver sections from late vitellogenic, ovulating and ovulated stages are shown in 21a, 21b and 21c respectively. Sections 21d, 21e and 21f were taken from male livers sampled at the same time as those for females. Female tissues were dominated by electron lucent hepatocytes (Lh) in all stages while male livers were dominated by electron dense hepatocytes (Dh). While vacuoles were found in most male and female livers sectioned, large secretory vacuoles (v) were found only in late vitellogenic female livers. Other structures observable in these sections are nuclei (N), nucleoli (Nu), mitochondria (m), and abundant rough endoplasmic reticulum (Rer).

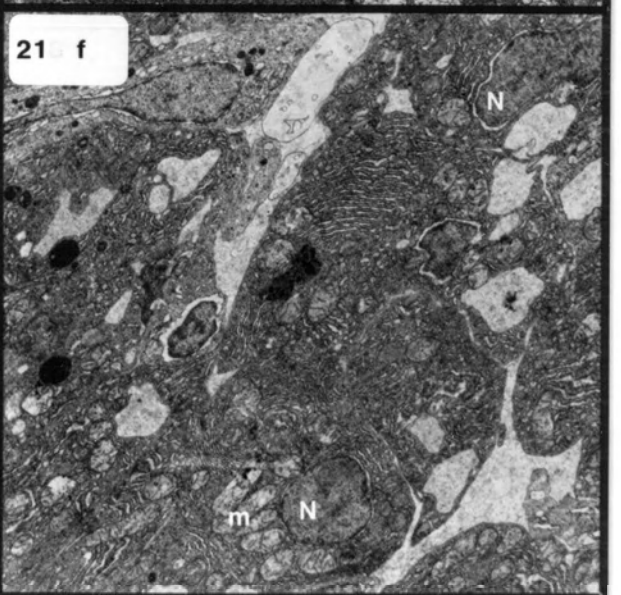
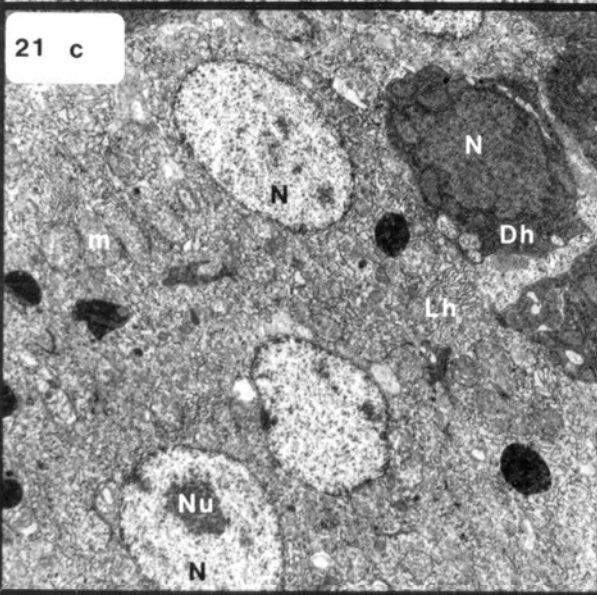
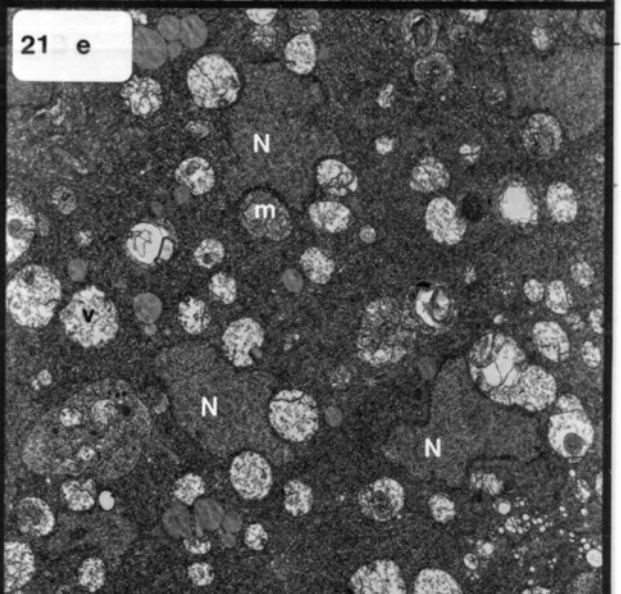
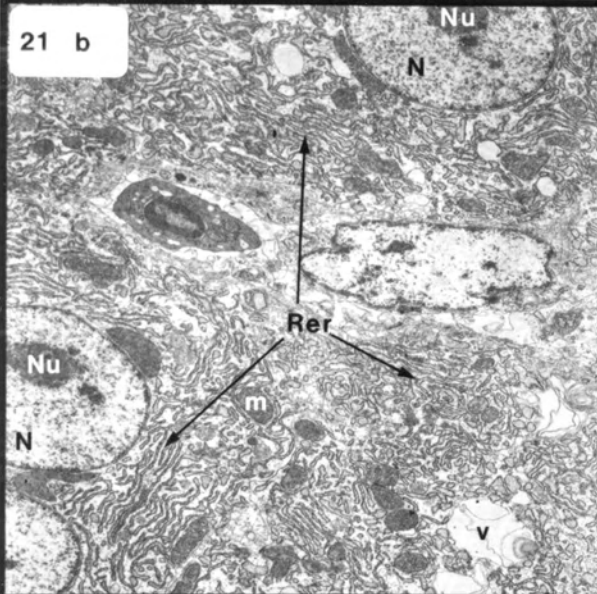
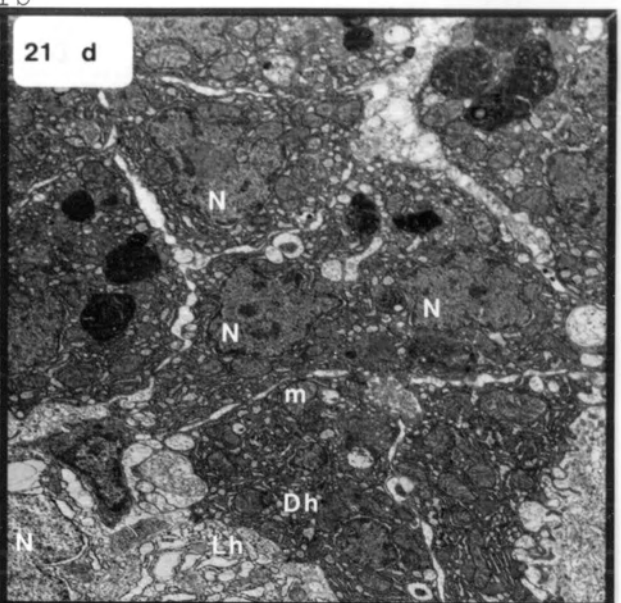
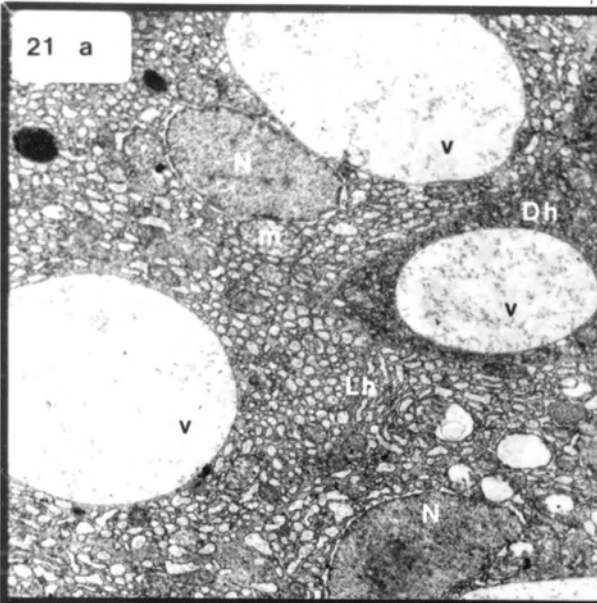
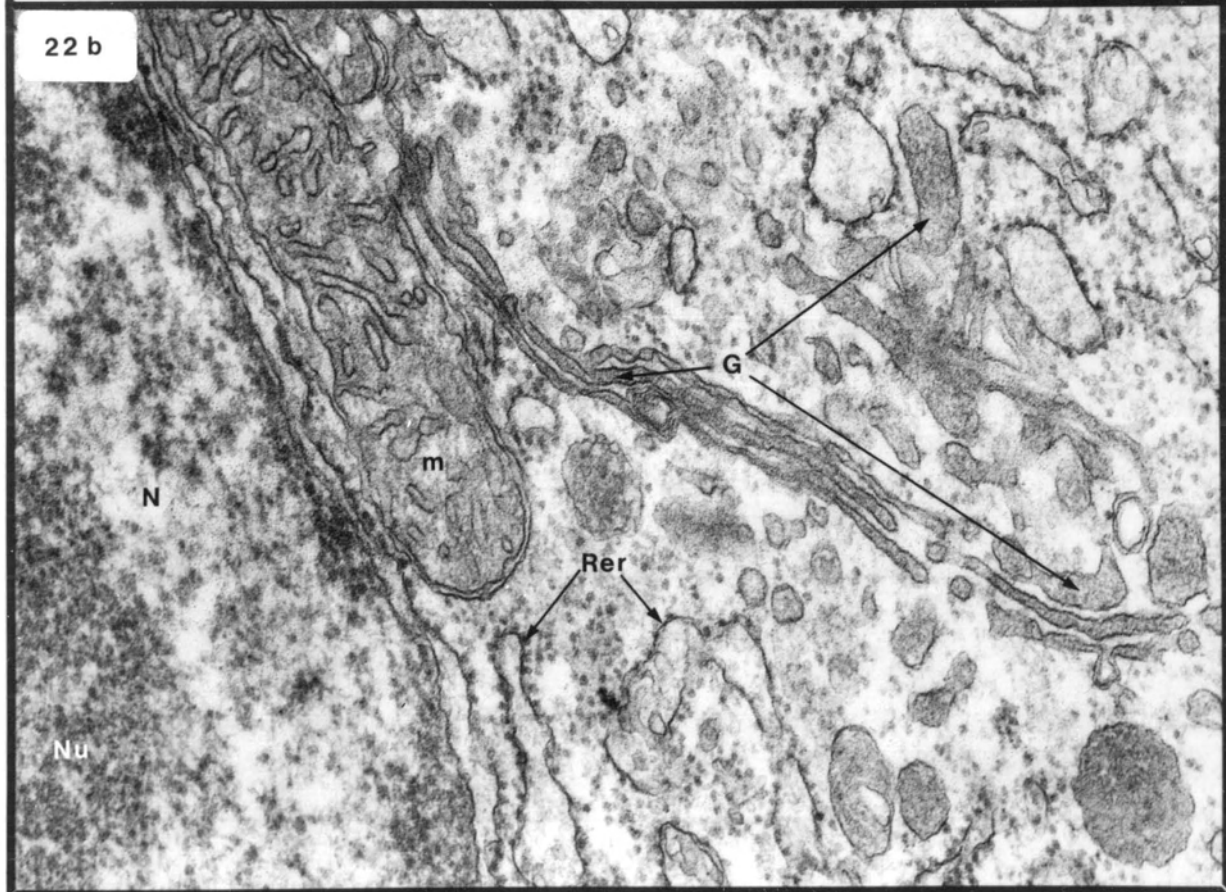
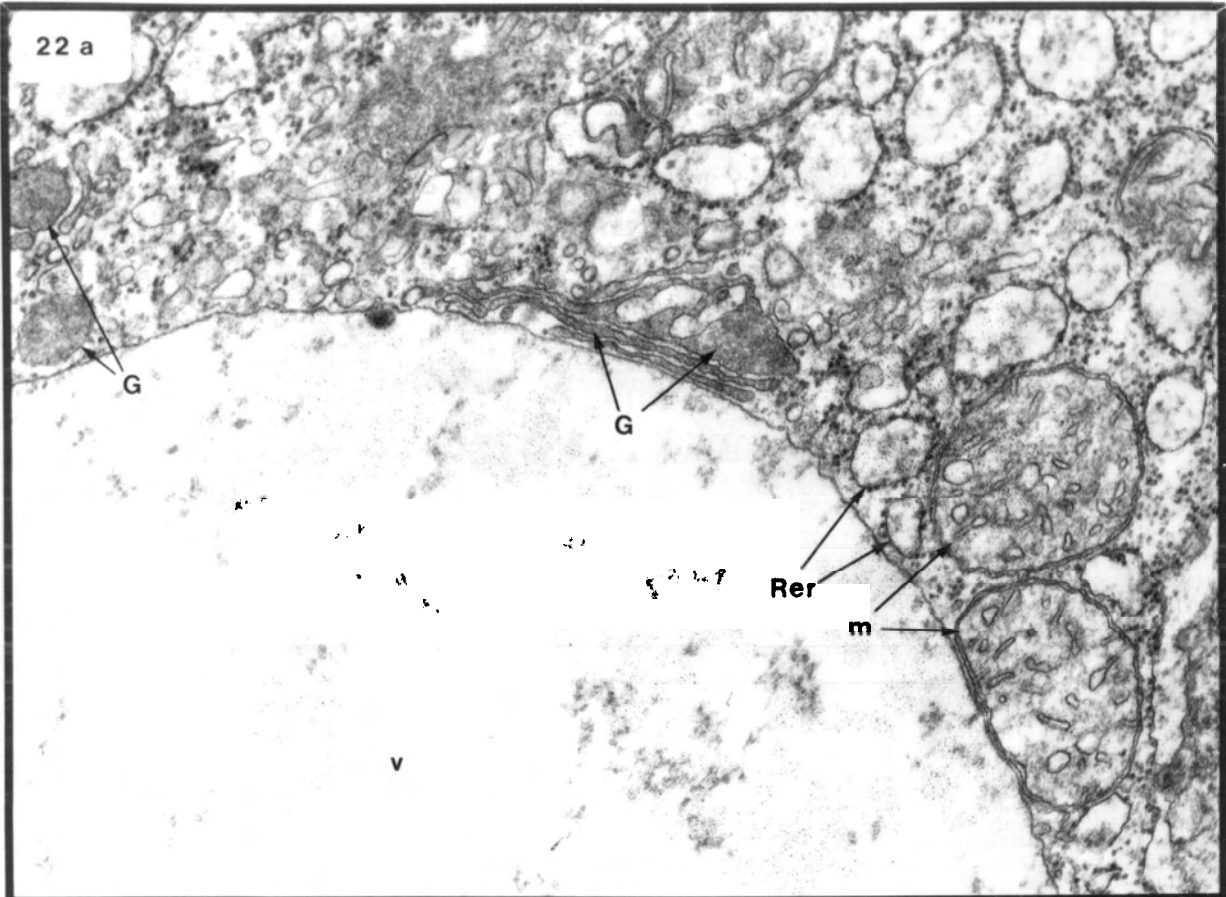


