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Title: Purification and characterization of a novel incomplete-type vitellogenin protein (VgC) in Sakhalin taimen (*Hucho perryi*)

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Abstract

A novel, incomplete-type vitellogenin (VgC) and its derived yolk lipovitellin (LvC) were immunologically detected in female serum and egg extracts, respectively, of Sakhalin taimen (*Hucho perryi*) using a subtype-specific antiserum against LvC of grey mullet (*Mugil cephalus*). The taimen VgC was purified from the sera of vitellogenic females by a combination of gel filtration, anion exchange, and immunoabsorbent column chromatography. Gel filtration of the purified VgC revealed that it had an apparent native mass of ~380 kDa, while the mass of the VgC polypeptide that appeared following SDS-PAGE was estimated to be ~140 kDa. An antiserum was raised against the purified VgC and utilized for the development of a subtype-specific immunoassay for VgC. Levels of VgC in the serum of female taimen increased from 25 µg/ml to ~1 mg/ml, with an increase of GSI. Levels of complete-type Vg and estradiol-17β (E₂) in the serum of E₂-administered juvenile taimen increased and reached peak levels similar to those found in vitellogenic females. Although VgC could be induced in the serum of E₂-administered taimen, it stayed at levels (35.5-73 µg/ml) lower than those obtained in females. This is the first report on the presence of serum VgC and yolk LvC in a salmonid species; these findings indicate that for Sakhalin taimen, like other highly-evolved teleost species, this minor subtype of Vg is significant in the formation of egg yolk.

Keywords: multiple vitellogenin, purification, quantification, Sakhalin taimen

1. Introduction

Vitellogenin (Vg) is the main precursor of egg yolk proteins in oviparous vertebrates (Wallace, 1985; Mommsen and Walsh, 1988; Specker and Sullivan, 1994). It is used as a biomarker for detecting the onset of puberty and the progression of maturation in female fish (Hiramatsu et al., 2005). It has been stated that, in teleosts, Vg is first sequestered in growing oocytes and then cleaved proteolytically into three major yolk proteins: lipovitellin (Lv), phosvitin (Pv), and β' -component (β' -c) (Matsubara and Sawano, 1995; Hiramatsu and Hara, 1996). This simple model in which one molecule of teleost Vg gives rise to three yolk proteins has been referred to as the “single Vg” model (see Hiramatsu et al., 2002c, 2005). However, recent gene cloning and immunobiochemical analyses have confirmed that the presence of multiple forms of Vg in fish is entirely normal, leading to the adoption of a new “multiple Vg model” for teleost oocyte growth (reviews: Hiramatsu et al., 2002c, 2005, 2006; Patiño and Sullivan 2002; Matsubara et al., 2003). As described in Hiramatsu et al. (2002c, 2005), members of advanced teleost taxa (*Paracanthopterygii* and *Acanthopterygii*) generally express three types of Vg at the transcription level, two of which (classified as VgA and VgB by Hiramatsu et al., 2005, 2006; VgAa and VgAb by Finn and Kristoffersen, 2007) have been referred to as a “complete” Vg form based on their complete structure with regard to yolk protein domains (Lv, Pv, and β' -c). On the other hand, one unique form of teleost Vg (VgC or Pv-less Vg), consisting largely of only the Lv domain, was referred to as an “incomplete” Vg form. It has been predicted that this VgC transcript would be detected in not only advanced teleosts but also in almost all fish (Matsubara et al., 2003; Hiramatsu et al., 2006), because VgC seems to be coded for by a

primitive form of Vg genes (Wang et al., 2000). However, this “incomplete” feature (e.g., the lack of Pv domain) of VgC may be a derived character and not an ancestral state (Babin, 2008), because VgC gene of evolved teleost species (e.g., *Acanthopterygii*) appeared to be an orthologue to a “complete” form of Vg in chicken (Vtg-I); these Vg genes were enclosed in a conserved Vg gene cluster in genomes of oviparous vertebrates. So far, the VgC protein was detected in only advanced teleosts (i.e., *Paracanthopterygii* and *Aconthopterygii*) and extent of its presence in primitive teleosts has not been verified yet (see reviews: Hiramatsu et al., 2005, 2006).

