



HOKKAIDO UNIVERSITY

Title	Enzyme Linked-Immunsorbent Assay (ELISA) of Vitellogenin in Whitespotted Charr, <i>Salvelinus leucomaenis</i>
Author(s)	KWON, Hyuk-Chu; 權, 熾枢; HARA, Akihiko et al.
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In several fish species, the female specific serum (or plasma) protein (Vg) has been characterized by electrophoretic, chromatographic, and immunological procedures (Emmersen and Petersen, 1976; Campbell and Idler, 1976; Hara and Hirai, 1978; Shigeura and Haschemeyer, 1985; Copeland et al., 1986). Induction of Vg in male and immature fishes by administration of estrogen or massive doses of aromatizable androgens have been reported by a number of authors (Aida et al., 1973; Hara and Hirai, 1978; Hori et al., 1979; Maitre et al., 1986). Estrogen also influences changes of the plasma lipid, protein and calcium levels in fishes (Plack et al., 1971; Mugiya and Watabe, 1977; Emmersen et al., 1979; Tinsley, 1985; Petersen and Korsgaard, 1989).

Avian and amphibian Vg incorporated by developing oocytes is enzymatically converted into the yolk proteins, lipovitellin and phosvitin (Wallace and Bergink, 1974; Christmann et al., 1977). In spite of much research on fish Vg, little has been known on a direct relationship between the serum Vg and its related yolk proteins. Hara and Hirai (1978) showed immunologically that rainbow trout Vg is cleaved into two egg yolk proteins, E1 (lipovitellin) and E2, and that these two proteins cross react with an antibody to serum Vg and also cross react with antisera raised against each other.

Vg levels has been measured in fish serum of plasma indirectly by determination of calcium or alkali-labile protein phosphorus (Emmersen and Petersen, 1976; Nath and Sundararaj, 1981) and directly by immunological methods such as immunodiffusion (Utter and Ridgeway, 1967; Plack et al., 1971; Hara and Hirai, 1978) or immunoelectrophoresis (Goedmakers and Verboom, 1974; Craik, 1978; Crim and Idler, 1978; Maitre et al., 1985). Recently, Idler et al. (1979) and Campbell and Idler (1980) have developed a radioimmunoassay (RIA) for more precise and sensitive quantification of fish Vg. Although RIAs are highly sensitive, they have some disadvantages as follows; a short half-life of isotope, instability of labeled conjugates, specialized equipment, waste disposal problems, and high cost.

The enzyme-linked immunosorbent assay (ELISA) has been developed as a method as sensitive as RIAs for quantification of hormones and proteins in tissue culture media (Goldsmith, 1981; Signorella and Hymer, 1984; Maitre et al., 1986). ELISA has several advantages over the RIA technique in its safety, simplicity, and speed. ELISAs for Vg have been developed for some invertebrates (Dumas et al., 1982; Derelle et al., 1986). However, the application of ELISA has not been attempted for fish Vg except for a recent report for sole (Nenez Rodriguez et al., 1989).

The present paper reports procedures for the purification of vitellogenin of whitespotted charr and for the development of an ELISA for charr Vg using the "sandwich" technique. Results of the ELISA measurements of serum Vg levels in charr after injection with estrogen and during the natural reproductive cycle are also reported here.

Materials and Methods

Fish

Whitespotted charr, *Salvelinus leucomaenis*, were obtained in 1988 and 1989 from stocks reared in outdoor fresh-water ponds at the Nanae Fish Culture Experi-

mental Station, Faculty of Fisheries, Hokkaido University. The fish were fed *ad libitum* with commercial trout food once a day.

Six to ten 2-3 year old fish were sampled each month throughout the year. Samplings were done during the spawning season to examine seasonal and maturational changes of serum Vg. After blood samples were collected by caudal section, the ovaries and the liver of each fish were weighed for determination of gonadosomatic index (ovary weight \times 100/body weight) and hepatosomatic index (liver weight \times 100/body weight). Serum and egg samples were collected from mature females for purification of Vg and the egg yolk proteins.

Experiments involving estrogen administration were performed as follows. Immature males weighing 180-200 g were selected and transferred to circular holding tanks. The fish were kept at a water temperature of $16\pm 0.2^{\circ}\text{C}$ and acclimated to the tanks for 1 week prior to the experiments. No food was given before or during the experiments. The fish were injected intraperitoneally with 1 mg estradiol-17 β per kg body weight dissolved in propylene glycol. Control fish were injected with propylene glycol only. Estradiol injections were made three times (days 0, 10, and 20). Blood samples were taken by syringe from the caudal vessels of each fish at various time intervals. Before sampling the fish were anesthetized with 0.01% p-aminobenzoate (benzocaine). Their blood was allowed to clot at room temperature and was then centrifugated to obtain serum. The serum samples were stored at -20°C until use.

Purification of Vg and its related egg yolk proteins

Whitespotted charr Vg and its related egg yolk proteins were isolated according to the procedure described by Hara and Hirai (1978). Ten milliliters of the vitellogenic serum was diluted with 10 vol. of cold distilled water (DW) and left at the cold room (4°C) for 1 hr. After centrifugation, the precipitate was collected and washed by suspension in cold DW and recentrifugation. This procedure was repeated twice. The final precipitate was dissolved in 1 ml of 0.02 M Tris-HCl buffer (pH 8.0) containing 2% NaCl and 0.1% NaN_3 . The solution was applied to a gel filtration column of Sepharose 6B (2.2×56 cm) in the Tris-HCl buffer described above. The column was eluted at a flow rate of 12.8 ml/h, and 2.7 ml fractions were collected. The eluate was monitored by absorbance at 280 nm. The presence of Vg during purification was assessed by single radial immunodiffusion using antiserum to E1 mentioned below. The fraction of one major peak was pooled as purified Vg.

Egg yolk was collected by perforation of ovulated eggs with a syringe fitted with a 20 gauge needle. Preliminary procedures for isolation and purification of two egg yolk proteins were the same as described above for Vg. The water-insoluble fraction of egg yolk was subjected to gel filtration on a Sephadex G-200 column (2.5×73 cm). The two peaks eluted were designated as egg protein 1 (E1) and egg protein 2 (E2), respectively, as previously described by Hara and Hirai (1978).

Preparation of antisera

Samples of the purified Vg (250 $\mu\text{g/ml}$), emulsified with a equal volume of Freund's complete adjuvant, were injected subcutaneously into New Zealand white rabbits 4 times at weekly intervals. Blood samples were taken from the ear veins of each rabbit 7 days after the last injection. After clotting for 1 hr at room

temperature, the blood samples were held in the cold room (4°C) overnight. Serum samples were obtained after centrifugation of the blood at 3,000 rpm for 20 min. Antisera to the two purified egg proteins, E1 and E2, were prepared in the same way.

A polyvalent antiserum against whitespotted charr female serum proteins was prepared by immunizing a rabbit with pooled mature female serum as described by Hara et al. (1984).

Electrophoresis and immunological procedure

Disc electrophoresis was carried out in 5% or 7.5% polyacrylamide gel by the method of Davis (1964). Gels were stained for proteins with Amido black 10B. Lipids were visualized in the gel by staining with Sudan black B according to the method of Prat et al. (1969) and for carbohydrates with periodic acid-Schiff's reagent according to the method of Zacharius et al. (1969).

