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Araştırma Makalesi/Research Article

**The Effects of 17 β –Estradiol on Vitellogenin, Total Protein,
Histochemical, and Some Morphological Indices on
*Chalcalburnus tarichi***

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Abstract: As in other oviparous vertebrates, 17 β -estradiol (E2) is a sex hormone present in fishes. E2 has a complex effects on the liver, female reproduction, sexual development and other organs. On the other hand, estrogenic compounds are also present in the aquatic environment by domestic waste and can adversely affect on aquatic organisms. The present study is aimed to investigate the effects of the injected E2 in a short period on the total weight, liver weight, gonad weight, hepatosomatic index (HSI), gonadosomatic index (GSI), plasma total protein, and vitellogenin (Vtg) levels on *Chalcalburnus tarichi*. It was found that the injected E2 caused an increase in all the measured values. However, only the liver weight, HSI, plasma total protein, and Vtg levels were found to be statistically significant ($P<0.05$). In addition, it was found that the liver glycogen content did not change the histological after E2 injection. According to SDS-PAGE and Western Blot analyses, the molecular weight of *C. tarichi* Vtg was found to be 145 kDa.

Key words: *Chalcalburnus tarichi*, Vitellogenin, 17 β -estradiol, Glycogen, Lake Van

**17 β –Estradiolün İnci Kefalinde Vitellogenin, Total
Protein, Histokimyasal ve Bazı Morfolojik İndeksler
Üzerine Etkisinin İncelenmesi**

Özet: 17 β -Estradiol (E2) diğer ovipar omurgalılarda olduğu gibi balıklarda da bir cinsiyet hormonudur. E2 karaciğerde, dişi üreme sisteminde, cinsel gelişiminde ve diğer organlar üzerinde kompleks etkilere sahiptir. Fakat östrojenik bileşikler evsel atıklar ile sucul ortamlarda bulunabilmekte ve sucul canlılar üzerine ters etkiler oluşturmaktadır. Bu çalışmada E2'nin inci kefalı (*Chalcalburnus tarichi*)'nde total ağırlık, karaciğer ağırlığı, gonad ağırlığı,

hepatosomatik indeks (HSI), gonadosomatik indeks (GSI), plazma total protein ve vitellogenin (Vtg) üzerine etkileri araştırıldı. E2 enjeksiyonu, ölçülen bütün değerlerde artışa neden olmasına rağmen bu artış sadece karaciğer ağırlığı, HSI, plazma total protein ve Vtg seviyelerinde istatistiksel olarak önemli bulundu ($P<0.05$). E2 enjeksiyonu karaciğer glikojeni üzerine histolojik olarak herhangi değişime neden olmadı. Ayrıca SDS-PAGE ve Western Blot analizleri sonucunda inci kefal Vtg proteinin moleküler ağırlığının 145 kDa olduğu belirlendi.

Anahtar kelimeler: *Chalcalburnus tarichi*, Vitellogenin, 17 β -estradiol, Glikojen, Van Gölü

INTRODUCTION

Like in other oviparous vertebrates, oocyte growth occurs by the uptake of the plasma egg yolk precursor protein (Vitellogenin) during the vitellogenin phase of oogenesis in fishes. This process is mainly coordinated under the control of 17 β -estradiol. The egg yolk is a significant source of nutrition for developing embryos and larvae (Wallace and Selman 1981; Arukwe and Goksoyr 2003). Vtg is not synthesized in the male fish. However, it can be induced in males by the exposure of exogenous estrogens (De Vlaming et al. 1980). Therefore, vitellogenin is an indicator of the presence of estrogen like chemicals in the aquatic environment (Sumpter and Jobling 1995).

Various synthetic chemicals are known to disrupt the endocrine systems of living organisms and are referred to as endocrine disrupting chemicals. Some of these chemicals are oestrogen mimicking. Examples of such chemicals include pesticides, polychlorinated biphenyls, polycyclic aromatic hydrocarbons,

surfactants, and plasticizers (Sumpter and Jobling 1995). Natural (17 β -estradiol, estriol and estrone) and synthetic oestrogens (17 α -Ethyhnl estradiol) through domestic wastes could also contaminate the aquatic environment (Pojana et al., 2007). The important sources of oestrogens are domestic effluents and livestock waste. Moreover, the estrogenic activities of natural 17 β -estradiol are considerably higher than that of endocrine disrupting chemicals in *in vivo* and *in vitro* assays (Folmar et al. 2002; Van den Belt et al. 2004).

