Bull. Fish. Sci. Hokkaido Univ.
53(3), 95-105, 2002.

Developmental Changes in Egg Yolk Proteins of Walleye Pollock, *Theragra chalcogramma*, and a Comparative Study of Immunoreactivity of Other North Pacific Teleosts and Invertebrate Eggs

Kevin M. BAILEY[1], Nazila MERATI[1], Michael HELSER[2], Naoshi HIRAMATSU[3] and Akihiko HARA[4]

(Received 3 September 2002 Accepted 10 October 2002)

Abstract

Changes in egg proteins during embryonic development in walleye pollock, *Theragra chalcogramma*, were shown by SDS-PAGE. Western blotting with a polyclonal antibody developed against proteins from hydrated eggs showed major reactive bands in serum of vitellogenic females at 175, 76 and 66 kDa. Vitellogenic ovaries had major reactive bands at 97 kDa, and extruded, hydrated eggs had bands at 94 kDa. Fertilized, late-stage developing eggs and yolked larvae had major bands at 66 kDa. These results suggested proteolytic cleavage of vitellogenin and other egg proteins upon uptake by oocytes and further digestion of egg proteins during development. Immunoblots run to test cross-reactivity potential between anti-pollock egg yolk protein antibodies and various proteins of invertebrate and other teleost species demonstrated that antigenic similarities exist between most teleosts and walleye pollock egg proteins, but not between pollock and invertebrate eggs. Subsequent Western blotting showed that several major immunoreactive egg proteins are shared in distantly related fish families. It is thought that egg-yolk proteins are antigenically conserved among teleosts.

Key words: Egg development, Egg proteins, Vitellogenin, Walleye pollock, *Theragra chalcogramma*, Cross-reactivity, Immunorecognition

Introduction

During the egg and early larval stages of marine fishes, maternally supplied yolk provides the components required for energy production, biosynthesis and maintenance. Vitellogenin, a lipoglycophosphoprotein (Bergink and Wallace, 1974; Christmann et al., 1977; Ng and Idler, 1983; Mommsen and Walsh, 1988; Specker and Sullivan, 1994), is the precursor of the major yolk proteins of fishes and many other oviparous animals. Vitellogenin is synthesized in the liver of females under the influence of estrogen, secreted into the blood, and then incorporated into egg yolk proteins. They are proteolytically cleaved into lipovitellin, phosvitin and proteins called YGP40 or β'-component in amphibians, birds and fishes (Wallace, 1978; Matsubara and Sawano, 1995; Yamamura et al., 1995; Hiramatsu and Hara, 1996). Previous studies using electrophoretic patterns have shown that several new protein bands appear in oocytes during vitellogenesis and because these proteins are smaller than vitellogenin, it has been suggested that vitellogenin is the precursor for the smaller egg proteins observed (Wallace and Selman, 1985). Greely et al. (1986) also observed changes in the protein component of teleosts during oocyte maturation and suggested that these changes result from the alteration of existing proteins. In marine fishes, information for the mechanisms of yolk breakdown and the fate of yolk products is limited to a few species (Matsubara et al., 1999; Carnevali et al., 1999; Harting and Kunkel, 1999).

Aside from the initial processing of vitellogenin, the occurrence of additional cleavage of yolk proteins along with final oocyte maturation has been discovered in some particular pelagic egg-laying marine fish (Wallace and Begovac, 1985; Wallace and Selman, 1985; Greely et al., 1986; Carnevali et al., 1993; Matsubara and

[1] National Marine Fisheries Service, Alaska Fisheries Science Center, 7600 Sand Point Way NE, BIN C15700, Seattle, WA 98115-0070, USA
[2] Department of Food Science, Cornell University, Ithaca, NY 14850, USA
[3] Department of Zoology, North Carolina State University, Raleigh, NC 27695-7617, USA
[4] Laboratory of Comparative Physiology, Graduate School of Fisheries Sciences, Hokkaido University, 3-1-1, Minato, Hakodate, Hokkaido 041-8611, Japan
(北海道大学大学院水産科学研究科生命機能学講座)

Sawano, 1995; Thorsen et al., 1996). The secondary processing of yolk proteins is thought to be an important event giving rise a pool of free amino acids that causes oocyte hydration for the acquisition of buoyancy. These free amino acids are also responsible for the rapid embryonic growth after fertilization. In teleosts, biochemical information on yolk degradation during embryogenesis is limited to a few species (Olin and Von Der Decken, 1990; Hartling and Kunkel, 1999; Matsubara et al., 1999), and the mechanisms of yolk breakdown and the fate of yolk products remains to be verified.

Walleye pollock is thought to have an annual spawning cycle and its oocyte maturation has been classified as partially synchronous (Hinckley, 1986). Walleye pollock currently supports a large commercial fishery in the North Pacific Ocean. It is the subject of an intensive study to determine abiotic and biotic factors critical to the recruitment of fish stocks. In this study, we used an antibody probe developed against egg yolk proteins of walleye pollock (*Theragra chalcogramma*) to show that the small proteins in egg yolk are derived from vitellogenin. Using SDS-PAGE and Western blots, we traced the major changes in egg proteins from sera of mature females through oocyte development and yolk absorption by the larvae. We also compared pollock egg protein components with eggs of other teleosts and invertebrate species. Immunoblots were initially used to quantify the amount of immunological specificity between walleye pollock and various other marine species. SDS-PAGE and Western blotting techniques were utilized to determine which specific egg proteins were immunoreactive with our anti-pollock egg yolk protein antibody.

Materials and Methods

Fish and biochemicals

Adult pollock were caught in the Tacoma Narrows vicinity of Puget Sound, Washington (U.S.A) by hook and line in autumn and winter months, and transported to the Manchester Aquaculture Facility of the Northwest Fisheries Science Center (U.S. National Marine Fisheries Service). Blood and ovaries of some of the fish were collected immediately after capture. Blood was collected by excising the tail. Blood samples were transported to the laboratory on ice and allowed to clot at approximately 4°C overnight. The resulting clot was removed and serum was separated by centrifugation at room temperature for 10 min. Ovaries were collected from approximately 40 cm fish and were frozen on dry ice for about 6 hr, after which time ovaries and blood sera were stored at −80°C. Other fish were held in net-pens and fed a diet of herring and squid until spawning commenced. Eggs were stripped, fertilized with milt, and then reared according to techniques in Bailey and Stehr (1986).

