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Developmental Fate of the Yolk Protein Lipovitellin in Embryos and Larvae of Winter Flounder, *Pleuronectes americanus*

RUTH C. HARTLING* AND JOSEPH G. KUNKEL
Program in Molecular and Cellular Biology, University of Massachusetts, Amherst, Massachusetts 01003

ABSTRACT The developmental fate of the vitellogenin-derived yolk protein, lipovitellin (Lv), was investigated in winter flounder embryos and yolk-sac larvae. Since Lv is present as only one major polypeptide in ovulated winter flounder eggs, unlike the multiple yolk polypeptides found in the mature eggs of most teleosts, this system is presented as a simpler model of yolk protein structure and utilization during teleostean development. Winter flounder Lv is cleaved during embryogenesis from a 94 kD polypeptide at fertilization to 67 kD and 26 kD polypeptides at hatching. The rate of this proteolytic processing is slow during early embryonic development, but enters a more rapid phase between days 8 and 12 post-fertilization in embryos reared at 4–5°C, and approaches 50% completion at day 10. Lv processing is essentially complete 3 days before hatching; nevertheless, major degradation of the Lv peptide by the developing winter flounder does not occur until after hatching. The Stokes radius of Lv changes only moderately following processing, from 4.50 nm in unfertilized eggs to 4.19 nm in late embryos and newly hatched larvae, whereas the processed Lv retains its heat stability relative to other yolk polypeptides. Nearly 50% of its lipid content, however, is released from the Lv particle during embryogenesis, concomitant with cleavage of the Lv 94 kD polypeptide. Lv processing may thus render a portion of the yolk protein-associated lipid more accessible to the developing embryo, whereas other yolk components are retained for later use by the winter flounder larva. Alternately, removal of lipid may lead to proteolytic vulnerability of the Lv polypeptide. In either case, only a portion of the lipid moiety of the Lv particle appears to play a significant nutritive role for the embryo, whereas its protein component is reserved for larval use. *J. Exp. Zool. 284:686–695, 1999.*

The majority of the yolk resources available to most oviparous animals is derived from vitellogenin (Vg), a large glycophospholipoprotein. In fishes, as in other vertebrates, Vg is hepatically synthesized and transported via the bloodstream to the ovaries, where it is rapidly and specifically taken up from the blood into maturing oocytes (Selman and Wallace, ’82, ’83; Tyler et al., ’88a,b, ’90, ’91; Kanungo et al., ’90; Tyler, ’93) by receptor-mediated endocytosis (Opresko and Wiley, ’87; Stifani et al., ’90). Within oocytes, Vg is proteolytically cleaved to form smaller yolk proteins (de Vlaming et al., ’80; Wallace and Begovak, ’85; Wallace and Selman, ’85; Tyler, ’93; Johanning and Specker, ’95; Matsubara and Sawano, ’95), of which the largest and most abundant, lipovitellin (Lv), contains the lipid-binding region of Vg (reviewed by Banaszak et al., ’91). Piscine oocytes show a remarkable degree of variation from species to species in the size and in the lipid and phosphorus content of yolk proteins (reviewed by Mommsen and Walsh, ’88). Such variability is further compounded in many teleosts by the additional proteolytic processing of yolk proteins late in oocyte maturation (Wallace and Begovac, ’85; Wallace and Selman, ’85; Greeley et al., ’86; Cerda et al., ’96; Thorsen and Fyhn, ’96). This phenomenon is more pronounced in marine fishes, and may help drive oocyte hydration in some (Wallace and Selman, ’85; Greeley et al., ’86; Carnevali et al., ’93; Cerda et al., ’96; Thorsen and Fyhn, ’96). The oocytes of marine, but not freshwater, fishes have been reported to decrease in protein phosphorus content in the course of maturation, and this process may be functionally tied to oocyte hydration (Craik, ’82; Craik and Harvey, ’84). Hydrolysis of yolk protein into free amino acids during final oocyte maturation also creates os-

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*Correspondence to: Ruth C. Hartling, Biology Department, Northland College, Ashland, WI 54806. E-mail: rhartlin@wheeler.northland.edu
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motic potential to drive oocyte hydration in marine pelagic eggs (reviewed by Fyhn, '93; Ronnestad and Fyhn, '93), although K^+ influx into maturing oocytes may be another important factor (Craik and Harvey, '84; LaFleur and Thomas, '91), particularly for the hydration of marine demersal eggs (McPherson et al., '89).

Although the proteolytic processing of ovarian yolk proteins has been documented among a growing list of teleosts, very little information is available regarding the fate of the Vg-derived proteins during embryonic and larval development. In this study, we have investigated the developmental fate of the yolk protein Lv in the demersal eggs of winter flounder, since only one major Lv polypeptide is present following ovulation in this species (Hartling et al., '97). The relative simplicity of winter flounder Lv suggests its use as a model system to investigate the fate of yolk protein during teleostean development. We have thus elucidated the further proteolytic processing of Lv in winter flounder embryos, characterized the protein before and after this processing event, and defined the timing of Lv depletion in yolk-sac larvae.

MATERIALS AND METHODS

Collection of eggs and rearing of embryos and larvae

Adult winter flounder were collected by otter trawl off the coast near Milford, CT, in March of 1994, 1995, and 1996 by members of the Milford Laboratory, National Marine Fisheries Service. Male and female winter flounder were maintained in separate tanks at the Milford Laboratory in flowing, ambient seawater (0–4°C) for up to 2 weeks. Eggs were stripped from ripe females; the eggs of each female were mixed with diatomaceous earth to prevent clumping (Smigielski and Arnold, '72), and fertilized with the combined milt of three males. Fertilized and unfertilized eggs were transported to the University of Massachusetts, Amherst on ice; unfertilized eggs were frozen at –20°C until use. Fertilized eggs were incubated at 4–5°C (in 1994), 5–6°C (in 1995), or 4.5–5.5°C (in 1996) in sand-filtered seawater containing 50 ppm each of penicillin G and streptomycin sulfate (Sigma, St. Louis, MO). Eggs were suspended in custom-made bags of Nytex netting (250 μm mesh) in 10-gallon tanks. Approximately 3000 eggs of a single female were placed in each bag; the tanks contained 1–2 ml seawater per egg. Dead material, when present, was removed on a daily basis. Embryos were incubated through hatching, and larvae were maintained in Nytex bags with no addition of exogenous food.