Chalcalburnus tarichi is an endemic cyprinid species indigenous to Lake Van in the Eastern Anatolia region of Turkey. In the lake, a parallel increase of pollution has been observed with an increase in the population. Abnormalities in the gonads of both male and female fishes are a dominant indicator of the pollution (Ünal et al. 2007). Ünal et al. (2007) explained that these abnormalities could originate from endocrine disrupting chemicals.

E2 has been accepted as one of the endocrine disrupting chemicals. Therefore,

the aim of this study was to determined the molecular weight of Vtg protein and assess the potential impact of E2 on vitellogenin, total protein, and the histochemical and morphological indexes on *C. tarichi*.

MATERIALS and METHODS

Experimental animal

Chalcalburnus tarichi (11.5-13.5 cm and 15.88-38.90 g), used in the present study, were caught from Lake Van. The adult fishes were kept in the municipal tap water until the process of E2 injection. Through the course of the experiment, the fishes were fed twice with commercial rainbow trout food. The fishes were maintained at an ambient temperature and in a normal photoperiod. All experimental procedures were carried on according to national animal care regulation.

C. tarichi were injected with 1 mg/kg E2 intraperitoneally. Bloods were drawn from the caudal vessel using a heparinized syringe after five days of E2 injection. Bloods were transferred to 1.5 mL eppendorf tubes that contained 25µl heparin and 25µL of the protease inhibitor aprotinin. Subsequently, the samples were centrifuged at 1.500xg for 15 minutes. The plasma was aspirated with a micropipette. Plasma were stored at -80 °C until further analysis. The fishes were sacrificed and dissected after the blood collection. The liver and the ovary were removed and weighed (g). The Gonadosomatic index (GSI) and

hepatosomatic index (HSI) values were calculated as follows:

$$\text{GSI} = (\text{total gonad weight} / \text{total body weight}) \times 100.$$

$$\text{HSI} = (\text{total liver weight} / \text{total body weight}) \times 100.$$

Histology

Ovary and hepatocyte tissue samples were fixed bouins fixative for least 24 h then transferred to 70 % ethanol. Fixed tissues were dehydrated in graded ethanol series, cleared in xylene and embedded in paraffin. Sections taken (5 µm) from ovary and liver tissues were stained with Hematoxylin-Eosin and Periodic Acid-Schiff (PAS). Sections were examined by light microscopy and then photographs were taken.

Electrophoresis and Western Blots

Plasma samples were analyzed using a 7.5% separating gel, and a 4.5 % stacking gel sodium dodecyl sulfate-polyacrilamide gel in the electrophoresis technique (SDS-PAGE) (Laemmli 1970). Plasma samples were then diluted with the sample buffer (0.25 M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 10 2%-Mercaptoetanol and 0.2% bromphenol blue). Electrophoresis was carried out at 25mA for 4 hours. The markers (212 kDa-55.5 kDa) of known molecular weight were used in the gel to determine the molecular weight of Vtg. Two different gels containing the same plasma sample were prepared in the course of the present study.

After electrophoresis, the first gel was stained with Coomassie Brilliant Blue for 10 minutes. The second gel was transferred to the Polyvinylidene Difluoride (PVDF) membrane for Western Blot analysis.