Gradient SDS-PAGE (4-22%) was carried out using a Tris-glycine buffer system (O'Farrell, 1975). Prior to electrophoresis, samples were incubated in a solution containing 1% SDS, 10% glycerol, and 0.01% bromphenol blue with or without 1% 2-mercaptoethanol (2-ME), at 60°C for 20 min. The gels were run at a constant voltage of 24 V for 16 hr and then stained with 0.1% Coomassie brilliant blue R 250 in ethanol, acetic acid and DW mixture (40 : 10 : 50). The gels were then destained in ethanol, acetic acid, glycerol and DW (200 : 50 : 25 : 725). The molecular weights of proteins were estimated using the following marker proteins (Sigma) : ferritin (MW 18,500), carbonic anhydrase (MW 29,000), ovalbumin (MW 45,000), bovine serum albumin (MW 66,000), phosphorylase B (MW 97,400), β -galactosidase (MW 116,000), myosin (MW 205,000), and catalase (MW 232,000).

Proteins separated by gradient SDS-PAGE were electroblotted onto a nitrocellulose (NC) membrane (Bio-Rad) by the method of Towbin et al. (1979). Before electroblotting, the gel and NC membrane were dipped briefly in transfer buffer (0.025 M Tris base, 0.192 M glycine, 20% methanol). Transfer of the proteins was carried out at 4 mA/cm² for 2 hr using a transfer chamber (Sartblot : Sartorius). After blotting, the NC membrane was blocked for 1 hr at 37°C with 5% skim milk to reduce non-specific reactions. The membrane was incubated in a 1 : 4,000 dilution of primary antiserum (rabbit anti-E1 or anti-E2) in 0.02 M Tris-HCl buffered saline (pH 7.5) (TBS) for 2 hr at 4°C. The NC membrane was washed 3 times for 30 min in TBS containing 0.05% Tween 20 to reduce background staining. Then the membrane was incubated in second antibody solution, goat anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad), for 2 hr in cold room. After washing 3 times in TBS as described above, antigen-antibody complexes were visualized with a HRP color development reagent (15 mg/20 ml TBS, 5 ml methanol and 15 μ l hydrogen peroxide) containing 4-chloro-1-naphthol (Bio-Rad).

Single radial immunodiffusion, double immunodiffusion and immunoelectrophoresis were conducted in 1% agarose gels using veronal buffer (pH 8.6) by routine procedures.

Serum samples and Vg standard solutions were diluted with 0.01 M phosphate buffered saline (pH 7.0) (PBS) containing 1% bovine serum albumin (BSA).

For single radial immunodiffusion, determination of the protein concentration of the Vg standard was performed using Bio-Rad protein assay kit (Bio-Rad), with BSA as a reference standard.

Double antibody sandwich ELISA

The procedure for labeling antibody (Fab' fragment) with enzyme was performed according to the method of Ishikawa (1987).

Preparation of the Fab' fragment from IgG : Rabbit IgG raised against whitespotted charr E1 was isolated from 10 ml antiserum by precipitation with ammonium sulfate at 35% saturation, followed by a diethylaminoethyl cellulose ion exchange chromatography (DE 52, Whatman). Purified IgG (20 mg/ml) was incubated with 0.8 mg pepsin (Sigma) with gentle stirring for 18 hr at 37°C. At the end of digestion period, the mixture was adjusted to pH 7.0 with 0.2 ml of 0.5 M Na₂HPO₄ and subjected to a gel filtration on Sephadex G-200 equilibrated with 0.1 M PBS (pH 7.0). The fraction containing F(ab')₂ fragment was concentrated to 16 mg/ml and then reduced with 50 μl of 0.1 M 2-mercaptoethylamine for 90 min at 37°C. After incubation, the Fab' fragments were isolated on a Sephadex G-75 column using 0.1 M PBS (pH 6.0) containing 5 mM ethylenediaminetetraacetic acid (EDTA).

Introduction of maleimide groups into HRP : Six mg of horseradish peroxidase (Toyobo C-1, RZ 3.33) dissolved in 0.6 ml of 0.1 M PBS (pH 7.0) was incubated with 2.4 mg of N-succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (Zieben chem.) dissolved in 30 μl of N, N-dimethylformamide for 60 min at 37°C with mild stirring. The precipitates formed were removed by centrifugation and the supernatant was subjected to a gel filtration on a column of Sephadex G-25 using 0.1 M PBS (pH 7.0). Fractions containing protein were pooled and concentrated by ultrafiltration.

Conjugation of maleimide-peroxidase and Fab' : The maleimide-peroxidase obtained (3.6 mg) in 0.5 ml of 0.1 M PBS (pH 7.0) was incubated with Fab' (4 mg) in 0.5 ml of 0.1 M PBS (pH 6.0) containing 2.5 mM EDTA at 4°C for 20 hr. To the reaction mixture was added 50 mM N-ethylmaleimide (10 μl). The mixture was then subjected to gel filtration on a column of Sephadex G-200 (2.5 × 73 cm) using 0.1 M PBS (pH 6.5). Each fraction was measured at absorbance 280 nm for protein and 403 nm for enzyme activity. The enzyme-antibody conjugates were stabilized by addition of 1% BSA containing 0.002% sodium merthiolate and stored at 4°C until use.

ELISA assay procedures : Assays were carried out in 96-well polystyrene ELISA microtiter plates (Titertex).

1) Antibody coating

The microtiter plates were coated with rabbit anti-E1 IgG from which was fractionated by DE 52 at a concentration of 40 μg/ml in PBS. A volume of 100 μl per well was dispensed into each well and incubated for 16 hr in the cold room (4°C).

2) Blocking

After 3 washes with 200 μl of PBS-0.1% Tween buffer per well, the plates were incubated with 200 μl of PBS-1% BSA overnight at 4°C or at 37°C.

3) Incubation of samples and standards

After 4 rinses with PBS-Tween, 100 μl of samples or standards, diluted serially with PBS-BSA, was added into microplates and incubated. The standard solutions (Vg from 1,000 ng/ml to 1 ng/ml) were prepared by dissolving a known amount of purified Vg.

4) Incubation with peroxidase-labeled antibody

After washing as described above, each well received 150 μl of peroxidase-

labeled antibody diluted 1 : 400 in PBS-BSA, followed by incubation for 1-4 hr.

5) Enzymatic color reaction

The plates were washed as described in step 2. Color was developed at room temperature for 30 min in the dark by adding 150 μ l of o-phenylenediamine (1 mg/ml 0.1 M citric acid-phosphate buffer (pH 5.0) containing 0.02% H₂O₂) to each well. The reaction was stopped by adding 100 μ l of 4N HCl per well. The absorbance at 492 nm was measured using as ELISA plate reader (Bio-Rad model 2550).

Results

Purification of Vg and its related egg yolk proteins

The water-insoluble fraction of mature female whitespotted charr serum showed a single symmetrical peak on Sepharose 6B column. It was collected as the purified Vg. Its elution position suggested that it has a molecular weight of approximately 540,000. After immunoelectrophoresis, this preparation of Vg gave rise to only one precipitin line when reacted against a polyvalent antiserum to mature female serum proteins or against a specific antiserum to E1 or E2 (data not shown). The purified protein gave a single band on 5% Disc-PAGE, and the band was stained by Sudan black B for lipid and by periodic acid-Schiff reagent (PAS) for polysaccharide (data not shown).

