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Incorporation and Utilization of Multiple Forms of Vitellogenin and their Derivative Yolk Proteins during Vitellogenesis and Embryonic Development in the Mosquitofish, *Gambusia affinis*

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ABSTRACT—We previously demonstrated the presence of three forms of vitellogenin (Vg), two 600 kDa Vgs (600Vg; VgA and VgB) and a 400 kDa Vg (400Vg; phosvitinless Vg) in plasma from maturing female viviparous mosquitofish, *Gambusia affinis*. For further quantitative elucidation of the accumulation and utilization of the multiple Vg-derived yolk proteins, two sandwich enzyme-linked immunosorbent assays (ELISA) were developed using antisera against 600Vgs and a 400 kDa yolk protein (400Yp; derived from 400Vg), respectively. Contents of 560 kDa yolk protein (560Yp; lipovitellins derived from 600Vg) and 400Yp measured by the ELISAs increased in accordance with the growth of vitellogenic oocytes, keeping their proportional ratio (mol/mol) at about 4:1. A similar ratio obtained for plasma Vgs suggests that the proportional accumulation of the multiple Vg-derived yolk proteins is regulated by the hepatic synthesis and secretion of their precursor Vgs. When egg homogenate was analyzed by gel chromatography, three peaks, consisting of 560Yp, 400Yp and 28 kDa native β'-component, were observed. The elution profile showed no change until embryos reached the early neurula stage, however, the relative height of the 560Yp peak as compared to the 400Yp one decreased after retinal pigmentation. Results from measurements of 560Yp and 400Yp at each embryonic stage supported the occurrence of unequal utilization of the two yolk proteins. The proportional ratios (mol/mol) of 560Yp content versus 400Yp content gradually decreased from 4.1 fold in early neurula embryo to 1.4 fold in larva just before parturition. The present study thus demonstrated unequal utilization of the multiple Vg-derived yolk proteins in developing embryos of mosquitofish.

Key words: vitellogenin, lipovitellin, phosvitinless vitellogenin, yolk protein, vitellogenesis, development

INTRODUCTION

Egg yolk proteins, which are nutrient stocks for embryonic development in oviparous vertebrates, are accumulated in oocytes during a phase of active oocyte growth called vitellogenesis (see Wallace, 1985). The precursor of the yolk proteins, vitellogenin (Vg), is a serum glycophospholipoprotein, which is synthesized in the liver of oviparous vertebrates under estrogen stimulation. Vg is incorporated into growing oocytes by receptor-mediated endocytosis and processed into smaller yolk proteins (for reviews see Specker and Sullivan, 1994; Hiramatsu *et al.*, 2002c; Patiño and Sullivan, 2002). These yolk proteins are classified as lipovitellin (Lv), phosvitin (Pv) and β'-component (βc). Lv is a large and highly lipidated protein and consists of two polypeptides, a heavy chain (LvH) and a light chain (LvL), which are derived from regions flanking the Pv domain of the Vg polypeptide (Byrne *et al.*, 1989; Hiramatsu *et al.*, 2002c). Phosvitin, a much smaller protein, is unique in that more than half of its amino acid residues are contained in highly phosphorylated polyserine domains (see Ng and Idler, 1983). The other small yolk protein, βc, contains neither lipid nor phosphorus (Markert and Vanstone, 1971; Campbell and Idler, 1980; Hara *et al.*, 1993; Matsubara and Sawano, 1995).

Recent molecular cloning of teleost Vg genes has verified the multiplicity of the Vg gene family in teleost species as well in higher oviparous vertebrates such as the chicken

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(Byrne *et al.*, 1989) and *Xenopus laevis* (Wahli *et al.*, 1979, 1980; Germond *et al.*, 1983). Evidence for multiplicity of the Vg gene in teleost species was first provided by molecular cloning of cDNAs encoding two completely distinct Vgs, VgI and VgII, in the mummichog, *Fundulus heteroclitus* (LaFleur *et al.*, 1995a, b). Subsequently, full-length cDNAs encoding two distinct Vgs were obtained from haddock, *Melanogrammus aeglefinus* (VgA and VgB; GenBank: AB284035 and AB284034) and medaka, *Oryzias latipes* (Vg1 and VgII; GenBank: AB064320 and AB074891). Duality of Vg proteins, VgA and VgB (Matsubara *et al.*, 1999) and their corresponding cDNA sequences (VgA and VgB; GenBank: AB181833 and AB181834), also ware demonstrated in the barfin flounder, *Verasper moseri*. These teleostean Vgs were classified into two groups, VgA and VgB, based on similarities in their primary structures, constituent yolk protein domains, and physiological functions (Matsubara *et al.*, 1999; Hiramatsu *et al.*, 2002a, b). The group A includes mummichog (Fun) VgI, haddock (Had) VgA, medaka (Med) Vg1, and barfin flounder (Bar) VgA, and the group B includes FunVgII, HadVgB, MedVgII, and BarVgB. The coding sequences of the both VgA and VgB groups are arranged in a linear fashion with respect to yolk protein domains as follows: NH_2-LvH (Lv1)-Pv-LvL (Lv2)-βc-C-terminal coding region-COOH (Hiramatsu *et al.*, 2002a, c). Besides the general type Vg, like VgA and VgB, an unusually small Vg protein having an approximate molecular mass of 300 kDa has also been discovered in two tilapia species (Ding *et al.*, 1989; Kishida and Specker, 1993; Takemura and Kim, 2001). Wang *et al.* (2000) identified a Vg cDNA named *vg3* in zebrafish *Danio rerio*, which encodes a novel Vg missing the polyserine Pv domain that had low similarities to other piscine Vg cDNAs. From these characteristics, the novel Vg was designated as phosvitinless Vg (PvlVg). Such a small Vg also has been identified immunologically and biochemically along with a typical VgA and VgB in the white perch, *Morone americana* (Hiramatsu *et al.*, 2002b). Subsequently, the PvlVg cDNA and protein have been discovered in estrogen-treated Japanese common goby, *Acanthogobius flavimanus* (Ohkubo *et al.*, 2003, 2004). In mosquitofish, *Gambusia affinis*, we demonstrated the presence of all the three forms of Vg proteins, VgA, VgB and PvlVg, in plasma from estrogen-treated females and identified their derivative yolk proteins in vitellogenic ovarian follicles (Sawaguchi *et al.*, 2005). Furthermore, full-length cDNAs encoding each Vg (VgA, VgB and PvlVg; GenBank: AB181835, AB181836 and AB181837) also were isolated from a liver cDNA library prepared from estrogen-treated mosquitofish.

As noted above, multiplicity of Vg is evident and may be the norm in teleost species. However, all of the previous studies focused on verification of the existence of multiple Vgs and qualitative analysis of these Vgs with regard to description of molecular alteration of Vgs and Vg-derived yolk proteins during different phases of oogenesis (Matsubara *et al.*, 1999; Reith *et al.*, 2001; Hiramatsu *et al.*, 2002b; Sawaguchi *et al.*, 2005). For further elucidation of the physiological functions of the multiple Vgs during teleost oogenesis, additional approaches including quantifications of the multiple Vgs and yolk proteins in each phase of ovarian and embryonic development are needed. The advances made in our previous study of mosquitofish (Sawaguchi *et al.*, 2005) enabled us to develop specific quantitative assays for each Vg gene transcript and its translated protein product. In the present study, we developed sandwich enzyme-linked immunosorbent assay (ELISA) for measurements of the major 560 kDa yolk proteins, which include Lvs derived from 600 kDa VgA and VgB, and a 400 kDa yolk protein derived from the 400 kDa PvlVg of mosquitofish. By using the ELISA systems, we measured both of the yolk proteins independently in vitellogenic oocytes and developing embryos to elucidate the properties of incorporation and utilization of multiple Vg-derived yolk proteins in this species.

