Determination of plasma vitellogenin levels and localization of vitellogenin in liver of Lake Van pearl mullet (*Chalcalburnus tarichi* Pallas, 1811)

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Abstract

This study focused on hepatosomatic and gonadosomatic index and levels of plasma 17β-estradiol (E_2), vitellogenin (Vtg) and total protein and determined the localization of Vtg in the liver during vitellogenesis in female C*halcalburnus tarichi*, an endemic cyprinid species living in the Lake Van, Turkey. The levels of plasma E_2, Vtg and total protein in female fish increased during vitellogenesis and they are positively correlated with each other. According to the results from the female fish, the lowest levels of E_2 and Vtg were measured in December at 120.60±23.38 pg ml^{-1} and 1.10±0.26 mg ml^{-1}, respectively. The highest levels of plasma E_2 and Vtg were in May (833.40±211.58 pg ml^{-1}) and April (31.31±4.9 mg ml^{-1}), respectively. The Vtg-positive hepatocytes were not uniform in liver of fishes. Generally, the hepatocytes surrounding the portal and central veins were strongly Vtg positive. These results showed that plasma Vtg levels in female fish increased during vitellogenesis related to plasma E_2 and gonadosomatic index.

Keywords: Vitellogenesis, 17β-estradiol, Gonadosomatic index, C*halcalburnus tarichi*, Lake Van

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Introduction

Vitellogenesis is an important stage of oocyte development in all oviparous animals and it is regulated by estrogens. In this stage, egg proteins are synthesized and pooled in oocytes. Vtg is a precursor protein, which is synthesized in liver in response to 17-β E_2 stimulation. This protein is transported to ovary through the circulatory system and then transformed into the egg proteins, lipovitellin and phosvitin (Arukwe and Goksøyr, 2003; Lubzens *et al*., 2010; Jena *et al*., 2013). Hence, Vtg is an important source of nutrition of the embryo and larvae.

C. *tarichi* is an endemic cyprinid species living in the Lake Van located in Turkey. It becomes sexually mature at three years old and it migrates into fresh water to spawn once a year (Ünal *et al*., 1999). After ovulation, young oocytes, which will be ovulated in the next year, start to grow up cortical-alveolar stage and they continue their development until October. Vitellogenic stage continues from October to April/May. Plasma E_2 increases and reaches maximum levels during vitellogenesis in *C. tarichi* (Ünal *et al*., 2005). Vtg is not synthesized in immature and male fish but it is induced by E_2 (Jena *et al*., 2013). The Vtg level increased in E_2-induced immature female and male fish and the Vtg immunoreactive cells were observed in liver, ovary and testis tissues (Pacoli *et al*., 1991; Bieberstain *et al*., 1999; Okumura *et al*., 2001; Mills *et al*., 2003; Van der Ven *et al*., 2003; Kobayashi *et al*., 2005).

The aims of the present study were to determine the levels of plasma E_2 and Vtg during vitellogenesis and distribution of the Vtg synthesizing cells in the liver in female *C. tarichi* sampled from Lake Van.

Materials and methods

Fish

Mature females of *C. tarichi* were sampled from Lake during vitellogenesis (October to May). The water temperature, dissolved oxygen and pH values were measured in fish sampling sites with a multiparameter device (Orion 5 Star; Thermo, Barrington, IL). The fish were transferred to laboratory in aerated containers. They were weighed totally and the blood samples were taken from the caudal vein using heparinized syringes containing aprotinin (25 µl) (Sigma St Louis, MO, USA). Blood samples were immediately centrifuged at 1500 *g* for 10 minutes and the plasma samples were stored at –76°C until analysis. After the blood was withdrawn, fish were sacrificed and dissected. The gonadosomatic index (GSI) and hepatosomatic index (HSI) were calculated as the ovary or liver weight/body weight ×100. All experimental procedures were carried out according to national animal care regulation.