The water-insoluble components of the egg yolk proteins of whitespotted charr were subjected to gel filtration on a column of Sephadex G-200. The elution profile appeared as two peaks, which were designated as E1 and E2, respectively. The molecular weights of these purified proteins were estimated to be approximately 300,000 for E1 and 30,000 for E2. These purified proteins were used as antigens for preparing antisera from rabbits. Purified Vg, E1 and E2 were analyzed by SDS-PAGE with or without 2-ME. The electrophoretic patterns are shown in Fig. 1. Vg polypeptides were resolved into four bands (MW 234,000, 152,000, 113,000, and 70,000, respectively). When reduced with 2-ME, Vg polypeptides were represented by three bands (MW 175,000, 118,000, and 82,000, respectively). E1 showed four major bands (MW 103,000, 84,000, 70,000, and 25,500) and some minor bands.

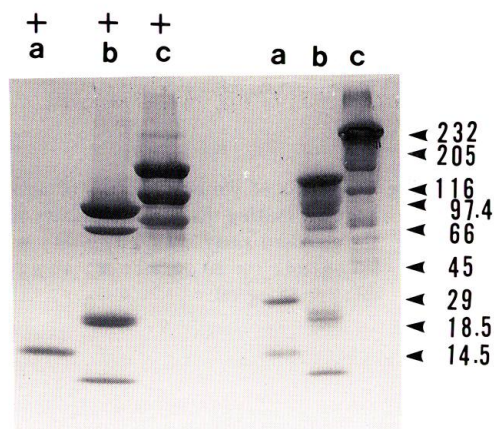


Fig. 1. Gradient SDS-PAGE of the purified E1 (b), E2 (a) and vitellogenin (c) stained with Coomassie brilliant blue R-250. +: reduced with 2-mercaptoethanol. Molecular weight values are expressed in kDa.

Reduced E1 showed three major bands (MW 98,000, 71,500, and 22,000, respectively) and one small peptide band. E2 revealed a single band corresponding to MW 30,000. In the presence of 2-ME, E2 showed a single band of MW 15,000. This suggests that E2 is a disulfide-linked dimer of MW 15,000 subunits.

Specificity of antisera to egg proteins

The specificity of antisera against E1 and E2 was demonstrated by the immunoblotting method. Figure 2 shows SDS-PAGE patterns of male and vitellogenic female sera and their immunoblotting. While the antisera against E1 and E2 showed different reactions with Vg subunits in the female serum, a main subunit of Vg corresponding to MW of 175,000 reacted with both the two antisera. Other small subunits of MW 95,000-105,000 and 60,000-85,000 reacted to anti-E1 and anti-E2, respectively.

ELISA for vitellogenin

1. *Concentration of antibody solution for coating the microtiter plates* : An appropriate concentration of antibody was incubated with 100 μ l of solution containing 1,

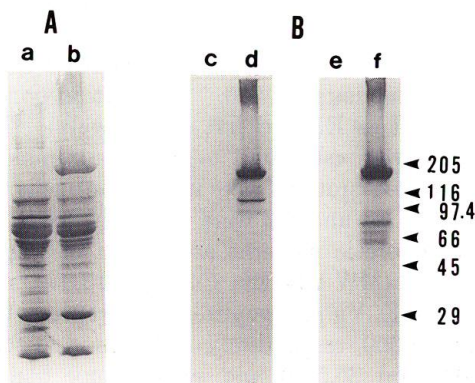


Fig. 2. Gradient SDS-PAGE (A) and immunoblotting pattern (B) of male and female serum proteins reduced with 2-mercaptoethanol. Antisera used for immunoblotting were anti-E1 (c, d) and anti-E2 (e, f). Molecular weight values are expressed in kDa. a, c, and e; male serum. b, d, and f; vitellogenic female serum.

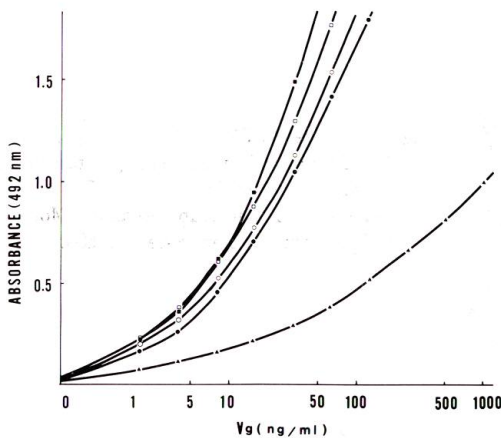


Fig. 3. Effects of various amounts of antibody for coating the microtiter plates on the standard assay. ▲: 1.0 μ g/ml; ●: 2.5 μ g/ml; ○: 10 μ g/ml; ■: 40 μ g/ml; □: 80 μ g/ml

2.5, 10, 40, and 80 $\mu\text{g/ml}$ of purified IgG for 16 hr at 4°C. The incubation time for the first and second reactions was set for 16 hr at room temperature. As shown in Fig. 3, a satisfactory calibration curve was obtained for antibody concentrations ranging from 2.5 to 40 $\mu\text{g/ml}$. The concentration of antibody solution was chosen for coating the plates 40 $\mu\text{g/ml}$ in order to obtain a maximum absorbance value.

2. *Effect of incubation time*: The incubation time necessary for the first and second antibody reactions (conjugates) was examined by the standard enzyme immunoassay procedure using various incubation times. Three different concentrations of Vg (10, 20 and 40 ng/ml) were used. As shown in Fig. 4, both reactions rapidly proceeded within 1 hr regardless of Vg concentrations. Although both reactions showed steady increases with time, incubation times for the first and second reactions were determined as 2 hr each at room temperature from a practical point of view. However, the sensitivity of the assay could be improved by longer incubation time. The calibration curves for various incubation times are shown in Fig. 5A (first reaction) and 5B (second reaction). These results showed that a shorter incubation time has a wider assay range. On the other hand, very short incubation times tended to yield less accurate results.

3. *Effect of temperature on the second reaction*: Influences of temperature on the second reaction were examined at room temperature (ca. 20°C) and 37°C. The reaction time was 2 hr in this experiment. The first reaction was performed overnight (16 hr) at room temperature. As shown in Fig. 6, the sensitivity decreased for assays at 37°C compared to those at room temperature (20°C). Hence, room temperature was selected for the second reaction as well as for the first reaction.