MATERIALS AND METHODS

Fish

Mosquitofish were collected from an irrigation canal at Nagashima, Mie, Japan, and were subsequently kept in a pond at Gifu University, Gifu, Japan under natural ambient conditions of photoperiod and water temperature. Adult fish were transferred to 50 L glass aquaria under 16 hr light and 8 hr dark photoperiod at 25°C. After 2 weeks acclimation, the fish were confirmed to have initiated the reproductive cycle. Fish were anesthetized with ethyl 4-aminobenzoate, then sampled. The values of standard length and body weight of adult females used in the present study varied from 33.5 mm to 46.1 mm and from 0.690 g to 1.585 g, respectively.

Samples

The ovaries were dissected out, and vitellogenic follicles were separated in a Ringer's solution designed for *Fundulus heteroclitus* oocytes (FO-solution: 113 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM Na_2HPO_4, 1 mM $NaHCO_3$, 1 mM sodium pyruvate, 5 mM glucose, 5 mM HEPES, 0.001% phenol red and 0.1 M NaOH for pH 7.5; Wallace and Selman, 1978). The developmental stages of embryos were classified according to Koya *et al.* (2000). The diameter and weight of each follicle and embryo was measured to 0.01 mm and 0.1 mg, respectively. For qualitative analysis of yolk proteins by chromatography and sodium-dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), yolk in follicles and fertilized eggs was withdrawn from each egg and embryo using microhematocrit tubes (Terumo, Tokyo, Japan) which were previously stretched using a gas burner to make sharp needle tips. The obtained yolk was diluted by adding 0.9% NaCl solution containing 0.005% phenylmethylsulfonylfluoride (PMSF) and 0.01% NaN_3 at a concentration of 10% (wt/vol). For measurement of yolk proteins by enzyme-linked immunosorbent assay (ELISA) as described below, freshly dissected vitellogenic follicles, fertilized eggs and embryos were homogenized in a 0.9% NaCl solution containing 0.005% PMSF. After centrifugation at 10,000 × *g* for 15 min at 4°C, the supernatants were collected as follicle extract (FO), egg extract (EG) and embryo extract (EM), respectively, and stored at −80°C until use.

Over sixty female mosquitofish were given an interperitoneal injection of 5 μl of estradiol-17β (E_2) solution (0.8 mg E_2/ml propylene glycol) to stimulate vitellogenin synthesis for collecting E_2 treated fish plasma (EP). Five days later, blood was taken from males, naturally vitellogenic females and E_2-treated females after anesthetization with ethyl-4-aminobenzoate. Blood samples were

taken from the severed caudal vein using heparinized microhematocrit tubes (Terumo) with sharpened tips, and centrifuged at 5000 × *g*, for 5 min to separate plasma. Collected male plasma (MP), female plasma (FP) and EP, were then frozen at -80°C until use.

Purified vitellogenins and phosvitinless vitellogenin

Purifications of Vgs from EP and yolk proteins from FO were the same as reported by Sawaguchi *et al.* (2005). Obtained Vg and major yolk protein preparations were termed 600Vg, 400Vg, 560Yp and 400Yp according to our previous report (Sawaguchi *et al.*, 2005). The 600Vg and 560Yp preparations with apparent molecular masses of 600 kDa and 560 kDa contain two different Vg proteins (600VgA and 600VgB) and their derivative Lv proteins (560LvA and 560LvB), respectively. On the other hand, 400Vg and 400Yp are phosvitinless Vg (PvlVg) and its derivative yolk protein, respectively, of this species having same the apparent molecular mass of ~400 kDa in native form.

Preparation of antibodies

Specific antisera to 600Vgs (ab-a-600Vg) and 400Yp (ab-a-400Yp) of the mosquitofish used in this study were prepared as in our previous report (Sawaguchi *et al.*, 2005). Purification of IgG and preparation of the $F(ab')_2$ of the ab-a-600Vg and ab-a-400Yp were carried out basically according to the method of Ishikawa *et al.* (1983) with minor modifications. The IgG was purified with ammonium sulfate precipitation followed by a DE52 (Whatman International Ltd., Kent, UK) ion-exchange chromatography. Each purified IgG preparation was digested with pepsin (P-6887, Sigma, MO, USA), then $F(ab')_2$ was collected with gel chromatography using Superose 12 HR 10/30 (Amersham Biotech, Buckinghamshire, England) equilibrated with phosphate buffered saline, pH 7.3 (PBS: Wako, Osaka, Japan). The obtained $F(ab')_2$ was then biotinylated by Biotin Labeling Kit (Roche, Mannheim, Germany).

Enzyme-linked immunosorbent assay

The 96 well polystyrene microtiter plate (Corning, NY, USA) was coated with 100 µl/well of ab-a-600Vg IgG or ab-a-400Yp IgG diluted in sodium bicarbonate buffer, pH 9.6, then incubated overnight at 4°C. The non-specific binding sites were saturated by incubating the plates with 250 µl of 1% BSA and 5% skimmed milk in the same buffer for 1 hr at room temperature. After two successive washes in 10 mM PBS, pH 7.3, containing 0.05% Tween 20 (PBST), 100 µl of serially diluted yolk protein standard solutions (39 ng/ml to 1250 ng/ml of purified 560Yp and 2.6 ng/ml to 163 ng/ml of purified 400Yp serially diluted with PBS containing 0.5% BSA) or FO, EG and EM samples (diluted 1:1,000 to 1:1,000,000,000 in