Eggs and invertebrate collections

Ovaries and egg samples from fish and invertebrates were collected in 1986-1987 from Puget Sound and stored at −80°C. Samples tested for potential cross-reactions are summarized in Table 1. Approximately 0.16 g of each sample was weighed and homogenized in a glass tissue grinder in 500 μl Tris-buffered saline (TBS) (20 mM Tris, 500 mM NaCl, pH 7.5). The homogenant was centrifuged for 3 min at 11,750×g at room temperature. The supernatant was drawn off and frozen at −80°C until needed. Protein stocks were diluted 1 : 100 in TBS and absorbance was read at 280 nm to measure protein concentration (Shimadzu UV-120-2). Final protein concentration used in the assay was 15 μg/100 μl. One hundred microliters of protein extract was applied to 0.45 μm nitrocellulose membrane in a microfiltration apparatus and allowed to gravity filter at 4°C. When filtering was complete and the membrane was dry, the membrane was removed from the filtering apparatus and blocked iu Blotto/Tween (5% nonfat milk, 0.05% Tween-20, 0.01% Antifoam A, and phosphate-buffered saline, pH 7.4). Immunoblotting procedures followed Theilacker (1986) except that 10% normal goat serum was added to the blocking solution before and during second antibody incubation. The primary antibody used in the assay was an unabsorbed polyclonal rabbit anti-pollock egg yolk protein IgG fraction (*see* below for antibody production in details). The titre used in these experiments was 1 : 5,000. The conjugate antibody used was a 1 : 3,000 dilution of alkaline phosphatase-labelled goat-rabbit IgG (Bio-Rad Lab.). Proteins were visualized using BCIP (p-nitro blue tetrazolium chloride) and NBT (5-bromo-4-chloro-3 indyl phosphate p-toluidine salt).

A series of positive and negative controls were run along with the rest of the samples to monitor the intensity of the color reaction and to calibrate the scale or cross-reactivity. The maximum amount of cross-reaction was observed as an intense purple-black color present in the homogenate of one pollock yolk-sac larvae. To this color we assigned a grade of +++, and scaled this as a very strong reaction. For strong reactions exhibiting a purple lavender color, a grade of ++ was assigned. Weak reactions displaying a light lavender were graded as +. Samples that showed no reaction were graded as −. Positive controls consisted of pollock egg and yolk-sac larvae extracts and female serum. Negative controls included normal pooled goat

Table 1. Samples used in cross-reactivity study

Sample	Common Name	Scientific Name	Family
1A	Pacific cod egg	*Gadus macrocephalus*	Gadidae
1B	Pacific tomcod ovary	*Microgadus proximus*	Gadidae
1C	Sand sole egg	*Psettichthys melanostictus*	Pleuronectidae
1D	Pacific tomcod egg	*Microgadus proximus*	Gadidae
1E	Pacific hake ovary	*Merluccius productus*	Gadidae
1F	Sablefish egg	*Anaplopoma fimbria*	Anoplopomatidae
1G	Pacific halibut egg	*Hippoglossus stenolepis*	Pleuronectidae
1H	Pacific herring egg	*Clupea harengus pallasi*	Clupeidae
1I	Rockfish embryo	*Sebastes sp.*	Scorpaenidae
2A	Padded sculpin egg	*Artedius fenestralis*	Cottidae
2B	Kelp greenling egg	*Hexagrammos decagrammus*	Hexagrammidae
2C	Angelfish egg		Cichlidae
2D	Cabezon egg	*Scorpaenichthys marmoratus*	Cottidae
2E	Whitespotted greenling egg	*Hexagrammos stelleri*	Hexagrammidae
2F	Decorated warbonnet egg	*Chirolophis decoratus*	Stichaeidae
2G	Lingcod egg	*Ophiodion elongatus*	Hexagrammidae
2H	Pollock flesh (female)	*Theragra chalcogramma*	Gadidae
2I	Pacific spiny lumpsucker egg	*Eumicrotremus orbis*	Cyclopteridae
3A	Grunt sculpin egg	*Rhamphocottus richardsoni*	Cottidae
3B	Coho salmon egg	*Oncorhynchus kisutch*	Salmonidae
3C	Chinook salmon egg	*Oncorhynchus tshawytscha*	Salmonidae
4A	Frilled dogwinkle egg	*Nucella lamellosa*	Muricidae
4B	Rosy tritonia egg	*Tritonia diomedea*	Tritoniidae
4C	Purple sea urchin egg	*Strongylecentrotus purpuratus*	Strongylecentrotidae
4D	Sand dollar egg	*Dendraster excentricus*	Dendrasteridae
4E	Lion nudibranch egg	*Melibe leonina*	Tethyidae
4F	Starfish egg	*Pisaster ochraceous*	Asteriidae
4G	Orange peel nudibranch egg	*Tochuina tetraqueta*	Tritoniidae
4H	Pacific sea-lemon nudibranch egg	*Anisodoris nobilis*	Discodoridiidae
4I	Hermissenda nudibranch egg	*Hermissenda crassicornis*	Facelinidae
5A	Northern shrimp egg	*Pandalus borealis*	Pandalidae
5B	Spot prawn egg	*Pandalus platyceros*	Pandalidae
5C	Gammarid roe	*Cyphocaris challengeri*	Lysianassidae
5D	Pacific Lyre crab	*Cancer oregonensis*	Cancridae
5E	Eyed crab roe	*Hyas lyratus*	Majidae
6A	Diatom	*Phaeodactylum tricornutum*	Cymbellaceae
6B	Dinoflaggelate	*Amphidium corteri*	Dinophyceae
6C	Green algae	*Platymonas suecia*	Chlamydomonadaceae
7A	1/10 pollock egg	*Theragra chalcogramma*	Gadidae
7B	1/100 pollock egg	*Theragra chalcogramma*	Gadidae
7C	1/1000 pollock egg	*Theragra chalcogramma*	Gadidae
7D	1 yolk-sac larvae	*Theragra chalcogramma*	Gadidae
7E	1/10 yolk-sac larvae	*Theragra chalcogramma*	Gadidae
7F	Female pollock blood serum	*Theragra chalcogramma*	Gadidae
7G	Male pollock blood serum	*Therogra chalcogramma*	Gadidae
7H	Protein extraction buffer		
7I	Normal goat serum pooled		

serum, TBS extraction buffer, and male pollock serum. All negative controls remained clear.