Preparation of egg extract and Lv standards

Yolk proteins were extracted from frozen unfertilized eggs by homogenization in ice-cold column buffer (0.4 M KCl, 0.05 M K_2 PO_4, pH 7.2), with 2 mM phenylmethylsulfonyl fluoride (PMSF, Sigma) added immediately before homogenization, using 4 μl buffer per mg of eggs. Yolk proteins from embryos and larvae were prepared by homogenizing 10 individuals in 125 μl of 2 mM PMSF. (Extracts intended for SDS-PAGE and densitometry included 0.2 mg/ml ovalbumin as an internal densitometric standard.) The homogenate was then diluted with 125 μl of ice-cold 2× column buffer. Insoluble material was removed from the homogenates by centrifugation at 3500*g* for 15 min at 4°C. In some cases, large-scale yolk protein extracts from cultures of embryos and larvae were made as described above, except that the homogenizations were performed using 4 μl column buffer (containing 2 mM PMSF) per mg embryos and larvae. Samples were kept at 4°C or on ice and either used within 24 hr of preparation or flash-frozen in liquid nitrogen and stored at –75°C for later use.

Lv standards for electrophoresis and immunoassays were prepared by gel permeation chromatography of unfertilized egg extracts as described (Hartling et al., '97), and were flash-frozen in liquid nitrogen and stored at –75°C until use. Determination of protein concentration was performed by the Bradford assay, modified for use on microtiter plates (Redinbaugh and Campbell, '85), using winter flounder Lv (determined by anhydrous weight) as a standard.

Heat treatment of egg extracts

Extracts prepared from unfertilized eggs and from day 16 embryos (1994 culture) were placed in an 85°C water bath for 7 min, then rapidly cooled in an ethanol/ice bath. The resulting precipitates were removed by centrifugation at 4°C for 15 min at 3500*g*, and the supernatants were examined by SDS-PAGE.

Gel permeation chromatography

Samples were applied to a Bio-Gel A-1.5 column (Bio-Rad, Hercules, CA) along with 5 mg blue dextran (Pharmacia Biotech, Uppsala, Sweden) and 7 mg potassium hydrogen phthalate (Fisher, Pittsburgh, PA) as void volume and total volume mark-

ers, respectively. Yolk extract prepared from unfertilized eggs was dialyzed (molecular weight cutoff = 30 kD) against column buffer containing 1 mM PMSF prior to column application in order to remove low molecular weight material which otherwise obscured the total volume peak. Standards of known Stokes radii (Sigma) were carbonic anhydrase (2.01 nm), ovalbumin (2.83 nm), bovine serum albumin (3.62 nm), alcohol dehydrogenase (4.55 nm), and thyroglobulin (8.6 nm). The column was run and column partition coefficients were determined as previously described (Hartling et al., '97).

Polyacrylamide gel electrophoresis and densitometry

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli ('70). Gels (100 × 80 × 1.5 mm) consisting of 10% and 4% acrylamide (10.2:1 acrylamide:bis) for the separating and stacking gels, respectively, were cast and run using a Bio-Rad Mini-PROTEAN II cell. Molecular weight standards (Bio-Rad) were phosphorylase B (97.4 kD), bovine serum albumin (66.2 kD), ovalbumin (45.0 kD), carbonic anhydrase (31.0 kD), trypsin inhibitor (21.5 kD), and lysozyme (14.4 kD). Proteins were resolved at constant voltage (150 V). Gels were stained with Coomassie Blue R-250 (Fisher) in 40% methanol, 10% acetic acid and destained in 40% methanol, 10% acetic acid. Densitometry of Coomassie blue-stained bands was performed by analysis of gel images taken by a video camera (MTI CCD72), using a Matrox PIP-640 frame buffer board to grab the image. The locations of Lv bands at 67 kD and 94 kD were determined for each lane using molecular weight standards, and densitometric integration of these bands was performed using custom software. The absorbance of a Coomassie-blue-stained Lv standard was found to be linear under these conditions between 0.5 μg and 8.0 μg ($r^2 = 0.974$). The calculated densitometric values were expressed as ratios of the 67 kD Lv band to the sum of the 67 kD and 94 kD Lv bands.

Immunoassays

Western blot analysis of proteins resolved by SDS-PAGE was performed as previously described, using a polyclonal antiserum raised against heat-stable (94 kD) Lv from unfertilized winter flounder eggs as the primary antibody (Hartling et al., '97).

A homologous enzyme-linked immunosorbant assay (ELISA) utilizing this antiserum was performed as described (Hartling et al., '97). Briefly, 100-μl aliquots of samples diluted in phosphate-buffered saline (PBS; 0.15 M NaCl, 0.10 M NaH_2PO_4, 0.05% EDTA, pH 7.0) were blotted onto a nitrocellulose membrane (Bio-Rad Dot Blot apparatus). The membrane was removed from the dot blot apparatus, blocked with 3% gelatin in PBS, and developed using anti-Lv primary antibody, horseradish peroxidase-conjugated secondary antibody (Bio-Rad), and 4-chloro-1-naphthol (Bio-Rad). Densitometric quantification was achieved by analysis of images taken by video camera as described above; integration of the individual dots was performed using custom software. Lv standards for this assay were prepared by Bio-Gel A-1.5 chromatography of winter flounder egg extracts (Hartling et al., '97), and were determined by weight.

Double immunodiffusion was adapted from the method of Ouchterlony ('49) for use on a microscope slide (Kunkel and Lawler, '74). Six wells for the antigen samples were cut in a 1% agarose gel at equidistance around a central well containing the anti-lipovitellin antiserum described above. The agarose gel was incubated for 52 hr in a humid chamber (to prevent gel desiccation) at 4°C while precipitin lines were allowed to form. The gel was then washed with several exchanges of PBS at 4°C; dried; stained with 0.5% Thiazine Red R (Allied Chemical) in 10% acetic acid, 40% methanol; and destained with 10% acetic acid, 40% methanol.