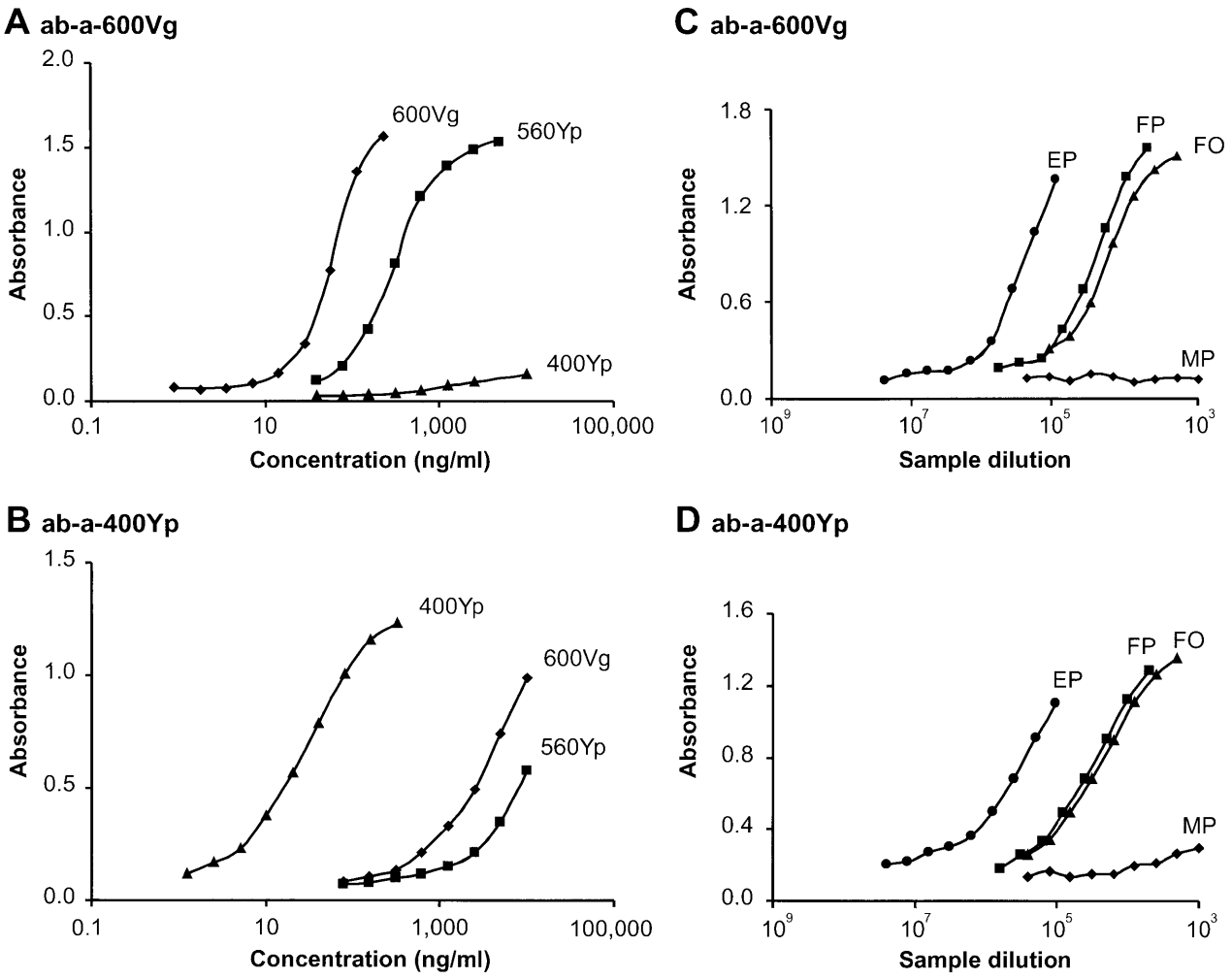


Fig. 1. Standard curves of 600 kDa vitellogenin preparation (600Vg), 560 kDa yolk protein (560Yp) and 400 kDa yolk proteins (400Yp) on ELISAs for 600Vg (A) and 400Yp (B), and the dilution curves of estradiol-17β treated fish plasma (EP), female plasma (FP), male plasma (MP) and vitellogenic follicle extract (FO) on the ELISA for 600Vg (C) and 400Yp (D). Each point represents the mean of duplicate determinations.

PBS containing 0.5% BSA) were introduced to the wells and incubated for 1.5 hr at room temperature. Each well was then washed five times with PBST, and received 100 μl of biotin labeled antibody, ab-a-600Vg F(ab')$_2$ or ab-a-400Yp F(ab')$_2$, diluted 1:3,000 in PBS-0.5% BSA. The plates were maintained for 1.5 hr at room temperature. After incubation, each well was washed again as above, and then received 100 μl of avidin–HRP (DAKO A/S, Denmark) diluted 1:5,000 with PBST-1% BSA. The plates were maintained for 1 hr at room temperature, and were then washed five times with PBST. For coloration, each well received 100 μl of TMB$^+$ (3,3',5,5'-tetramethylbenzidine) Substrate (Dako, Glostrup, Denmark). Color development was stopped by adding 50 μl of 2 M sulfuric acid. The colorimetric data of absorbance at 450 nm is measured using MTO-100 microplate reader (Corona, Ibaraki, Japan).

Chromatography

Gel chromatographic analysis for separation of Vgs and yolk proteins was performed using prepacked column of Superose 6 HR 10/30 (Amersham Biotech) as described by Matsubara and Sawano (1995). Native molecular masses of Vgs and yolk proteins were estimated with elution positions of standard proteins: HMW and LMW Gel Filtration Calibration Kit (Amersham Biotech).

Polyacrylamide gel electrophoresis

Sodium-dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on precast acrylamide gels with a gradient of total acrylamide concentrations from 5–20% (Atto, Tokyo, Japan). The molecular masses of protein bands were estimated by SDS-PAGE molecular weight standards of wide range (Sigma). The gels were stained with Silver Stain Kit (Wako).

Statistical analysis

Data in all line graphs is presented as mean±SEM. The data were analyzed by one-way analysis of variance (ANOVA) to test for differences among stages. The means were subsequently compared by Fisher's protected least significant difference test. Differences were considered statistically significant at $p \leqq 0.05$.

RESULTS

Establishment of ELISA

A sandwich ELISA for measuring 600Vg (a mixture of native 600 kDa VgA and VgB proteins) and 560Yp (a mixture of VgA- and VgB-derived native 560 kDa Lv proteins) was developed using a specific antiserum against 600Vg (ab-a-600Vg). A result from comparison of absorbance curves of serially diluted standard protein preparations of 600Vg, 560Yp and 400Yp when tested with this ELISA system is shown in Fig. 1A and B. The sensitivity range of this ELISA was from 7.2 ng/ml to 229 ng/ml for the 600Vg under the conditions used in the present report. When 560Yp standard was tested by this ELISA, sufficient cross-reactivity (18.8%) against the ab-a-600Vg was observed, and the sensitivity range was from 39 ng/ml to 1250 ng/ml. Moreover, a lower but considerable cross-reactivity (0.69%) was also observed in the test of 28Yp, and the sensitivity range was from 625 ng/ml to 10,000 ng/ml (data not shown). In contrast, very low cross-reactivity (0.15%) was found with 400Yp. The ELISA developed for measuring 400Yp using the specific antiserum against 400Yp (ab-a-400Yp) revealed a sensitivity range from 2.6 ng/ml to 163 ng/ml for 400Yp. Very low cross-reactivities, 0.64% and 0.21%, were observed for 600Vg and 560Yp, respectively.

Precisions of these ELISA systems were validated by assessing intra- and inter-assay coefficients of variation (CV). The CV of the intra- and inter-assay in the ELISA for 600Vg were 3.6% (n=4) and 7.7% (n=12), respectively, and those in the ELISA for 400Yp were 5.3% (n=4) and 13.1% (n=10), respectively. The specification of performance of each ELISA system is summarized in Table 1.