Antibody preparations

Egg yolk proteins were extracted as follows. Hydrated, ovulated and unfertilized eggs were extruded from a ripe female, frozen over dry ice and transferred to liquid nitrogen. Five grams of eggs were thawed, rinsed with 0.9% NaCl and homogenized using a ground-glass tissue grinder over ice. The egg homogenate was centrifuged at 8,160×g for 4 min. The supernatant solution contained some floating particles that were collected and filtered through a 0.20 μm filter membrane (Plack et al., 1971; Hara and Hirai, 1978). The clear solution was dripped into 30 ml chilled, distilled water and allowed to flocculate. The precipitate, collected by centrifugation. was dissolved in 1 ml 0.02 M Tris-HCl buffer pH 8.0 containing 0.2% NaCl, dripped into chilled, deionized distilled water and allowed to stand at 4°C for 2 hr. The supernatant was decanted, and rhe precipitate was dissolved in Tris-HCl buffer (above mentioned) and precipitated into distilled water again. The flocculate was centrifuged at 1,200×g at 4°C for 15 min, the supernarant was discarded, and precipitate was dissolved in 2.5 ml Tris-HCl buffer. A stock solution for rabbit injection was diluted to 2.3 mg protein/ml, mixed with an equal volume of Freund's complete adjuvant, and then it was frozen. A New Zealand male rabbit was injected four times at 10 day intervals. Blood was collected about 6 weeks after the initial injecrion. Blood was allowed to stand for 1 hr at room temperature, it was then stirred once, allowed to stand overnight at 4°C, and then centrifuged ar 8,160×g at 4°C for 10 min. The IgG fraction was salted out from the blood sera by using a 40% solution of saturated ammonium sulfate; the precipitate was dissolved in 0.01 M phosphate buffer at pH 8.0 and was purified by passing the solution through a DEAE column using DE52 (Whatman). A faint reactivity of anti-egg protein antisera with male sera detected on dot-blots and Western blots was removed by affinity purification. Male blood sera were atrached to a column of Sepharose 4B (Phamacia) activated wirh cyanogen bromide, and the IgG solution was passed through it. The resulting absorbed IgG fraction showed no cross-reactivity to male sera.

Electrophoresis

Preparation of samples for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed as follows. Ovaries, eggs and larvae were homogenized in sample buffer (0.16 g/0.5 ml TBS) with a ground glass rissue grinder on ice. The homogenate was centrifuged at room temperature for 3 min at 11,750×g: the supernatant was collected and stored at −80°C. Protein concentrations of sera, ovary, egg, and larval homogenates were determined by absorbance at 280 nm.

Prior to electrophoresis, the homogenates were diluted to the same concentrations in sample buffer and heated to 95°C for 4 min. Sample wells were loaded with 20 μg/5 μl protein. The SDS-PAGE gradient gels (10–20%) were run at constant voltage until the dye front migrated to the bottom. Coomassie Brilliant Blue R-250 stain or silver stain was used for protein visualization. Molecular weights of egg proteins were determined by plotting the molecular weight of protein standards (Bio-Rad Lab.) versus the distance they migrated from the interface of the stacking and separating gel.

Western blotting

For Western blots, 3.5 μg/5 μl protein per well was loaded for each sample on SDS-PAGE gels which were constructed and run as described above. Duplicate gels were silver stained. After running the gels, they were equilibrated in Tris-glycine buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3) for 30 min. The proteins were transferred to nitrocellulose paper at 50 V for the first hour and at 100 V for the second hour. The nitrocellulose paper with the transferred proteins was blocked with BLOTTO block solution (5% nonfat dry milk, 0.05% Tween-20 in TBS) overnight. Following two 10 min washes in TTBS (0.05% Tween-20 in TBS), the nitrocellulose paper was incubated with the anti-pollock egg yolk antibody (diluted 1 : 1,000 in BLOTTO block solution) for 2 hr. The egg protein development blots used the absorbed antibody, and the cross-reactivity blots used the unabsorbed antibody. The membrane was washed in BLOTTO wash solution (5% nonfat dry milk, 0.3% Tween-20%, in TBS), three times for 10 min each, and in TTBS two times for 10 min each. The reactive proteins were rhen probed by incubation in the second antibody solution (alkaline phosphatase-labelled goat anti-rabbit IgG) diluted 1 : 3,000 in BLOTTO block solution. Excess antibody was removed by washing in BLOTTO wash solution four times, TTBS two times and TBS two times, all for 10 min each. The reactive proteins were then visualized by color development as described above.

Results

Egg protein development

A composite gel of sera, ovarian oocytes, hydrated fertilized eggs, yolk-sac and post-yolk-sac feeding larvae is shown in Fig. 1. This gel was used ro calculate