Sakhalin taimen (*Hucho perryi*) is distributed in Hokkaido (the largest island in northern Japan), the Southern Kuril Islands, Sakhalin, and Primore of Far Eastern Russia (Holcik et al., 1988; Kawamura, 1989). The sexual maturation of this species appears to be late compared to that of other salmonids; female taimen first spawn at 6-8 years of age. This species is depleted in Hokkaido streams from overfishing, loss of spawning grounds, and water pollution, and recently came to the brink of extinction in Japan. In order to obtain basic information about the reproductive physiology of this fish, Hiramatsu and Hara (1996) characterized Vg and yolk proteins and subsequently developed an enzyme-linked immunosorbent assay (ELISA) to measure Vg in the serum of female taimen for monitoring their maturation (Hiramatsu et al., 1997). The type of Vg quantified in that study appeared to be the complete-type Vg owing to its biochemical features and immunological relation with yolk proteins (Hiramatsu and Hara, 1996); thus, the VgC protein and its derivative yolk proteins have not been verified in Sakhalin taimen (nor in any other salmonids).

In Japanese goby (*Acanthogobius flavimanus*), two subtypes of Vg protein (Vg-530 and Vg-320; they were identified as VgA and VgC, respectively, by Matsubara et al. (2003)) were quantified and levels of Vg-320 were found to be higher than those of the Vg-530 in the serum of females when they began vitellogenesis (Ohkubo et al., 2003). In Japanese medaka (*Oryzias latipes*) (Hiramatsu et al., 2008), ratios of two subtypes of Vg (VgA/B:VgC) in the serum of females varied depending on their reproductive stages (ratios ranged from ~2:1 to ~8:1). Like in the goby, however, in medaka, VgC might be the dominant Vg subtype at an early vitellogenic stage and perhaps is induced earlier than complete-type Vg (VgA/B) under a low level of estrogenic stimulation because: 1) VgC alone was detected in the serum of males after a very short (2 h) exposure to estradiol-17 β (E₂), and 2) in pre-vitellogenic oocytes, an antiserum against VgC (a-VgC) immunostained yolk granules, however no immunoreactivity was found when a-VgA/B was used. These findings suggest that VgC could be used as a sensitive and early biomarker for detecting the initiation of puberty in females, although levels of complete-type Vg generally become much higher than those of VgC in fish in active vitellogenic phases (Ohkubo et al., 2003; Fujiwara et al., 2005; Hiramatsu et al., 2008). However, no information is currently available for VgC in salmonids with this regard.

The objectives of this study were to obtain basic knowledge on the biochemical and physiological properties of the VgC protein in salmonids. Therefore, we specifically aimed to: (1) detect, purify, and characterize the VgC protein in Sakhalin taimen, (2) develop a type-specific immunoassay (single-radial immunodiffusion; SRID) for VgC, and (3) verify the properties of this protein in the circulation of juvenile taimen following E₂

administration, as well as in serum and oocytes (or eggs) of females at different reproductive stages.

2. Materials and methods

2.1. Experimental animals, tissue samples, and hormone treatment

Sakhalin taimen were reared at the Nanae Fresh-Water Laboratory, Field Science Center for Northern Biosphere, Hokkaido University, Hakodate, Japan in outdoor concrete ponds supplied with a continuous flow of river water, at ambient temperature and photoperiod. The fish were fed to satiation with a commercial trout food once a day.

Three to ten female taimen were sampled each month from January 2001 to August 2002 (5- and 6-year-old groups). Blood samples were collected from their caudal vessels and allowed to stand at 4°C for several hours. Serum was then separated by centrifugation and stored at -30°C until use. Ovaries were collected and weighed for the estimation of gonad somatic index (GSI).

In 2006, additional samples of blood and ovaries (or ovulated eggs) were obtained from five vitellogenic females and two females at ovulation in order to quantify the levels of the two subtypes of Vg and Lv in those samples.

For the estrogen administration experiments, juvenile taimen (2 years old) were selected and transferred to circular holding tanks supplied with a continuous flow of artesian well water. The fish were fasted before or during the experiments, in which they were injected (intra-peritoneal route) with E₂ dissolved in propylene glycol at a dose of 1 mg/kg body weight. A second dose was given 15 days following the first injection. Control fish were

injected with propylene glycol only. Fish were sampled 3, 7, 14, 21, 28, and 35 days after the first injection. Four fish were sampled each time, except at day 35 (seven fish sampled). Besides these sample sets, four fish that had not been injected were sampled and designated as the initial control group. Serum was prepared and stored as described above until use for the quantification of complete Vg, VgC, and E₂.