Specificities of these ELISAs were evaluated by measuring EP, FP, MP and FO diluents which were serially diluted from 100,000 to 25,600,000, 5,000 to 640,000, 1,000 to 256,000 and 2,000 to 128,000, respectively (Fig. 1C, D). The absorbance curves of the 600Vg ELISA obtained from EP and FP diluents showed full parallelism with each other and with the standard curve of 600Vg (Fig. 1C). Moreover, close parallelism with the absorbance curve was also observed when FO diluents were assessed in this ELISA. Considering the cross-reactivities of the 600Vg ELISA against 560Yp (18.8%) and 28Yp (0.69%) coupled with information about the ratio of their molecular masses (560:28) and molar ratio (1:1), about 0.19% of the absorbance value of FO was thought to be yielded by the reaction of the 28Yp. However, the error (0.19%) is thought to be negligible since it corresponds much lower than both intra- and inter-assay CVs. In the evaluation of the specificity of the 400Yp ELISA, fully parallel absorbance curves were obtained from EP, FP and FO diluents to the standard curve of 400Yp (Fig. 1D). However, both ELISAs did not show any considerable cross-reactivity with MP diluents.

Table 1. Performance and quality of ELISAs for vitellogenins and yolk proteins

	ab-a-600Vg		ab-a-400Yp
	600Vg	**560Yp**	**400Yp**
Assay limit (ng/ml)			
Maximum	229	1250	163
Minimum	7.2	39	2.6
Cross reactivity (%)			
600Vg	100		0.64
560Yp	18.8		0.21
28Yp	0.69		0.24
400Yp	0.15		100
Intra assay (CV)	3.60%		5.30%
Inter assay (CV)	7.70%		13.10%

600Vg: 600 kDa vitellogenin preparation (a mixture of 600VgA and 600VgB), 560Yp: 560 kDa yolk protein preparation (a mixture 560LvA and 560LvB derived from 600VgA and 600VgB, respectively), 28Yp: 28 kDa yolk protein preparation (a mixture 28βcA and 28βcB derived from 600VgA and 600VgB, respectively), 400Yp: 400 kDa yolk protein preparation derived from 400 kDa phosvitinless vitellogenin. Details are described in Sawaguchi *et al.* (2005). CV: coefficient of variation.

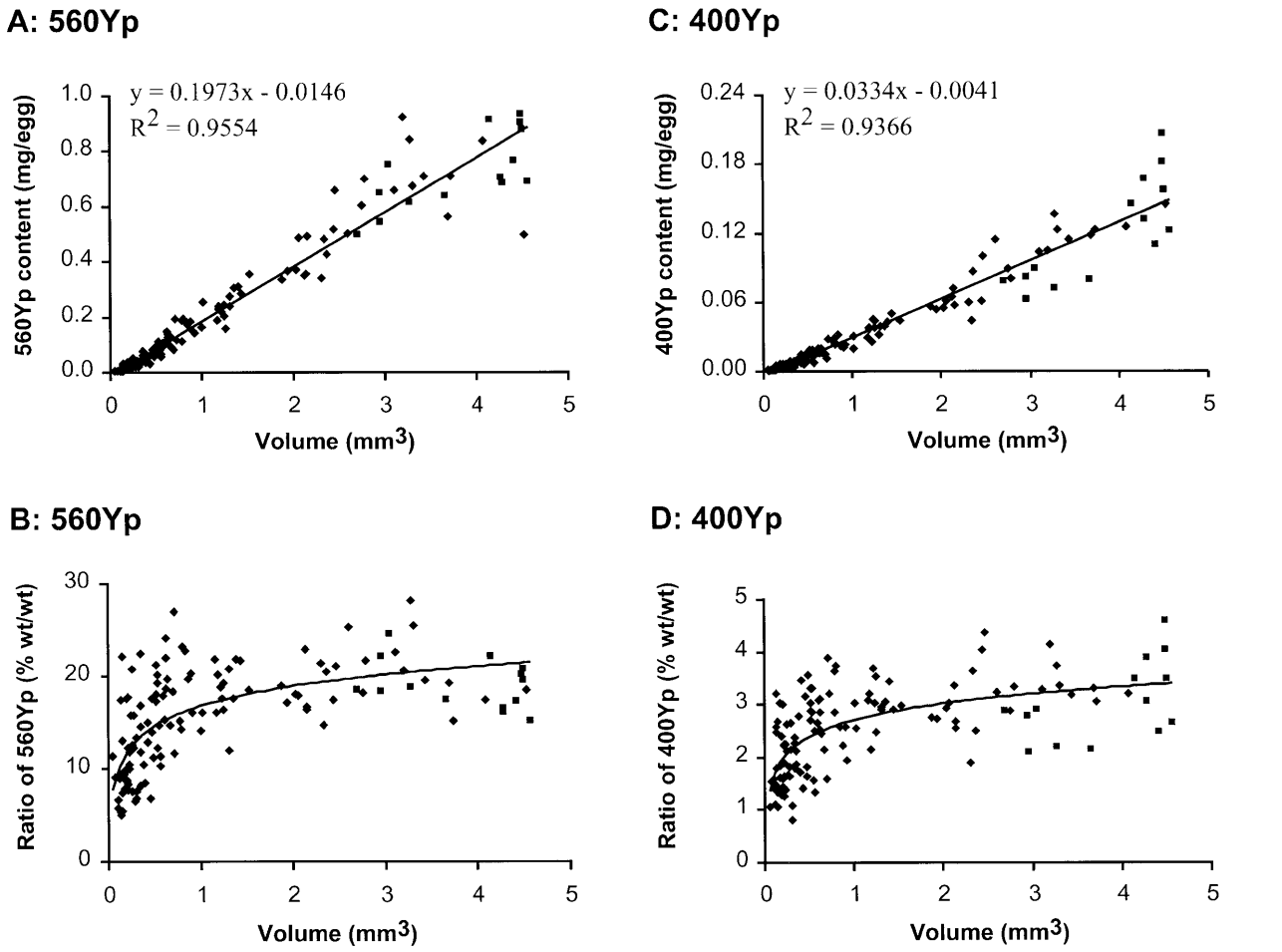


Fig. 2. Changes in 560Yp contents (A) and 400Yp contents (C), and their proportional ratios (B and D) of follicles and eggs in accordance with the increment of their volumes in mosquitofish during the reproductive cycle and embryonic development. Diamonds and squares indicate vitellogenic follicles and eggs, respectively.

Contents of the 560Yp and 400Yp in vitellogenic follicles and eggs

Contents of the 560Yp and 400Yp in follicles at various vitellogenic phases and eggs just after fertilization were measured using the established two ELISA systems. Figure 2 shows changes in the 560Yp and 400Yp contents, and their percent proportions in various sized vitellogenic follicles and eggs of the mosquitofish. The contents of both 560Yp and 400Yp linearly increased to 0.932 mg/egg and 0.206 mg/egg on maximum values, respectively, in accordance with the increment of follicle volume (Fig. 2A, C). The proportional ratios (wt/follicle wt) of both yolk proteins increased during the early stage of vitellogenesis corresponding to a value of about less than 1 mm^3 (Fig. 2B, D). However, after growing to larger than 1 mm^3, the ratios of 560Yp and 400Yp weights versus follicle volumes were maintained almost constant levels at about 20% and 3%, respectively (Fig. 2B, D). When the proportional ratio of 560Yp content versus 400Yp content in mol/mol was calculated for individual vitellogenic follicle and egg at various developmental stages, the ratios centered around 4.1 fold during vitellogenesis and oocyte maturation, although the values varied especially in early vitellogenesis (Fig. 3).