Lipid analysis

Quantitative lipid determination was performed by an adaptation of the procedure of Wallace ('65). This technique employed the lipid extraction method of Bligh and Dyer ('59) to assess the percent lipid content of avian and amphibian yolk proteins. In the current study, lipids were extracted from winter flounder Lv prepared by Bio-Gel A-1.5 chromatography as described above. Lv for this assay was isolated from 13.5-hr embryos (1995 cultures) or from late embryos and larvae (1995 cultures at approximately 40% hatch). One milliliter of Lv in column buffer was placed in a flask with 1.25 ml chloroform and 2.50 ml methanol. The solution, which formed a single layer, was thoroughly mixed, and an additional 1.25 ml of chloroform was added. After further mixing, two phases were allowed to form, with the precipitated protein layered between the aqueous and organic phases. The organic phase was removed by pipette to a separate flask, and the aqueous phase and

protein layers were subjected to two additional extractions with water-equilibrated chloroform/methanol (1:1). The three organic fractions were pooled and evaporated to dryness under vacuum. The resulting organic residue was extracted with 1 ml chloroform/methanol (9:1). Insoluble material was removed by brief centrifugation, and the chloroform and methanol were evaporated to dryness under vacuum.

The aqueous phase was removed by pipette from the precipitated protein, and the protein was washed with n-butanol, then with methanol, and finally with diethyl ether. Both the protein and the lipid residues were prepared for dry weight determination by baking at 65°C for 16 hr under vacuum.

One-dimensional thin layer chromatography of the lipid fraction was performed according to Murakami et al. ('91) in chloroform/methanol/water (65:25:4). Standards (Sigma) included phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, sphingomyelin, cholesterol, and triglyceride (trilinolein). Lipids were visualized with iodine vapor, ninhydrin spray (0.2% in ethanol/acetone, 1:1), or phosphomolybdenum blue spray (Vaskovsky et al., '75).

RESULTS

Proteolytic cleavage of Lv during embryonic development

We examined the fate of Lv over the course of winter flounder embryonic development by SDS-PAGE (Fig. 1A). Electrophoresis of extracts of unfertilized winter flounder eggs reveals a predominant 94 kD Lv polypeptide, along with several minor bands. By day 10 following fertilization, however, a decline in the amount of this polypeptide is accompanied by a concomitant rise in the amount of a 67 kD polypeptide and the appearance of a faint 26 kD band. At day 15 following fertilization, when approximately half of the winter flounder culture has hatched, the 67 kD yolk polypeptide is predominant, and the 94 kD polypeptide has nearly disappeared. A Western blot of extract from day 10 embryos probed with a polyclonal antiserum raised against Lv from unfertilized eggs demonstrates that both the 67 kD and the 26 kD polypeptides are derived from the 94 kD Lv polypeptide (Fig. 1B). Two other bands visible on the blot (60 kD and at the gel front) are also present in extracts of unfertilized eggs (Fig. 1A); together with the 67 kD and 26 kD polypeptides, they cross-react with a portion of the anti-Lv antiserum which was affinity-purified against the 94 kD polypeptide (data not shown). In addition, Ouchterlony double immunodiffusion using this antiserum reveals immunologic identity between Lv from unfertilized eggs and from embryos at day 10 and day 15 after fertilization (Fig. 2).

In order to elucidate the timing of this proteolytic event during winter flounder embryonic development, we employed densitometric analysis of SDS-PAGE to quantify the relative amounts of the 94 kD and 67 kD bands in embryo extracts of flounder raised in the 1994 season (Fig. 3A). Lv

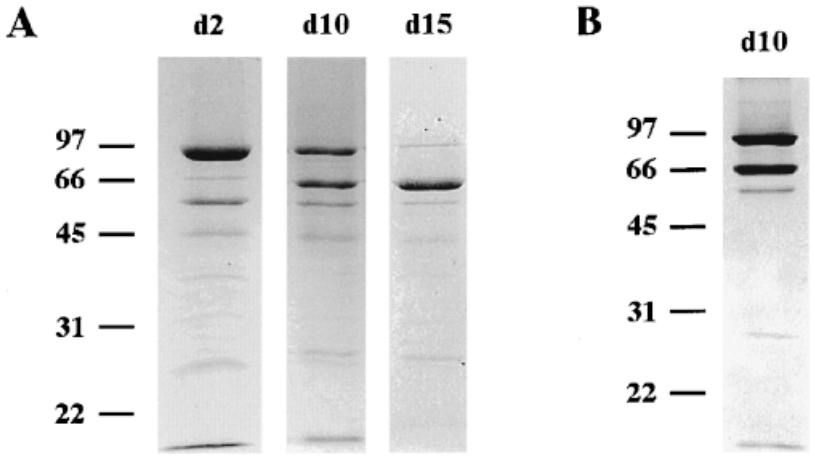

Fig. 1. Proteolytic processing of Lv during winter flounder development. (**A**) SDS-PAGE of whole extracts of embryos (1994 cultures). Extracts were prepared at: d2, day 2; d10, day 10; and d15, day 15 after fertilization; 50% hatch was at day 18. Ovalbumin (45 kD) was added to each extract as a densitometric standard. (**B**) Western blot of whole extract of day 10 embryos (1996 culture), using an antiserum raised against Lv from unfertilized eggs. Molecular weight standards in kD are indicated.

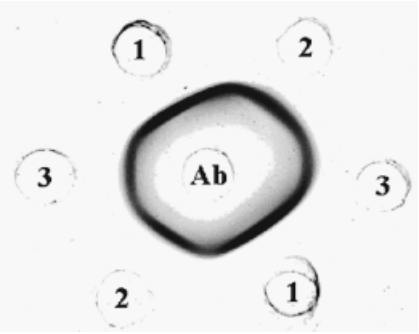

Fig. 2. Ouchterlony double immunodiffusion using a polyclonal antiserum raised against Lv from unfertilized eggs (Ab). 1, Unfertilized egg extract; 2, day 10 embryo extract; 3, day15 embryo extract (1994 cultures).

