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Circannual variation in plasma vitellogenin and gonadotropin II levels in relation to annual ovarian cycle in female mrigal, *Cirrhinus mrigala*

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Abstract

Two forms (HA I and HA II) of vitellogenin (Vg), the yolk-precursor protein, were purified from the plasma of estradiol-17β (E_2)-treated Indian major carp, mrigal (*Cirrhinus mrigala*), by gel filtration on Ultrogel AcA 34 followed by adsorption chromatography on Hydroxylapatite (HA)-Ultrogel. Native HA I and HA II had molecular weights of 500 kDa and 550 kDa, respectively. The apparent masses of purified HA I and HA II after SDS-PAGE under reducing condition were 75 kDa and 85 kDa, respectively. HA I was found to be lipid rich whereas HA II was phosphorous rich. The co-purified Vg containing both HA I and HA II was used to raise polyclonal antisera (a-Vg) and its specificity was assessed by Western blot analysis. In a double immunodiffusion test, the plasma of vitellogenic females and E_2-treated males as well as crude egg yolk protein (CYP), cross-reacted with a-Vg giving two precipitin lines in each case, thereby indicating the presence of two forms of Vg. HA I and HA II each gave single precipitin lines. No cross-reaction was observed with control male plasma. A competitive enzyme-linked immunosorbent assay (ELISA) was developed using a-Vg and HA I. The detection limit of the assay was 6.25 ng/ml, and the intra- and inter-assay variations were 4.48 and 7.57%, respectively. Displacement curves parallel to the standard (HA I) were obtained with plasma samples from vitellogenic female and E_2-treated male mrigal. The assay was validated by estimating Vg in plasma samples from adult female mrigal captured in the field throughout the year and compared with ovarian development. Annual profiles of plasma Vg and gonadotropin (GTH II: estimated by common carp GTH II ELISA) levels presented a good correlation with gonadosomatic index (GSI) and mean number of vitellogenic oocytes during different reproductive phases, i.e., preparatory (Feb–Apr), pre-spawning (May–Jun), spawning (Jul–Aug) and post-spawning (Sep–Jan).
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1. Introduction

In fish, as in other oviparous vertebrates, the synthesis of the yolk-precursor protein vitellogenin (Vg) is the prerequisite for oocyte growth during oogenesis (vitellogenesis) and thus contributes vitally to egg quality and reproductive success. Pituitary gonadotropin (GTH) and

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ovarian steroid hormone (estradiol-17β, E_2) regulate vitellogenesis in fish (Nagahama, 2000). Two gonadotropins, GTH I (similar to follicle stimulating hormone, FSH) and GTH II (similar to luteinizing hormone, LH) have been purified from the pituitaries of many fish species (Swanson, 1991; Van Der Kraak et al., 1992; Tanaka et al., 1993). Studies made in salmonid fish revealed that GTH I is primarily involved in vitellogenesis and spermatogenesis whereas GTH II triggers maturation and ovulation and spermiation. However, both GTHs are equally potent in stimulating E_2 production (see Swanson et al., 1991; Gen et al., 2000). Van Der Kraak et al. (1992) reported that no clear functional differences exist between GTH I and GTH II of common carp and salmon when tested in both *in vitro* and *in vivo* bioassays (Suzuki et al., 1988a; Swanson et al., 1991). These findings suggest that both GTHs are involved in fish reproduction. In response to GTH-induced ovarian estrogen release, hepatocytes synthesize and release Vg into the bloodstream, from where it is taken up and incorporated into growing oocytes via receptor-mediated endocytosis (Sawaguchi et al., 2006 and references therein). Vg(s) undergo limited proteolytic cleavage to form lipovitellin (Lv), phosvitin (Pv) and β′-component (β′-c) prior to deposition as egg yolk proteins in the ooplasm (reviewed in Specker and Sullivan, 1994; Nath, 1999; Hiramatsu et al., 2002a). Vg is considered as a female specific protein but similar proteins have been identified in males of many fish species (Ding et al., 1989; Kishida and Specker, 1993). However, the exact function of Vg in male is yet to be established.

In teleosts, Vg is a high molecular weight (300–600 kDa) glycolipophosphoprotein that circulates as a dimer (Specker and Sullivan, 1994). Although the number of molecular forms of Vg is not confirmed, two forms have been identified in various teleost species including tilapia (Ding et al., 1989; Kishida and Specker, 1993; Buerano et al., 1995), mummichog (LaFleur et al., 1995a), zebra fish (Wang et al., 2000), rainbow trout (Trichet et al., 2000), haddock (Reith et al., 2001) and medaka (Shimizu et al., 2002) and three molecular forms in white perch (Hiramatsu et al., 2002b), mosquito fish (Sawaguchi et al., 2005) and red sea bream (Sawaguchi et al., 2006). The isolation, characterization and specific assay development for different forms of Vg have become more important to understand better the evolution of multiple piscine Vgs and their physiological significance in reproduction (Sawaguchi et al., 2005).

In female fish significant levels of Vg are present in circulation during vitellogenesis and Vg synthesis can be induced by E_2 in both males and immature females. Thus, measurement of plasma Vg is widely practiced to monitor the reproductive status of females. Sensitive assays like radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA) and single radial immunodiffusion have been adopted for quantification of fish Vgs, of which ELISA is believed to be the safest option for routine work (reviewed in Specker and Sullivan, 1994).

The Indian major carp, mrigal, *Cirrhinus mrigala*, is one of the most common carps in the Indian subcontinent and is a very important candidate for freshwater aquaculture. Previously we had reported the role of two different mrigal Vgs in the process of synthesis and incorporation of Vg in Indian catfish, C*larias batrachus*, through a series of *in vivo* experiments (see Nath and Maitra, 2001). However, circannual variations in plasma Vg and GTH levels in relation to ovarian growth are lacking in this species. Therefore, the major objectives of the present investigation were (i) to purify and partially characterize Vg from the plasma of E_2-treated mrigal, (ii) development of an ELISA for mrigal Vg and (iii) to determine the circannual variations in plasma Vg and GTH II levels in relation to different phases of ovarian growth in female mrigal.

2. Materials and methods

2.1. Collection and care of fish

Sexually mature specimens of *C. mrigala* (body weight range: 0.5–0.75 kg) were collected around Santiniketan (Lat. 23°41′30″ N and Long. 87°30′47″ E) and maintained in the laboratory in cement tanks (240 cm × 150 cm × 120 cm) under natural photoperiod and temperature. Fish were fed *ad libitum* with laboratory-made fish food, containing rice bran, oil cake, fortified with vitamin C and B-complex and rabbit food pellets [Lipton, India]. Water in the tanks was replenished daily and was circulated with the help of motor pumps for aeration at regular time intervals. Fortified procaine penicillin (Allembic Chemical Works, Vadodora, India) was added to tank water (1:1000) occasionally as a prophylactic against skin infection. Fish were acclimated to laboratory conditions for 7 days prior to their use in experiments.

2.2. Preparation of mrigal Vg

2.2.1. Estradiol-17β (E_2) injection for vitellogenin synthesis

E_2 (Sigma, USA) was dissolved in ethanol (10 mg in 0.2 ml) and at the time of injection a suspension was

made with 0.64% saline. Ten males were injected (0.1 ml/fish/day) intramuscularly on alternate days at the E_2 dose level of 50 μg/100 g body weight for 30 days. Control fish were maintained in separate tanks and received equal volume saline injections.