Histology and Immunohistochemistry

The ovary and liver tissues were fixed in Bouin's solution for routine histological processing. Liver fragments were also fixed in formalin (4%) and 4% paraformaldehyde solutions for immunohistochemical studies. Sections taken (5 µm) from ovary and liver tissues were stained with hematoxylin-eosin. Liver tissues fixed with paraformaldehyde were washed in phosphate buffered saline (PBS) and maintained in 30% sucrose

solution until saturation. The frozen liver sections (4 µm) were taken using a Leica CM 1900 cryostat (Leica Microsystems, Numsloch, Germany) and stained with streptavidin-biotin complex method and immunofluorescent method for detection of Vtg (Bancroft and Gamble, 2002). Also, some paraffin sections taken from liver were stained using the same method. Negative control sections were incubated under the same conditions without primary antibody.

For immunohistochemical detection of Vtg, liver sections were incubated overnight with the primary antibody, carp Vtg polyclonal antibody (1:150, CT-1 Biosense Laboratories, Norway), at 4 °C. After washing in Tris buffered saline (TBS, 0.025 M Tris, 0.15 M NaCl, pH 7.5), the sections were treated with biotinylated anti rabbit IgG (Sigma St Louis, MO, USA) for 5 hours at 4°C, washed in TBS again and incubated with peroxidase conjugated streptavidin for 2 hours at room temperature. The sections were then washed in TBS and the peroxidase activity was detected with 3,3′ diaminobenzidine tetrahydrochloride (Sigma St Louis, MO, USA). The samples were washed in distilled water and stained with Mayer's hematoxylin (Sigma St Louis, MO, USA) for counter staining. The sections were examined under a microscope (Nikon, Eclipse E600, Tokyo, Japan) and photographs were taken.

For immunofluorescence detection of Vtg, the liver sections were washed in TBS after incubation with the primary antibody (1:150, CT-1 Biosense Laboratories, Norway) and incubated with anti rabbit IgG labeled with Alexa 488 (1:1000, Invitrogen, Carlsbad, CA) as a secondary antibody at room temperature for 5 hours. The sections were washed in TBS and mounted. The preparations were examined using a confocal laser scanning microscope (Zeiss LSM-510 Meta, Germany) and photographs were taken.

Plasma total protein, Vtg and E_2 analyses

Plasma total protein levels were determined with Bradford method (Bradford, 1976) using bovine serum albumin (µg ml^{-1}) as a standard protein.

Plasma Vtg levels of female fish were measured using a carp-vitellogenin ELISA Kit according to manufacturer's instruction (Biosense Laboratories, Norway; working range 0.24-250 ng Vtg ml^{-1}). Plasma samples were diluted 1:100. All samples were analyzed in duplicate. Absorbance was recorded at 492 nm using a microplate reader (DAS Instruments, Rome, Italy).

The levels of plasma E_2 were measured using a solid-phase competitive chemiluminescent enzyme immunoassay by Immulite 1000 Systems (Siemens, Los Angeles, CA, USA). The minimum and maximum detection limits were 20 pg ml^{-1} and 2000 pg ml^{-1}, respectively.

Data analysis

Data are expressed as the mean±SEM. Differences between means were analyzed using one way analysis of variance (ANOVA) followed by a Duncan's test comparison of the differences between groups. Statistical significance was inferred at $p<0.05$.

Results

Plasma levels of total protein, Vtg and E_2

We sampled *C. tarichi* in October to May (Fig. 1). The water temperature, pH and dissolved oxygen values of the sampling sites are given in Table 1. The temperature, pH and dissolved oxygen levels were measured as +1 to +16 °C, 9.40–9.71 and 8.5–12.1 mg l^{-1}, respectively.