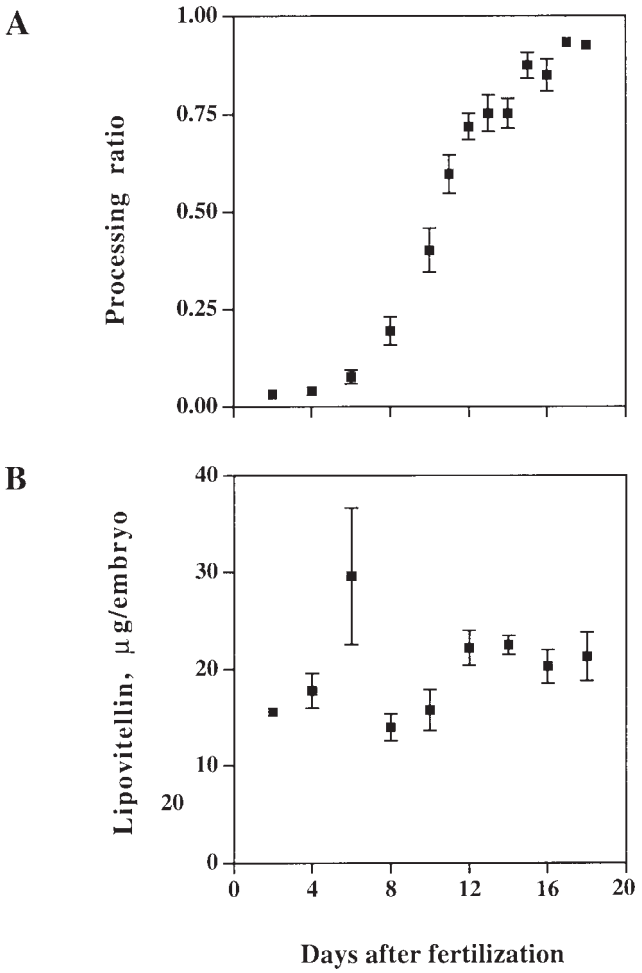

Fig. 3. Lv content in winter flounder embryos (1994 cultures). (**A**) Time course of Lv processing, expressed as the densitometric ratio of 67 kD Lv to the sum of 94 kD and 67 kD Lv on SDS-PAGE of whole embryo extracts. Each point represents the mean of three extracts of 10 embryos each, where each extract comprises the offspring of a different female. (**B**) Total Lv content in winter flounder embryos, determined by ELISA. Each point represents the mean of the same embryo extracts used in (A).

processing takes place slowly during the first few days of embryonic development, then occurs at a rapid rate on days 8–12, approaching 50% completion on day 10 (tail-bud stage). Lv processing then continues at a slower rate, nearing complete processing of the 94 kD polypeptide by day 15.

Lv utilization

In order to determine the relationship of Lv processing to its utilization by developing winter flounder, we used ELISA to quantify the amount of Lv in extracts of embryos and larvae raised in the 1994 season. This immunoassay (Hartling et al., '97) specifically quantifies Lv using the anti-Lv antiserum mentioned previously (Figs. 1B and 2). Using this technique, we found that the amount of Lv in winter flounder eggs does not decline significantly during embryonic development (Fig. 3B). However, the amount of Lv in yolk-sac larvae decreases rapidly following hatching (Fig. 4), when Lv processing has been essentially complete for several days.

Stability of Lv following proteolytic cleavage

As described previously (Hartling et al., '97), the 94 kD polypeptide of Lv is heat stable relative to

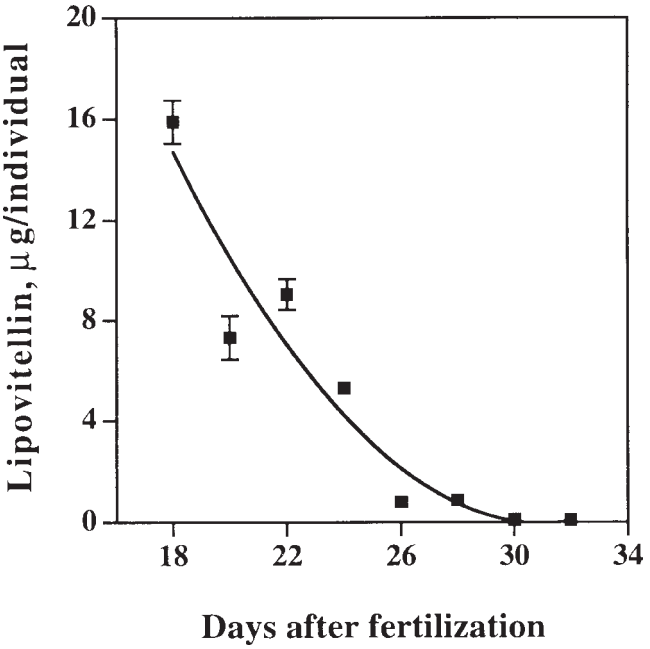

Fig. 4. Lv utilization by winter flounder larvae (1994 culture). Total Lv content per individual was determined by ELISA of extracts of 10 larvae each, except day 18, which was an extract of 10 embryos (culture at approximately 50% hatch). Each point represents the mean of four replicates of a single extract and represents the offspring of one female.

most other polypeptides present in extracts of winter flounder unfertilized eggs. Since these other polypeptides are immunologically related to the 94 kD polypeptide and appear to be fragments of Lv produced by limited proteolytic cleavage (Hartling et al., '97), we investigated the heat-stability of the 67 kD and 26 kD polypeptides by heating late embryo extract to 85°C for 7 min and examining the soluble fraction by SDS-PAGE (Fig. 5). Like Lv from unfertilized eggs, the processed Lv also displays heat stability under these conditions.

We also investigated to what extent Lv processing alters the overall size of the molecule. Figure 6 depicts the Stokes radius of Lv from unfertilized eggs and from day 16 embryos. The processed Lv exhibits a moderate shift in Stokes radius from 4.50 nm in unfertilized eggs to 4.19 nm in late embryos.

This change in Stokes radius is accompanied by a net loss of associated lipid. Lv isolated from 13.5-hr embryos or from late embryos and newly hatched larvae (1995 cultures at approximately 40% hatch) was subjected to quantitative lipid extraction in chloroform/methanol; the percentage of lipid in the Lv complex was then determined by weight. This percentage drops from 25.0% in Lv from 13.5-hr embryos to 14.5% in Lv from late embryos and newly hatched larvae. Since no protein is lost from the Lv particle, we calculated the change in lipid composition relative to the original Lv particle from 13.5-hr embryos. Expressed in this way, the lipid content falls from 25.0% to 12.7%, representing a loss of 49.2% of the original lipid. When the lipid extracts were examined by thin layer chromatography, the major components both before and after processing were neutral lipids (comigrating with the trilinolein standard) and phospholipids, predominantly phosphatidylcholine (data not shown).

DISCUSSION

Developing embryos and larvae of winter flounder provide a simple and convenient model for examining yolk utilization in teleosts. Winter flounder Vg (Nagler and Idler, '87, '90; Hartling et al., '97) and Lv from oocytes (Nagler and Idler, '90; Nagler et al., '91) and ovulated eggs (Hartling et al., '97) have already been well characterized. Since only one major Lv polypeptide exists at fertilization, it is easier to determine the developmental fate of Lv in this species than in other teleosts, which possess multiple yolk polypeptides in the mature egg.