Following the SDS-PAGE process, the Western Blot analysis of PVDF membrane (Bio-Rad) was performed using a transfer buffer (Transfer buffer: 25 mM Tris, 192 mM glycine and 200 mL methanol for 1L). The transfer of proteins was realized at 3 hours for 300 mA. To prevent a non specific combination of the membrane, 0.05% Tween 20 (TBS-T) and 25mM Tris including 5% milk powder was maintained at room temperature. The primer antibody (rabbit anti carp vitellogenin polyclonal antibody, Biosence, Norway) was diluted with the PVDF membrane TBS-T at the ratio of 1:1.000 and incubated for an hour at room temperature. After the incubation, the membrane was washed 3 times with TBS-T and incubated with alkaline phosphatase conjugated with goat anti-Rabbit IgG antibody (1:10.000 Sigma). The membrane was further incubated in the solution of 5-bromo-4chloro-3-indolyl phosphate and nitrobluetetrazolium including 100 mM NaCl and 5 mM MgCl, (pH 9.5) in 0.1 M Tris-HCl

buffer and the color formation was observed. The color reactions stopped after the membrane was taken into the water.

Total protein content

The total plasma protein concentration was determined by the method of Bradford (Bradford 1976) using bovine serum albumin as the protein standard.

Enzyme-linked immunosorbent assay (ELISA)

The concentration of Vitellogenin in the plasma was determined using the Carp VTG enzyme-linked immunosorbent assay (ELISA) kit supplied by Biosense Laboratories (Bergen, Norway). The plasma was diluted at the ratio of 1:500.000. All assay procedures were followed in accordance with the manufacturer. In addition, all plasma samples were analyzed in duplicate.

Statistical analysis

The unpaired student test method was used for comparing the data among the different binary groups. The data was expressed as a mean \pm standard error on the mean (SEM). The statistical significance was inferred at $P<0.05$.

RESULTS

The effect of E2 on Vitellogenin, total protein, and some morphological indices

The total height, total weight, liver weight, gonad weight, HSI, GSI, plasma total protein, and the Vtg levels of injected with E2 and the control group fishes are given in

Table 1. It was found that, there was an increasing in all the measurements as shown in Table 1. However, it was determined that this increase was statistically significant only in the liver weight (0.65 ± 0.08 g), HSI value (5.87 ± 0.86 %), plasma total protein (48.274 ± 3.845 mg/mL), and Vtg (46.427 ± 1.346 mg/mL) levels ($P < 0.05$).

Table 1. Total height, total weight, liver weight, gonad weight, hepatosomatic index (HSI), gonadosomatic index (GSI), plasma total protein and vitellogenin levels in fishes of control group and E2 treatment group. The values which are measured were given as Mean \pm Standard error of mean. n: number of the animals, *: $P < 0.05$

	Control group (n:5)	E2 treatment group(n:5)
Fork lenght (cm)	12.20 \pm 0.25	13.20 \pm 0.51
Total weight (g)	18.33 \pm 1.19	25.70 \pm 3.09
Liver weight (g)	0.34 \pm 0.040	0.65 \pm 0.08*
Gonad weight (g)	0.77 \pm 0.11	1.57 \pm 0.41
HSI (%)	4.20 \pm 0.47	5.87 \pm 0.86*
GSI (%)	1.86 \pm 0.11	2.56 \pm 0.10
Total protein (mg/mL)	20.904 \pm 2.279	48.274 \pm 3.845*
Vitellogenin (mg/mL)	1.015 \pm 0.740	46.427 \pm 1.346*

Histology

Following the histological investigation, all of the fishes injected with E2 and the members of the control group were determined as females. The samples taken from the livers of these fishes were stained with the periodic acid-Schiff. It was

observed that there was no clear and significant difference in the density of glycogen content in the liver tissues of the E2 injected fishes and that of the control group fishes (Figure 1A and B). It was observed that the glycogen ingredient in the hepatocyte varied individually