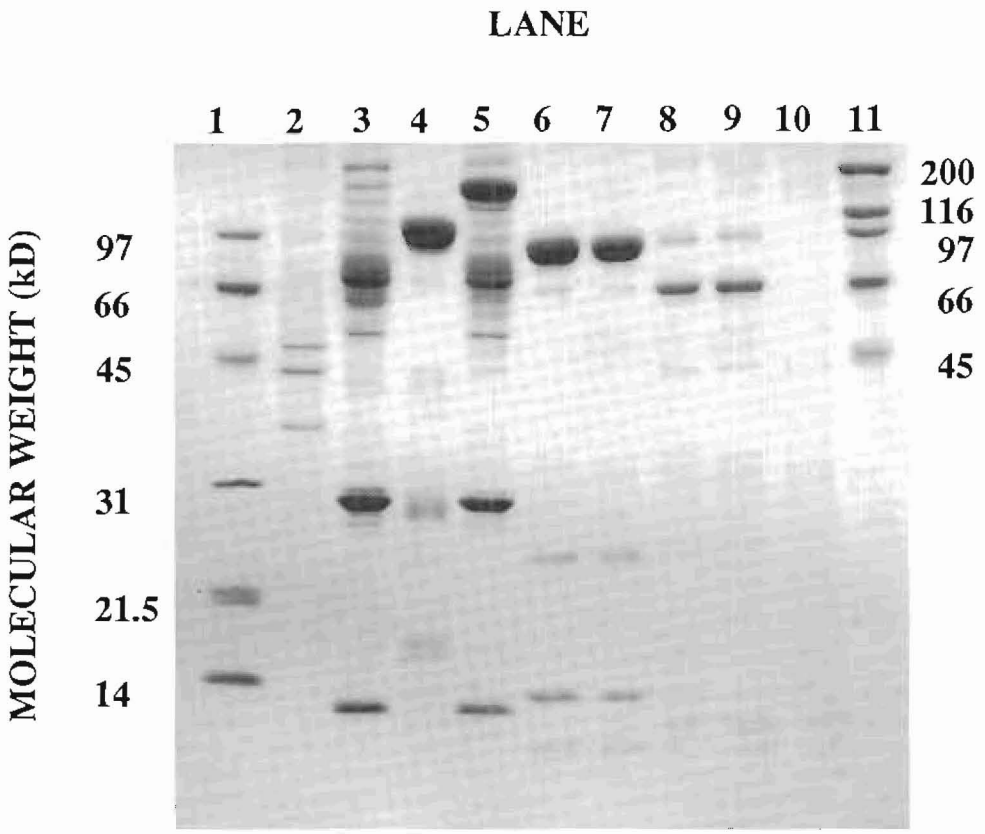


Fig. 1. SDS-PAGE of serum, ovarian and egg proteins of walleye pollock. Gradient gel, 10–20% stained with Coomassie Blue. Lane: 1, low molecular weight marker; 2, pre-vitellogenic ovary extract; 3, pre-vitellogenic female serum; 4, vitellogenic ovary extract; 5, vitellogenic female serum; 6, crude purified unfertilized egg extract; 7, early fertilized egg extract; 8, late fertilized egg extract; 9, yolk-sac larva extract; 10, post-yolk-sac larva extract; and 11, high molecular weight marker.

molecular weights for comparison with Western blots and their duplicate silver-stained gels. Bands were assigned to either major or minor categories based on their initial staining intensity. The molecular weights of the main bands of yolk protein were observed to be 97 kDa for vitellogenic ovary extract and 94 kDa for hydrated fertilized eggs (Fig. 1, lanes 4 and 6).

Western blots indicated that the polyclonal antibodies developed against crude, purified pollock egg proteins were immunoreactive with egg yolk protein and its derivatives in sera and ovary extracts (Fig. 2). Ovaries from female walleye pollock collected in December that were in the early stages of egg development did not react with the egg yolk protein antibodies (Fig. 2, lane 1), indicating that the individuals were previtellogenic. However, sera collected during this month exhibited three minor bands which reacted with the antibodies at 175, 76 and 66 kDa (Fig. 2, lane 7). As oocytes matured from February to April, ovaries reacted to the antibodies revealing major proteins of 97, 58, 25 and 14. During these months blood sera samples showed major bands of 175, 76 and 66 kDa in addition to the minor proteins observed in December. Individuals that had completed spawning in May had no reactive proteins in their ovaries, but weak immunoreactions in the May sera sample were observed that corresponded ro the major bands found in the blots of sera from preceding months. These weak bands did not correspond closely to those of the egg extracts, indicating that in walleye pollock, either egg yolk proteins are not being resorbed directly or that resorption had already taken place. Sera from male walleye pollock collected in March and May did not react with the antibody (Fig. 2, lanes 12 and 13).

Fertilized walleye pollock eggs up to age 4 days had major reactive bands of 94, 76, 58 and 25 kDa and another just under 14 kDa (referred to as 14 kDa-1; 14 kDa was the smallest molecular weight marker used) (Fig. 3). Minor bands were found at 96 and 66 kDa. Late-stage eggs and yolked larvae had major bands of 94, 66 and 25 kDa, minor bands of 76 and 14 kDa-1. As well as another minor band which migrated slightly beyond 14 kDa-1. First-feeding pollock larvae which had resorbed their yolk had no reactive yolk proteins (Fig. 3, lane 7).

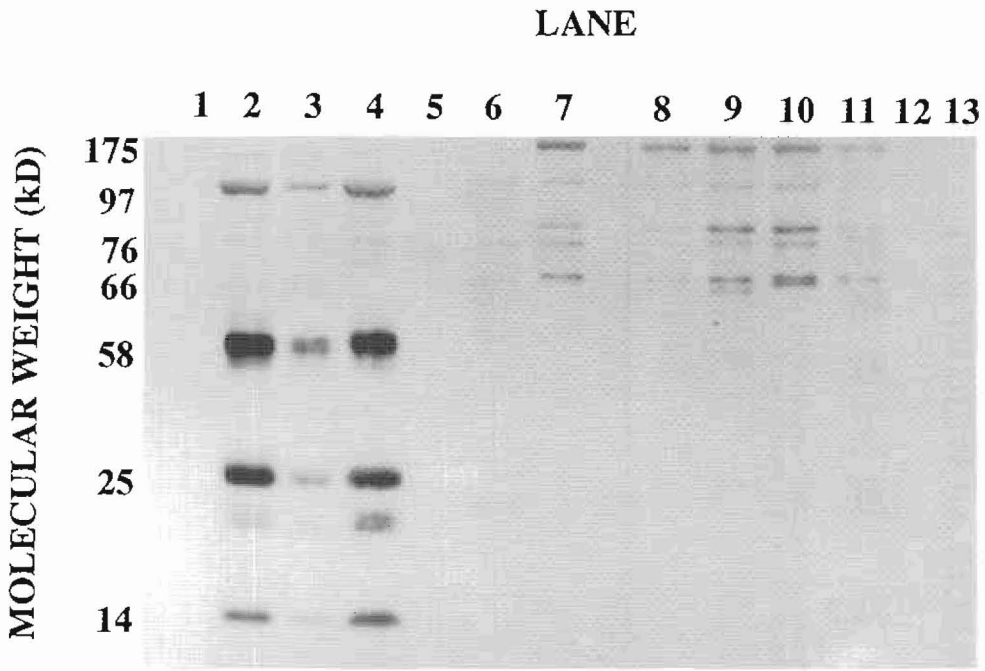
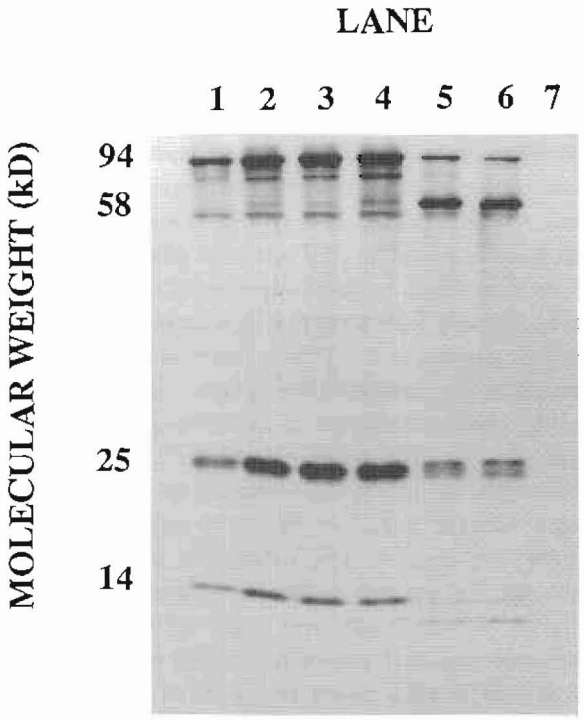


Fig. 2. Western blotting of ovarian and serum proteins of walleye pollock. SDS-PAGE linear gradient 10–20%; proteins transferred to nitrocellulose paper for immunodetection with rabbit antibody developed against crudely purified egg proteins. Lane: 1, pre-vitellogenic ovary extract; 2, developing ovary extract; 3, vitellogenic ovary extract; 4, spawning ovary extract; 5, spent ovary extract; 6, pre-vitellogenic female serum; 7, pre-vitellogenic female serum; 8, developing female serum; 9, vitellogenic female serum; 10, spawning female serum; 11, spent female serum; 12, mature male serum; and 13, spent male serum.