Nevertheless, the fate of Vg in winter flounder is complex (summarized in Fig. 7). Winter flounder Vg has a native molecular mass of 500 kD (Nagler and Idler, '87); the Vg polypeptide is reported to be 180 kD (Nagler and Idler, '87) or 175 kD (Hartling et al., '97), and appears to exist as a homodimer in serum (Hartling et al., '97). Within the oocyte, Vg is proteolytically processed to form polypeptides of 101.4 kD, 94.4 kD, and 22.5 kD, of which the 101.4 kD polypeptide is predominant (Nagler and Idler, '90). Following oocyte maturation and ovulation, however, the majority of the Vg-derived yolk protein exists as a 94 kD polypeptide monomer, which appears to correspond to the 94.4 kD polypeptide observed by Nagler and Idler ('90) in oocytes; several smaller polypeptides are

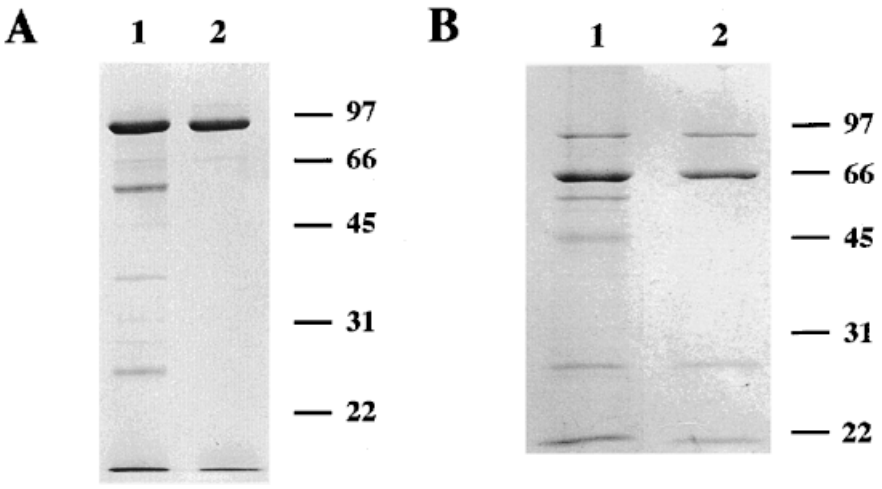

Fig. 5. Heat stability of Lv before and after proteolytic processing. SDS-PAGE of: (**A**) unfertilized egg extract; (**B**) day 16 embryo extract (1994 culture). Lane 1, whole extract; lane 2, soluble fraction of heat-treated extract. Molecular weight standards in kD are indicated. Ovalbumin (45 kD) was added to lane B1 as a densitometric standard.

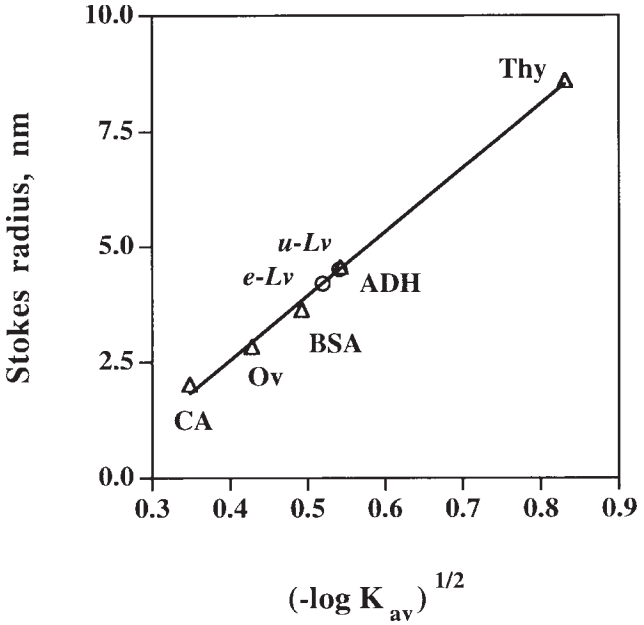

Fig. 6. Stokes radius determination of Lv before and after proteolytic processing. The values for Lv (circles) from unfertilized eggs (u-Lv; 1994 culture) and day 18 embryos and larvae (e-Lv; 1996 culture) were obtained by plotting Stokes radius vs. the square root of the negative log of the column partition coefficient for calibration standards (triangles). The standards were: Thy, thyroglobulin (8.6 nm); ADH, alcohol dehydrogenase (4.55 nm); BSA, bovine serum albumin (3.62 nm); Ov, ovalbumin (2.83 nm); and CA, carbonic anhydrase (2.01 nm). Note that u-Lv overlaps the ADH standard.

also present, but most seem to result from the proteolytic nicking of the 94 kD polypeptide (Hartling et al., '97). In addition, winter flounder ovaries incorporate a non-Vg serum lipoprotein of 1170 kD native molecular mass, termed VHDL II (Nagler and Idler, '87). This protein, or its derivative, is present in oocytes as a 68.7 kD polypeptide (Nagler and Idler, '90). VHDL II, however, accumulates in oocytes to a much lesser extent than does Vg (Nagler and Idler, '90; Nagler et al., '91), and the 68.7 kD polypeptide is undetectable by SDS-PAGE in mature, ovulated eggs (Hartling et al., '97). However, gel permeation chromatography of winter flounder egg extract on Bio-Gel A-1.5 reveals a peak that absorbs strongly at 280 nm and co-migrates with the total volume peak (R. Hartling, unpublished observations). Such low molecular weight material (<10 kD) may consist at least in part of small peptides and/or free amino acids derived from Vg and/or VHDL II, and may be an important source of nutrients for the embryo, as well as an important regulator of oocyte hydration (reviewed by Fyhn, '93).

In this study, we report the further proteolytic processing of Lv during winter flounder embryonic development. The 94 kD Lv polypeptide in unfertilized eggs is cleaved to form 67 kD and 26 kD polypeptides (Fig. 1). This proteolytic processing takes place very slowly in early embryos, but becomes more rapid between days 8 and 12 of embryonic development in flounder reared at 4–5°C, reaching 50% processed at tail-bud stage and nearing completion three days before hatching (Fig. 3B).