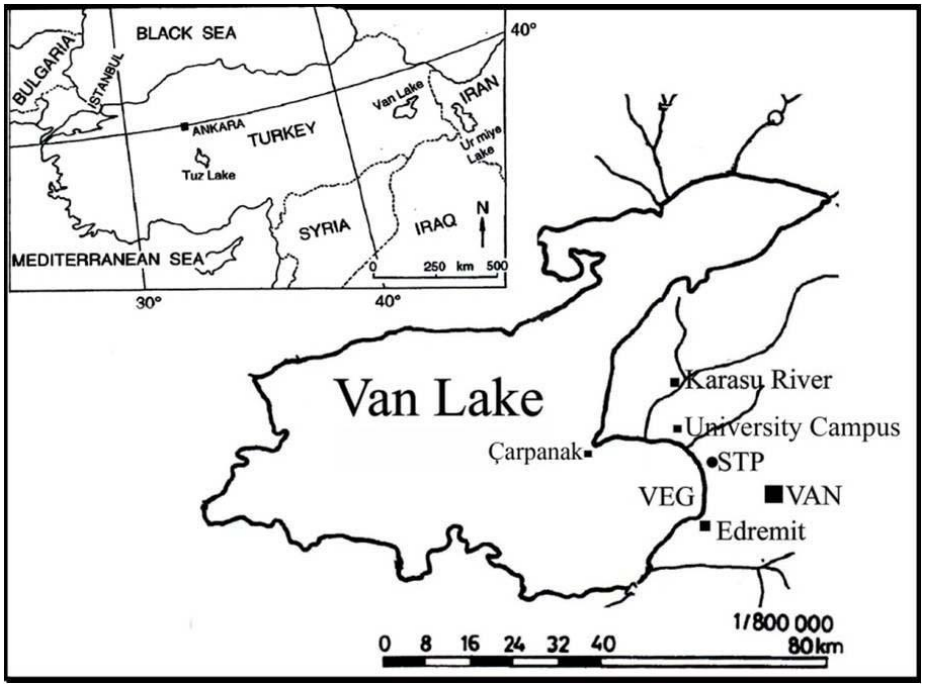

Figure 1: Sampling sites of *Chalcalburnus tarichi* in the Lake Van (VEG: Van-Edremit Gulf; STP: Sewage treatment plant).

Table 1: Temperature (T), pH and dissolved oxygen (DO) of water in the *Chalcalburnus tarichi* sampling sites. Data are expressed as the mean ± SEM.

Months	T (°C)	pH	DO (mg l^{-1})
October	+15±0.5	9.52±0.2	11±1.2
November	+12±0.2	9.40±0.1	11±0.9
December	+5±0.2	9.50±0.3	12.1±0.6
January	+5.7±0.3	9.71±0.1	10±1.2
February	+5±0.4	9.53±0.3	10.5±0.3
March	+1±0.2	9.54±0.2	12.1±1.4
April	+11.8±0.5	9.53±0.2	10.2±0.4
May	+16±0.5	9.56±0.2	8.5±1.6

Total, liver and ovary weights of vitellogenic female fish were measured for HSI and GSI in vitellogenic females (Table 2). Although there were some individual differences, HSI and GSI increased during vitellogenesis. The lowest and highest HSI were measured in December (1.787±0.103%) and April (3.228±0.161%), respectively. The GSI was 5.616±0.270%, in October and it increased during vitellogenesis and peaked in May (12.850 ± 0.19 %).

The levels of plasma total protein, E_2 and Vtg were measured for vitellogenic females (Fig. 2). The lowest level of plasma total protein was measured in

October (15.598±0.249 mg ml^{-1}). It significantly increased and peaked up in April (29.489±0.795 mg ml^{-1}) (p<0.05).

Table 2: Hepatosomatic (HSI) and gonadosomatic index (GSI) of vitellogenic *Chalcalburnus tarichi* from October to April. n: number of samples. Data are expressed as the mean±SEM. Different letters indicate statistical differences at p<0.05.