Figure 22.

Transmission electron micrographs of Golgi associations in late vitellogenic and ovulated hepatocytes.

22a. In this section of a hepatocyte from a late vitellogenic liver, the Golgi apparatus (**G**) is associated with a large secretory vacuole (**v**). Golgi bodies at this stage contain clusters of small, electron-dense granules. Also evident in this section are mitochondria (**m**) and tubular rough endoplasmic reticulum (**Rer**) with expanded lumens. (40,000X)

22b. Golgi structures in hepatocytes sectioned from ovulating and ovulated livers were not associated with large secretory vacuoles. Also visible in this section are the nucleus (**N**) and nucleolus (**NU**). (67,000X)



mean of 17 to 19 in late vitellogenic and ovulated stages respectively. In both instances, the presumptive increase was not significant.

HEPATIC CHANGES DURING OVULATION

Liver tissue from both male and female fish contained two distinct types of hepatocytes (Figure 21). Electron-dense hepatocytes generally had abundant but compressed RER and small, poorly-defined Golgi structure. Electron-lucent hepatocytes had abundant RER with dilated profiles and large, well-defined Golgi bodies. The nuclei of both cell types contained one or more nucleoli. Those in female cells were larger, and well-defined. Nuclei from both male and female hepatocytes contained chromatin accumulations immediately beneath the nuclear membranes. Female liver tissue differed from that of males principally in the ratio of electron-lucent to electron-dense hepatocytes. Electron-dense hepatocytes dominated male liver tissue. Female liver tissues were dominated by electron-lucent hepatocytes for all three stages (Figures 21a, 21b and 21c). Female vitellogenic hepatocytes contained large, well-developed vacuoles while ovulating and ovulated hepatocytes contained small, irregular vacuoles. Golgi structures in late vitellogenic hepatocytes were always associated with vacuoles (Figure 22a) while those from ovulated hepatocytes were not associated with vacuoles and were more commonly found near the nucleus (Figure 22b).