Our results suggest that Lv remains intact as a single native particle following proteolytic cleavage. Ouchterlony double immunodiffusion using

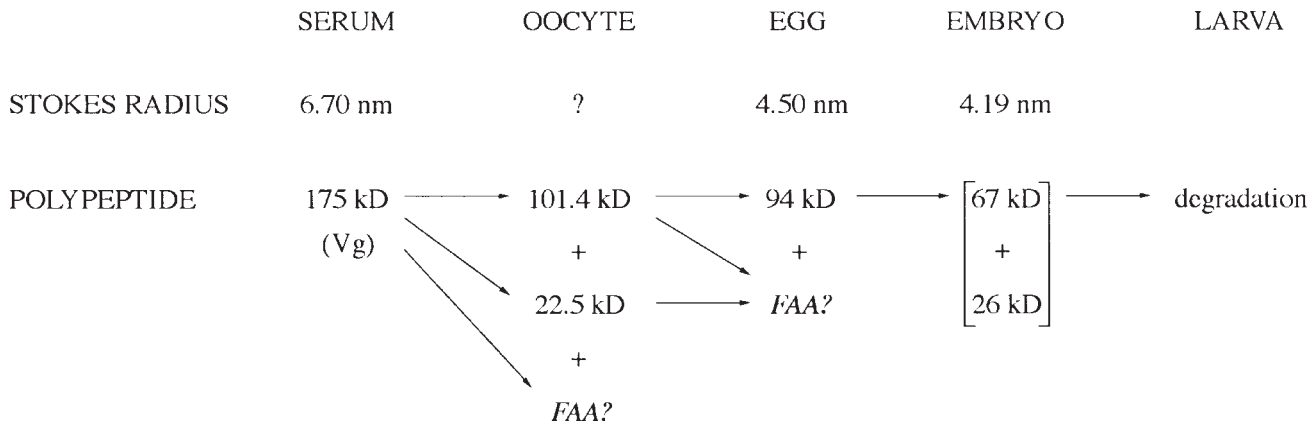

Fig. 7. The fate of Lv in winter flounder. Stokes radius of the entire lipoprotein particle is shown for Vg and for Lv from eggs and embryos. The Vg polypeptide (175 kD) is cleaved in oocytes to form 101.4 kD (Lv) and 22.5 kD polypeptides. Lv then undergoes successive proteolytic processing in maturing oocytes and in embryos, and is finally degraded in yolk-sac larvae. While the 67 kD + 26 kD Lv is reserved for larval use, the rest of the Vg polypeptide (~80 kD) is potentially available for embryonic growth, perhaps in large part as a pool of free amino acids (FAA). Oocyte data are from Nagler and Idler ('90), and Vg data are from Hartling et al. ('97).

a polyclonal antibody raised against 94 kD Lv shows identity between Lv from unfertilized eggs and from embryos at day 10 and day 15 after fertilization (Fig. 2). In contrast, Ouchterlony gels of barfin flounder (*Verasper moseri*) yolk proteins, which undergo proteolytic processing during oocyte maturation, show only partial identity between yolk proteins from vitellogenic oocytes and from ovulated eggs when an anti-Vg antiserum is used (Matsubara and Sawano, '95). Similarly, quantitative immunoelectrophoresis of American cockroach (*Periplaneta americana*) yolk proteins, which also undergo proteolytic processing during embryogenesis, yields a reaction of partial identity between yolk proteins from ovulated eggs and from day 9 embryos (Storella et al., '85). Both the results of Matsubara and Sawano ('95) and Storella et al. ('85) indicate that the proteolytic cleavage of these yolk proteins is accompanied by the loss of antigenic determinants. Our results with winter flounder Lv, however, indicate that the embryonic processing of Lv leaves its molecular structure sufficiently intact that few, if any, antigenic determinants are lost. Moreover, the processed Lv retains its heat stability, unlike the Lv fragments found in unfertilized eggs (Fig. 5). Furthermore, the overall size of Lv changes only moderately, from 4.50 nm in unfertilized eggs to 4.19 nm in late embryos and newly hatched larvae (Fig. 6). The loss of 12.3% of the weight of the Lv particle in the form of lipid can account for the majority of this decrease in size.

The embryonic processing of the Lv polypeptide does not immediately lead to its degradation by developing winter flounder. We found that the amount of Lv per individual does not decline substantially until after hatching, which occurs 3 days after Lv processing is essentially complete (Fig. 3). Thus the 94 kD Lv polypeptide, although the predominant yolk polypeptide in unfertilized eggs, does not contribute significantly to the nutrition of the winter flounder embryo, but is instead reserved for larval use. These results are consistent with the findings of Ronnestad et al. ('93), who demonstrated that free amino acids in the yolk compartment of Atlantic halibut (*Hippoglossus hippoglossus*) larvae are utilized prior to any significant degradation of yolk protein.

Although reports on the fate of individual yolk proteins during teleostean development are few, Olin and von der Decken ('90) have shown that proteolytic processing of Vg-derived proteins occurs during the early development of Atlantic salmon, *Salmo salar*. In this report, they describe the specific cleavage of several yolk polypeptides during both oocyte maturation and embryonic and larval development. Since they examine only two developmental stages (eyed egg and 7-day-old alevin), the timing of this processing is unclear, although it is not complete in the larva. Indeed, alevins in which yolk protein processing is more advanced have a greater mortality rate than alevins in which proteolytic processing occurs more slowly. However, the longer developmental time of salmonids, compounded by their unusually large supply of yolk, may account for this discrepancy between the timing of Lv processing in salmon and in winter flounder.

In contrast, Murakami et al. ('90) describe the fate of yolk proteins in medaka, *Oryzias latipes*. In this fish, several yolk phosphoproteins are degraded during embryonic development and are nearly depleted before hatching, while other yolk proteins are retained for larval use. This proteolytic degradation is gradual throughout embryogenesis, and is preceded by dephosphorylation of the yolk phosphoproteins. However, these investigators did not observe the specific cleavage of medaka yolk proteins into stable but smaller polypeptides, as we describe in winter flounder.

The enzymes responsible for the specific processing and eventual degradation of Lv in winter flounder remain undefined. The initial processing of Vg into yolk proteins in vertebrate oocytes appears to be catalyzed by the aspartic protease cathepsin D (Retzek et al., '92; Sire et al., '94; Yoshizaki and Yonezawa, '94). In rainbow trout (*Oncorhynchus mykiss*) embryos, the activities of acid phosphatase and of the thiol protease cathepsin L have been implicated in yolk protein degradation (Sire et al., '94). Such enzyme activity, however, is almost entirely localized to the yolk syncytial layer and not to the yolk mass itself. It is therefore unlikely that these enzymes are directly involved in the proteolytic processing of Lv prior to its degradation in rainbow trout embryos, if indeed such a proteolytic cleavage event exists in this species.