2.2. Antisera

Polyclonal antiserum against whole serum proteins of male chum salmon, *Oncorhynchus keta*, (anti-male) was raised in a rabbit by intra-dermal injection of the male serum emulsified with an equal volume of Freund's complete adjuvant (Merck, Darmstadt, Germany). This emulsified male serum (250 µl per injection) was injected four times at weekly intervals. For immunization with purified taimen VgC, a rabbit was injected with the antigen into the lymph nodes, followed by two additional booster injections into the back. Blood was collected from the ear vein of immunized rabbits one week after the final injection and used to prepare the antiserum. Antisera raised against taimen egg extracts (anti-Egg), purified taimen Lv (anti-Lv), purified masu salmon (*Oncorhynchus masou*) Lv (anti-masu Lv) and purified grey mullet (*Mugil cephalus*) LvC (anti-mullet LvC) were prepared and characterized in the same manner as in our previous studies (see details in Hiramatsu and Hara, 1996; Fukada et al., 2001; Amano et al. 2007a).

2.3. Electrophoresis and immunological procedures

Immunoelectrophoresis (IEP) was conducted by a routine procedure in 1% agarose gel prepared with 0.05 M sodium barbital buffer, pH 8.6. Single radial immunodiffusion (SRID) using 1% agarose gels containing a specific antiserum (anti-taimen VgC or anti-masu Lv) was performed according to the method described by Mancini et al. (1965). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with a 3% stacking gel and 7.5% separating gel was performed according to Laemmli (1970). Gels were stained with 0.1% Coomassie Brilliant Blue R-250 (CBB; Bio-Rad, Hercules, CA, USA). Relative molecular masses of polypeptides were estimated using Precision Plus Protein Standards All Blue Prestained (Bio-Rad). Western blotting was carried out according to the method of Towbin et al. (1979) using the polyclonal rabbit antisera described earlier.

2.4. Purification of vitellogenin C

All purification procedures were performed at 4°C. Starting samples for column chromatography were prepared by water-precipitation methods as described by Hara et al. (1993). Briefly, serum from vitellogenic female fish was precipitated by adding it dropwise to a 10 times volume of ice-cold distilled water and stored overnight at 4°C. After centrifugation, the resulting precipitate was collected and dissolved in 0.02 M Tris-HCl (pH 8.0) containing 2% NaCl and 0.1% NaN₃.

Following water-precipitation, gel filtration using Sephadex G-200 (GE Healthcare UK Ltd, Little Chalfont, UK) was performed to begin purifying the VgC. The Sephadex G-200 media was loaded into a 5 × 90 cm glass column and equilibrated with the Tris-HCl buffer.

Samples were eluted at a flow rate of 40 ml/h. Eluted fractions were collected at a volume of 13 ml per tube.

Anion-exchange chromatography was performed using a DE-52 media (GE Healthcare UK Ltd). The DE-52 media was loaded into a 2.5×18 cm column. The column was equilibrated with a starting buffer of 0.02 M Tris-HCl (pH 8.0) containing 0.05 M NaCl. Samples were eluted by step-wise addition of 0.02M Tris-HCl buffer (pH 8.0) containing 0.1, 0.2, and 2.0 M NaCl at a flow rate of 60 ml/h. Eluted fractions were collected at a volume of 8.0 ml per tube.

Immunoabsorbent column chromatography (2.5×8 cm) was performed using Sepharose 4B (GE Healthcare UK Ltd) coupled with anti-male. The column was equilibrated with phosphate buffered saline (PBS; 0.01 M sodium phosphate buffer, pH 7.0, containing 0.25 M NaCl). The pass-through and bound fractions were eluted by PBS and 8.0 M urea, respectively, at a flow rate of 20 ml/h and 60 ml/h, respectively. Eluted fractions were collected at a volume of 5 ml per tube.