Discussion

OOCYTE PROVISIONING

Central to life is the necessity to reproduce. Natural selection favours traits that maximize the production of fit zygotes. Organisms have evolved to adopt two general approaches to the production of zygotes. The first approach assumes that the number of zygotes produced is improved by investing in the largest possible number of adequately provisioned gametes. Because organisms have a finite amount of energy available to invest in gametes, a critical balance must be maintained between gamete numbers and the provisioning afforded to each gamete. Some species have evolved to produce many small eggs. Zygotes produced by this means are generally just adequately provisioned, small, and vulnerable to adverse environmental conditions. Post-zygotic mortality is high.

Other species place larger investment in post-zygotic care. The chance of producing large numbers of fit zygotes is sacrificed for the advantage of assured production of at least a few. The trend for species in this group is to reduce the investment in gametes. The trend is apparent in the seaperch Cymatoqaster aggregata where 5 to 20 eggs are fertilized and incubated internally until several large, fully developed juveniles emerge (Darling et al., 1980). For the rock bass, Ambloplites rupestris about 500 eggs are spawned by the female, fertilized externally in a nest constructed by the male, and incubated and defended in the nest for five days until hatching (Gross and Nowell, 1980). There are exceptions such as Sebastes ruberrimus where fertilization of hundreds of thousands of eggs is internal, as is incubation until hatching (Hart, 1973; pp. 442-443, fecundity of 2.7 million in an 8.7 kg specimen).

Pacific herring produce about twenty thousand or more eggs. After spawning, both males and females leave the spawning area and migrate to offshore summer feeding areas. Zygotes are left to develop unattended. The energy for metabolism and growth required to sustain the zygote until it begins feeding must be contained within their reservoir of yolk.

For Pacific herring the matter of appropriate gamete provisioning is of critical importance. Because Pacific herring cease feeding prior to final gamete maturation (Gillis, 1979), and will not start feeding again until after spawning, the raw materials for oocyte provisioning during late vitellogenesis must come from stored energy.

VITELLOGENESIS IN PACIFIC HERRING

Vitellogenin is an alkali-labile phosphorylated protein found in females and estrogenized males (Emersen and Petersen, 1976; Wallace, 1978; Nath and Sundararaj, 1981). The values reported in Figure 3 for male tissues resulted from non-vitellogenin sources of alkali-labile phosphorus. Hepatocytes have previously been identified as the site of exogenous vitellogenesis in response to 17-beta estradiol secretion in the ovary and the vascular system has been identified as the transportation route from the liver to the ovary (Wallace, 1978; Sundararaj and Nath, 1981; Ng and Idler, 1983). The low ALPP levels in liver tissue (Figure 4) suggested that vitellogenin was secreted into the vascular system very quickly following its synthesis and phosphorylation. Higher ALPP levels in female serum suggest that the sequestering mechanism was less efficient than the hepatocyte secretory mechanism, or that the vascular system acted as a store for vitellogenin reducing the synthetic inhibition that storing vitellogenin in hepatocytes may have generated. Ultrastructural configurations in hepatocytes sampled from late vitellogenic livers were consistent with changes described for zebrafish (Peute et al., 1978) and rainbow trout (van Bohemen et al., 1981). For Pacific herring, electron-lucent rather than electron-dense hepatocytes appear to have been responsible for vitellogenin synthesis. This conclusion was supported by their abundance in female livers during late vitellogenesis; their scarcity in male livers during all stages examined; their abundant dilated RER; and the presence of large secretory vacuoles with which well-developed and large Golgi bodies were associated. Golgi bodies have recently been shown to be the structure that concentrates and secretes vitellogenin in frog hepatocytes (Herbener et al.,

1984).