Our results indicate that the proteolytic processing of Lv in winter flounder embryos is accompanied by a net loss of nearly 50% of its associated lipid. It is unclear whether the cleavage of 94 kD Lv is directly responsible for this loss, or if removal of part of its lipid moiety renders Lv more vulnerable to proteolytic cleavage. If the former is true, however, the molecular changes in Lv following proteolytic cleavage may lead to a greater accessibility of a portion of its lipid moiety to the

developing embryo. In this way, a subset of Lv-associated lipids may be released in a developmentally specified manner, while other yolk components are retained for later use. Since different species of yolk lipids are utilized at different rates during the embryonic and larval development of teleostean fishes (reviewed by Wiegand, '96), Lv processing may be a significant mechanism for providing availability of particular lipids at a specific developmental time. Such a mechanism appears to exist in embryos of the sea urchin *Hemicentrotus pulcherrimus*, where proteolytic processing of yolk protein is accompanied by a decrease in the amount of lipids in yolk granules (Yokota et al., '93). Specifically, the yolk granule content of triacylglycerol and the phospholipids phosphatidylcholine and phosphatidylethanolamine decreased by over 50%, while only 30% of yolk granule cholesterol was lost during yolk protein processing. Although our analysis of the lipid fraction of winter flounder Lv by thin layer chromatography does not reveal a similar pattern of preferential loss of one or more particular classes of lipid, further experiments with such techniques as gas chromatography are necessary to quantify what changes, if any, occur among specific types of lipid within Lv concomitant with proteolytic processing. Another question for further work is whether lipophilic hormones such as thyroid hormones (Brown and Bern, '89) and cortisol (Sampath-Kumar et al., '95), which are present in the yolk and may be transported to oocytes by vitellogenin (Specker and Sullivan, '94), are associated with Lv in developing embryos, and, if so, whether their content changes within the lipid moiety of Lv following its proteolytic cleavage.

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LITERATURE CITED

Banaszak L, Sharrock W, Timmins P. 1991. Structure and function of a lipoprotein: lipovitellin. Annu Rev Biophys Chem 20:221–246.

Bligh EG, Dyer WJ. 1959. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917.

Brown CL, Bern HA. 1989. Thyroid hormones in early development, with special reference to teleost fishes. In: Schreibman MP, Scanes CG, editors. Development, maturation and senescence of neuroendocrine systems: a comparative approach. San Diego: Academic Press. p 289–306.

Carnevali O, Mosconi G, Roncarati A, Belvedere P, Limatola E, Polzonetti-Magni AM. 1993. Yolk protein changes during oocyte growth in European sea bass *Dicentrarchus labrax* L. J Appl Ichthyol 9:175–184.

Cerda J, Selman K, Wallace RA. 1996. Observations on oocyte maturation and hydration in the black sea bass, *Centropristis striata* (Serranidae). Aquat Living Resources 9:325–335.

Craik JCA. 1982. Levels of phosphoprotein in the eggs and ovaries of some fish species. Comp Biochem Physiol 72B: 507–510.

Craik JCA, Harvey SM. 1984. Biochemical changes occuring during final maturation of eggs of some marine and freshwater teleosts. J Fish Biol 24:599–610.

de Vlaming VL, Wiley HS, Delahunty G, Wallace RA. 1980. Goldfish (*Carassius auratus*) vitellogenin: induction, isolation, properties and relationship to yolk proteins. Comp Biochem Physiol 67B:613–623.

Fyhn HJ. 1993. Multiple functions of free amino acids during embryogenesis in marine fishes. In: Walther BT, Fyhn HJ, editors. Physiological and biochemical aspects of fish development. Bergen, Norway: University of Bergen. p 299–308.

Greeley MS Jr, Calder DR, Wallace RA. 1986. Changes in teleost yolk proteins during oocyte maturation: correlation of yolk proteolysis with oocyte hydration. Comp Biochem Physiol 84B:1–9.

Hartling RC, Pereira JJ, Kunkel JG. 1997. Characterization of a heat-stable fraction of lipovitellin and development of an immunoassay for vitellogenin and yolk protein in winter flounder (*Pleuronectes americanus*). J Exp Zool 278:156–166.

Johanning KM, Specker JL. 1995. Characterization of yolk proteins during oocyte development of tilapia, *Oreochromis mossambicus.* Comp Biochem Physiol 112B:177–189.

Kanungo J, Petrino TR, Wallace RA. 1990. Oogenesis in *Fundulus heteroclitus*: VI. Establishment and verification of conditions for vitellogenin incorporation by oocytes in vitro. J Exp Zool 254:313–321.

Kunkel JG, Lawler DM. 1974. Larval-specific serum protein in the order Dictyoptera: I. Immunologic characterization in larval *Blattella germanica* and cross-reaction throughout the order. Comp Biochem Physiol 47B:697–710.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 277:680–687.

LaFleur GJ Jr, Thomas P. 1991. Evidence for a role of Na^+, K^+-ATPase in the hydration of Atlantic croaker and spotted sea trout oocytes during final maturation. J Exp Zool 258:126–136.

Matsubara T, Sawano K. 1995. Proteolytic cleavage of vitellogenin and yolk proteins during vitellogenin uptake and oocyte maturation in barfin flounder (*Verasper moseri*). J Exp Zool 272:34–45.

McPherson R, Greeley MS Jr, Wallace RA. 1989. The influence of yolk protein proteolysis on hydration in the oocytes of *Fundulus heteroclitus.* Dev Growth Differ 31:475–483.

Mommsen TP, Walsh PJ 1988. Vitellogenesis and oocyte assembly. In: Hoar WS, Randall DJ, editors. Fish physiology, volume 11A. New York: Academic Press. p 347–406.

Murakami M, Iuchi I, Yamagami K. 1990. Yolk phosphoprotein metabolism during early development of the fish, *Oryzias latipes.* Dev Growth Differ 32:619–627.

Murakami M, Iuchi I, Yamagami K 1991. Partial characterization and subunit analysis of major phosphoproteins of

egg yolk in the fish, *Oryzias latipes.* Comp Biochem Physiol 100B:587–593.