2.2.2. Collection of plasma samples

Both E_2- and saline-treated fish were bled separately by caudal puncture using heparinized syringes. Blood was collected in tubes (kept in ice) each containing 0.05% PMSF (phenylmethylsulfonylfluoride, SRL, India) and aprotinin (0.5 TIU/ml of blood) (Sigma, USA) dissolved in 0.01 M phosphate buffer (pH 7.4) containing 0.9% NaCl. Plasma was collected by centrifugation at 1500 ×*g* for 10 min at 4 °C and either used immediately or kept at −30 °C until its use for the purification of Vg.

2.2.3. Purification, characterization of mrigal Vg and preparation of antiserum

Vg was purified from the plasma of E_2-treated male mrigal by gel filtration on Ultrogel AcA 34 and adsorption chromatography on Hydroxylapatite-Ultrogel (HA-Ultrogel from LKB, Sweden). The plasma sample (~5 ml) was loaded on an Ultrogel AcA 34 column (73×2.6 cm) and eluted with 0.05 M Tris–HCl buffer (pH 8.0) containing 2% KCl, PMSF and aprotinin at a flow rate of 30 ml/h. 5-ml fractions were collected and absorbance read at 280 nm. Alkali-labile phosphorous (ALP) was estimated as an index of Vg in every alternate fraction as described by Nath and Sundararaj (1981a). Fractions of peak I (henceforth ULT I) containing ALP were pooled and dialyzed extensively against chilled 0.01 M phosphate buffer (pH 8.0). Protein content was estimated by the method of Lowry et al. (1951). Dialyzed sample (~30 mg protein) was loaded on HA-Ultrogel column (12×1.5 cm) previously equilibrated with 0.01 M phosphate buffer (pH 8.0) and bound proteins were eluted by increasing concentration of potassium phosphate buffer (0.1 M and 0.5 M, pH 8.0) in a stepwise manner.

To determine the molecular mass of purified proteins, gel filtration was performed on Sepharose 4B column (22×1.4 cm) following the methods of Andrews (1965). The column was calibrated with the following marker proteins (Pharmacia): aldolase (156 kDa), catalase (232 kDa), ferritin (440 kDa) and thyroglobulin (669 kDa).

The total phosphorous, lipid and carbohydrate content of HA I and HA II were estimated following the methods of Bartlett (1959), Folch et al. (1957) and Umbreit et al. (1958), respectively. In each determination, 0.8-ml aliquots containing 5 mg each of purified HA I and HA II were used.

Anti-Vg antisera (hereafter a-Vg) were raised by using the co-purified mrigal Vg (HA I and HA II, eluted together from the HA-Ultrogel column by employing 0.01–1 M continuous phosphate buffer, pH 8.0, gradient, data not shown) in rabbits by intra-dermal injection with 1 mg of protein emulsified with Freund's complete adjuvant (SRL, India). The rabbits were re-challenged 1 month after the first injection with 500 μg of Vg emulsified in Freund's incomplete adjuvant (SRL, India). Booster injection of 100 μg of Vg was given after 3 weeks through the ear vein. Blood was collected from the ear vein 1 week after the booster injection and allowed to clot at room temperature for 30 min and kept at 4 °C overnight. Antiserum was collected by centrifugation at 1500 ×*g* (4 °C) for 10 min and stored in aliquots at −30 °C.

2.3. Electrophoresis

Native PAGE of plasma samples from control male, vitellogenic female and E_2-treated male mrigal was performed in 4–15% gradient gels using electrophoresis buffer (0.025 M Tris, 0.192 M glycine, pH 8.3). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 4–15% gradient separating gels was performed for purified Vg (HA I and HA II) according to Laemmli (1970) under reducing [β−mercaptoethanol] condition. Gels were run at 150 V (constant) at 4 °C and stained with Coomassie Brilliant Blue R250 (SRL, India) for protein and with Sudan black B (BDH, India) for lipid.

2.4. Western blot analysis

Plasma samples from control male, vitellogenic female and E_2-treated male mrigal, as well as purified mrigal Vgs (HA I and HA II), were separated by native PAGE (4–15% gradient gel) and electroblotted onto a nitrocellulose membrane according to the method of Towbin et al. (1979). After electroblotting, the membrane was blocked with 3% BSA and 5% casein in Tris-buffered saline (0.02 M Tris and 0.5 M NaCl, pH 7.5; TBS) for 2 h with constant shaking. Membranes were incubated with primary antiserum (a-Vg) diluted 1:1000 in TBS for 3 h followed by incubation with the secondary antibody (goat anti-rabbit IgG conjugated with horseradish peroxidase, PAP (Sigma, USA) diluted 1:4000 for 2 h at room temperature). After washing thrice with TBS containing 0.05% Tween 20, the antigen–antibody reactions were visualized by DAB–H_2O_2.

2.5. Ouchterlony immunodiffusion test

Double immunodiffusion test was performed for cross-reaction studies following the method of Ouchterlony (1953). Plasma samples and protein fractions, as indicated in Section 2.4, as well as crude yolk proteins (CYP) of mrigal (prepared according to Bhakta and Nath, 1996) were allowed to cross-react with the a-Vg antiserum.

2.6. Enzyme-linked immunosorbent assay (ELISA) for mrigal Vg (HA I)

The antigen-capture competitive ELISA for mrigal Vg (HA I) was developed following the method of Burzawa-Gerard et al. (1991) and Nuñez-Rodriguez et al. (1989). Ninety six-well microtitre plates (Costar, Cambridge, MA, USA) were coated with 200 μl/well containing either 1 μg/ml of HA I or BSA (non-specific binding) in 0.05 M sodium carbonate buffer, pH 9.6. Plates were incubated overnight at 4 °C. The non-specific binding sites were saturated by incubating the plates with 200 μl of 1% BSA in 0.01 M phosphate buffer pH 7.4 containing 0.15 M NaCl, 0.05% Tween 20 (PBST) for 1 h at room temperature (30 °C). Blocking solution was removed by washing the plates three times with PBST. Serially diluted Vg standard solution (100 μl) containing 1.56 to 3200 ng/ml of HA I or individual plasma samples (diluted 1:50 to 1:30,000 in PBST-BSA) were mixed with equal volume of 1:25,000 of primary antibody (a-Vg: 1:25,000) in separate tubes and incubated overnight at 4 °C. Wells were loaded with 200 μl of sample-primary antibody solution and incubated for 3 h at room temperature (~25 °C). Standards and plasma samples were assayed in triplicate. After the plates were washed as above, each well received 200 μl of PAP (1:4000 in PBST-BSA), and incubated for 2 h at room temperature (30 °C). For color development, each well received 200 μl of freshly prepared coloring reagent containing 10 mg *o*-phenylenediamine, OPD (Sigma, USA) in 25 ml of 0.1 M citrate–phosphate buffer, pH 5.0, and 3 μl of H_2O_2. After 30 min of incubation in the dark at room temperature, the reaction was stopped by adding 50 μl/well of 2 N H_2SO_4 and the absorbance was measured at 492 nm using an Anthos 2001 microplate reader (Anthos Labtec, Austria).