Measurements of serum Vg in male and immature fish treated with estrogen

1. *Primary response to a single injection of estrogen*: Figure 7 shows the changes of serum Vg levels after a single injection of estradiol in male whitespotted charr. The Vg level was measured by the standard ELISA procedure. Vg was not detected in control fish treated with vehicle (propylene glycol) only. Significant serum Vg levels ($0.5 \pm 0.1 \mu\text{g/ml}$) were not detected within 8 hr after the estrogen treatment. The appearance of Vg was detected in the serum between 8 and 10 hr after the estrogen administration and thereafter Vg showed an almost linear increase up to 100 $\mu\text{g/ml}$

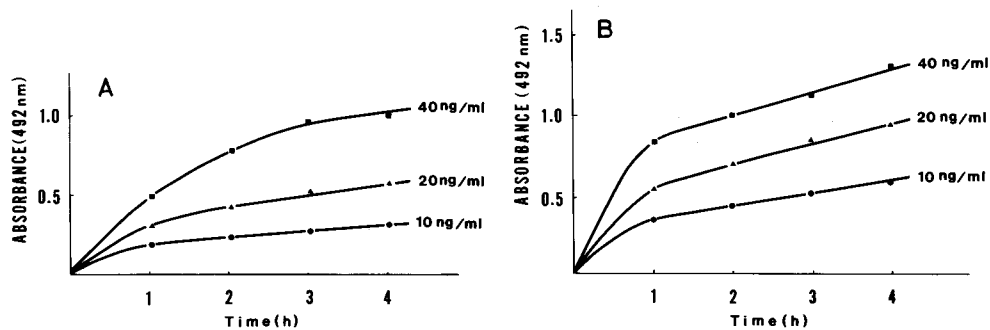


Fig. 4. Effects of the incubation time on the first reaction (A) and the second reaction (B) in three different levels of Vg.

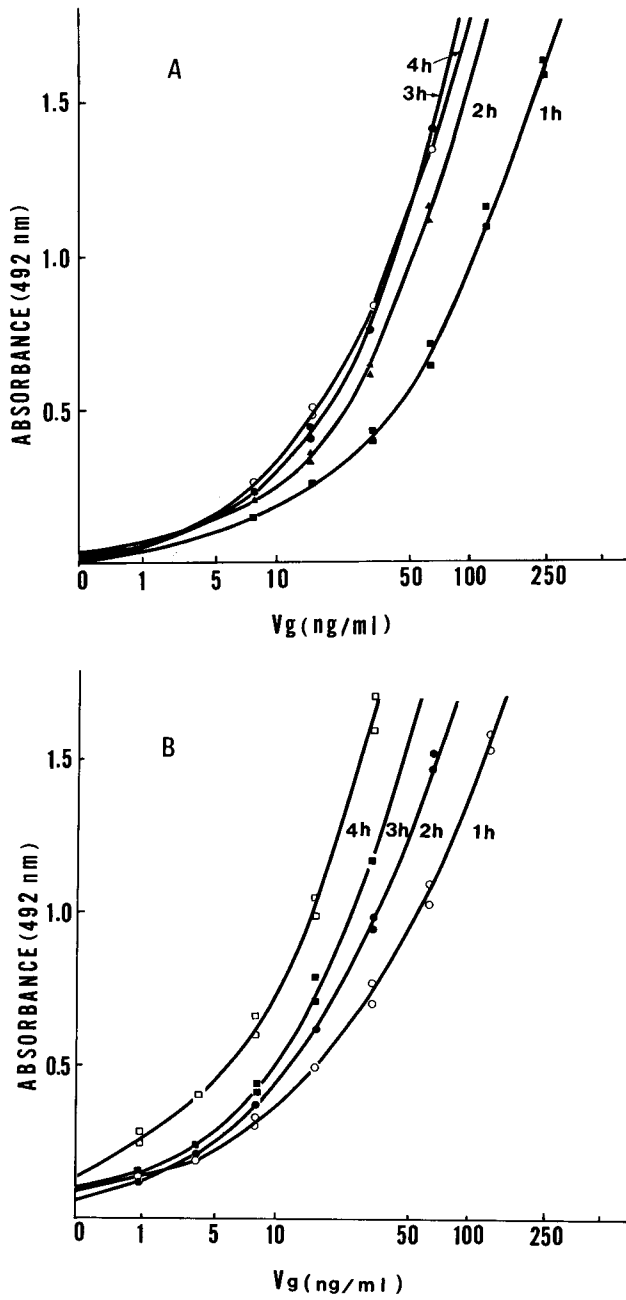


Fig. 5. Calibration curves for incubation time on the first reaction (A) and the second reaction (B).

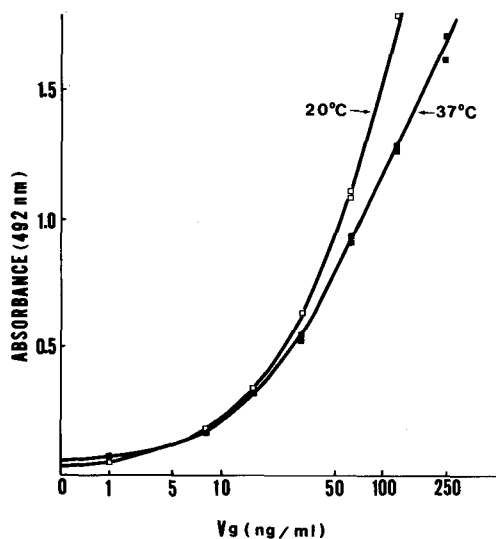


Fig. 6. Effect of temperature on the second reaction. Temperature for the first reaction was set at room temperature (ca. 20°C). Incubation times for the first and second reaction were 16 hr and 2 hr, respectively.

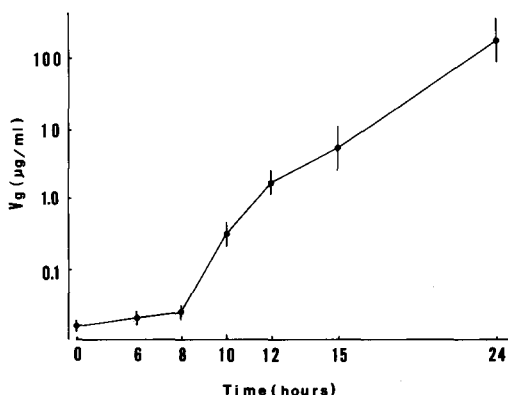


Fig. 7. Changes of serum vitellogenin levels of whitespotted charr male after a single injection of estradiol-17 β . The rearing water temperature was set at 16°C during the experimental period. Vertical bars represent S.E. of means.

throughout a 24 hr period.

2. *Induction of Vg following successive injection of estrogen*: The Vg levels of male whitespotted charr after the first, second, and third injection of estradiol are shown in Fig. 8. Peak levels of serum Vg were observed 10 days after the first injection, and 7-8 days after the second or third injection. When Vg levels reacted a plateau after the second estrogen injection, they were about twofold the peak value following the first injection. These results showed a markedly enhanced response after each successive injection of estradiol. Furthermore, the decline of Vg in the blood after the first injection was faster than those after the second and third injections. Control fish treated with propylene glycol only did not show any Vg in the serum.

Annual changes of serum vitellogenin level

Annual changes in the level of serum Vg, gonadosomatic index (GSI) and

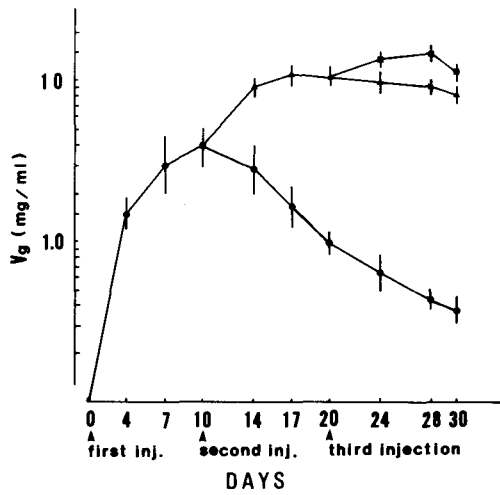


Fig. 8. Amplification of vitellogenin synthesis following three injections of estradiol-17 β as primary, secondary, and tertiary responses in male whitespotted charr. Vertical bars represent S.E. of means.