Months	n	Mean of body weight (g)	Mean of liver weight (g)	Mean of ovary Weight (g)	HSI (%)	GSI (%)
October	16	100.10±2.41^{b}	2.17±0.16^{b}	5.65±0.37^{a}	2.17±0.14^{ab}	5.62±0.27^{a}
November	17	101.72±4.30^{b}	2.27±0.16^{b}	6.11±0.32^{a}	2.22±0.11^{ab}	6.06±2.27^{a}
December	13	94.65±3.68^{a}	1.70±0.13^{a}	5.62±0.26^{a}	1.79±0.10^{a}	6.01±0.30^{a}
January	9	106.49±5.68^{b}	2.16±0.13^{b}	9.26±0.41^{b}	2.05±0.12^{ab}	8.82±0.47^{b}
February	12	97.38±9.19a^{b}	2.33±0.25^{b}	8.51±0.69^{b}	2.43±0.19^{bc}	9.24±0.98^{b}
March	9	100.10±6.13^{b}	2.73±0.26^{b}	8.05±0.67^{b}	2.729±0.19^{cd}	8.481±0.86^{b}
April	15	125.06±5.35^{c}	4.04±0.24^{c}	11.27±0.83^{c}	3.23±0.161^{d}	8.88±0.39^{b}
May	9	107.81±7.66^{b}	3.09±0.30^{c}	13.87±1.35^{c}	2.87±0.20^{cd}	12.85±0.19^{c}

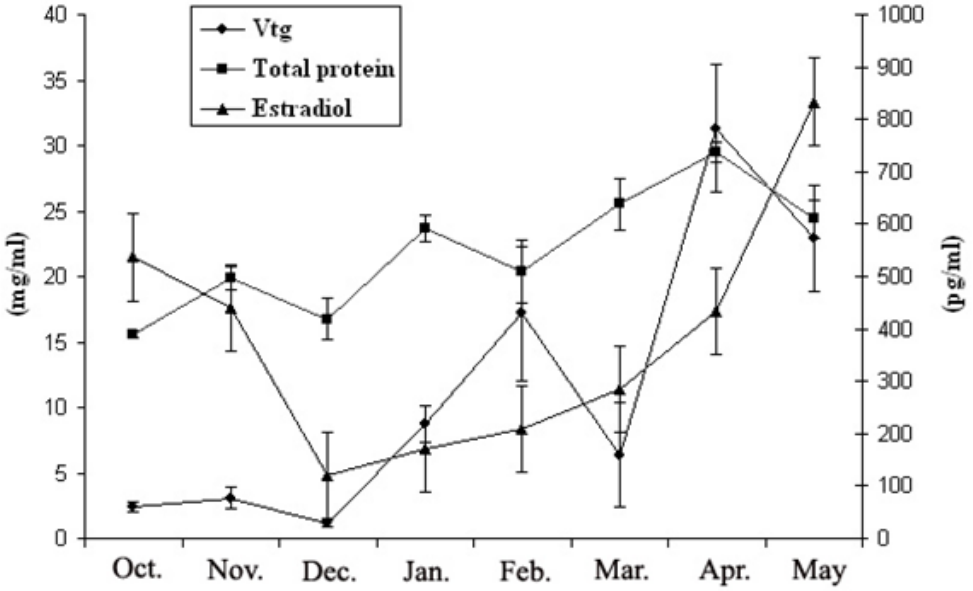

Figure 2: The levels of plasma total protein, 17β- E_2 and vitellogenin Vtg of vitellogenic (October to April) and mature (May) *Chalcalburnus tarichi* from the Lake Van. All values are the mean± SEM.

Plasma E_2 level was measured as 536.4±127.95 pg ml^{-1} in October and it significantly decreased in November and December (p<0.05). However, its level increased and reached the maximum level in May (833.4±211.58 pg ml^{-1}).