Absorbance measurements were expressed by a non-linear model $[(Bi-\mathrm{NSB}/B_0-\mathrm{NSB})\times 100=f\,(\log\,(\mathrm{dose}$ or dilution))], where B_i, represented the binding of sample or standard Vg; B_0, the maximum binding and NSB, the non-specific binding. The analysis of competition curves was performed after linearization of the binding percentage values (B) through logit–log transformation [logit $B=\log_e\,(B/100-B)$]. The parallelism between regression lines was tested by analysis of covariance (ANCOVA) following the method described by Snedecor and Cochran (1957). Parallelism of pure Vg to circulating Vg was demonstrated by serially diluting the plasma samples from control male, vitellogenic female and E_2-injected male.

2.7. Assay of plasma GTH II

Plasma GTH II levels were estimated by heterologous GTH II ELISA (unpublished data) using common carp GTH II and anti-GTH II antibodies (kindly gifted by Dr. R.E. Peter, Biological Sciences, University of Alberta, Edmonton, Canada). Briefly, 20 ng/well of GTH II coating and antiserum dilution at 1:25,000 were used for routine assay. Under such conditions the detection limit was 78 pg/ml. The intra- and inter-assay coefficients of variance ranged from 2 to 8% ($n=6$) and 1.5 to 10% ($n=12$), respectively. Plasma samples were diluted 1:10 or more for estimation of GTH II. In this ELISA, a good parallelism was observed between carp GTH II standard preparation and plasma samples as well as pituitary homogenate from mrigal (unpublished data).

2.8. Monthly sampling of female mrigal

Sexually mature female mrigal (body weight range: 150–200 g) were obtained throughout the year at the middle of every month and sacrificed on the day of collection. Each fish was weighed to the nearest g, bled by caudal puncture and plasma separated as described earlier. Plasma samples were diluted 1:10 with PBS (0.01 M phosphate buffer, pH 7.4, containing 0.15 M NaCl), divided into small aliquots, and stored at −30 °C for estimation of plasma Vg and GTH II. After blood collection fish were killed by decapitation, ovaries dissected out, and weighed to the nearest 0.1 g and then fixed in aqueous Bouin's fixative (24 h) for histological studies. Gonadosomatic index (GSI) was calculated by using the following formula:

$$\frac{(\text{Gonad weight})}{(\text{Body weight})}\times 100$$

2.9. Histological studies

Transverse sections of ovaries were cut at 6 μm and stained with Earlich's haematoxyline and eosine. Three different stages of oogenesis were characterized and identified in the ovarian sections of mrigal following

a method described by Ghosh and Nath (2005): stage-I (S-I), the non-yolky oocytes present throughout the year; stage-II (S-II), the vitellogenic oocytes characterized by the appearance of cortical alveoli (CA) and/or yolk vesicle (YV) in cytoplasm; and stage-III (S-III), the fully formed yolky oocytes. Differential counts of these three stages were done from stained sections following the technique of Nath and Maitra (2001).

2.10. Expression of results and statistical analysis

For the comparison of data, the ovarian weight changes were expressed on 100 g body weight basis (GSI), the different stages (S-I, -II and -III) of oocytes as percentage and plasma levels of GTH II as ng/ml and Vg as μg/ml. *P* values were calculated by Student's '*t*' (Snedecor and Cochran, 1957) test for plasma Vg levels between the late pre-spawning (Jun) and early spawning (Jul) and between spawning (Jul) and post-spawning (Sep).

3. Results

3.1. Purification and partial characterization of the Vg molecule

Fig. 1 shows the elution profile of plasma from E_2-treated and control male mrigal after gel filtration on

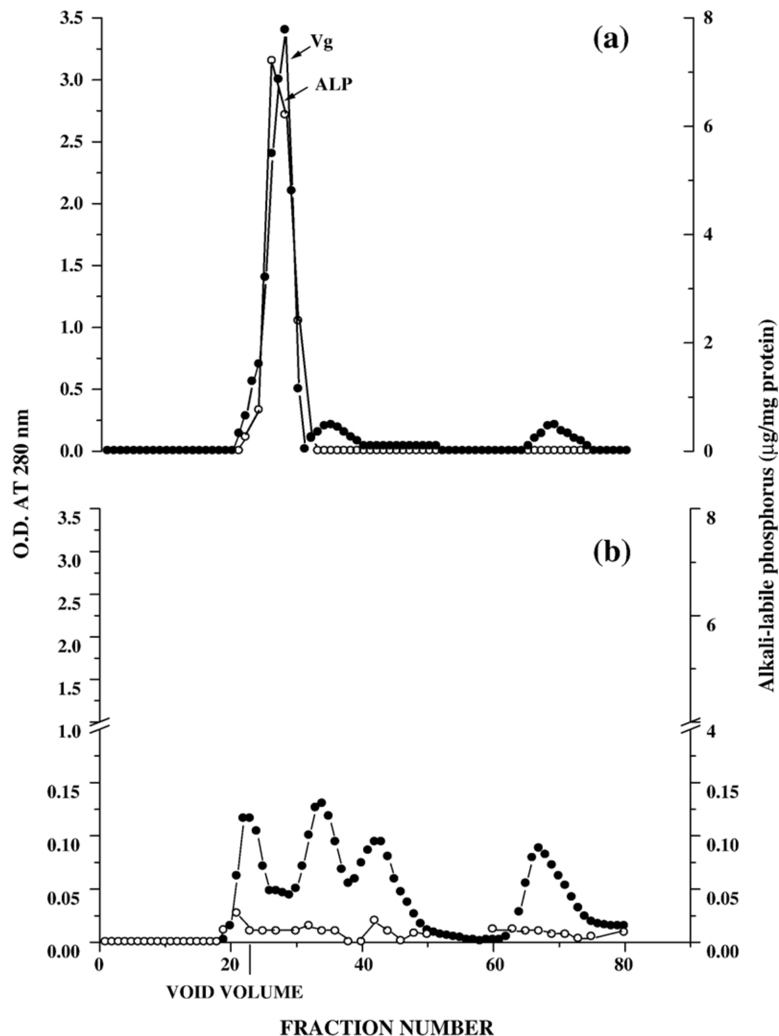

Fig. 1. Elution profile of plasma samples from E_2-treated male (a) and control male (b) mrigal, after gel filtration on Ultrogel AcA 34 column (73 × 2.6 cm). In each case 3 ml of plasma was loaded and 5-ml fractions were collected in each tube with 0.05 M Tris–HCl buffer (pH 8) containing 2% KCl at a flow rate of 30 ml/h. The optical density of each fraction was monitored at 280 nm (closed circle) and the fractions containing ALP (open circle), ULT I were pooled and used for adsorption chromatography.