Immunoblotting and immunospecificity studies

Anti-pollock egg proteins cross-reacted most intensely with proteins from members of the Gadidae (Table 2, Fig. 4). Strong reactions were also noted with proteins of Pacific halibut (*Hippoglossus stenolepis*), rockfish (*Sebastes* spp.), sablefish (*Anoplopoma fimbria*), and sand sole (*Psettichthys melanostictus*) eggs or yolked embryos. Very weak or no reactions occurred with members of the Salmonidae, Hexagrammidae, Cottidae and Stichaedidae. A weak reaction was noted with female pollock flesh tested using unabsorbed antibody. For a complete summary, see Table 2 and Fig. 4.

Gel electrophoresis and immunoblots of fertilized 24-hour old walleye pollock eggs and eggs of selected marine fish species revealed that they possess several bands in common. The molecular weights of egg proteins observed in SDS-PAGE gels (Fig. 5) were

Fig. 3. Western blotting of egg, yolk-sae larva and post-yolk-sac larva proteins of walleye pollock. SDS-PAGE linear gradient 10–20%; proteins transferred to nitrocellulose paper for immunodetection with rabbit antibody developed against crudely purified egg protein. Lanes: 1, crudely purified unfertilized egg extract; 2, unfertilized egg extract; 3, 1-day-old fertilized egg; 4, 4-day-old fertilized egg; 5, 8-day-old fertilized egg; 6, yolk-sac-larva ; and 7, post-yolk-sac larva.

Table 2. Results of dot-blots testing potential cross-reactivity between selected invertebrate and fish eggs. Reactions are scored by comparison to positive control. In this case, we used one walleye pollack yolk-sac larvae as the guide and assigned grades based on the intensity of reactivity.

Sample	Type	Cross-reactivity
Pacific cod egg	Teleost	+++
Pacific tomcod ovary	Teleost	+++
Sand sole egg	Teleost	++
Pacific tomcod egg	Teleost	+++
Pacific hake ovary	Teleost	++
Sablefish egg	Teleost	++
Paelfic halibut egg	Teleost	+++
Pacific herring egg	Teleost	+
Sebastes sp. embryo	Teleost	++
Padded sculpin egg	Teleost	+
Kelp greenling egg	Teleost	+
Angelfish egg	Teleost	+
Cabezon egg	Teleost	++
Whitespotted greenling egg	Teleost	+
Decorated warbonnet egg	Teleost	+
Lingcod egg	Teleost	+
Pollock flesh (female)	Teleost	+
Pacific spiny lumpsucker egg	Teleost	−
Grunt sculpin egg	Teleost	−
Coho salmon roe	Teleost	+
Chinook salmon roe	Teleost	+
Pollock yolk-sac larvae	Teleost	(+++)
Sea snail egg	Invertebrate	−
Giant orange nudibranch egg	Invertebrate	−
Sea urchin egg	Invertebrate	−
Sand dollar egg	Invertebrate	−
Melibe leonina	Invertebrate	−
Starfish egg	Invertebrate	−
Orange peel nudibranch egg	Invertebrate	−
Sea lemon nudibranch egg	Invertebrate	−
nudibranch egg	Invertebrate	−
Pink shrimp egg	Invertebrate	−
Spot prawn egg	Invertebrate	−
Gammarid roe	Invertebrate	−
Pygmy rock crab roe	Invertebrate	−
Pacific lyre crab roe	Invertebrate	−
Diatom	Algae	−
Dinoflagellate	Algae	−
Green algae	Algae	−

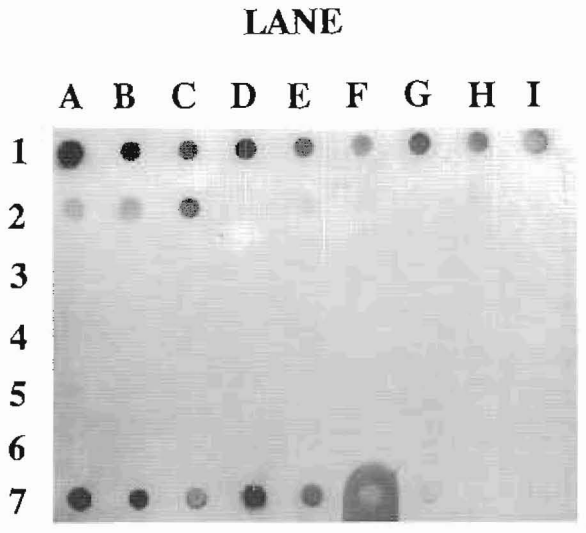


Fig. 4. Immunoblot of teleost and invertebrate eggs to determine cross-reactivity potential. Samples were exposed to a 1 : 5,000 dilution of rabbit anti-pollock egg yolk protein antibody. Secondary antibody used was a 1 : 3,000 dilution of goat anti-rabbit alkaline phosphatase. See Table 1 for immunoblot legend.