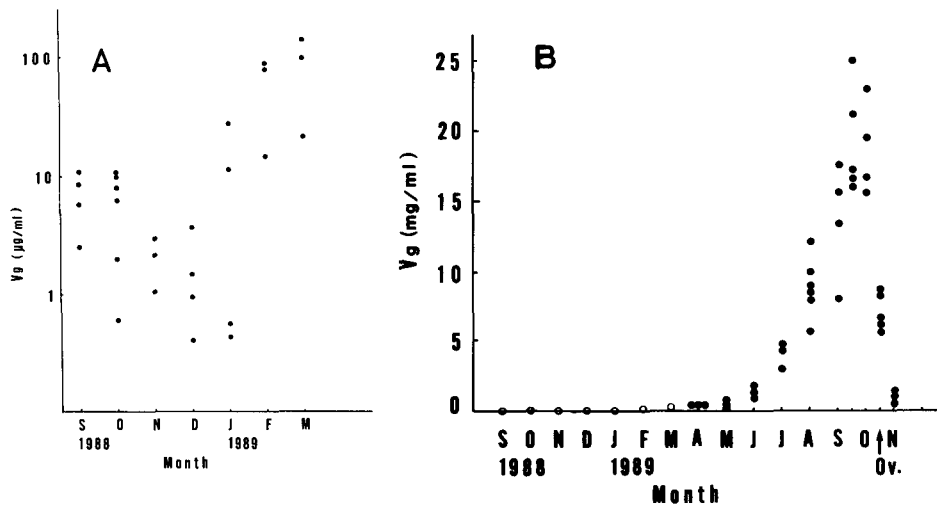


Fig. 9. Seasonal changes of the serum vitellogenin levels in whitespotted charr. Vitellogenin levels were measured by the ELISA (A) and a single radial immunodiffusion method (B).

hepatosomatic index (HSI) are shown in Figs. 9 and 10. During the period between September 1988 and March 1989, when the fish were in previtellogenic stages, serum Vg levels were measured by the newly developed ELISA method (Fig. 9A). Between April and November 1989, serum Vg levels were measured with the single radial immunodiffusion method (Fig. 9B). During the earlier part of the former period (September to December 1988), Vg level showed a slight decrease. The level during this period was within a range of 0.5-11 $\mu\text{g/ml}$ and GSI was maintained at less than 0.5. The Vg level and GSI rose slowly but gradually from February to

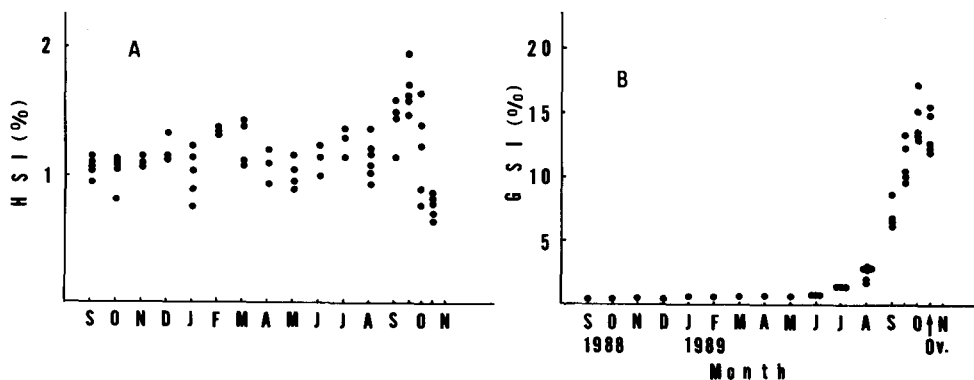


Fig. 10. Seasonal changes of hepatosomatic index (A) and gonadosomatic index (B) in female whitespotted charr.

June 1989. A sharp increase of the Vg level was observed in early July and it reached a maximum value (25 mg/ml) in mid-September, one month before ovulation. Maximum GSI and HSI were observed in early October and mid-September, respectively (Fig. 10). After ovulation, serum Vg levels rapidly dropped to a level less than 200 μ g/ml.

Discussion

In many teleostean fishes, several different procedures such as gel filtration and ion exchange chromatography have been employed for the isolation and purification of Vg. Prior to these procedures, Vg-containing serum proteins were selectively precipitated using distilled water with or without Ca^{2+} , Mg^{2+} , Fe^{2+} and/or EDTA (Wiley et al., 1979; de Vlaming et al., 1980; Campbell and Idler, 1980; Maitre et al., 1985). In the present study, whitespotted charr Vg was purified by precipitation with distilled water, followed column chromatography on Sepharose 6B according to the method of Hara and Hirai (1978). The method using distilled water precipitation for Vg is very easy, simple and efficient for the isolation of salmonid Vg, although its effectiveness depends on the serum Vg concentration. Hara and Dickhoff (unpublished) found that Vg precipitation was not observed by this technique in female coho salmon when the serum Vg concentration was below 5 mg/ml.

It is well known that Vg is subjected a degradation during and/or after isolation procedures (Wiley et al., 1979; de Vlaming et al., 1980). To avoid heterogeneous elution profiles, a serine inhibitor, PMSF, and a trypsin inhibitor, aprotinin, usually are added to blood samples and elution buffers. These inhibitors largely prevent proteolysis, but their use is inconvenient for routine analysis because of their low solubility and high toxicity (Norberg and Hank, 1985). Although proteolysis inhibitors were not used in the present study, the purified Vg was intact as observed by its electrophoretic and immunoelectrophoretic profiles.

The molecular weight of the purified whitespotted charr Vg was 540,000 by Sepharose 6B column chromatography. This molecular weight is similar to the Vg's

of Atlantic salmon (MW 520,000 by So et al., 1985), rainbow trout (MW 535,000 by Maitre et al., 1985) and flounder (MW 550,000 by Emmersen and Petersen, 1976). The range of the molecular weights of native vitellogenins reported for different fish species is wide (MW 326,000–600,000). Mommsen and Walsh (1988) suggested that the large variation in apparent molecular weight of Vg within the same species may be due to different methodologies used, different degrees of proteolytic breakdown, or dephosphorylation during isolation.

It is well known that egg yolk proteins generally consist of lipovitellin and phosvitin. Several studies have shown the presence of these proteins in different fish species (Hara and Hirai, 1978; Campbell and Idler, 1980; de Vlaming et al., 1980). In the present study, whitespotted charr yolk proteins were separated into two components, E1 and E2, according to the procedures of Hara and Hirai (1978). They suggested that, in rainbow trout, E1 is lipovitellin and E2 might be a compound of phosvitin and the β -component of egg yolk. The β -component is characteristic of the yolk of salmonid eggs (Jared and Wallace, 1968; Markert and Vanstone, 1971). Although charr E2 might be a complex of phosvitin and the β -component, two different antigenic proteins could not be detected in this fraction in a previous report (Hara and Hirai, 1978).