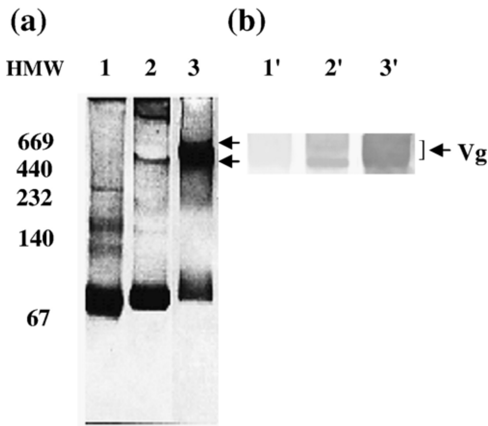

Fig. 2. (a) Plasma samples of control male (2 μl, lane 1), vitellogenic female (2 μl, lane 2) and E_2-treated male (2 μl, lane 3) mrigal were separated by 4–15% native PAGE and stained with Coomassie Brilliant Blue R250. At extreme left, migration position of high molecular weight marker proteins (Pharmacia) are shown. Arrowheads indicate migration position of putative Vg. (b) Duplicate lanes were electroblotted to nitrocellulose membrane and plasma samples of control male (lane 1′), vitellogenic female (lane 2′) and E_2-treated male (lane 3′) mrigal were analyzed by Western blot using a-Vg (1:1000) as primary antibody. The secondary antibody was goat anti-rabbit IgG-PAP conjugate (1:4000). Note that the Vg bands were fused together (arrow).

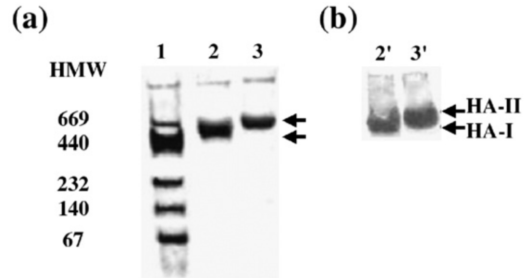

Fig. 4. (a) Native PAGE (4–15% gel) and (b) Western blot analysis of HA I (lane 2 and 2′) and HA II (lane 3 and 3′). After the run, one set of gels was stained for protein with Coomassie Brilliant Blue R250 and the other was blotted onto a nitrocellulose membrane and Western blot analysis performed as described in Fig. 2.

Ultrogel AcA 34. The E_2-treatment produced an increase in a single and symmetric protein peak (ULT I) containing ALP (index of Vg) eluted just after the void volume (Fig. 1a). A protein peak eluted at a similar position from control male plasma contained very little to no ALP (Fig. 1b). Electrophoresis of plasma samples from control male, vitellogenic female and E_2-treated male, along with corresponding Western blot analysis, is shown in Fig. 2 (a and b). In normal vitellogenic female plasma (lane 2) two protein bands (arrowheads) were present, but absent in normal male plasma (lane 1). These two protein bands were also present in E_2-treated mrigal plasma but remained closely spaced because of their closeness in molecular mass and high concentration of protein (Fig. 2a, lane 3). These E_2-induced proteins, also present in vitellogenic females, were strongly recognized by a-Vg in Western blot analysis (Fig. 2b, lanes 2′ and 3′) and also stained for lipid and carbohydrate (data not shown).

When fractions containing the ULT I proteins were subjected to adsorption chromatography on HA-

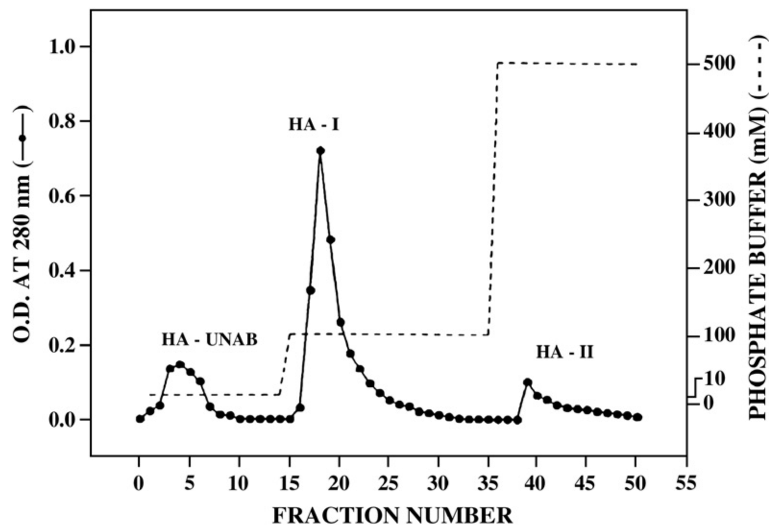

Fig. 3. Elution profile of 30 mg Vg protein (ULT I) on HA-Ultrogel column (12 × 1.5 cm) equilibrated with 0.01 M phosphate buffer, pH 8. Protein was eluted with 0.1 M (HA I) and 0.5 M (HA II) phosphate buffer, pH 8.

Ultrogel, two bound protein peaks, HA I and HA II, were eluted by 0.1 M and 0.5 M phosphate buffer (pH 8.0), respectively (Fig. 3). On native PAGE, both HA I and HA II resolved into a single protein band (Fig. 4a) and in Western blot analysis using a-Vg, each protein band was immunostained (Fig. 4b).

In double immunodiffusion (Fig. 5) using a-Vg, CYP (well 1) and plasma from vitellogenic female (well 7) each formed two distinct but very closely placed precipitin lines. E_2-treated male plasma (well 2) and semi-purified Vg, ULT I (well 3) cross-reacted with a-Vg giving diffused precipitin lines in each case. Plasma from control male (well 6) did not show any cross-reaction. HA I (well 4) and HA II (well 5) each formed single precipitin line.

The apparent molecular masses of HA I and HA II were 500 kDa and 550 kDa, respectively, as determined by gel filtration on Sepharose 4B (data not shown) and native PAGE (4–15%) analysis (Fig. 4a). These two purified Vgs differ considerably in lipid, carbohydrate and phosphorous content. The total lipid content of HA I was 260 μg/mg protein as against 103 μg/mg of HA II. The carbohydrate content of HA I and HA II were 27 and 117 μg/mg protein, respectively. Nonetheless, the ALP and total phosphorous content presented most striking variations as HA I contained 5 μg and 7 μg/mg protein whereas HA II contained 23 μg and 24 μg/mg protein, respectively.

Fig. 6 shows the SDS-PAGE analysis of HA I and HA II under reducing condition along with marker proteins. The electropherogram indicated that HA I contained one major band of 75 kDa and three faint bands of approximately 110, 25 and 14 kDa (lane 2) whereas HA II gave one major band of 85 kDa and three minor bands of approximately 140, 120 and 25 kDa (lane3). Both HA I and HA II showed some similarities in banding pattern, however, the 14 kDa peptide of HA I and the 140 kDa of HA II are unique to the respective Vg forms (Fig. 6).

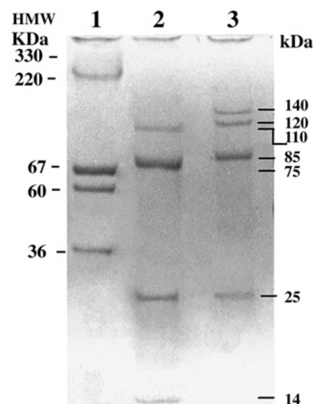

Fig. 6. SDS-PAGE (0.1% SDS) in 4–15% gel, of the purified Vg preparations, HA I (lane 2) and HA II (lane 3) along with molecular weight markers (lane 1) under reducing condition. Gels were stained for protein with Coomassie Brilliant Blue R250.