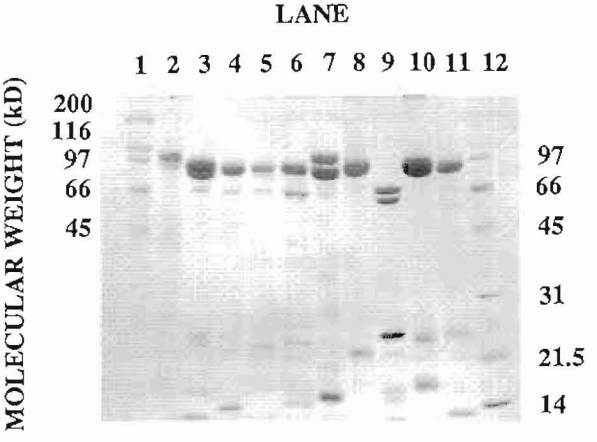


Fig. 5. SDS-PAGE of comparative egg proteins. Gradient gel 10–20%, stained with Commassie Blue. Lanes: 1, high molecular weight marker; 2, Pacific cod egg; 3, Pacific tomcod egg; 4, Pacific halibut egg; 5, Pacific hake ovary; 6, sablefish egg; 7, *Sebastes* sp. embryo; 8, Pacific herring egg; 9, Grunt sculpin egg; 10, coho salmon egg; 11, 24-h fertilized walleye pollock egg; and 12, low molecular weight marker.

similar to those seen in the Western blots (Fig. 6). Western blots of walleye pollock egg proteins (Fig. 6, lane 10) showed peptide bands of 94 kDa, several bands between 94 and 66 kDa, 25 kDa, and 14 kDa. Western blots of egg proteins from Pacific cod (*Gadus macrocephalus*), tomcod (*Microgadus proximus*), sablefish, Pacific halibut, Pacific hake (*Merluccius productus*), Pacific herring (*Clupea harengus*), coho salmon (*Oncorhynchus kisutch*), and embryo proteins of rockfish (*Sebastes* spp.) also possessed the approximately 94 kDa band. Grunt sculpin (*Rhamphocottus richardsoni*) egg extract reacted with the walleye pollock egg yolk antibodies only in the approximately 20 kDa band (Fig. 6, lane 8).

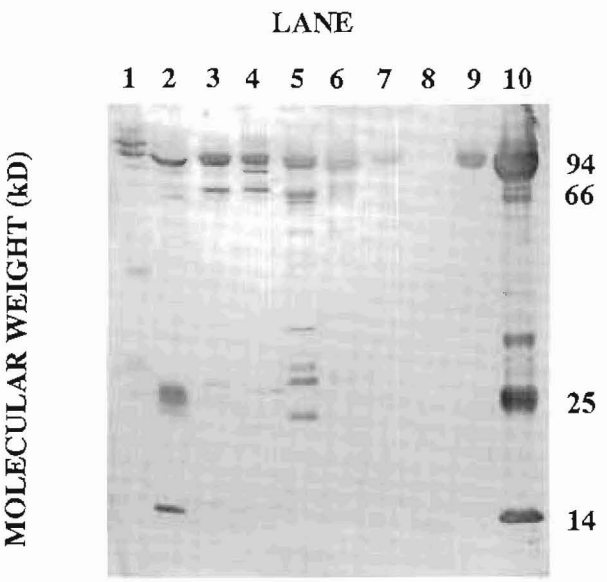


Fig. 6. Western blotting of various egg, ovary and yolk-sacs of teleost species. SDS-PAGE linear gradient 10–20%; proteins transferred to nitrocellulose paper and incubated with rabbit anti-pollock egg yolk protein antibody (1 : 5,000). The secondary antibody used was a 1 : 3,000 dilution of goat anti-rabbit alkaline phosphatase. The reaction was visualized using an NBT/BCIP conjugate reaction. Lanes: 1, Pacific cod egg; 2, Pacific tomcod egg; 3, Pacific halibut egg; 4, Pacific hake ovary; 5, sablefish egg; 6, *Sebastes* sp. embryo; 7, Pacific herring egg; 8, Grunt sculpin egg; 9, coho salmon egg; 10, 24-h fertilized walleye pollock egg.

Discussion

Our results showed that there was egg yolk-related protein in the serum of maturing female walleye pollock because of its immunoreactivity with antibody developed against pollock egg yolk proteins. As shown by SDS-PAGE, followed by Western blotting, the serum egg-related proteins are comprised of a major monomeric protein with a molecular weight of 175 kDa and minor proteins with 97, 76 and 66 kDa. Conversely, Hamazaki et al. (1987) and Babin (1987) found that the antibody developed against purified vitellogenin reacted with the smaller proteins found in egg yolk. The value of 175 kDa is somewhat smaller than that of 200 kDa found for the major serum vitellogenin in the killifish *Fundulus heteroclitus* (Selman and Wallace, 1983) and for the medaka *Oryzias latipes* (Hamazaki et al., 1987), and larger than that of the 140–147 kDa protein found in the sera of goldfish *Carassius auratus* (de Vlaming et al., 1980). It is identical in weight to the protein found in the sera of *Oncorhynchus mykiss*, formerly *Salmo gairdneri* (Babin, 1987). Considering other investigations about subunit structure of fish vitellogenins (Specker and Sullivan, 1994), the band with molecular mass of 175 kDa could be a monomeric form of pollock vitellogenin.

Female sera and maturing unhydrated oocytes both had an immunostaining band indicating a 97 kDa protein. This band was much more intense in the oocyte homogenate blot. Traces of the 97 kDa protein found in the sera suggest that it could result from processing of the 175 kDa serum peptide. The other major immunostaining proteins in the pollock oocytes were 58 and 25 kDa and thus much smaller than those found in sera. Wallace and Selman (1985) found major proteins in *Fundulus* oocytes at 122, 103, 75, 45, 42, 26 and 20 kDa. De Vlaming et al. (1980) found proteins at 105, 110 and 19–25 kDa originating from lipovitellin and phosvitin-derived proteins of 7.6 and 14.5 kDa. Greeley et al. (1986) found that the major yolk protein of homogenates of premature marine pelagic fish eggs is 90–108 kDa and stated that, generally, lipovitellin is 100–130 kDa and phosvitin is 33–40 kDa.

Matsubara et al. (1999) provided a multiple vitellogenin model in barfin flounder (*Verasper moseri*), and two lipovitellins were identified electrophoretically and immunologically in postvitellogenic oocytes. Each appeared to be composed of distinct heavy chains (107 kDa and 94 kDa) and light chains (30 kDa and 28 kDa) when analyzed by SDS-PAGE. As to β'-component and phosvitin in the vitellogenic oocytes of barfin flounder, their molecular weights on SDS-PAGE were estimated as 17 kDa and 38 kDa, respectively (Matsubara and Sawano, 1995). Although the proteins from walleye pollock oocytes were generally smaller than these, our results are similar, with the 97 kDa protein probably representing a heavy chain of lipovitellin.