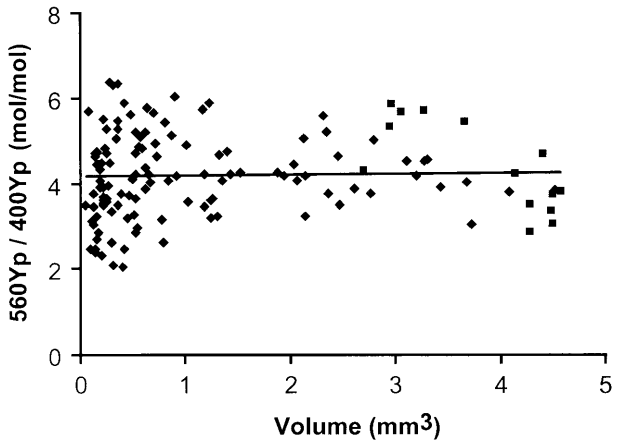


Fig. 3. Change in ratio of 560Yp versus 400Yp with the increase in volume of mosquitofish follicles and eggs during the reproductive cycle. Diamonds and squares indicate vitellogenic follicles and eggs, respectively.

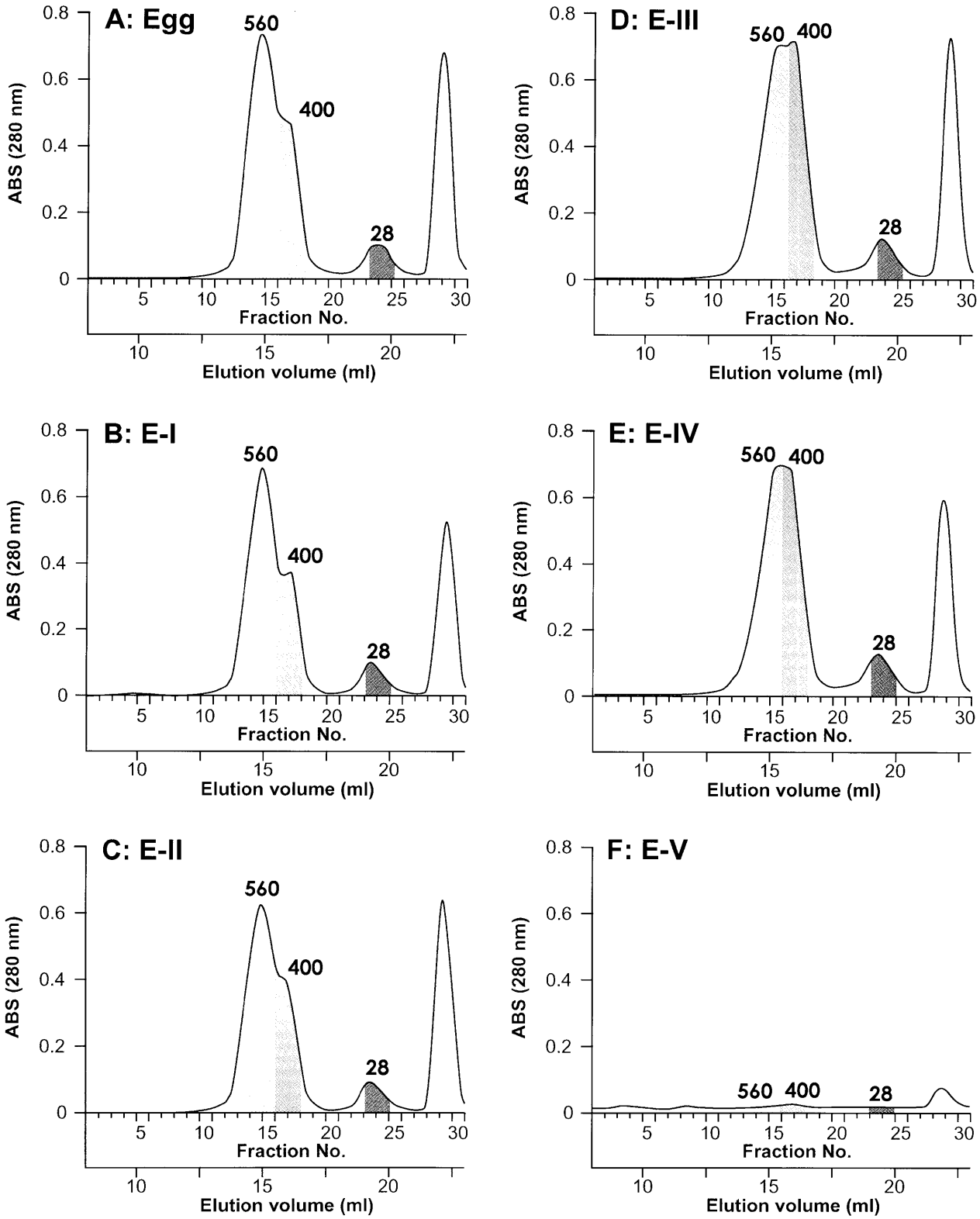
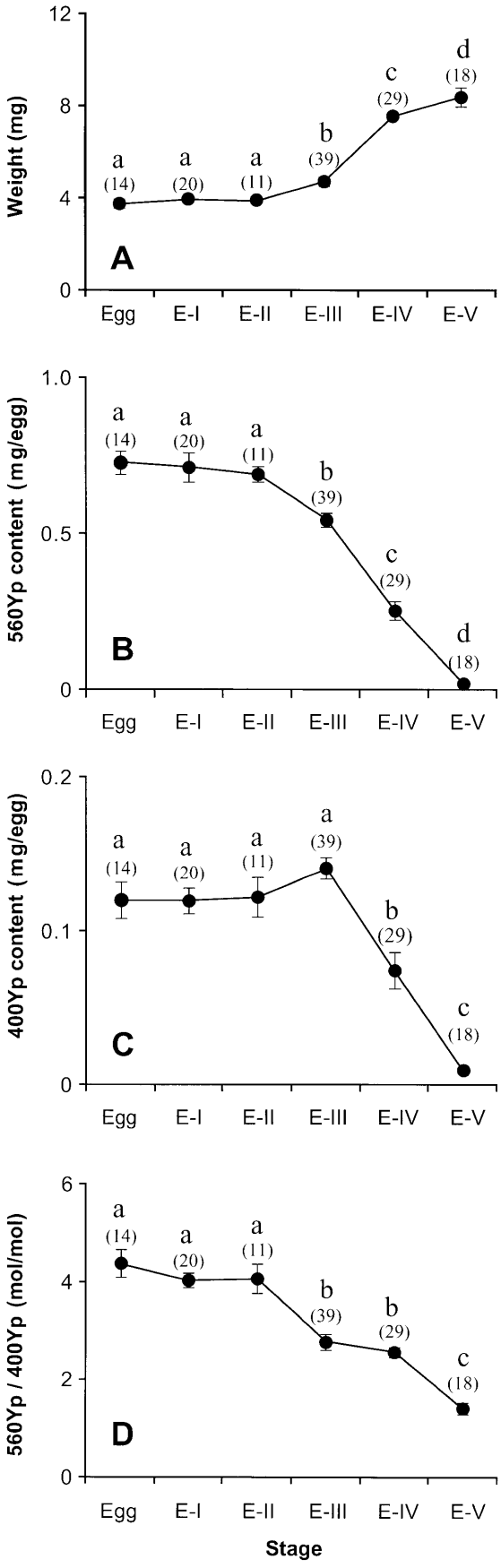


Fig. 4. Elution profiles of homogenates of eggs and developmental embryos after Superose 6 gel chromatography. Egg: eggs, E-I: embryogenesis stage I, E-II: embryogenesis stage II, E-III: embryogenesis stage III, E-IV: embryogenesis stage IV, E-V: embryogenesis stage V. Shaded areas and numbers on the peaks indicate the elution positions of major yolk proteins and the molecular masses, respectively.