Nagler JJ, Idler DR. 1987. Evidence for another yolk precursor protein, distinct from vitellogenin, in winter flounder (*Pseudopleuronectes americanus*). In: Idler DR, Walsh JM, Crim LW, editors. Proceedings of the Third International Symposium on the Reproductive Physiology of Fish. St. John's, Newfoundland: Memorial University of Newfoundland. p 209–210.

Nagler JJ, Idler DR. 1990. Ovarian uptake of vitellogenin and another very high density lipoprotein in winter flounder (*Pseudopleuronectes americanus*) and their relationship with yolk proteins. Biochem. Cell Biol 68:330–335.

Nagler JJ, Murrin F, Idler DR. 1991. Plasma protein incorporation and localization within the developing ovary of winter flounder. In: Scott AP, Sumpter JP, Kime DE, Rolfe MS, editors. Proceedings of the Fourth International Symposium on the Reproductive Physiology of Fish. Norwich, UK: University of East Anglia. p 303–305.

Olin T, von der Decken A. 1990. Yolk proteins in salmon (*Salmo salar*) oocytes, eyed eggs, and alevins differing in viability. Can J Zool 68:895–900.

Opresko LK, Wiley HS. 1987. Repeptor-mediated endocytosis in *Xenopus* oocytes: I. Characterization of the vitellogenin receptor system. J Cell Biol 262:4109–4115.

Ouchterlony O. 1949. Antigen-antibody reaction in gels. Acta Pathol Microbiol Scand 26:507–515.

Redinbaugh MG, Campbell WH. 1985. Adaptation of the dye-binding protein assay to microtiter plates. Anal Biochem 147:144–147.

Retzek H, Steyrer E, Sanders EJ, Nimpf J, Schneider WJ. 1992. Molecular cloning and functional characterization of chicken cathepsin D, a key enzyme for yolk formation. DNA Cell Biol 11:661–672.

Ronnestad I, Fyhn HJ. 1993. Metabolic aspects of free amino acids in developing marine fish eggs and larvae. Rev Fish Sci 1:239–259.

Ronnestad I, Groot EP, Fyhn HJ. 1993. Compartmental distribution of free amino acids and protein in developing yolk-sac larvae of Atlantic halibut (*Hippoglossus hippoglossus*). Mar Biol 116:349–354.

Sampath-Kumar R, Byers RE, Munro AD, Lam TJ. 1995. Profile of cortisol during ontogeny of the Asian seabass, *Lates calcarifer*. Aquaculture 132:349–359.

Selman K, Wallace RA. 1982. Oocyte growth in the sheepshead minnow: uptake of exogenous proteins by vitellogenic oocytes. Tissue Cell 14:555–571.

Selman K, Wallace RA. 1983. Oogenesis in *Fundulus heteroclitus*: III. Vitellogenesis. J Exp Zool 266:441–457.

Sire M-F, Babin PJ, Vernier J-M. 1994. Involvement of the lysosomal system in yolk protein deposit and degradation during vitellogenesis and embryonic development in trout. J Exp Zool 269:69–83.

Smigielski AS, Arnold CR. 1972. Separating and incubating winter flounder eggs. Prog Fish Cult 34:113.

Specker JL, Sullivan CV. 1994. Vitellogenesis in fishes: status and perspectives. In: Davey KG, Peter RE, Tobe SS, editors. Perspectives in comparative endocrinology: invited papers from XII International Congress of Comparative Endocrinology. Ottawa: National Research Council of Canada. p 304–315.

Stifani S, Le Menn F, Rodriguez JN, Schneider WJ. 1990. Regulation of oogenesis: the piscine receptor for vitellogenin. Biochim Biophys Acta 1045:271–279.

Storella JR, Wojchowski DM, Kunkel JG. 1985. Structure and embryonic degradation of two native vitellins in the cockroach, *Periplaneta americana*. Insect Biochem 15: 259–275.

Thorsen A, Fyhn HJ. 1996. Final oocyte maturation in vivo and in vitro in marine fishes with pelagic eggs: yolk protein hydrolysis and free amino acid content. J Fish Biol 48:1195–1209.

Tyler C. 1993. Electrophoretic patterns of yolk proteins throughout ovarian development and their relationship to vitellogenin in the rainbow trout, *Oncorhynchus mykiss*. Comp Biochem Physiol 106B:321–329.

Tyler CR, Sumpter JP, Bromage NR. 1988a. In vivo ovarian uptake and processing of vitellogenin in the rainbow trout, *Salmo gairdneri*. J Exp Zool 246:171–179.

Tyler CR, Sumpter JP, Bromage NR. 1988b. Selectivity of protein sequestration by vitellogenic oocytes of the rainbow trout, *Salmo gairdneri.* J Exp Zool 248:199–206.

Tyler CR, Sumpter JP, Handford RM. 1990. The dynamics of vitellogenin sequestration into vitellogenic ovarian follicles of the rainbow trout, *Salmo gairdneri.* Fish Physiol Biochem 8:211–219.

Tyler CR, Sumpter JP, Campbell PM. 1991. Uptake of vitellogenin into oocytes during early vitellogenic development in the rainbow trout, *Oncorhynchus mykiss* (Walbaum). J Fish Biol 38:681–689.

Vaskovsky VE, Kostetsky EY, Vasendin IM. 1975. A universal reagent for phospholipid analysis. J Chromatography 114:129–141.

Wallace RA. 1965. Resolution and isolation of avian and amphibian yolk-granule proteins using TEAE-cellulose. Anal Biochem 11:279–311.

Wallace RA, Begovac PC. 1985. Phosvitins in *Fundulus* oocytes and eggs: preliminary chromatographic and electrophoretic analyses together with biological considerations. J Biol Chem 260:11268–11274.

Wallace RA, Selman K. 1985. Major protein changes during vitellogenesis and maturation of *Fundulus* oocytes. Dev Biol 110:492–498.

Wiegand MD. 1996. Composition, accumulation and utilization of yolk lipids in teleost fish. Rev Fish Biol Fish 6:259–286.

Yokota Y, Kato KH, Mita M. 1993. Morphological and biochemical studies on yolk degradation in the sea urchin, *Hemicentrotus pulcherrimus.* Zool Sci 10:661–670.

Yoshizaki N, Yonezawa S. 1994. Cathepsin D activity in the vitellogenesis of *Xenopus laevis*. Dev Growth Differ 36:299–306.