3.2. ELISA for mrigal Vg (HA I)

As HA I represents quantitatively the major form of mrigal Vg (see Fig. 3), a competitive ELISA for mrigal HA I was developed using the a-Vg antiserum to measure plasma Vg levels in adult female mrigal during

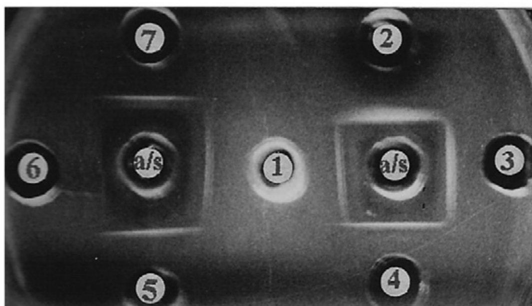

Fig. 5. Immunodiffussion test of mrigal crude yolk protein (well 1), plasma samples from E_2-treated male (well 2), Vg protein obtained after gel filtration, ULT I (well 3) and adsorption chromatography, HA I (well 4), HA II (well 5), control male plasma (well 6) and vitellogenic female plasma (well 7) with antisera (a/s) raised against the co-purified Vg containing both HA I and HA II. Note the formation of two closely placed precipitin lines against the wells 1, 2, 3 and 7 and one precipitin line each against 4 and 5.

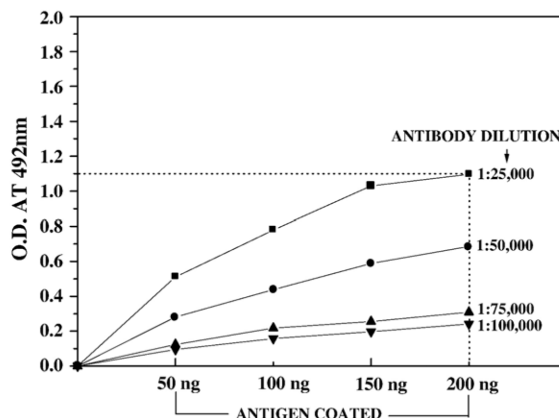

Fig. 7. Determination of optimal assay concentrations for Vg (HA I) and a-Vg by coating wells with serial dilutions of purified HA I, (50–200 ng) and incubating with different antibody dilutions (1:25,000 to 1:100,000). The antibody dilution of 1:25,000 and HA I coating at 200 ng were chosen as routine conditions.

different phases of its annual breeding cycle. The optimal assay concentrations for HA I and a-Vg were determined by testing four dilutions (1:25,000; 1:50,000; 1:75,000 and 1:100,000) of a-Vg with different concentrations (50–200 ng/well) of HA I coating (Fig. 7). Finally 200 ng of HA I coating and 1:25,000 dilution of antiserum were chosen for routine ELISA. Under such conditions the assay produced consistently the routine calibration curve (Fig. 8a) that ranges from 3.125 ng/ml to 320 ng/ml in diluted samples, with approximate linearity between 6.25 and 200 ng/ml (91–30% binding). The specificity of the assay was monitored by comparing the slopes of six different standard curves (data not shown). A good parallelism among the standard curves was observed by ANOVA ($F_{obs}=0.085<F_{0.05}=2.77$ with *d.f.* 5, 18) by comparing the results from different plates. The intra- and inter-assay coefficients of variance (CV) around 50% binding were 4.48% ($n=6$) and 7.57% ($n=9$), respectively. The ability of the antiserum to recognize native Vg was assessed by comparing binding displacement curves for standard preparation and increasing serial dilutions of plasma from vitellogenic female, E_2-treated male and control male mrigal (Fig. 8b). Linearization of the displacement curves by logit/log transformation (Fig. 8c and d) revealed that serial dilutions of plasma from vitellogenic female and E_2-treated male mrigal had slope, which were not statistically different from the standard preparation ($F_{obs}=1.268<F_{0.05}=3.369$ with *d.f.* 2, 26). This observed parallelism indicates the specificity of the assay. In contrast, plasma from control male mrigal showed no significant displacement at dilutions 1:50 and above. For subsequent assay, plasma samples were diluted to at least 1:100 to avoid possible non-specific effects of other plasma proteins, and to ensure that displacement values fell within the linear range of

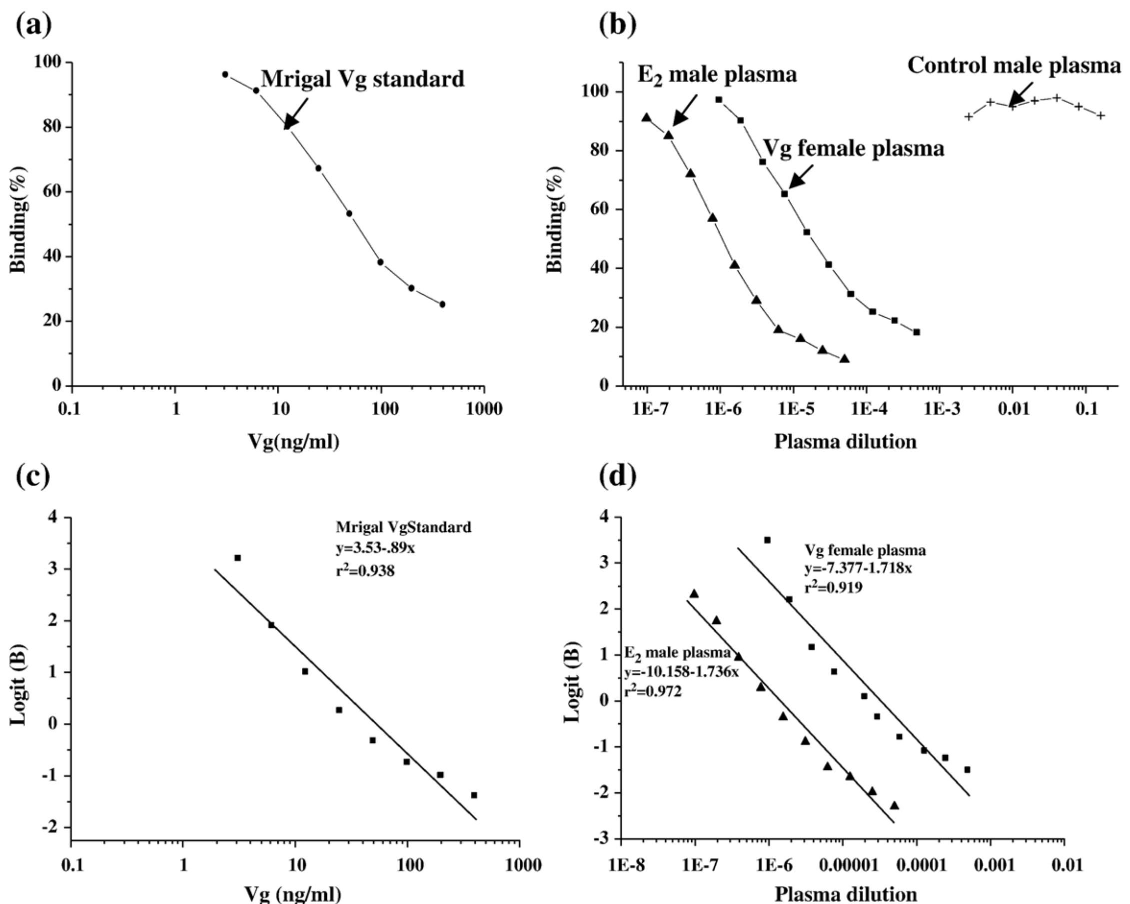

Fig. 8. Binding displacement curves obtained with (a) Vg standard (●) and (b) serial dilutions of plasma from E_2-treated male (▲), vitellogenic female (■) and control male mrigal (+). Panels (c) and (d) present the same data following linearization. All points are the mean of triplicate determinations.

the standard curve. Under these conditions the minimum effective detection limit of Vg in plasma was 2.5 µg/ml.