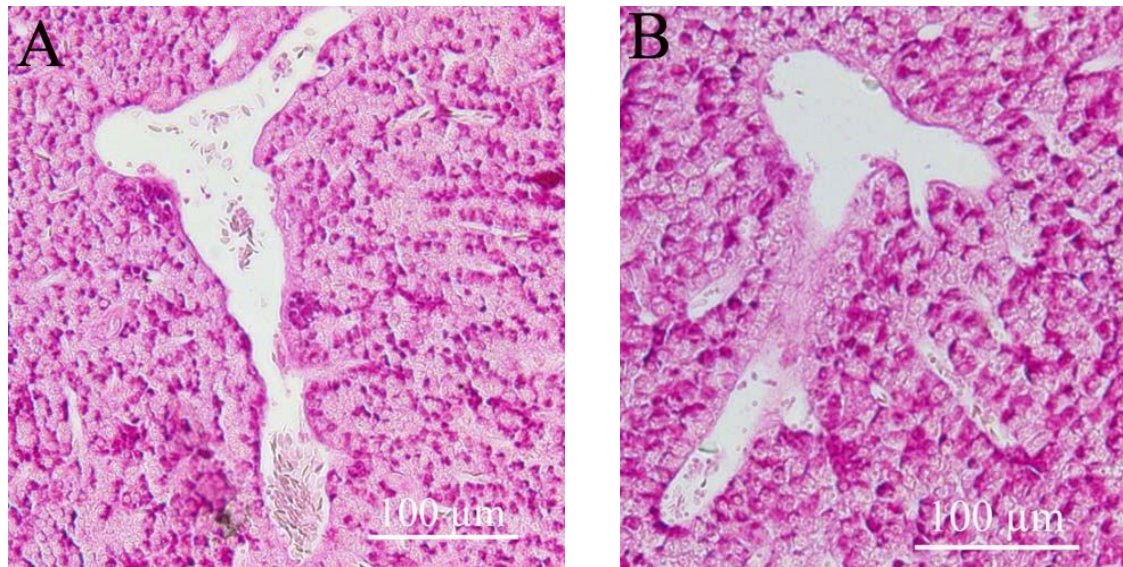


Figure 1. Glycogen content in the liver of *Chalcalburnus tarichi* **A)** Control and **B)** E2 treatment group. (PAS).

Electrophoresis and Western Blot

To determine the molecular weight of Vtg in *C. tarichi*, the SDS-PAGE electrophoresis technique was performed on the plasma samples of the E2 injected females, the control females, male, and juvenile fishes (Figure 2A). The obtained Vtg bands were verified using western blot and carp polyclonal antibody (Rabbit anti-carp Vitellogenin polyclonal antibody, Biosense, Norway) (Figure 2B). Accordingly, the Vtg molecular weight in *C. tarichi* was determined as 145 kDa. In this study, both electrophoresis and Western Blot analysis were used. However, the vtg band was observed only in the E2 injected female fishes. The vtg band was not observed in the plasma samples of the male and juvenile fishes.

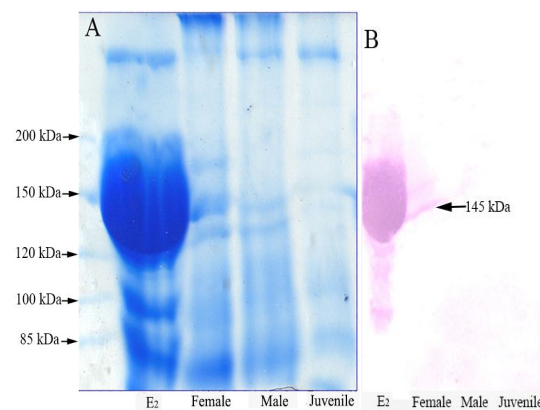


Figure 2. Determination of the molecular weight of vitellogenin in plasma samples on *Chalcalburnus tarichi* **A)** SDS-PAGE separation of serum samples from E2 injected, female, male and juvenile fish collected from Lake Van **B)** Western blot analysis with the same plasma samples using carp polyclonal antibody (Rabbit anti-carp Vitellogenin polyclonal antibody, Biosense, Norway).

DISCUSSION

In the present study, the gonadosomatic index (GSI), hepatosomatic index (HSI), plasma total protein, Vtg levels, and glycogen content in liver and the molecular weight of Vtg protein were determined after E2 injection in *Chalcalburnus tarichi*.