The site of dephosphorylation of vitellogenin was the layer of follicular cells surrounding each oocyte as indicated by alkaline phosphatase staining results (Figures 5 and 6). Alkaline phosphatase was localized in thecal and granulosa cells. Staining was heaviest in the proximal apex of the granulosa cells and granulosa granules. These findings suggested that the apex of the cell is the site of most of the dephosphorylation of vitellogenin. In addition, granulosa RER was well developed during the late vitellogenic period and appeared closely associated with membrane-bound granulosa granules. There is some evidence that vitellogenin rather than its cleavage products may be incorporated by oocytes (Selman and Wallace, 1983). In Pacific herring however alkaline phosphatase activity in follicular cells and widespread exocytotic activity from the bases of granulosa cells supported the conclusion that at least during late vitellogenesis, vitellogenin was dephosphorylated within the follicular cells.

The site of cleavage of vitellogenin into its constituents (phosvitin and lipovitellin) has not been fully explained in other studies, nor did the present study provided any supplementary insights. Bergink and Wallace (1974) have demonstrated that the conversion of vitellogenin into yolk proteins involves proteolytic splitting in susceptible regions of the molecule. In addition, Inoue *et al.*, (1971) have shown that vitellogenin-derived phosphate is monesterified to serine and that alkali liberation of phosphate includes the loss of serine from the vitellogenin molecule. Liberation of alkali-labile phosphorus may accompany proteolytic cleavage. If this is so then for late vitellogenic Pacific herring, follicular cells become possible sites for the cleavage of vitellogenin into yolk proteins. This speculation is supported by Selman and Wallace's (1983) finding that macromolecules are rapidly incorporated into yolk globules. Also, protein synthetic structures were absent from the ooplasm of Pacific herring. If, however, separate enzymes are responsible for dephosphorylation and proteolytic splitting of

vitellogenin, then the site of the cleavage of vitellogenin into phosvitin and lipovitellin remains speculative.

RER was absent from the ooplasm. Ooplasm mitochondria, when observed, contained dilated cisternae and the space between inner and outer limiting membranes was also dilated. Granular material within the ooplasm consisted of glycogen granules. Oocyte reticulum was always smooth. Concentrically arranged membranes were commonly observed in the ooplasm. Ooplasm organelle configurations and structures are in keeping with those reported for steroid producing tissues (Nagahama et al., 1976), suggesting that for Pacific herring, the ooplasm is a possible site for the synthesis of 17-beta estradiol. Steroid producing ultrastructure was not observed in granulosa cells. Although a few isolated fragments of SER were observed in some thecal cells, steroid producing ultrastructure was absent. Thecal cells specialized for the production of steroids were not observed during this study for Pacific herring.

Yolk proteins are shunted to the microvilli of the oocyte by the granulosa cells by means of micropinocytosis (Droller and Roth, 1966; Anderson, 1968; Shackley and King, 1977). Micropinocytosis was observed in all sections of vitellogenic oocytes in Pacific herring and appears to be associated with the fibrillar network of the granulosa cells.

At the start of ovulation, vitellogenin production ceased and the hepatocytes began to assume their pre-vitellogenic organelle configurations. During this period the gonads of both males and females enlarged markedly. Female gonads enlarged more rapidly than those of males. Two phenomena were associated with this rapid increase for ovaries. Firstly, by comparing the gonadosomatic index for females (Figure 1) with ovarian ALPP levels (Figure 3), it was evident that for the population, some vitellogenesis continued to occur over the period of population ovulation. Because ovarian ALPP levels appeared to reach a minimum at the same time that female gonadosomatic indices reached a maximum, it was concluded that for an individual female herring, vitellogenesis ceases near the time of

the onset of ovulation. This conclusion was supported by ultrastructural evidence. The granulosa cells became detached from the externus and began to degrade early in the process of ovulation. If vitellogenesis continued during the early stages of ovulation, then it would be expected that ALPP levels would stay at the higher values beyond the time when all of the females in the population had ovulated. Secondly, moisture uptake during the first hydration accounted for most of the increase (discussed subsequently).