3.3. Annual changes in plasma Vg, GTH II and ovarian growth in female mrigal

Fig. 9 shows the plasma Vg and GTH II changes in relation to ovarian growth of the female mrigal during one complete breeding cycle, which was divided into four periods, preparatory (Feb–Apr), pre-spawning (May–Jun), spawning (Jul–Aug) and post-spawning (Sep–Jan). There was a gradual increase in plasma Vg level from early preparatory period in Feb (37±2.5 µg/ml) reaching the peak value (1500±1.89 µg/ml) in pre-spawning period (Jun). During spawning period (Jul) Vg levels decreased to 298.7±13.4 µg/ml ($P<0.001$, compared to the level in Jun) before dropping significantly ($P<0.001$) to basal or undetectable levels during Sep–Oct, the post-spawning period (see Fig. 9a). Increase in plasma Vg levels was well correlated with increased GSI due to the incorporation of Vg into oocytes forming S-II and S-III oocytes (Figs. 9b and 10), through pre-spawning period (May–Jun) reaching the peak in spawning period (Jul) during which all S-II oocytes were converted into S-III yolky oocytes (Fig. 10c). Thereafter GSI decreased sharply and the ovary underwent regression after spawning (Figs. 9b and 10d).

On the other hand, plasma GTH II level (Fig. 9a) showed a peak value during preparatory period in March (39.2±0.17 ng/ml), thereafter declined steadily till early post-spawning (Sep) but pulsatile changes were noticed during May and July. During the post-

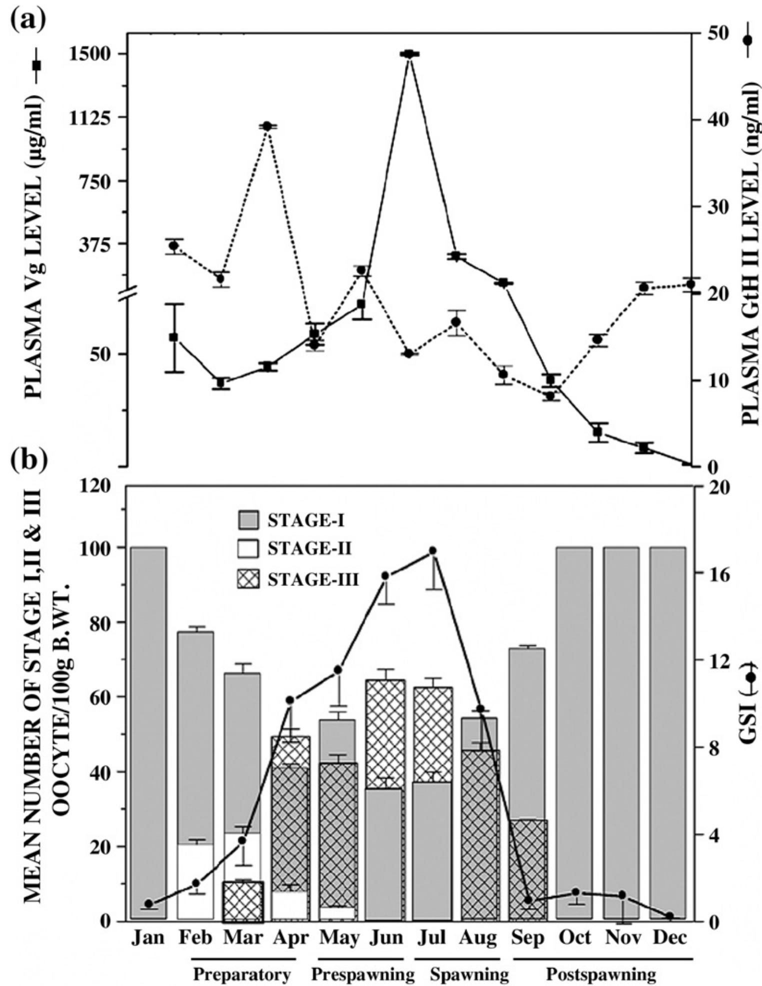

Fig. 9. Circannual changes in plasma Vg and GTH II levels (a) and ovarian weight (gonadosomatic index, GSI) with relative percentages of stage S-I, S-II and S-III oocytes (b) of female mrigal. Values are mean ± S.E. n=5 for each month.

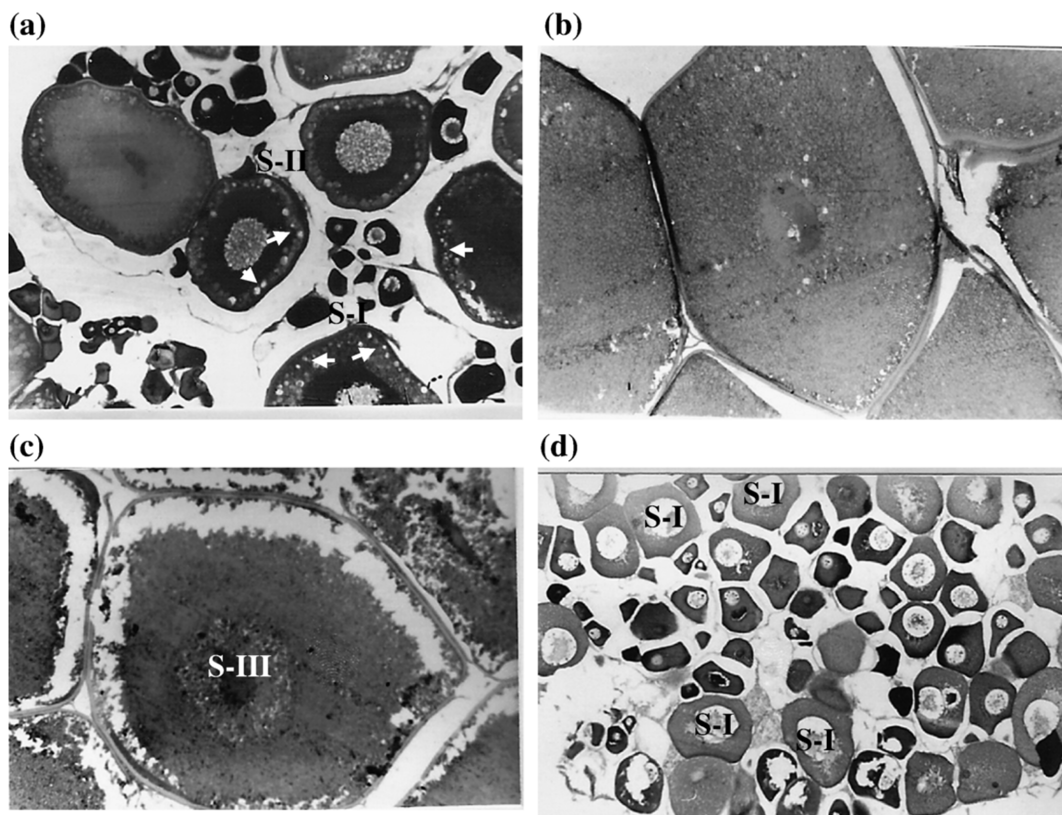

Fig. 10. Photomicrograph of transverse section of ovary during different phases of breeding cycle (original magnification × 4). (a) Preparatory period (February) showing the appearance of stage-II (S-II) oocytes. Note the presence of cortical alveoli (CA, arrow) at the periphery of the oocyte cytoplasm. (b) Fully form S-III yolky oocytes during pre-spawning period (May). (c) S-III oocyte during spawning period (August). (d) S-I non-yolky oocytes during post-spawning period (December).

spawning period (Dec) plasma GTH II level begins to increase (Fig. 9a).