Gel filtration was performed for the estimation of native mass with a Superose 6 column (GE Healthcare UK Ltd) fitted to the fast protein liquid chromatography (FPLC) system (GE Healthcare UK Ltd). Samples were eluted with 0.02 M Tris-HCl, pH 8.0, containing 2% NaCl and 0.1% NaN₃. The column was eluted at a flow rate of 0.5 ml/min and fractions were collected at a volume of 0.5 ml per tube. The following marker proteins were used to calibrate the Superose 6 column: immunoglobulin G (150 kDa), aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa), and thyroglobulin (669 kDa).

Determination of the protein concentration of the purified VgC solutions was performed

using BCA protein assay kit (Pierce, Rockford, IL, USA) with bovine serum albumin (BSA; Pierce) as the reference standard.

2.5. Purification of complete vitellogenin

Complete-type Vg was prepared from the serum of vitellogenic taimen according to the procedure described by Hiramatsu and Hara (1996). Protein concentration of the purified complete-type Vg was determined as described above.

2.6. Enzyme immunoassay (EIA) for estradiol-17 β

Quantification of E₂ in the serum samples was carried out using an estradiol immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions.

3. Results

3.1. Detection of vitellogenin C and its-derived lipovitellin

Typical IEP patterns of serum and egg extracts are shown in Fig. 1. No precipitin line was formed against the male control serum when two antisera (i.e., anti-Egg and anti-mullet LvC) were used. The former antiserum formed two precipitin lines with female serum and three precipitin lines with egg extracts. In contrast, the latter antiserum generated only one precipitin line against each of both samples; the immuno-reactive products in the female serum and eggs were tentatively referred to as VgC and LvC, respectively.

3.2. Purification of vitellogenin C

Detection of VgC at each step of the purification procedure was performed using an absorbed antiserum, which was prepared from anti-Egg by being pre-absorbed with purified complete-type Vg (ab.anti-Egg). It should be stated here that this ab.anti-Egg is expected to exhibit the same property in immunoreactivity as an antiserum against LvC in theory. At the initial step of purification, the water-precipitate fraction of the vitellogenic female serum underwent gel filtration on the Sephadex G-200 (Fig. 2A). Fractions (tubes numbered 69-76) eluted during the later part of the major peak appeared to contain mainly VgC by the immunological detection. These fractions were pooled and dialyzed against the starting buffer before being applied to a DE-52 column. Figure 2B shows an elution pattern of the anion-exchange column chromatography using DE-52 media. Following step-wise elutions by adding various concentrations of NaCl (4 steps; see Fig. 2B for details), a peak corresponding to elution of the pass-through fractions (0.05 M NaCl; fraction numbers 1-32) contained predominantly VgC with a trace amount of other serum proteins. Following dialysis against PBS, these fractions were applied as a pooled VgC fraction onto an immunoabsorbent column. Figure 2C shows the elution pattern of the immunoabsorbent column coupled with anti-male. The pass-through fractions (numbers 8-20) produced a major peak and were pooled and collected as purified VgC. The native mass of VgC was estimated to be ~380 kDa by the gel filtration on Superose 6 (data not shown). The purified VgC was subjected to IEP using anti-male, anti-Egg, and ab.anti-Egg (Fig. 3). Only one precipitin line was generated against anti-Egg and ab.anti-Egg, while the purified

VgC did not react with anti-male. Figure 4 shows patterns of purified VgC and serum samples following SDS-PAGE and its corresponding Western blotting using anti-mullet LvC, anti-Lv, and anti-male. In SDS-PAGE under reducing conditions, purified VgC appeared as two bands (~140 kDa and ~115 kDa). These two bands were specifically stained with anti-mullet LvC, but not stained with other two antisera, indicating that purified VgC was highly separated from complete-type Vg and other serum proteins.

3.3. Antiserum against purified vitellogenin C

Antiserum was raised in a rabbit against purified VgC (anti-VgC) and its specificity was examined by IEP (Fig. 5). The antiserum reacted with female serum and egg extracts by forming only one precipitin line, but did not react with male serum, confirming the specificity of anti-VgC against its antigen; this, in turn, confirmed the purity of the VgC. The specificity of the antiserum was further confirmed by SRID (data not shown). In an SRID plate containing anti-VgC, a precipitin ring was formed only with the targeted antigen, but neither with the male serum nor with complete Vg. Serial dilutions of purified VgC formed standard precipitin rings in SRID, resulting in a practical standard curve ($R^2 > 0.99$) at a range between 25 and 400 $\mu\text{g/ml}$.