Greeley et al. (1986) found that hydrated eggs of marine pelagic fishes had a major yolk protein of 90 kDa. Theilacker et al. (1986) reported seven reactive bands to antisera developed against Northern anchovy (*Engraulis mordax*) egg extract corresponding to proteins at 91.2, 80.7, 72.9, 30.3, 28.2, 18.2 and 16.7 kDa. Matsubara et al. (1999) found that one lipovitellin heavy chain decreased in size from 94 kDa to 92 kDa, while another heavy chain showed degradation into free amino acids during oocyte maturation in barfin flounder. Lipovitellin light chains were also processed into smaller peptides (22 kDa and 15 kDa) along with the oocyte maturation. Similar proteolytic cleavege of lipovitellin associated with the final oocyte maturation, and followed by embryogenesis was observed in another flounder, *Pleuronectes americanus* (Hartling and Kunkel, 1999). Carnevali et al. (1999) suggested that cathepsin D and L seemed to be involved in the proteolysis of vitellogenin and yolk proteins in red sea bream (*Sparus aurata*). In our study, we obtained similar

results with the major reactive bands in hydrated unfertilized and fertilized eggs of 94 and 25 kDa and other minor bands of 76, 66 and 14 kDa. Based on strong immunostaining and the molecular mass, the 25 kDa band could be a β'-component or smaller molecule of lipovitellin fragments (a light chain of lipovitellin). The decreased intensity of this band in late-stage embryos indicates that this component was being rapidly catabolized. The shifting distribution of polypeptide molecular weight towards smaller polypeptides from hydrated eggs through late-stage embryos indicates rhar vitellogenin-derived polypeptides are being utilized to meet anabolic and maintenance requirements of developing embryos. Our results generally support the concept rhat proteolytic cleavage of native vitellogenin occurs during receptor mediated endocytosis into yolk globules of the developing oocytes (Wallace, 1985; Busson-Mabillot, 1984). Trace presence of the major 76 kDa egg protein in sera could have resulted from proteolytic processing that results in smaller polypeptides as a result of lysosomal activity (Perona er al., 1988; Wallace and Selman, 1985) and hydration (Greeley et al., 1986). Further proteolytic activity through the fertilized egg and yolk-sac larval stages that results in a shift from large to smaller polypeptides is a function of molecular cleavage at specific stages in development. The products of this activity yield quite special functions in the development process (Indrasith et al., 1987), whereas others may act as energy reserves (Zhu et al., 1986).

Utter and Ridgway (1967) reported that antisera developed against serum vitellins of several teleost species cross-reacted at the family level, and in some cases, the antibodies reacted outside of the family group. Utter and Ridgway (1967) also showed thar rhe amount of specificity between the serum vitellins decreased as the families tested became more genetically distant. Theilacker et al. (1986) reported thar antisera raised against anchovy egg proteins cross-reacted among members of the clupeidae. Covens et al. (1987) observed high levels of immunorecognition when subunits of stickleback (*Gasterosteus aculeatus*) and seabass (*Dicentrarchus labrax*) vitellogenin were tested against estradiol-induced vitellogenic plasma of fish of different orders. From our results and those of others (Covens et al., 1987; Hara et al., 1983; Utter and Ridgway, 1967), it seems that teleost egg proteins are conserved. There may be some changes in configuration, amino acid composition and molecular weight of the egg proteins, but the critical determinants have remained intact. Invertebrate eggs tested showed no specificity to anti-pollock egg yolk proteins. Although studies of vitellogenin coding sequences in genomic DNA of insects, amphibians and birds by hybridization to cDNA probes indicate a high degree of conservation (James et al., 1982; Nardelli et al., 1987), similarities in antigenic determinants between fish and invertebrates have been lost. Algal extracts were tested against pollock egg-yolk protein antibodies because they serve as common foods for invertebrare species tesred (Crook and Sunderland, 1984), and no cross-reaction was noted.

Immunoblotting of egg proteins showed that matured oocytes of other North Pacific fishes exhibited similar banding patterns as hydrated walleye pollock eggs. Different developmental stages i.e., Pacific hake ovary with maturing oocytes, possessed the same banding pattern as 24-hour walleye pollock egg. The occurrence of immunological cross-reactivity between walleye pollock egg protein and distantly related teleost families as demonstrated by Western blots reflects the highly conservative nature of teleost egg-yolk protein.

Acknowledgements

We would like to thank R.A. Wallace and P. Babin for their helpful comments. C. Mills, M. Orellana and P. McMahon provided samples for the cross-reactivity studies. We also thank W.W. Dickhoff for facilities and support. This article is contribution FOCI-0116 to the Fisheries-Oceanography Coordinated Investigations.

References

Babin, P.J. (1987) Apolipoproteins and the association of egg yolk proteins with plasma high density lipoproteins after ovulation and follicular atresia in the rainbow trout (*Salmo gairdneri*). *J. Biol. Chem.*, **262**, 4290-4296.

Bailey, K. and Stehr. C.L. (1986) Laboratory studies on the early life history of the walleye pollock, *Theragra chalcogramma* (Pallas). *J. Exp. Mar. Biol. Ecol.*, **99**, 233-246.

Bergink, E.W. and Wallace, R.W. (1974) Precursor-product relationship between amphibian vitellogenin and the yolk proteins, lipovitellin and phosvitin. *J. Biol. Chem.*, **249**, 2897-2903.

Busson-Mabillot, S. (1984) Endosomes transfer yolk proteins to lysosomes in the vitellogenic oocyte of the trout. *Biol. Cell*, **51**, 53-66.

Carnevali, O., Carletta, R., Cambi, A., Vita, A. and Bromage, N. (1999) Yolk formation and degradation during oocyte maturation in seabream Sparus aurata: involvement of two lysosomal proteinase. *Biol. Reprod.*, **60**, 140-146.

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

Christmann, J.L., Grayson, M.J. and Huang, R.C.C (1977)

Comparative study of hen yolk phosvitin and plasma vitellogenin. *Biochemistry,* **16**, 3250-3256.

Covens, M., Covens, L., Ollevier, F. and De Loof, A. (1987) A comparative study of some properties of vitellogenin (Vg) and yolk proteins in a number of freshwater and marine teleost fishes. *Comp. Biochem. Physiol.,* **88B**, 75-80.

Crook, N.E. and Sunderland. KDA. (1984) Detection of aphid remains in predatory insects and spiders by ELISA. *Ann. Appl. Biol.,* **105**, 413-422.

Greeley, M.S., Calder, D.R. and Wallace. R.A. (1986) Changes in teleost yolk proteins during oocyte maturation: correlation of yolk proteolysis with oocyte hydration. *Comp. Biochem. Physiol.,* **84B**, 1-9.