There remains the question of the fate of the phosphate liberated from vitellogenin in the follicular cells. From the work of Mano and Lipman (1966a and 1966b), Inoue *et al.*, (1971) and Craik (1982) it is apparent that vitellogenin arrives at the oocyte with more phosphorus than can be accounted for in yolk phosphatins and lipovitellin. This is especially true for marine spawning teleosts which have less yolk phosphorus than fresh-water spawning teleosts by a factor of 10 (Craik, 1982). These authors offered some speculation on the fate of the residual phosphate. Some of the phosphate is evidently used for the phosphorylation of phosphatins before incorporation into yolk reserves (Mano and Lipman, 1966b). In addition, some of the residual phosphate may be associated with the first hydration. The evidence for this contention came from the present study. Presumably, the osmotic pressure within the ovary during the first hydration was similar to that of the surrounding water. The process of the first hydration was therefore presumed to be energy consuming. Some of the residual phosphate may have been associated with water uptake during the first hydration. Other energy consuming processes that occurred during ovulation were the degradation of atretic oocytes, follicles and stroma. Some of the phosphate may have been associated with these processes (Wallace, 1978; Wallace and Selman, 1981). Pacific herring eggs dehydrated on contact with sea water (in the absence of cortical alveolar release of colloids), therefore Craik's (1982) contention that residual phosphate may be associated with the massive water uptake during the second hydration is not supported by the findings of this study.

Craik (1982) also contends that phosphate reserves are not required in marine-spawning teleosts because most essential ions are readily available from sea-water, particularly in coastal environments. His contention is supported by the presence in spawned echinoid eggs of a phosphate active transport mechanism (Brookbank, 1978). It is expected that similar mechanisms may occur in marine-spawning teleosts such as Pacific herring.

OVULATION HYDRATION

Late vitellogenesis may account for some of the weight increase in female ovaries during ovulation but the first, or ovulation hydration accounts for most of the increase. By comparing female gonadosomatic indices with ovarian moisture levels it was evident that increases in ovarian moisture closely paralleled increases in the gonadosomatic index. The gonadosomatic index for females and egg moisture were positively correlated ($n = 170$; $r = 0.68$; $P < 0.01$). It was therefore concluded that vitellogenesis is a relatively constant phenomenon and that the acceleration in the rate of increase in GSI evident in this study and those of Brett and Solmie (1982) and Kreiberg et al., (1982), resulted mostly from moisture uptake. This conclusion was supported by ultrastructural changes in female electron-lucent hepatocytes which regressed during ovulation and by the negative correlation between female hepatosomatic and gonadosomatic indices ($n = 170$; $r = -0.60$; $P < 0.01$).

Results of TEM investigations suggested the time of the ovulation hydration. The swelling accompanying hydration tends to smooth egg surface texture. By comparing the surface texture of ovulating eggs (Figure 12c) with that of ovulated eggs (Figures 13b and 13c) it was concluded that hydration may have begun at the time when the oocyte started to emerge from the follicle, but did not run to completion until the eggs had fully emerged.

FERTILIZATION HYDRATION

The second egg hydration accompanied fertilization and water-hardening. Hart and Yu (1980) have shown that this hydration is caused by the high osmotic pressure associated

with the release of colloids from the cortical alveoli immediately after fertilization. This process was demonstrated in the present study. Interestingly, unfertilized eggs dehydrated on exposure to sea water. Additional experimentation over a wider salinity range than that used in the present study will be required to investigate correlation between hydration/dehydration of fertilized/unfertilized eggs.

EGG MEMBRANES

The structure of the Pacific herring oocyte membrane differs from those reported elsewhere. Dumont and Brummet (1980) report 9 strata in the egg membrane of Fundulus heteroclitus, but many of these appear to be similar to the laminations of the Pacific herring internus and sub-internus. Groot and Alderdice (1985) report three layers in salmonid eggs. These appear to be analogous to the three major layers of herring egg membranes. For this reason, Groot and Alderdice's terminology (externus, internus and sub-internus) have been used to describe Pacific herring oocyte and egg membranes. The proximal and distal interlayers (descriptive terminology) do not appear to have been described elsewhere. Names for these layers should await some elucidation concerning their function(s).

Light and electron microscopy have suggested the origins of the egg membrane layers. Similar staining characteristics between the atretic oocyte membrane and the internus and sub-internus of vitellogenic oocytes suggested that the latter two layers were secreted by the oocyte. These observations support Anderson's (1967) conclusions. The proximal interlayer lies between the internus and sub-internus and must therefore also have been secreted by the oocyte. The marked difference in staining characteristics between the internae and the externus, and the presence of externus material on the basal sides of granulosa cells suggested that the externus was secreted by the granulosa cells. Histological studies of early oocyte development will be required to confirm the origins of these layers.