4. Discussion

In the present study, mrigal Vgs were purified from E_2-induced plasma, by using a two-step procedure involving gel filtration on Ultrogel AcA 34 followed by adsorption chromatography on HA-Ultrogel. The first step was not sufficient to separate Vg because of slight contamination from other plasma proteins (data not shown). Therefore, adsorption chromatography was performed and two forms of Vg, HA I and HA II, were eluted. A similar procedure was used to purify two forms of Vg from the ascites fluid of E_2-treated medaka (Shimizu et al., 2002). A double chromatography procedure was adopted to purify Vg from many fish (Hara et al., 1980; Tyler and Sumpter, 1990; Burzawa-Gerard and Dumas-Vidal, 1991; Mananos et al., 1994a; Johnsen et al., 1999; Takemura and Kim, 2001; Hiramatsu et al., 2002b; Ohkubo et al., 2003).

The apparent molecular weights of two forms of mrigal Vg appeared to be identical in their native condition (HA I: 500 kDa and HA II: 550 kDa). Matsubara et al. (1999) also purified two Vgs similar to each other in barfin flounder (VgA: 530 kDa, VgB: 550 kDa in native condition and VgA: 168 kDa, VgB: 175 kDa on SDS-PAGE). Similarly, in white perch both FSPP1 and FSPP2 at native state resolved into 532 kDa, and on SDS-PAGE under reducing conditions at 180 kDa (Hiramatsu et al., 2002b). Two Vgs exhibiting similar molecular masses were also reported in mummichog (LaFleur et al., 1995a,b), haddock (Reith et al., 2001) and mosquito fish (Sawaguchi et al., 2005). However, two heteromolecular forms of Vg have been reported from tilapia (Ding et al., 1989; Kishida and Specker, 1993; Buerano et al., 1995; Takemura and Kim, 2001), Japanese common goby (Ohkubo et al., 2003) and medaka (Shimizu et al., 2002).

Addition of SDS to PAGE under reducing conditions caused dissociation of native HA I and HA II to four peptide bands in each case, from which 75 kDa band in

HA I and 85 kDa band in HA II were considered as Vg monomers based on their staining intensity (see Fig. 6). The resulting multiple bands may be either due to dissociation of multimeric Vg molecule into their parent subunits or due to proteolytic cleavage which is facilitated by SDS-induced unfolding of protein chains as reported earlier in many fish (Mananos et al., 1994a; Korsgaard and Pedersen, 1998; Johnsen et al., 1999 and references therein). The molecular weights of monomeric form of mrigal Vg (HA I: 75 kDa and HA II: 85 kDa) are in contrast to those (ranging from 130 to 180 kDa) reported for other teleost fish (Shimizu et al., 2002; Hiramatsu et al., 2002b; Sun et al., 2003; see also Specker and Sullivan, 1994). These finding and reports from other fish suggest that the fish Vgs are heterogeneous in nature (see Sawaguchi et al., 2005, 2006).

HA I was lipid rich but less phosphorylated than HA II. The occurrence of two such differentially phosphorylated Vg molecules in mrigal can be correlated with the findings of Shigeura and Haschemeyer (1984) who isolated nine different yolk proteins on the basis of phosphorous content from Antarctic fish, *Chenocephalus aceratus*. Further, the phosphorous contents of HA I and HA II, either in lipidated (total phosphorous) or delipidated (ALP) conditions, are almost similar, suggesting that most of the phosphorous present is protein-bound and not phospholipids.

Because of its low phosphorous content, HA I seems to be like tilapia VTG: 130 (Kishida and Specker, 1993) or medaka Vg2 (Shimizu et al., 2002) and may be lacking the phosvitin (Pv) domain. However, as reported by Hiramatsu et al. (2002b), two distinct medaka Vg cDNAs (MED Vg1 and MED Vg2) contained a Pv domain (residues 1060–1166 for MED Vg1, 1078–1209 for MED Vg2). Recently Wang et al. (2000) have deduced the primary structure of zebra fish Vg gene (*vg3*) which encodes a novel Vg without a Pv domain and the molecular mass of Pv-less Vg (PvlVg) does not belong to any distinct cluster of vertebrate Vgs (see Sawaguchi et al., 2005, 2006). Without knowing the structure of the HA I gene it is not possible to conclude whether it lacks a phosvitin domain.

Both HA I and HA II are high molecular weight proteins containing carbohydrate, lipid and phosphorous indicating their glycolipophosphoprotein nature. The presence of ALP (Nath and Sundararaj, 1981a) further indicates that HA I and HA II are two forms of plasma Vg. Further, in immunodiffusion tests the a-Vg did not cross-react with male plasma whereas it cross-reacted with the plasma of vitellogenic females and E_2-treated males, and also with CYP, giving two closely spaced precipitin lines indicating the occurrence of two Vgs in circulation which took part in the formation of yolk. In Western blot analysis, a-Vg cross-reacted strongly with high molecular weight proteins present in the plasma of vitellogenic female and E_2-induced male mrigal as well as with purified HA I and HA II. Based on these characteristics, which entail that HA I and HA II are E_2 inducible, high molecular weight, glycolipophosphoprotein yolk-precursor proteins, they are hence considered as Vgs.

The main aim behind development of an ELISA for mrigal Vg was to establish the relationship between plasma Vg levels and reproductive status of female mrigal during its annual breeding cycle. Since the a-Vg was raised against a co-purified mrigal Vg and the antiserum recognized equally both HA I and HA II, ELISA was developed by using HA I as standard to estimate plasma Vg. ELISA, either for one or two forms of Vg, has been developed to correlate plasma Vg levels with ovarian growth in other teleosts (Takemura and Kim, 2001; Ohkubo et al., 2003; see Specker and Sullivan, 1994 for earlier references).

The HA I ELISA's working range was 6.25 to 200 ng/ml. This sensitivity is comparable to the values reported for Vg ELISAs of other species including 1 ng/ml for *Dicentrarchus labrax* (Mananos et al., 1994b), 2.5 ng/ml for *Solea vulgaris* (Nuñez-Rodriguez et al., 1989), 10 ng/ml for *Pleuronectes vetulus* (Lomax et al., 1998), 160 ng/ml for *Morone sexatilis* (Kishida et al., 1992) and also comparable Vg levels estimated by RIA for coho salmon, *Onchorhynchus kisutch* (50 ng/ml; Benfey et al., 1989); for *Salmo salar* (2.5 ng/ml; So et al., 1985); for *Anguilla anguilla* (1.1 ng/ml; Burzawa-Gerard and Dumas-Vidal, 1991) and for *Cyprinus carpio* (2 ng/ml; Tyler and Sumpter, 1990).