The molecular weight of the native lipovitellin (E1) of whitespotted charr was found to be 300,000 by gel filtration. This value is close to those of the putative lipovitellins of rainbow trout (300,000 by Hara and Hirai, 1978; 290,000 by Campbell and Idler, 1980; 350,000 by Riazzi et al., 1988). In this study, the lipovitellin was dissociated into four subunits (MW 98,000, 71,500, 22,000, and a small peptide) by gradient SDS-PAGE in the presence of 2-ME. Some workers have reported that the rainbow trout lipovitellin consists of two subunits (90,000 and 15,000 by Hara and Hirai, 1978; 95,000 and 24,000 by Chen, 1983; 92,000 and 20,000 by Babin, 1987). They demonstrated that it is present as a dimer in its native state. On the other hand, goldfish lipovitellin (de Vlaming et al., 1980) is resolved into two high molecular weight bands (105,000 and 110,000) and four lower molecular weight bands (19,000–25,000).

Phosvitin is known to be a serin-rich phosphoprotein, which can comprise 3% of the egg yolk protein of fishes (Campbell and Idler, 1980). This protein is soluble in trichloroacetic acid and cannot be stained with Coomassie blue (Wallace and Begovac, 1985). Estimates of the molecular weight of fish phosvitin are very variable (19,000 by Mano and Yoshida, 1969; 24,000 by Wallace et al., 1966; 43,000 by Campbell and Idler, 1980). Phosvitin could not be identified immunologically in whitespotted charr. On the other hand, E2 of the charr was estimated to have a molecular weight of 30,000 by gel filtration. SDS-PAGE indicated that the molecular weight of E2 was 30,000 without reduction and 15,000 after reduction with 2-ME. These components appear similar to E2 from rainbow trout egg yolk protein (Hara and Hirai, 1978).

In the present study, the antibody against E1 was used for ELISA. This was done for the following reason. Hara et al. (1984) identified two female-specific serum proteins in mature female and estrogen-treated whitespotted charr by immunoelectrophoresis using antiserum against charr Vg. One of the female-specific serum proteins was considered to be identical with Vg, composed of a complex of E1 and E2. The other seemed to be a fragment of Vg, because of its deficient E1

antigenicity and strong E2 antigenicity. They concluded that the two female-specific proteins found in the charr serum represent an E1 + E2 (Vg) complex and a free E2 fragment of Vg. Therefore, precise amount of Vg in the serum can be determined with antiserum to E1. In this study, the results of Western blotting supported the above mentioned hypothesis.

Idler et al. (1979) have developed a RIA for measurement of fish Vg. However, this technique has not found widespread application because of the difficulty in isotopic labeling of Vg and the instability of the labeled Vg. These problems were partially improved by using plasma Vg as the standard and an egg yolk fraction (lipovitellin or β -component) as the tracer (Campbell and Idler, 1980). Suitable RIAs for the determination of fish Vg were developed using mild iodogen methods (Salacinski et al., 1979) for labeling Vg (Sumpster, 1985; So et al., 1985; Copeland et al., 1986). Nevertheless, the problem of instability of the labeled Vg still remains to be solved.

Recently, an ELISA for fish Vg was described using a competitive binding method (Nunuz Rodriguez et al., 1989). In the present study, a specific and sensitive ELISA for whitespotted charr Vg was developed utilizing a non-competitive (sandwich) method in polystyrene microtiter plate as the solid phase.

For conjugating antibody and enzyme, there are several methods in which glutaraldehyde, periodate, maleimide and pyridyl disulfide are used as coupling reagents. The maleimide method was chosen in this study. The peroxidase-Fab' fragment of antibody conjugates made by this method was found to have a superior performance compared to those generated by other methods (Yoshitake et al., 1982). The conjugates prepared were less polymerized and less heterogenous after coupling the enzyme to the antibody. Moreover, the maleimide method can be performed under mild conditions and does not impair either enzyme or antibody. The conjugate prepared in this way was so stable that it could be used for more than 10 months without significant loss of activity.

To optimize the assay conditions, various experiments were performed in this study. Engvall and Perlmann (1972) indicated that low concentrations of antigens may not be detected if the concentration of antibody used for coating the plates is either too high or too low. In the present experiments, a concentration of 40 $\mu\text{g}/\text{ml}$ was chosen for coating the plates with antibody to insure maximum sensitivity.

The optimum incubation time and temperature for the assay were also investigated. To shorten the incubation time in the second reaction, the effect of a high temperature (37°C) was tested. However, this temperature did not increase the reaction rate compared to a room temperature (20°C). This is probably due to an increase in the rate of the reverse reaction, i.e., dissociation of antigen-antibody complex (Hibi, 1978). Hence, the plates in these experiments were sensitized at room temperature. The incubation time of samples and conjugates was usually 2 hr for the sake of convenience. When a more sensitive assay was necessary, the incubation time for the first reaction was prolonged to 4 hr and that for the second reaction was set overnight (16 hr). The sensitivity of the present study was almost equivalent to that of RIAs for fish Vg by Campbell and Idler (1980).

Although Vg is not detectable in plasma of males and immature females, Vg synthesis can be induced by treating these animals with estrogen (Korsgaard and Petersen, 1976; Hori et al., 1979; Campbell and Idler, 1980; Nath and Sundararaj,

1981). The time course of Vg synthesis after a single injection of estrogen had been well established in chicken and frog; the first detectable levels of Vg in the blood was induced 10 hr after injection in roosters (Bergink and Wallace, 1974), 9-12 hr in *Xenopus laevis* (Clemens, 1974), and 24-48 hr in cod (Plack et al., 1971). These data were obtained by measuring the rates of isotope incorporation into proteins secreted by liver slices or explants and by immunochemical identification of serum Vg synthesized *in vivo*. In whitespotted charr, Vg was detected 8-10 hr after a single injection of estrogen, *i.e.* much faster than in cod (Plack et al. 1971).

The amplification effect of successive estrogen treatments on vitellogenesis has been studied in detail in the amphibian (Tata and Smith, 1979). A similar phenomenon was observed in the present study of whitespotted charr. Our observations on this phenomenon in whitespotted charr, made using a direct measurement of Vg by ELISA, were similar to those reported by Sundararaj and Nath (1981) for catfish. They used an indirect measurement of plasma alkali-labile phosphorus as an index of Vg. However, in whitespotted charr, the time required (7-10 days) to reach the maximum Vg level after each successive estrogen treatment was longer than in catfish (2 days). According to Bohemen and Lambert (1981), the annual reproductive cycle of female rainbow trout is divided into four physiological periods: 1) previtellogenesis, 2) endogenous vitellogenesis, 3) exogenous vitellogenesis, and 4) ovulation and spawning. This model has been generally accepted for other salmonid species. During exogenous vitellogenesis distinct increases of GSI, HSI, and serum Vg levels are observed, but the serum Vg concentration is the most interesting parameter for studying this maturing process. By application of ELISA for Vg to whitespotted charr, it was possible to quantify serum Vg and to determine the sex of fish in early gonadal stages of development gonads. Vg levels in whitespotted charr varied from 5-10 $\mu\text{g/ml}$ in September 1988 to 15-25 mg/ml at the end of exogenous vitellogenesis in mid-September 1989. GSI also increased in accordance with Vg levels. It is very interesting to note that serum Vg levels tended to decrease slightly during September to December 1988. The occurrence of Vg during fish previtellogenesis has not been reported previously. Further investigations on the vitellogenesis during this period are needed to fully understand the physiology of vitellogenesis.