3.4. Maturational changes in serum levels of dual vitellogenins

Serum Vg levels in female Sakhalin taimen were measured using SRID (Fig. 6). Complete-type Vg was detected in the serum of 25 females, with a large variation in levels (from 62 $\mu\text{g/ml}$ to 19.8 mg/ml). Complete-type Vg was detected from June 2001 and high

serum levels (mg/ml order) were sustained to August 2002, except in four individuals obtained from April to June 2002. In contrast, VgC was detected in fewer fish ($n=13$) at lower levels (25-934 $\mu\text{g/ml}$) than complete-type Vg throughout the experiment. It became detectable in the serum from July 2001 (in 5-year-old fish) and was thereafter present during most of the sampling period except for a few months.

Figure 7 shows the correlation of GSI against serum levels of two Vgs (i.e., complete-type Vg and VgC) using a dataset of all 2001 and 2002 samples. Detection of complete-type Vg and VgC appeared to be possible in individuals with GSIs greater than 0.3% and 1%, respectively.

3.5. Relationship between two types of vitellogenin and lipovitellin

In addition to the above investigations on groups of female taimen (2001-2002), levels of dual Lvs, as well as dual Vgs, were quantified in vitellogenic females ($n=5$) and females at ovulation ($n=2$), which were also sampled in 2006 to determine the deposition profiles of the Vg derivatives in the ovaries and eggs (Fig. 8). The complete-type Vg was detected in the serum of all females, with levels ranging from 330 $\mu\text{g/ml}$ to 16750 $\mu\text{g/ml}$. In contrast, VgC was detected in only five of the seven females sampled, those five having oocytes/eggs with large diameters and high GSIs, with levels (146-650 $\mu\text{g/ml}$) lower than those of females with complete-type Vg. These Vg production levels were similar to those found in fish sampled in 2001-2002 (see above section). The derivative of complete-type Vg (i.e., Lv) was detected in all samples (41.5-1055.7 $\mu\text{g/mg}$ proteins), while LvC stayed at low levels (41.2-80.6 $\mu\text{g/mg}$) and was detected in only five individuals; thus, the pattern of

the detectability of Lv and LvC in the ovaries and eggs was also similar to that found for parental Vgs. The levels of Lv in the ovaries and eggs seemed to reach a plateau at mid-vitellogenesis (November) and remained at that level until ovulation (April and May), while those of LvC revealed a tendency to be slightly higher at ovulation, resulting in a slight decrease in Lv/LvC ratios at this stage..

3.6. Serum levels of dual vitellogenins following estrogen-administrations

Changes in the serum levels of dual Vgs in juvenile taimen following E₂-administration are shown in Fig. 9. Neither Vg subtype was detected in either the initial control or control group. Injection of E₂ induced the appearance of complete-type Vg in the serum of all juvenile taimen. As expected, a typical time-dependent increase in the level was found from 0 (initial control; none-detectable) to 21 days (5925.0±2652.3 µg/ml) after the first injection. Although there was an apparent increase in the levels after the second injection (between day 14 and day 21), thereafter the levels remained high (~5-6 mg/ml) until the end of the experimental period. In contrast, VgC induction was not observed in the serum of the E₂-administered group for the first injection. Following the second injection at day 15, VgC became evident in the serum; levels of VgC (35.5-73.0 µg/ml), however, were much lower than those of complete-type Vg and did not show any time-dependency.

4. Discussion

In the present study, VgC was purified from the serum of vitellogenic taimen by a combination of water-precipitation, gel filtration, anion-exchange (DE-52), and