The plasma Vtg level was measured as 2.406±0.436 mg ml^{-1} in October. No change was indicated in November and December. It significantly increased in January and February to 8.767±1.376 and 17.171±5.122 mg ml^{-1}, respectively (p<0.05). Its level decreased in March (6.366±3.952 mg ml^{-1}). The highest plasma Vtg (31.308±4.913 mg ml^{-1}) was measured in April and it decreased again in May (22.899±4.040 mg ml^{-1}) (p<0.05).

Distribution of vitellogenin in liver

The localization and Vtg intensity were not uniform within the hepatocyte population (Figs. 3a, b). Some hepatocytes were more

strongly stained in the liver than the others and these cells were more abundant in some regions (Fig. 3a). Especially, the hepatocytes surrounding the hepatic portal branches and central veins were strongly immune-reactive for Vtg (Figs. 3a, b, c). The non-stained or weakly stained hepatocytes were interspersed among the strongly stained cells (Fig. 3b). Vtg staining was observed only in cytoplasm of hepatocytes and no immune reaction was observed in bile duct, endothelium, blood cells, lumens of hepatic portal and central veins and sinusoids.

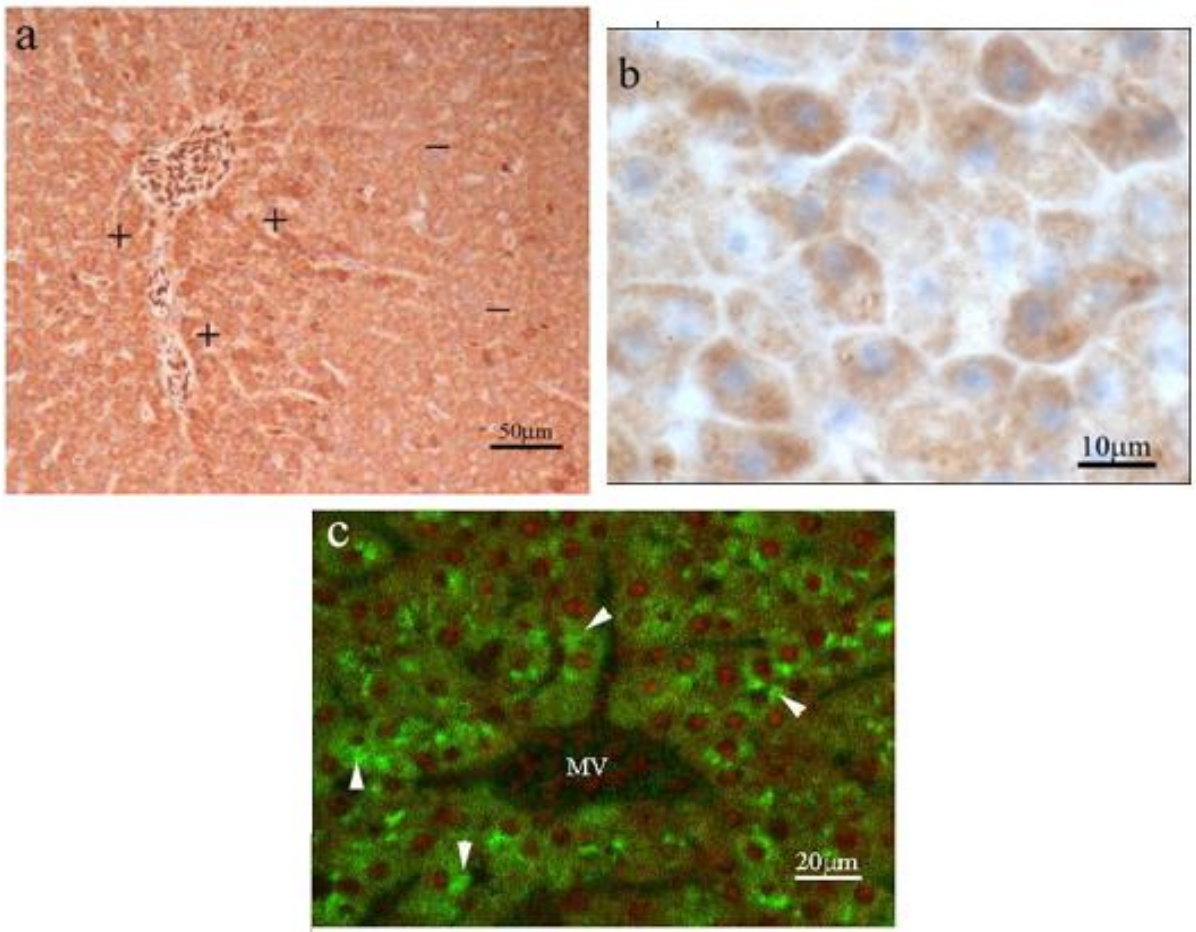

Figure 3: Immunohistochemical localization of the vitelogenin (Vtg)-positive and -negative hepatocytes in the liver sections of female *Chalcalburnus tarichi.* a) Vtg-positive, (+) and Vtg-negative, (–) regions in the hepatocyte populations surrounding the central vein. b) Magnified views of Vtg-immuno positive and negative hepatocytes c) Immunofluorescent detection of Vtg-positive hepatocytes (arrowhead) surrounding the central vein (MV).

Discussion

In the present study, we measured the GSI and HSI and the levels of total protein in plasma, E2 and Vtg and the localization of Vtg immunoreactive hepatocytes in *C.tarichi* from Lake Van. In the sampling sites, temperature, pH and dissolved oxygen were measured. The water temperature changed from +1 (in March) to +16°C (in May) depending on the season. Water pH values were indicated between 9.40–9.71. The water of Lake