To compare the proportional ratio of 560Yp versus 400Yp with that of 600Vg versus 400Vg in vitellogenic female plasma, measurements of concentrations of 600Vg and 400Vg were carried out for plasma from five vitellogenic females. The average plasma concentrations of 600Vg and 400Vg were 3.3±051 mg/ml and 0.8±0.09 mg/ml, respectively. The ratio (mol/mol) of 600Vg versus 400Vg calculated from the concentration for each individual was 3.3±0.21 on average.

Utilization of yolk proteins during embryonic development

Figure 4 shows the elution profiles of extracts from eggs and developing embryos after Superose 6 gel chromatography. A major UV-absorbing peak with a smaller shoulder corresponding to the apparent molecular masses of 560 kDa and 400 kDa, respectively, and a minor peak at 28 kDa were observed in the elution profile of EG (Fig. 4A). From our previous study (Sawaguchi *et al.*, 2005), the 560 kDa, 400 kDa and 28 kDa peaks are believed to involve Lv-Pv complexes derived from VgA and VgB, a PvlVg-derived yolk protein, and a βc being cut off from VgA and/or VgB, respectively. As embryonic development progressed, the elution profiles did not show any obvious changes until stage II (Fig. 4B, C). Considerable changes were observed in the 560 kDa and 400 kDa peaks in elution profiles of embryo homogenates at developmental stages III and IV, although there seemed to be no change in the 28 kDa peak (Fig. 4D, E). In stage III and IV, height of the 560 kDa peak relative to that of the 400 kDa peak decreased when compared to those in EG seen at stage II. All three peaks were not observed in stage V embryos just before parturition (Fig. 4F).

Utilization of 560Yp and 400Yp during embryonic development were quantitatively analyzed by using the two ELISA systems (Fig. 5). Figure 5A shows the change in weight of embryo with its surrounding follicle layer at each developmental stage. The average weight of eggs just before or after fertilization was 3.8 mg and showed no change until embryonic stage II. The weight then rapidly increased to 8.4 mg embryo stage V. Figure 5B and C show the changes in the contents of 560Yp and 400Yp, respectively, during embryonic development. The average content of 560Yp gradually decreased from 0.73 mg/ind. in eggs to 0.69 mg/ind. in stage II embryos and showed a rapid decline to 0.02 mg/ind. by stage V. On the other hand, the average 400Yp content was maintained at a stable level, around 0.12

Fig. 5. Changes in weight (A), 560Yp content (B), 400Yp content (C), and proportional ratio of 560Yp and 400Yp (D) of eggs and developmental embryos of mosquitofish. Closed circle indicates mean ± SEM. Egg: eggs, E-I: embryogenesis stage I, E-II: embryogenesis stage II, E-III: embryogenesis stage III, E-IV: embryogenesis stage IV, E-V: embryogenesis stage V. Numbers in parentheses indicate sample size. Different letters indicate significant differences ($p<0.05$) from each other.

mg/ind., until embryonic stage III. Then, the values rapidly decreased to 0.01 mg/ind. in stage V embryos. When proportional ratios (mol/mol) of the contents of 560Yp versus 400Yp were evaluated, the ratios were kept at about 4.1 fold in eggs to stage II embryos. Subsequently, the ratio gradually decreased to 1.4 fold in stage V embryos (Fig. 5D).

DISCUSSION

The present study firstly focused on the establishment of ELISA systems for measurements of two distinct types of Vg, a general complete-type of VgA and VgB, and an incomplete-type of PvlVg, and their derivative yolk proteins in the mosquitofish. By using the ELISA systems, we were able to measure the two types of Vg-derived yolk proteins in ovarian follicles, eggs and embryos throughout vitellogenesis and embryonic development. In our previous report (Sawaguchi *et al.*, 2005), three distinct forms of Vg cDNA and their product proteins were discovered and identified in E_2-treated females. With regard to our classification of multiple teleost Vgs (Hiramatsu *et al.*, 2002b), the three mosquitofish Vgs were categorized as VgA and VgB each with an apparent native molecular mass of 600 kDa, and a VgC (PvlVg) of 400 kDa. In vitellogenic follicles, the VgA and VgB molecules are cleaved into two yolk proteins having apparent molecular masses of 560 kDa (560Yp) and 28 kDa. The former is thought to be a dimeric molecule consisting of two sets of LvH and Pv-LvL complexes with molecular masses of 126 kDa and 66 or 55 kDa, respectively, and the later is thought to be a βc. On the other hand, although the 400 kDa PvlVg (400Vg) is accumulated into follicles without undergoing any apparent alteration to its native molecular mass, its product yolk protein (400Yp) is suggested to undergo proteolytic nicking and consists of two sets of 112 kDa LvH and 33 or 26 kDa LvL (Sawaguchi *et al.*, 2005).

We targeted the 600Vg and 400Yp to establish ELISAs for measuring 600Vg and 560Yp, and 400Vg and 400Yp, respectively. Until present, ELISAs for two forms of Vg have been reported for the tilapia (Kishida and Specker, 1993; Takemura and Kim, 2001) and for the Japanese common goby (Ohkubo *et al.*, 2003). The sensitivity ranges of our ELISAs established in the present study were 7.2 to 229 ng/ml for 600Vg, 39 to 1250 ng/ml for 560Yp and 2.6 to 163 ng/ml for 400Yp, respectively. These sensitivity ranges are similar to those reported for Vg ELISAs for other fish (e.g. Takemura and Kim, 2001; Ohkubo *et al.*, 2003). The parallelism observed between each dilution curve of the purified Vg, EP, FP and FO in both ELISAs demonstrates the precision and accuracy of our ELISAs to specifically measure the targeted Vg and its derivative yolk protein. The low cross-reactively to alternate Vgs in each ELISA ensures independent measurement of the different types of Vg and yolk proteins. Such high sensitivities are thought to be even enough to detect the presence of Vgs in serum from male fish that were exposed to environmental estrogen. Currently, Vg has become accepted as a biomarker of fish exposure to exogenous environmental estrogens (reviews in Sumpter, 1997; Arcand-Hoy and Benson, 1998; Hiramatsu *et al.*, 2005). The viviparous mosquitofish has been established as a valuable bioindicator species for monitoring estrogenic contamination of aquatic environments (Bortone *et al.*, 1989; Drysdale and Bortone, 1989; Howell and Denton, 1989; Tolar *et al.*, 2001), thus, our Vg ELISAs should also be useful for such applications.