During the early stages of ovulation an additional (sixth) layer was deposited on the external surface of the externus. This electron-dense layer (Figure 20b and 22c) was similar to a thin layer identified in salmonid eggs by Groot and Alderdice (1985). They concluded that this layer that they called externus caused the transition from a porous oocyte membrane to a semi-permeable one. Their conclusion was supported by the appearance of annulated plugs in the external openings of the membrane pores. In Pacific herring eggs the thin epilayer was not associated with plugs or other occluding mechanisms. Discontinuities in the epilayer were frequently observed. Surface examination of the externus revealed that the pores were still open during ovulation (Figure 17a).

Changes occurred in the thin epilayer on contact with sea water. The electron-dense epilayer expanded. This process accompanied the development of the adhesive nature of herring eggs. Because of the location of the epilayer, the concurrence of the development of adhesiveness and the structural changes in the epilayer, it was concluded that the expanded epilayer constituted the adhesive substance. The adhesive substance stained PAS negative. However, the externus in the vicinity of the epilayer stains PAS positive in some sections. These observations suggested that the activated layer itself was proteinaceous, but that the unactivated epilayer was mucosaccharide in nature. TEM observation of fertilized water-hardened egg revealed that the activation of the epilayer resulted in the adhesion of debris to the surface of the externus (Figure 14). Closer examination of this debris revealed that it was comprised almost entirely of sperm cells. These eggs were fertilized in a beaker in the laboratory. Sperm densities were much higher than those anticipated for natural spawning waters. For wild spawned eggs, it is less likely that large accumulations of sperm would occur.

EGG SEMI-PERMEABILITY

It was concluded from this study that the achievement of semi-permeability in the Pacific

herring egg occurred in the internus. Semi-permeability was achieved during the first hydration that accompanied ovulation and occurred as follows. During vitellogenesis, the pores in the internus took indirect, possibly helical paths through the internus (Figures 20a 20b and 22b). During the first hydration following expulsion from the follicle, the egg swelled slightly, stretching and compressing the total egg membrane. In response to this stretching and compression, the pores in the internus collapsed, probably rendering the membrane semi-permeable. This suggestion was supported by the following evidence. First, the pores of the internus were fully open in late vitellogenic and ovulating oocytes, but fully occluded in all ovulated eggs examined. Second, semi-permeability was indirectly demonstrated twice in the course of this study, during the process of paraffin infiltration for LM and infiltration with epoxy resin for TEM. In both instances vitellogenic and ovulating oocytes infiltrated easily indicating that the porous structure of the oocyte membrane was still open. The relatively large paraffin and epoxy molecules moved easily into the oocyte through the pores as the transition vehicle diffused out. Infiltration of ovulated eggs by both media occurred very slowly. While the relatively light transitional vehicles diffused out of the egg, the large molecules of paraffin and epoxy diffused in less readily. The eggs shrivelled. Epoxy infiltrating eggs frequently popped, or resumed their former dimensions in an explosive (audible) manner. Evidently, the pressure inside the egg was so reduced that the vapour pressure of the transition vehicle (propylene oxide) was reached resulting in a violent 'pop'. If the epilayer was responsible for the achievement of semi-permeability, then infiltration difficulties would have been encountered in ovulating as well as ovulated eggs.

However, if we assume that an increase in moisture content produced an identical increase in egg volume then the calculated increase in diameter for a 10% increase in moisture was only 3%. This increase seemed (intuitively) to be insufficient to collapse the pore canals in the internus. Additional changes that accompanied late ovulation were the

development of the spongy texture in the externus and the merging of yolk globules to form larger ones. The former event may have facilitated the expansion of the internus in response to water uptake and chemical changes associated with changes in internal egg structure may have resulted in an increase in egg diameter larger than that calculated. Further experimentation, including the accurate measurement of egg diameter increases that accompany ovulation, will be required to further elucidate the transition to semi-permeability.

POLYSPERMY

External and internal micropyle diameters, and sperm equatorial diameters were 1.2, 0.5 and $0.8\mu\text{m}$ respectively. Although sperm penetration was not observed during this study, it was apparent that sperm cells entering the micropyle canal would become lodged and held fast by the annular ridges, rendering the passage impassable to other sperm. The lodged sperm cell would be required to donate its DNA from its within the canal. This mechanism may be partly responsible for the preclusion of polyspermy.

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