immunoabsorbent column chromatography. The purified product was identified as the incomplete-type Vg in Sakhalin taimen based on its biochemical properties: 1) the masses of taimen VgC (~380 kDa and ~140 kDa in gel filtration and SDS-PAGE, respectively) were smaller than those of the complete-type Vg (~540 kDa and ~165 kDa, respectively; Hiramatsu and Hara, 1996), which is consistent with results commonly found for other teleosts, 2) the masses of taimen VgC were similar to those reported for the VgC of other teleosts (Hiramatsu et al., 2002b; Shimizu et al., 2002; Amano et al., 2007b), 3) taimen VgC hardly bound to anion-exchangers in the presence of NaCl (0.05 M), a property found for VgC of other teleosts (Hiramatsu et al., 2002b; Amano et al., 2007b), and 4) taimen VgC was immunoreactive to anti-mullet LvC, which was the same antiserum preparation that has been utilized to detect VgC and/or LvC in grey mullet, Pacific saury (*Cololabis saira*), and marbled sole (*Pleuronectes yokohamae*) (Amano et al., 2007b, 2008, 2009). Collectively, these biochemical and immunochemical properties revealed that the purified product was VgC of Sakhalin taimen.

A simple SRID system was developed using purified VgC and its subtype-specific Vg antiserum (i.e., anti-VgC). This VgC SRID appeared to be specific to its targeted antigen (VgC) and showed no cross-reactivity to other protein components in the serum of Sakhalin taimen, nor to complete-type Vg. Thus, this simple protocol is the first procedure developed to quantify VgC in a salmonid species.

Levels of complete-type Vg have been quantified in the serum of female Sakhalin taimen (Hiramatsu et al., 1997) and they appear to change with the season and reproductive stage. When the complete-type Vg was quantified by an enzyme-linked immunosorbent assay, it

was detected at low levels (<200 ng/ml) in 3-year-old fish in May; thereafter, its levels increased rapidly (from July samples of 5-year-old fish). By October, the levels reached approximately 20-30 mg/ml and remained high for 7 months until April (samples of 6-year-old fish). Following the first ovulation in May (data from 6-year-old fish), serum levels of complete-type Vg decreased suddenly (the abrupt decrease stopped in June). In the present study, two subtypes of Vg were measured in the serum of female taimen using subtype-specific Vg SRID. Five-year-old fish ($n=61$) were divided into two groups, one exhibiting high levels (>1 mg/ml) and the others exhibiting non-detectable (<25 $\mu\text{g/ml}$) of complete-type Vg. Based on the results of the previous study, the high level group, with large GSI values, was expected to mature and ovulate in the next May (when the fish were 6 years old).

A large portion of the female taimen had undetectable serum VgC levels; VgC became detectable only when GSI reached ~1%. In contrast, levels of complete-type Vg were detectable in 25 females and were positively correlated with GSI values where they were less than 1%. A few individuals that exhibited high levels of VgC also had constantly high levels of complete-type Vg; such individuals had high GSIs (more than 1%), indicating they would probably have been ready to spawn in the coming spring (May, when fish were 6 years old). Since the SRID assay is simple to perform, VgC, in conjunction with complete-type Vg, can be used as a predictive marker for the spawning of Sakhalin taimen. Vitellogenic oocytes may accumulate multiple Vgs in specific ratios during the period preceding follicle maturation. This accumulation reflects disparate rates of deposition into growing oocytes of the yolk proteins derived from each form of parent Vg, as suggested by

previous studies of marine or estuarine pelagophils (Matsubara et al., 1999; Reith et al., 2001; Hiramatsu et al., 2002c; Sawaguchi et al., 2006; Finn, 2007; Kolarevic et al., 2008; Amano et al., 2008). The composition of yolk proteins derived from multiple Vg subtypes can be regulated, theoretically, by: 1) the ratios in circulating levels of multiple Vg subtypes, 2) the numbers and/or affinity of ovarian receptor(s) for Vg, and 3) both of these two factors. In the present study, the ratio of complete-type Vg to VgC tended to decrease as GSI increased from early- to mid-vitellogenesis and thereafter stabilized at ~20:1 during late-vitellogenesis. This ratio was not largely different from the ratio of Lv deposition derived for complete-type Vg to LvC (~23:1, ~21:1, and ~22:1) in mid- to late- vitellogenic oocytes, indicating that the ratios of circulating dual Vgs influence the ratio of their deposition to a certain extent. The complete-type Vg is known to be taken up specifically by growing oocytes *via* endocytosis that is mediated by a membrane receptor (VgR). Recent findings indicate a possible multiplicity in fish VgR, although a specific receptor for VgC has not been verified in any teleosts (Reading et al., 2008). An endocytotic system mediated *via* multiple VgRs might be involved in the process of ovarian uptake of dual Vgs in order to maintain the specific deposition ratio of dual Lvs in the oocytes and eggs of female taimen.