As noted above, although measurements of two distinct forms of Vg in plasma have been reported in some species, there has been no information about the quantities of their derivative yolk proteins in follicles and eggs. In tilapia and Japanese common goby, the molecular ratio of the two form Vgs contained in blood plasma of maturing females are 1.9 fold and 2.7 fold (mol/mol), respectively (Kishida and Specker, 1993; Ohkubo *et al.*, 2003). The ratio (mol/mol) of 600Vg versus 400Vg in plasma from female mosquitofish during active vitellogenesis was about 3.3. Thus, PvlVg-type proteins are maintained at lower levels than the complete forms of Vg in all the three species analyzed. The ratio, 3.3 fold, in mosquitofish female plasma is similar to the ratio, 4.1 fold, in follicles and eggs. Therefore, as the liver does not store substantial quantities of Vg, the ratio of the two distinct types of Vg-derived yolk proteins is suggested to be ultimately controlled by the process of transcription and translation of the Vg genes.

The site of Vg that binds to the Vg receptor (VgR) of teleosts is located in the Lv domain of the Vg molecule (Stifani *et al.*, 1990; Hiramatsu *et al.*, 2002a). A recent study of interactions of tilapia Vg with its receptor demonstrated that an 85- amino acid fragment (VtgSE) located in the LvH domain mediates receptor binding (Li *et al.*, 2003). Site-directed mutagenesis of a conserved short motif (HLTKTKDL) indicated that its first lysine residue (^{181}lysine) likely plays a critical role in receptor binding. Although the ^{181}lysine is conserved among so far available deduced amino acid sequences of general complete-type piscine Vgs, including mosquitofish VgA and VgB, substitution of another basic residues, arginine or glutamine for lysine, occurs in PvlVg of mosquitofish (Sawaguchi *et al.*, 2005) as well in the PvlVgs of zebrafish (Wang *et al.*, 2000) and Japanese common goby (Ohkubo *et al.*, 2003). The impact on receptor binding affinity of such substitutions at a key functional residue in the ligand warrants further investigations with regard to regulation in incorporation of multiple Vgs into oocytes.

Results from measurements of 560Yp and 400Yp in eggs and embryos at every stage of development demonstrated that considerable consumption of both yolk proteins started after embryo stage II. This stage in mosquitofish corresponds neurula stage to pre-pigmentation retina stage according to definition of Chambolle *et al.* (1970) and Koya *et al.* (2000). In contrast, embryo weights drastically increased stage III onward as previously reported by Chambolle *et al.* (1970) and Koya *et al.* (2000). Considering our quantitative analysis of yolk proteins in embryos, there is no evidence

that an additional Vg uptake occurs during this period. Further investigations are necessary to elucidate the contribution of the nutrient supply other than Vgs during gestation in the mosquitofish.

In elution profiles of gel chromatographies of eggs and embryos, changes in the proportion of 560Yp versus 400Yp peaks were observed in accordance with the progression of embryonic development. Although the 560Yp is likely utilized prior to 400Yp, the proteolysis of 560Yp does not seem to occur inside of the yolk mass since there are no other smaller protein peaks, which involve the transit of degraded polypeptides by proteolysis, even in the gel chromatograms of the stage III and IV embryos in which yolk proteins are actively consumed. The yolk syncytial layer (YSL) is known to play important roles in proteolytic yolk protein digestion by teleost embryos (Walzer and Schonenberger, 1979a, b; Trinkaus, 1993; Krieger and Fleig, 1999). Generally, yolk processing during embryogenesis occurs pinocytotically in the YSL, which is derived from collapsed marginal blastomeres in early cleavage stages and forms a syncytium between the egg yolk and the inner cell mass (Trinkaus, 1993). In the mummichog *Fundulus heteroclitus*, which is a closely related species to mosquitofish, the YSL was formed at the blastula stage, and active pinocytosis of the yolk mass was observed at the margin of YSL especially in mid to late embryogenesis (Trinkaus, 1993). Unequal consumption of 560Yp and 400Yp, in which the 560Yp was selectively utilized, also was supported by the result of immunological measurements of the yolk proteins in the present study. Nothing is presently known about the mechanism of the differential consumption of the 560Yp and 400Yp during embryonic development with regard to selective pinocytosis by yolk syncytial layer (YSL). Further investigation is necessary to explore this mechanism, including the possibility of receptor mediation.

The present study established two ELISA systems for general-type Vg and PvlVg, and quantified their derivative yolk proteins in follicles, eggs and embryos in mosquitofish. This is the first report of quantitative elucidation of the incorporation and utilization of multiple Vg-derived yolk proteins in any oviparous vertebrate, including viviparous teleosts. So far as we know, there have not been any established immunological assay systems for quantifying VgA and VgB, or their derivative Lvs in teleosts, including the mosquitofish, probably, because of difficulties in isolation of the two types of complete Vg due to their similarities in biochemical characteristics. Nevertheless, although multiplicity of Vg is evident in various species, functional properties of two (VgA and VgB) of the three forms of Vg have been verified only in a few marine or brackish water teleosts (barfin flounder, Matsubara *et al.*, 1999; haddock, Reith *et al.*, 2001; white perch, Hiramatsu *et al.*, 2002a). Advances in the development of quantitative assay systems for different types of Vg should enable us to elucidate further details of the physiological mechanisms involved in the regulated accumulation and utilization of multiple Vg-derived yolk proteins in highly evolved teleosts including the viviparous mosquitofish.

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REFERENCES

Arcand-Hoy LD, Benson WH (1998) Fish reproduction: An ecologically relevant indicator of endocrine disruption. Environ Toxicol Chem 17: 49–57

Bortone SA, Davis WP, Bundrick CM (1989) Morphological and behavioral characters in mosquitofish as potential bioindication of exposure to kraft mill effluent. Bull Environ Contam Toxicol 43: 370–377

Byrne BM, de Jong H, Fouchier RAM, Williams DL, Gruber M, Ab G (1989) Rudimentary phosvitin domain in a minor chicken vitellogenin gene. Biochem 28: 2572–2577

Campbell CM, Idler DR (1980) Characterization of estradiol-induced protein from rainbow trout serum as vitellogenin by the composition and radioimmunological cross reactivity to ovarian yolk fraction. Biol Reprod 22: 605–617

Chambolle P, Cambar R, Tran D (1970) Table chronologique du développement embryonnaire de *Gambusia* sp. (Poisson Teleosteen). Bull Biol 104: 443–452

Ding JL, Hee PL, Lam TJ (1989) Two forms of vitellogenin in the plasma and gonads of male *Oreochromis aureus*. Comp Biochem Physiol B 93: 363–370

Drysdale DT, Bortone SA (1989) Laboratory induction of intersexuality in the mosquitofish, *Gambusia affinis*, using paper mill effluent. Bull Environ Contam Toxicol 43: 611–617

Germond JE, ten Heggeler B, Schubiger JL, Walker P, Westley B, Wahli W (1983) Vitellogenin B2 gene in *Xenopus laevis*: isolation, in vitro transcription and relation to other vitellogenin genes. Nucleic Acids Res 11: 2979–2997

Hara A, Sullivan CV, Dickhoff WW (1993) Isolation and some characterization of vitellogenin and its related egg yolk proteins from coho salmon (*Oncorhynchus kisutch*). Zool Sci 10: 245–256

Hiramatsu N, Hara A, Hiramatsu K, Fukada H, Weber GM, Denslow ND, Sullivan CV (2002a) Vitellogenin-derived yolk proteins of white perch, *Morone americana*: purification, characterization, and vitellogenin-receptor binding. Biol Reprod 67: 655–667