Van is alkaline and its pH is 9.5–9.8 (Çetinkaya and Duyar, 1996). We found that the dissolved oxygen varied between 8.5–12.1 mg/l.

The GSI and HSI and plasma total protein, E_2 and Vtg levels increased during vitellogenesis in *C. tarichi* in October-May and their levels were also correlated with each other. We previously reported that changes of GSI were related with oocyte development (Ünal *et al*., 1999) and the level of plasma E_2 increased during vitellogenesis (Ünal *et al*., 2007). In English sole (*Parophrys vetulus*) (Johnson *et al*., 1991; Lomax *et al*., 1998), some cyprinids (Rinchard *et al*., 1997), *Oncorhynchus masou* (Fujita *et al*., 2005) and *Clupea pallasii* (Koya *et al*., 2003) the levels of GSI, HSI, plasma E_2 and Vtg increase during vitellogenesis. In *C. tarichi*, the lowest levels of HSI, plasma E_2 and Vtg were measured in December, while no change in GSI was detected. The water temperature also decreased to 5°C (from 12°C) in December. Fish migrate to inside and deeper parts of the lake according to water temperature. The lowest water temperature (+1°C) was measured in March and Vtg levels also significantly decreased to 6.37±3.95 mg ml^{-1} (from 17.17±5.12 mg ml^{-1}) in this month while the E_2 level increased. We can say that the decrease of HSI, plasma E_2 and Vtg levels may relate with feeding of fish.

Histological observations showed that the distribution of Vtg immunopositive cells was not homogenous and the immunopositive cells surrounding the central vein were stained strongly. These results are consistent with other fish species (Pacoli *et al*., 1991; Arukwe *et al*., 1999; Bieberstain *et al*., 1999; Van der Ven *et al*., 2003; Kobayashi *et al*., 2005). Okumura *et al*., (2001) also demonstrated that Vtg immunostaining increased during the vitellogenic stage in the liver of female Japanese fish.

In summary, this study showed that the GSI, HSI and the plasma's total protein, and E_2 and Vtg levels increased during vitellogenesis and the distribution of Vtg synthesizing cells are not uniform in *C. tarichi*.

Acknowledgements

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