Hamazaki, T.S., Iuchi, I. and Yamagami, K. (1987) Purification and identification of vitellogenin and its immunohistochemical detection in growing oocytes of the teleost, *Oryzias latipes. J. Exp. Zool.,* **242**, 333-341.

Hara, A. and Hirai, H. (1978) Comparative studies on immunochemical properties of female-specific serum protein and egg yolk proteins in rainbow trout (*Salmo gairdneri*). *Comp. Biochem. Physiol.,* **59B**, 339-343.

Hara, A., Takano, K. and Hirai, H. (1983) Immunochemical identification of female-specific serum protein, vitellogenin. in the medaka, *Oryzias latipes* (Teleosts). *Comp. Biochem. Physiol.,* **76A**, 135-141.

Hartling, R.C. and Kunkel, J.G. (1999) Developmental fate of the yolk protein lipovitellin in embryos and larvae of winter flounder, *Pleuronectes americanus. J. Exp. Zool.,* **284**. 686-696.

Hinckley, S. (1986) Spawning dynamics and fecundity of walleye pollock (*Theragra chalcogramma*) in the Bering Sea. M.S. thesis, Univ. Washington, Seattle, 104 p.

Hiramatsu, N. and Hara. A. (1996) Relationship between vitellogenin and its related egg yolk proteins in Sakhalin taimen (*Hucho perryi*). *Comp. Biochem. Physiol.,* **115A**. 243-251.

Indrasith, L.S., Furusawa, T., Shikata, M. and Yamashita, O. (1987) Limited degradation of vitellin and egg-specific protein in *Bombyx* eggs during embryogenesis. *Insect Biochem.,* **17**, 539-545.

James, T.C., Boud, Y.M., Maack, C.A., Applebaum, S.W. and Tata, J.R. (1982) Evolutionary convervation of vitellogenin genes. *DNA,* **I**. 345-351.

Matsubara, T. and 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.

Matsubara, T., Ohkubo, N., Andoh, T., Sullivan. C.V. and Hara, A. (1999) Two forms of vitellogenin, yielding two distinct lipovitellins, play different roles during oocyte maturation and early development of barfin flounder, *Verasper moseri*, a marine teleost that spawns pelagic eggs. *Dev. Biol.,* **213**, 18-32.

Mommsen, T.P. and Walsh, P.L. (1988) Vitellogenesis and oocyte assembly. pp. 347-406, Hoar, W.S. and Randall, D.J. (eds), *Fish Physiology,* **Vol. XIA**, Academic Press, New York :

Nardelli, D., Gerber-Huber, S., van het Schip, F.D., Gruber, M., Geert, A.B. and Whali, W. (1987) Vertebrate and nematode genes coding for yolk proteins are derived from a common ancestor. *Biochemistry,* **26**, 6397-6402.

Ng, T.B. and Idler, D.R. (1983) Yolk formation and differentiation in teleost fishes. pp. 373-404, Hoar, W.S , Randall, D.J. and Donaldson, E.M (eds), *Fish Physiology,* **Vol. IX**, Academic Press. New York.

Olin, T. and 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.

Perona, R., Bes, J.C. and Vallejo, C.G. (1988) Degradation of yolk in the brine shrimp *Artemia*. Biochemical and morphological studies on the involvement of the lysosomal system. *Biol of the Cell,* **63**, 361-366.

Plack, P.A., Pritchard, D.J. and Fraser. N.W. (1971) Egg proteins in cod serum. Natural occurrence and induction by injections of oestradiol 3-benzoate. *Biochem. J.,* **121**, 847-856.

Selman. K. and Wallace, R.A. (1983) Oogenesis in *Fundulus heteroclitus*. Vitellogenesis. *J. Exp. Zool,* **226**, 441-457.

Specker, J.L. and Sullivan, C.V. (1994) Vitellogenesis in fishes : status and perspectives. pp. 304-315, Davey, K.G., Peter, R E. and Tobe, S.S. (eds), *Perspectives in Comparative Endocrinology*, National Reserch Council. Ottawa.

Theilacker, G.H., Kimball, A.S. and Trimmer, J.S. (1986) Use of an ELISPOT immunoassay to detect euphausiid predation on larval anchovy. *Mar. Ecol. Prog. Ser.,* **30**. 127-131.

Thorsen, A., Kjesbu. O., Fyhn, H.J. and Solemdal, P. (1996) Physiological mechanisms of byoyancy in eggs from brackish water cod. *J. Fish Biol.,* **48**. 457-477.

Utter, F.M. and Ridgway, G.J. (1967) Cross-reactive properties of antisera prepared in rabbits by stimulations with teleost vitellins. *Fish. Bull. U.S.,* **66**, 203-207.

de Vlaming, V.L , Wiley, H.S., Delahunty, G. and Wallace, R.A. (1980) Goldfish (*Carassius auratus*) vitellogenin: induction, isolation, properties and relationship to yolk proteins. *Comp. Biochem. Physiol.,* **67B**, 613-623.

Wallace, R.A. (1978) Oocyte growth in nonmammalian vertebrates. pp. 469-502, Jones, R.E. (ed). *The Vertebrate Ovary*, Plenum Press, New York.

Wallace, R.A. (1985) Vitellogenesis and oocyte growth in non-mammlian vertebrates. pp. 127-177, Browder. L. (ed), *Developmental Biology*, **Vol. 1**,. Pergamon Press, New York.

Wallace, R.A. and Begovac, P.C. (1985) Phosvitins in *Fundulus* oocytes and eggs. Preliminary chromatographic and electrophoretic analyses together with biological considerations. *J. Biol. Chem.,* **260**, 11268-11274.

Wallace, R.A. and Selman, K. (1985) Major protein changes during vitellogenesis and maturation of *Fundulus* oocytes. *Devel. Biol.*, **110**, 492–498.

Yamamura, J.I., Adachi, T., Aoki, N., Nakajima, H., Nakamura, R. and Matsuda, T. (1995) Precursor-product relationship between chicken vitellogenin and the yolk proteins: The 40 kDa yolk plasma glycoprotein is derived from the C-terminal cysteine-rich domain of vitellogenin II. *Biochim. Biophys. Acta*, **1244**, 384–394.

Zhu, J., Indrasith, L.S. and Yamashita, O. (1986) Characterization of vitellin, egg-specific protein and 30 kDa protein from *Bombyx* eggs, and their fates during oogenesis and embryogenesis. *Biochim. Biophys. Acta*, **882**, 427–436.