Hiramatsu N, Matsubara T, Hara A, Donato DM, Hiramatsu K, Denslow ND, Sullivan CV (2002b) Identification, purification and classification of multiple forms of vitellogenin from white perch (*Morone americana*). Fish Physiol Biochem 26: 355–370

Hiramatsu N, Matsubara T, Weber GM, Sullivan CV, Hara A (2002c) Vitellogenesis in aquatic animals. Fish Sci 68: 694–699

Hiramatsu N, Cheek AO, Sullivan CV, Matsubara T, Hara A (2005) Vitellogenesis and endocrine disruption. In "Biochemistry and Molecular Biology of Fishes" Ed by TP Mommsen, T Moon, Elsevier Science Press, Amsterdam, pp 431–471

Howell WM, Denton TE (1989) Gonopodial morphogenesis in female mosquitofish, *Gambusia affinis affinis*, masculinized by exposure to degradation products from plant sterols. Env Biol Fish 24: 43–51

Ishikawa E, Imagawa M, Hashida S, Yoshitake S, Hamaguchi Y, Ueno T (1983) Enzyme-labeling of antibodies and their fragments for enzyme immunoassay and immunohistochemical

staining. J Immunoassay 4: 209–327
Kishida M, Specker JL (1993) Vitellogenin in tilapia (*Oreochromis mossambicus*): Induction of two forms by estradiol, quantification in plasma and characterization in oocyte extract. Fish Physiol Biochem 12: 171–182
Koya Y, Inoue M, Naruse T, Sawaguchi S (2000) Dynamics of oocyte and embryonic development during ovarian cycle the viviparous mosquitofish *Gambusia affinis*. Fish Sci 66: 63–70
Krieger J, Fleig R (1999) Yolk mobilization in perch, *Perca fluviatilis* L., embryos. Fish Physiol Biochem 21: 157–165
LaFleur GJ Jr., Byrne BM, Haux C, Greenberg RM, Wallace RA (1995a) Vitellogenins and vitelline envelope protein precursors (chorogenins). In "Proceedings of the Fifth International Symposium on the Reproductive Physiology of Fish." Ed by FW Goetz, P Thomas, University of Texas, Austin, pp 336–338
LaFleur GJ Jr., Byrne BM, Kanungo J, Nelson L, Greenberg RM, Wallace RA (1995b) *Fundulus heteroclitus* vitellogenin: The deduced primary structure of a piscine precursor to noncrystalline, liquid-phase yolk protein. J Mol Evol 41: 505–521
Li A, Sadasivam M, Ding JL (2003) Receptor-ligand interaction between vitellogenin receptor (VtgR) and vitellogenin (Vtg), implications on low density lipoprotein receptor and apolipoprotein B/E. J Biol Chem 31: 2799–2806
Markert JR, Vanstone WE (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 Canada 28: 1853–1856
Matsubara T, Sawano K (1995) Proteolytic cleavage of vitellogenin and yolk proteins during vitellogenin uptake and oocyte maturation in barfin flounder (*Verasper moseri*). J Exp Zool 272: 34–45
Matsubara T, Ohkubo N, Andoh T, Sullivan CV, Hara A (1999) Two form 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
Ng TB, Idler DR (1983) Yolk formation and differentiation in teleost fishes. Fish Physiol 9: 373–404
Ohkubo N, Mochida K, Adachi S, Hara A, Hotta K, Nakamura Y, Matusbara T (2003) Development of enzyme-linked immunosorbent assays for two forms of vitellogenin in Japanese common goby (*Acanthogobius flavimanus*). Gen Comp Endocrinol 131: 353–364
Ohkubo N, Andoh T, Mochida K, Adachi S, Hara A, Matsubara T (2004) Deduced primary structure of two forms of vitellogenin in Japanese common goby (*Acanthogobius flavimanus*). Gen Comp Endocrinol 137: 19–28
Patiño R, Sullivan CV (2002) Ovarian follicle growth, maturation, and ovulation in teleost fish. Fish Physiol Biochem 26: 57–70
Reith M, Munholland J, Kelly J, Finn RN, Fyhn HJ (2001) Lipovitellins derived from two forms of vitellogenin are differentially processed during oocyte maturation in haddock (*Melanogrammus aeglefinus*). J Exp Zool 291: 58–67
Sawaguchi S, Koya Y, Yoshizaki N, Ohkubo N, Andoh T, Hiramatsu N, Sullivan CV, Hara A, Matsubara T (2005) Multiple vitellogenins (Vgs) in mosquitofish (*Gambusia affinis*): Identification and characterization of three functional Vg genes and their circulating and yolk protein products. Biol Reprod 72: 1045–1060
Specker JL, Sullivan CV (1994) Vitellogenesis in fishes: status and perspectives. In "Perspectives in comparative endocrinology" Ed by KG Davey, RG Peter, SS Tobe, National Research Council Canada, Ottawa, pp 304–315
Stifani S, Le Menn F, Rodriguez JN, Schneider WJ (1990) Regulation of oogenesis: the piscine receptor for vitellogenin. Biochim Biophys Acta 3: 271–279
Sumpter JP (1997) Environmental control of fish reproduction: a different perspective. Fish Physiol Biochem 17: 25–31
Takemura A, Kim BH (2001) Effects of estradiol-17β treatment on *in vitro* and *in vivo* synthesis of two distinct vitellogenin in tilapia. Comp Biochem Physiol A 129: 641–651
Tolar JF, Mehollin AR, Watson RD, Angus RA (2001) Mosquitofish (*Gambusia affinis*) vitellogenin: identification, purification, and immunoassay. Comp Biochem Physiol C 128: 237–245
Trinkaus JP (1993) The yolk syncytial layer of *Fundulus*: Its origin and history and its significance for early embryogenesis. J Exp Zool 265: 258–284
Wahli W, Dawid IB, Wyler T, Jaggi RB, Weber R, Ryffel GU (1979) Vitellogenin in *Xenopus laevis* is encoded in a small family of genes. Cell 16: 535–549
Wahli W, Dawid IB, Wyler T, Weber R, Ryffel GU (1980) Comparative analysis of the structural organization of two closely related vitellogenin genes in *X. laevis*. Cell 20: 107–117
Wallace RA (1985) Vitellogenesis and oocyte growth in nonmammalian vertebrates. In "Oogenesis" Ed by LW Browder, Plenum Press, New York, pp 127–177
Wallace RA, Selman K (1978) Oogenesis in *Fundulus heteroclitus*. I. Preliminary observations on oocyte maturation *in vivo* and *in vitro*. Dev Biol 62: 354–369
Walzer C, Schönenberger N (1979a) Ultrastructure and cytochemistry of the yolk syncytial layer in the alevin of trout (*Salmo fario trutta* L. and *Salmo gairdneri* R.) after hatching. I. The vitellogenesis zone. Cell Tiss Res 196: 59–73
Walzer C, Schönenberger N (1979b) Ultrastructure and cytochemistry of the yolk syncytial layer in the alevin of trout (*Salmo fario trutta* L. and *Salmo gairdneri* R.) after hatching. II. The cytoplasmic zone. Cell Tiss Res 196: 75–93
Wang H, Yan T, Tan JTT, Gong Z (2000) A zebrafish vitellogenin gene (*vg3*) encodes a novel vitellogenin without a phosvitin domain and may represent a primitive vertebrate vitellogenin gene. Gene 256: 303–310

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