The results of the present study suggested that VgC is taken up by growing oocytes and that it forms at least a portion, albeit relatively minor, of yolk proteins in taimen. The proportion of VgC and LvC in total Vg and Lv proteins, respectively, and their induction and accumulation levels, vary depending on species or reproductive stage. Namely, in comparison with other teleosts, the ratio of complete-type Vg to VgC in taimen (~20:1) was

higher than ratios obtained for goby (~4.5:1, Ohkubo et al., 2003), mosquitofish (*Gambusia affinis*) (~4.1:1, Sawaguchi et al., 2005), and medaka (~8:1, Hiramatsu et al., 2008). In contrast, the ratio of Lvs (21~23:1 in vitellogenic oocytes, ~12:1 and ~17:1 in ovulated eggs) was similar to those reported for marine pelagophils, barfin flounder (*Verasper moseri*) (LvA+LvB:LvC=~24:1; Sawaguchi et al., 2008) and grey mullet (LvA+LvB:LvC=~17:1; Amano et al., 2008a), but was higher than it reported for a fresh water species, mosquitofish (~4.5:1, Sawaguchi et al., 2005). Although the present study does not provide any explanation of why taimen oocytes achieve such a specific dual Lv ratio or whether it differs from or is consistent with those of other fish, recent research has given rise to speculation about the physiological significance of a “multiple Vg” system in marine pelagophils. Briefly, cleavage of yolk proteins results in the generation of free amino acids (FAAs) in amounts that reflect the ratio of incorporated VgA to VgB to VgC during follicular maturation. If the suggestion that the resulting FAA pool plays important roles in the regulation of oocyte hydration, egg buoyancy, and embryonic nutrition (reviews: Hiramatsu et al., 2005, 2006) is correct, processes involved in the proper deposition of multiple Vg derivatives may need to be tightly regulated, and any disruption of such processes may reduce the quality of eggs and offspring. It is possible, then, that the specific ratio (21~23:1 in vitellogenic oocytes, ~12:1 and ~17:1 in ovulated eggs) in taimen supplies the appropriate balance of nutrients for the developing embryos and thus could be important for their vital activities. It does not seem to be involved in oocyte hydration or the acquisition of egg buoyancy, as the complete-type Vg in salmonids has not diverged into two subtypes (VgA nor VgB) (Matsubara et al., 2003), and its derived yolk proteins

are not likely to be degraded into FAA during follicle maturation (Hiramatsu et al., 2002a).

In fact, salmonids produce demersal eggs in a freshwater environment and their oocytes undergo little hydration during follicle maturation.

Like in other teleosts, both complete-type Vg and VgC were induced in the serum of juvenile taimen by estrogen administration. Serum levels of complete-type Vg (5925.0 ± 2652.3 $\mu\text{g/ml}$ at day 21) and estrogen (333.4 ± 311.0 pg/ml at day 21) were similar to those of reproductive females. However, levels of VgC ($35.5\text{-}73$ $\mu\text{g/ml}$) in the serum of estrogen-treated fish seemed to be much lower than the maximal physiological levels ($25\text{-}934$ $\mu\text{g/ml}$ in reproductive females), suggesting that other factors (e.g., growth hormone, growth factors, etc) might be involved in the natural production of VgC in this species. Definitive causes of this phenomenon remain to be determined. The induction of VgC by estrogen has been found in only a few species, such as tilapia, *Oreochromis mossambicus* (Kishida and Specker, 1993; Takemura and Kim, 2001), Japanese goby (Ohkubo et al., 2003), and medaka (Fujiwara et al., 2005). In general, VgC appeared to be a minor Vg component when these species were exposed to or injected with various doses of estrogens, which agrees with results of the present study. However, VgC seemed to be induced as the major Vg subtype under low levels of estrogen, as is the case in some studies of medaka and goby (Hiramatsu et al., 2008; Ohkubo et al., 2003). Salmonids (e.g., *Oncorhynchus mykiss*) exhibit a cluster of complete-type Vg gene (stated as Vtg-A type in salmonid by Babin et al., 2008) located on a single chromosomal region, containing a set of twenty highly-similar, tandemly-arranged genes and ten pseudogenes (Trichet et al., 2000; Babin et al., 2008) and this seems to cause massive induction of complete-type Vg protein.