In the present study two methods, immunodiffusion and ELISA, were used for the quantification of fish Vg. The immunodiffusion method has found general application for measuring fish Vg. Although its sensitivity (20 $\mu\text{g/ml}$) is inferior to that of RIA and ELISA (less than 10 ng/ml), this technique is sufficient to quantify circulating serum Vg during the later stages of exogenous vitellogenesis. The ELISA is so sensitive that even a trace amount of Vg in serum samples can be measured. Therefore, the samples should be diluted more than one million-fold, if necessary. In this regard, use of both immunodiffusion method and ELISA may be very effective in studies of the dynamics of serum Vg during gametogenesis.

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References

- Aida, K., Ngan, P.V. and Hibiya, T. (1973). Physiological studies of gonadal maturation of fishes. I. Sexual differences in composition of plasma protein of ayu in relation to gonadal maturation. *Bull. Japan. Soc. Sci. Fish.* **39**, 1107-1115.
- 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.
- Bergink, E.W. and Wallace, R.A. (1974). Estrogen-induced synthesis of yolk proteins in roosters. *Am. Zool.* **14**, 1177-1193.
- Bohemen, Ch. G. van and Lambert, J.D.G. (1981). Estrogen synthesis in relation to estrone, estradiol, and vitellogenin plasma level during the reproductive cycle of the female rainbow trout, *Salmo gairdneri*. *Gen. Comp. Endocrinol.* **45**, 105-114.
- Campbell, C.M. and Idler, D.R. (1976). Hormonal control of vitellogenesis in hypophysetomized winter flounder (*Pseudopleuronectes americanus* Walbaum). *Ibid.* **28**, 143-150.
- Campbell, C.M. and Idler, D.R. (1980). Characterization of an estradiol induced protein from rainbow trout serum as vitellogenin by the composition and radioimmunological cross-reactivity by ovarian yolk fractions. *Biol. Reprod.* **22**, 605-617.
- Chen, T.T. (1983). Identification and characterization of estrogen-responsive gene products in the liver of rainbow trout. *Can. J. Biochem. Cell Biol.* **61**, 802-810.
- Christmann, J.L., Grayson, M.J. and Hwang, R.C.C. (1977). Comparative study of hen yolk phosvitin and plasma vitellogenin. *Biochemistry* **16**, 3250-3256.
- Clemens, M.J. (1974). The regulation of egg yolk protein synthesis by steroid hormones. *Progress. Biophys. Mol. Biol.* **28**, 71-107.
- Copeland, P.A., Sumpter, J.P., Walker, T.J. and Croft, M. (1986). Vitellogenin levels in male and female rainbow trout (*Salmo gairdneri* Richardson) at various stages of the reproductive cycle. *Comp. Biochem. Physiol.* **83B**, 487-493.
- Craik, J.C.A. (1978). The effect of estrogen treatment on certain plasma constituents associated with vitellogenesis in the elasmobranch *Scyliorhinus canicula* L. *Gen. Comp. Endocrinol.* **35**, 455-464.
- Crim, L.W. and Idler, D.H. (1978). Plasma gonadotropin, estradiol and vitellogenin and gonad phosvitin levels in relation to the seasonal reproductive cycle of female brown trout. *Ann. Biol. Anim. Biochim. Biophys.* **18**, 1001-1005.
- Davis, B. (1964). Disc electrophoresis-II. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.* **121**, 404-427.
- Derelle, E., Grosclaude, J., Meusy, J.-J., Junera, H. and Martin, M. (1986). ELISA titration of vitellogenin and vitellin in the freshwater prawn, *Macrobrachium rosenbergii*, with monoclonal antibody. *Comp. Biochem. Physiol.* **85B**, 1-4.
- 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. *Ibid.* **67B**, 613-623.
- Dumas, M., Buschor, J. and Lanzrein, B. (1982). Development of an enzyme-linked immunosorbent assay (ELISA) for measurement of vitellogenin concentrations in cockroaches. *Experientia* **38**, 874-875.
- Emmersen, B.K. and Petersen, I.M. (1976). Natural occurrence, and experimental induction by estradiol-17 β , of a lipophosphoprotein (vitellogenin) in flounder (*Platichthys flesus*, L.). *Comp. Biochem. Physiol.* **55B**, 315-321.
- Emmersen, J., Korgsaard, B. and Petersen, I.M. (1979). Dose response kinetics of serum vitellogenin, liver DNA, RNA, protein and lipid after induction by estradiol-17 β in male flounders (*Platichthys flesus* L.). *Ibid.* **63B**, 1-6.

- Engvall, E. and Perlmann, P. (1972). Enzyme linked immunosorbent assay, ELISA. III. Quantification of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. *J. Immunol.* **100**, 129-135.
- Goedmakers, A. and Verboom, B.L. (1974). Studies on the maturation and fecundity of the pike, *Esox lucius*. *Aquaculture* **4**, 3-12.
- Goldsmith, P.K. (1981). A highly sensitive enzyme-linked immunosorbent assay for human immunoglobulin E: Comparison of microtiter plate and disk methodologies. *Anal. Biochem.* **117**, 53-60.
- 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., Matsubara, T., Saneyoshi, M. and Takano, K. (1984). Vitellogenin and its derivatives in egg yolk proteins of whitespotted charr (*Salvelinus leucomaenis*). *Bull. Fac. Fish. Hokkaido Univ.* **35**, 144-153.
- Hibi, N. (1978). Enzyme-immunoassay of human α -fetoprotein. *Gann.* **69**, 67-75.
- Hori, S.H., Kodama, T. and Tanahashi, K. (1979). Induction of vitellogenin synthesis in goldfish by massive doses of androgen. *Gen. Comp. Endocrinol.* **37**, 306-320.
- Idler, D.R., Hwang, S.J. and Crim, L.W. (1979). Quantification of vitellogenin in Atlantic salmon, *Salmo salar* plasma by radioimmunoassay. *J. Fish. Res. Bd. Can.* **36**, 574-578.
- Ishikawa, E. (1987). Methods for enzyme-labeling; antigens and antibodies. p. 75-126. In Ishikawa, E., Kawai, T. and Miyai, K. (eds.), *Enzyme Immunoassay* 410 p. Igaku Shoin, Tokyo.
- Jared, D.W. and Wallace, R.A. (1968). Comparative chromatography of the yolk proteins of teleosts. *Comp. Biochem. Physiol.* **24**, 437-443.
- Korsgaard, B. and Petersen, I.M. (1976). Natural occurrence and experimental induction by estradiol-17 β of a lipophosphoprotein (vitellogenin) in flounder (*Platichthys flesus*, L). *Ibid.* **54B**, 443-446.
- Maitre, J.L., Le Guellec, C., Derrien, S., Tenniswood, M. and Valotaire, Y. (1985). Measurement of vitellogenin from rainbow trout by rocket immunoelectrophoresis: Application to the kinetic analysis of estrogen stimulation in the male. *Can. J. Biochem. Cell Biol.* **63**, 982-987.
- Maitre, J.L., Valotaire, Y. and Guguen-Guillouzo, C. (1986). Estradiol-17 β stimulation of vitellogenin synthesis in primary culture of male rainbow trout hepatocytes. *In Vitro Cell. Develop. Biology* **22**, 337-343.
- Mano, Y. and Yoshida, M. (1969). A novel composition of phosvitins from salmon and trout roe. *J. Biochem. (Tokyo)* **66**, 105-108.
- Markert, J.R. and Vanstone, W.E. (1971). Egg proteins of coho salmon (*Oncorhynchus kisutch*): Chromatographic separation and molecular weights of the major proteins in the high density fraction and their presence in salmon plasma. *J. Fish. Res. Bd. Can.* **28**, 1853-1856.
- Mommsen, T.P. and Walsh, P.J. (1988). Vitellogenesis and oocyte assembly. p. 347-406. In Hoar, W.S., and Randall, D.J. (eds.), *Fish Physiology* **11A**, 546 p. Academic Press, New York.
- Mugiya, Y. and Watabe, N. (1977). Studies on fish scale formation and resorption. II. Effect of estradiol on calcium homeostasis and skeletal tissue resorption in the goldfish, *Carassius auratus* and the killifish, *Fundulus heteroclitus*. *Comp. Biochem. Physiol.* **57A**, 197-202.
- Nath, P. and Sundararaj, B.I. (1981). Isolation and identification of female-specific serum phospholipoproteins (vitellogenin) in the catfish *Heteropneustes fossilis*. *Gen. Comp. Endocrinol.* **43**, 184-190.
- Nenez Rodriguez, J.N., Kah, O., Geffard, M. and Le Menn, F. (1989). Enzyme-linked immunosorbent assay (ELISA) for sole (*Soles vulgaris*) vitellogenin. *Comp. Biochem. Physiol.* **92B**, 741-746.
- Ng, T.B. and Idler, D.R. (1983). Yolk formation and differentiation in teleost fishes. p. 373-404. In Hoar, W.I., Randall, D.J. and Donaldson, E.M. (eds.), *Fish Physiology* **9**, 477 p. Academic Press, New York.
- Norberg, B. and Haux, C. (1985). Induction, isolation and a characterization of the lipid content of plasma vitellogenin from two *Salmo* species: Rainbow trout (*Salmo gairdneri*) and sea trout (*Salmo trutta*). *Comp. Biochem. Physiol.* **81B**, 869-876.