The parallelism observed between the HA I standard curve and plasma dilution curves of vitellogenic females and E_2-treated males demonstrated that mrigal a-Vg recognized circulating Vg antigens in a similar fashion and did not show any significant cross-reaction with the plasma from control male mrigal at dilution 1:50 and above. However, the low level of Vg (0.55 μg/ml) detected in males, at dilutions lower than 1:50, may be non-specific as in rainbow trout Vg ELISA where anti-Vg cross-reacted with Vg-free pig serum to the same extent as normal male serum (Bon et al., 1997).

The Vg ELISA was used to evaluate the annual profile of plasma Vg in mrigal. A definite seasonal breeding cycle was demonstrated and the marked increase in ovarian weight, with concomitant formation of yolky oocytes, during the breeding season was shown to be due to the hormonal (GTH and E_2) regulation of synthesis and incorporation of Vg into the oocytes. On

this basis the annual ovarian cycle of mrigal can be divided into four periods: preparatory (Feb to Apr), pre-spawning (May to Jun), spawning (Jul to Aug) and post-spawning (Sep to Jan). During preparatory period, the significant increase in ovarian weight was due to the formation of vitellogenic (S-II) oocytes, coinciding with increased plasma Vg levels. As active vitellogenesis occurred during pre-spawning period there was a rapid conversion of S-II to S-III oocytes along with a high plasma Vg level (1.5 mg/ml). The peak ovarian growth observed during spawning period (Jul), with a significant decrease in plasma Vg level, indicated the termination of vitellogenesis. Interestingly, S-II oocytes were by and large absent in June and July. This may be due to rapid conversion of S-II to S-III oocytes which is a unique feature in this carp. No report is available in this respect in other fish studied so far. Thereafter the GSI reduced sharply due to spawning and the ovary remained regressed containing only oogonia and S-I oocytes during the post-spawning period. This is in accordance with the findings made in many other fish species (Van Bohemen et al., 1981; Mukherjee et al., 1989; see also Sundararaj and Vasal, 1976).

The seasonal profile of plasma Vg in female mrigal was similar to those reported in the literature for other teleost species. The peak value (1.5 mg/ml) was obtained 1 month earlier than the spawning period and was in accordance with the findings of others (Mananos et al., 1994a; Matsubara et al., 1994; Lomax et al., 1998). However, the maximum value obtained in plasma of different fish varies considerably and is species specific. This may well be due to the consequence of different reproductive strategies, duration of vitellogenesis and maximum size attained by the gravid oocytes (see Lomax et al., 1998 and references therein).

In the present study, a correlation between the ovarian growth (GSI) and plasma GTH II levels was established during one complete annual ovarian cycle. With respect to plasma GTH II level, the peak value was attained in March and thereafter steadily declined till September. Nonetheless significant increase in plasma GTH II level was noticed in May (pre-spawning) and July (spawning) compared to values of respective previous months. These observations suggest that GTH II from pituitary may be secreted in a pulsatile manner which in turn regulates reproduction as reported earlier in trout (Zohar et al., 1986) and carp (Bieniarz et al., 1992). However, at this moment, it is not possible to rule out the role of GTH I, since no work has been done due to non-availability of GTH I. In this context, it is to be mentioned that there is no unanimous opinion regarding the role of GTH I and GTH II in female reproduction because the differential role of GTHs has been worked out mostly in salmonids and a few non-salmonids, common carp, red sea bream and stripped bass. Findings from salmonid fish suggest that GTH I is associated with vitellogenesis whereas GTH II regulates final oocyte maturation, although both GTHs are equipotent in E_2 production (Swanson et al., 1991; Prat et al., 1996). On the contrary, in red sea bream FSH (GTH I) may have no significant role in female but LH (GTH II) may regulate complete reproduction (ovarian growth and maturation) (Gen et al., 2000, 2003). Even in African catfish (*Clarias gariepinus*) GTH II is supposedly the key regulator of reproduction in females (Koide et al., 1992). Therefore it is assumed that in the present study GTH II may regulate female reproduction in mrigal.

The rise in plasma GTH II levels in March coincided with a progressive increase in plasma Vg levels with a slight increase in GSI, and the ovary showed the presence of yolk vesicles in the cytoplasm of growing oocytes (the onset of vitellogenesis). This is in accordance with the findings of Gen et al. (2003) but in contrast to those of Prat et al. (1996). During this period GTH II may be required to stimulate ovarian E_2 production, which in turn acts on the liver to synthesize Vg. It is already established by both *in vivo* and *in vitro* studies that GTH II and also GTH I can induce ovarian E_2 production (Suzuki et al., 1988b; Kagawa et al., 2003; Swanson et al., 1991). Further administration of SG-G100 and carp GTH (equivalent to GTH II) has been shown to induce synthesis and incorporation of Vg in the catfish, *Heteropneustes fossilis* (Nath and Sundararaj, 1981b; Sundararaj et al., 1982).

GTH II concentration in the plasma of females decreased sharply after the start of vitellogenesis in April and then increased significantly in May (pre-spawning period) when there was active vitellogenesis. Therefore, GTH II may be responsible for the incorporation of Vg into oocytes. This is in accordance with the earlier observations made in many teleosts (see Burzawa-Gerard, 1982; Ng and Idler, 1983) and also in red sea bream (Gen et al., 2000, 2003). In contrast, Tyler et al. (1991) demonstrated that in rainbow trout GTH I but not GTH II facilitates Vg uptake. The third rise in plasma GTH II was in July, the spawning period during which maturation, ovulation and spawning takes place. Since the plasma GTH II level was not significantly more than the levels in June, this may indicate the initiation of maturation. It is already known that GTH II induces maturation, ovulation and spawning in many teleosts (Swanson et al., 1991; Van Der Kraak et al., 1992). The rise in plasma GTH II levels at the end of post-spawning period (Nov–Dec) may have a function

in initiating the next cycle of ovarian growth. A post-ovulatory rise in GTH levels has been described in many fish (Kobayashi et al., 1986).

These observations in female mrigal suggest that GTH II may be capable of stimulating almost all aspects of female reproduction including steroidogenesis, vitellogenesis, oocyte maturation and spawning, as in red sea bream. However, it is necessary to find out the role of GTH I in mrigal reproduction.

In conclusion, (i) HA I and HA II isolated in this study can be considered as two forms of mrigal Vg on the basis of the characteristics described above and those reported for Vg in the literature. Further, the procedure employed to purify two forms of mrigal Vg provided pure forms of HA I and HA II as judged by electrophoresis and immunological studies, and therefore, could be used to purify Vg from other fish species. (ii) A sensitive and reproducible ELISA for mrigal Vg (HA I) was developed for the measurement of total Vg in mrigal and it is adequate to measure Vg from ng/ml to mg/ml level. The assay was further validated by quantification of Vg during one complete annual breeding cycle of female mrigal. (iii) Based on ovarian growth changes, plasma Vg and GTH II levels, the annual breeding cycle of mrigal could be divided into preparatory, pre-spawning, spawning and post-spawning periods which made it clear that female mrigal could be obtained during preparatory and post-spawning periods for reproductive manipulation.

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