In the present study however, the prevalence of dual Vgs among salmonids was not fully determined as a more sensitive quantitative assay for VgC, which can detect the induction of Vg following weak estrogenic stimulation, has yet to be developed.

In summary, we have detected, purified, and characterized a novel incomplete-type Vg in Sakhalin taimen and identified it as the VgC of this species. This is the first time a VgC has been identified in salmonids. We also prepared a subtype-specific Vg antiserum against purified VgC, which was utilized to develop a type-specific VgC immunoassay (VgC SRID). Immunological detection and quantification revealed that taimen VgC was an E₂-inducible serum protein and it was detected in the serum of reproductive females that exhibited relatively advanced gonadal development. Levels of circulating and deposition VgC (or its derivative, LvC) appeared to be always lower, as well as less responsive to estrogenic stimulation, than complete-type Vg (or its derivative, Lv). Results thus far indicate that, when dual Vgs are detected by SRID, complete-type Vg perhaps is the better marker for the detection and discrimination of puberty and gender, respectively, of this species. However, VgC, as detected by SRID in the serum of female taimen, can be a predictive marker for the discrimination of individuals that are expected to spawn within a year. A dual marker system (complete-type Vg and VgC) would be an excellent tool to help render commercial aquaculture more efficient, especially for fishes that take a long time to complete ovarian growth, such as Sakhalin taimen.

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Legends

Figure 1. Immunoelectrophoresis of male serum (M), female serum (F), and egg extracts (E) of Sakhalin taimen. Antisera used in the analysis were raised against taimen egg extracts (anti-Egg), and purified mullet lipovitellin C (anti-mullet LvC).

Figure 2. Chromatograms obtained during the purification of vitellogenin C (VgC). A water-insoluble fraction of female serum components underwent gel filtration on Sephadex G-200 (panel A). Fractions from the shaded area indicated in chromatogram A were pooled and subsequently subjected to anion-exchange chromatography using DE-52 (panel B). The shaded area in chromatogram B represents the crude VgC fraction that was applied to an immunoabsorbent column coupled with antiserum against male serum of chum salmon (panel C). The pass-through fractions indicated by the shaded area in chromatogram C were pooled and collected as purified VgC.

Figure 3. Immunoelectrophoresis of female serum and purified vitellogenin C (VgC). An antiserum was raised against taimen egg extracts (anti-Egg) and used in the analysis with (ab.anti-Egg) or without pre-absorption by purified complete-type Vg.

Figure 4. The 7.5% SDS-PAGE and its corresponding Western blot of male serum (M), female serum (F), and purified vitellogenin C (VgC). The SDS-PAGE gel was stained with Coomassie Brilliant Blue (CBB). Arrows indicate the estimated mass (kDa) of the major bands that appeared in the purified VgC. Western blotting was performed using antisera raised against mullet purified lipovitellin C (anti-mullet LvC), taimen purified Lv (Lv derived from complete-type Vg; anti-Lv), and male chum salmon serum (anti-male).

Figure 5. Immunoelectrophoresis of male serum (M), female serum (F), and egg extracts (E) using an antiserum against purified taimen vitellogenin C (anti-VgC) and one against taimen egg extracts (anti-Egg).

Figure 6. Changes in serum levels of dual vitellogenins (Vg and VgC) in female taimen. Numbers enclosed inside white and black circles indicate the number of fish that exhibited undetectable levels of complete-type Vg and VgC, respectively.

Figure 7. Correlation of gonad somatic index (GSI) and concentration of vitellogenins (Vgs) in female taimen. Numbers enclosed inside white and black circles indicate the number of fish that exhibited undetectable levels of complete-type Vg and VgC, respectively.

Figure 8. Levels of circulating and deposited dual vitellogenins (Vg and VgC; panel A) and their derived lipovitellins (Lv and LvC, respectively; panel B) in early vitellogenic ($n=2$), mid-late vitellogenic ($n=3$), and post-ovulatory ($n=2$) female taimen.

Figure 9. Induction levels of dual vitellogenins (Vg and VgC) in the serum of estrogen-administered taimen. Injection was performed twice either at day 0 or day 15.