- O'Farrell, P.H. (1975). High resolution two dimensional electrophoresis of protein. *J. Biol. Chem.* **250**, 4007-4021.
- Pan, M.L., Bell, W.J. and Telfer, W.H. (1969). Vitellogenic blood protein synthesis by insect fat body. *Science* **165**, 393-394.
- Petersen, I. and Korsgaard, B. (1989). Experimental induction of vitellogenin synthesis in eel (*Anguilla anguilla*) adapted to seawater or freshwater. *Comp. Biochem. Physiol.* **93B**, 57-60.
- 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.
- Prat, J.P., Lamy, J.N. and Weill, J.D. (1969). Coloration des lipoproteins apres electrophorese en gel de polyacrylamid. *Bull. Soc. Chim. Biol.* **51**, 1367.
- Riazi, A., Fremont, L. and Gozzelino, M.T. (1988). Characterization of egg yolk proteins from rainbow trout *Salmo gairdneri* (RICH). *Comp. Biochem. Physiol.* **89B**, 399-407.
- Salacinski, P., Hope, J., Clement-Jones, V., Sykes, J. and Lowry, P.J. (1979). A new simple method which allows theoretical incorporation of radio-iodine into proteins and peptides without damage. *J. Endocrinol.* **81**, 131.
- Shigeura, H.T. and Haschemeyer, A.E.V. (1985). Purification of egg yolk proteins from the Antarctic fish *Chaenocephalus aceratus*. *Comp. Biochem. Physiol.* **80B**, 935-949.
- Signorella, A.P. and Hymer, W.C. (1984). An enzyme-linked immunosorbent assay for rat prolactin. *Anal. Biochem.* **136**, 372-381.
- So, Y.P., Idler, D.R. and Hwang, S.J. (1985). Plasma vitellogenin in the landlocked Atlantic salmon, *Salmo salar* Ouananiche: Isolation, homologous radioimmunoassay and immunological cross-reactivity with vitellogenin from other teleosts. *Comp. Biochem. Physiol.* **81B**, 63-71.
- Sumpter, J.P. (1985). The purification, radioimmunoassay and plasma levels of vitellogenin from the rainbow trout, *Salmo gairdneri*. p. 355-357. In Lofts, B. and Holmes, W.N. (eds.), *Current Trends in Comparative Endocrinology*. Hong Kong University Press, Hong Kong.
- Sundararaj, B.I. and Nath, P. (1981). Steroid-induced synthesis of vitellogenin in the catfish, *Heteropneustes fossilis* (Bloch). *Gen. Comp. Endocrinol.* **43**, 201-210.
- Tata, J.R. and Smith, D.F. (1979). Vitellogenesis: A versatile model for hormonal regulation of gene expression. *Rec. Prog. Horm. Res.* **35**, 47-95.
- Tinsley, D. (1985). A comparison of plasma levels of phosphoprotein, total protein and total calcium as indirect indices of exogenous vitellogenesis in the crucian carp, *Carassius carassius* (L.). *Comp. Biochem. Physiol.* **80B**, 913-916.
- Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gel to nitro cellulose sheets: procedure and some application. *Proc. Natl. Acad. Sci. USA* **76**: 4350-4354.
- Utter, F.M. and Ridgeway, G.J. (1967). A serologically detected serum factor associated with maturity in the English sole, *Parophrys vetulus* and Pacific halibut, *Hippoglossus stenolepis*. *Fish. Bull.* **6**, 47-48.
- Wallace, R.A. (1985). Vitellogenin and oocyte growth in nonmammalian vertebrates. p. 127-177. In Browder, L.W. (ed.), *Developmental Biology 1*, Plenum, New York.
- Wallace, R.A. and Begovac, P.C. (1985). Phosvitin in *Fundulus* oocytes and eggs. *J. Biol. Chem.* **260**, 11268-11274.
- Wallace, R.A. and Bergink, E.W. (1974). Amphibian vitellogenin: Properties, hormonal regulation of hepatic synthesis and ovarian uptake, and conversion to yolk proteins. *Am. Zool.* **14**, 1159-1175.
- Wallace, R.A., Jared, D.W. and Eisen, A.Z. (1966). A general method for the isolation and purification of phosvitin from vertebrate eggs. *Can. J. Biochem.* **44**, 1647-1655.
- Wiegand, M.D. (1982). Vitellogenesis in fishes. p. 136-146. In Richter, C.J.J. and Goos, H.J.T. (eds.), *Reproductive Physiology of Fish*, 256 p. Pudoc, Wageningen.
- Wiley, H.S., Opresko, L. and Wallace, R.A. (1979). New methods for the purification of vertebrate vitellogenin. *Anal. Biochem.* **97**, 145-152.
- Yoshitake, S., Imagawa, M. and Ishikawa, E. (1982). Efficient preparation of rabbit Fab'-horseradish peroxidase conjugate using maleimide compounds and its use for enzyme im-

munoassay. *Anal. Lett.* **15** (B2), 147-160.

Zacharius, R.M., Zell, T.E., Morrison, J.H. and Woddlock, J.J. (1969). Glycoprotein staining following electrophoresis on acrylamide gels. *Anal. Biochem.* **30**, 148-152.