Vitellogenin is a precursor egg protein in the glycopospholipoprotein structure that is synthesized in the liver. In the studies conducted on several fishes, the increasing values of HSI, plasma total protein, and Vtg levels were defined during the vitellogenesis phase (Johnson et al. 1991; Koya et al. 2003). Similarly, an increase in the HSI value, plasma total protein, and the levels of Vtg were defined in E2 injected *Salmo gairdneri* (Van Bohemen et al. 1982; Haux and Norberg 1985; Flet and Leatherland 1989) and *Zoarcetes viviparus* (Korsgaard and Petersen 1979). In addition, after E2 injection, it was reported that there was an increase in the liver lipid level of *Carassius auratus* (De Vlaming et al. 1980) and the water content in the liver of *Channa punctatus* (Sehgal and Goswami, 2001). In the present study, it was clearly defined that there was an increase in the HSI value, plasma total protein, and Vtg levels of *C. tarichi* after E2 injection (Table1.). It was observed that E2 clings to the receptors in the liver cells and causes an increase in the Vtg and total protein levels, which are precursors of egg proteins (Flouriot et al. 1997). No

significant increase in the length, total weight, gonad weight, and GSI value in *C. tarichi* after E2 injection was observed ($P>0.05$). The length and weight of fishes were found to vary according to the age and feeding patterns. The GSI value is obviously related to oocyte growth and increases during the vitellogenesis and maturing phases (Johnson et al. 1991). The increase in the HSI and GSI values is parallel with the plasma E2 and Vtg levels during the Vitellogenesis phase (Koya et al. 2003). However, it was determined that the increase of the gonad weight and GSI values in the E2 injected fishes was not significant. Similarly, it was observed that the GSI value did not change 14 days after 17α -Ethinyl estradiol was added to water and 7 days after injection in *Oncorhynchus mykiss* (Verslycke et al. 2002). Thus, it can be mentioned that the insignificant difference of GSI values after E2 injection in *C. tarichi* could be a result of the short duration of the experimental period (5 days).

As in other vertebrates, glycogen is the first energy source used by fishes. At the same time, glycogen participates in the Vtg structure. Histological investigation indicated that the content of glycogen decreases with the injection of E2, *Ictalurus punctatus*, in the cells during the synthesis of Vtg (Pacoli et al. 1991). In addition, it was seen that the content of glycogen decreases in the liver of *Carassius auratus* (De Vlaming et al. 1980),

Salmo gairdneri, and *Platichthys flesus* (Petersen et al. 1983) when biochemical methods were used. In this study, it was observed that no difference in the content of glycogen in hepatocytes compared to injected E2 fishes and control fishes. The reason could be due to a result of the short duration of the experimental time. Nevertheless, to arrive at a definite conclusion, the glycogen level in the liver tissue extract must be measured.

The molecular weight of Vtg in fishes varies according to the species. The molecular weight of Vtg was observed as 150kDa in *Ictalurus punctatus* (Goodwin et al. 1992), 154kDa in *Oncorhynchus mykiss* (Watts et al. 2003), 150kDa in *Pimephales promelas* (Zhang et al. 2004), 130 kDa in *Pleuronectes vetulus* (Roubal et al. 1997), 190 and 156kDa two bands in *Cyprinus carpio* (Fukada et al. 2003), 180kDa in *Sparus aurata* (Mosconi et al. 1998), and 134 kDa in *Phoxinus oxycephalus* (Park et al. 2003). The antibodies of the specific fishes were used in these studies. However, it was demonstrated that the antibodies against carp (*Cyprinus carpio*) Vtg showed good cross-reactivity with Vtgs of several other cyprinids (Tyler et al. 1996; Nilsen et al. 2004). Nilsen et al. (1998) pointed out that polyclonal and monoclonal antibodies, which they created against *Salmo salar*, made cross-reactions with Vtg of the other fish species. In this

study, carp Vtg polyclonal antibody was used and the molecular weight of Vtg of the *C. tarichi* was identified as 145 kDa. It was observed that the molecular weight in *C. tarichi* was within the defined limit of values in the Cyprinid species which belong to the same family as well. Thus, it indicates that the molecular weight could change from one species to another. In this study, the Vtg band could not be determined in the plasma of the male and juvenile fishes caught in Lake Van. The absence of vitellogenin in plasma of both male and juvenile fish may have indicated that Lake Van is clean in terms of estrogen mimicking chemicals.

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