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Determination of hormones inducing oocyte maturation in *Chalcalburnus tarichi* (Pallas, 1811)

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Abstract *Chalcalburnus tarichi* is an endemic cyprinid species living in the Lake Van basin, in eastern Anatolia, Turkey. The present study was undertaken to determine which hormones induce oocyte maturation in *C. tarichi*. The levels of 17α,20β,21-trihydroxyprogesterone (20β-S), progesterone (P), 17α-hydroxyprogesterone (17α-HOP), 11-deoxycortisol (11-DOC), and 17α-hydroxy-20β-dihydroprogesterone (17,20β-P) were measured in fish caught from Lake Van and the Karasu River, and injected with human chorionic hormone (hCG) (1,000 and 1,500 IU/kg). Oocytes of fish caught from the lake were also incubated in vitro with different doses (50, 200, and 1,000 ng/ml) of 20β-S, 17α-HOP, 11-DOC, and 17,20β-P. 11-DOC was found to be the most effective hormone among those measured for inducing oocyte maturation in vivo and in vitro. 17,20β-P could not be determined in the plasma of any fish in vivo ($P < 0.05$). 1,000 IU/kg dose of hCG given by injection caused a statistically significant increase in all plasma hormone levels ($P < 0.05$). It was found that there was a significant decrease in the P level only at 1,500 IU/kg dose of hCG injected ($P < 0.05$), while the level of other hormones increased at this dose ($P < 0.05$). It was also determined that all the hormones were effective in germinal vesicle breakdown (GVBD) in in vitro oocyte culture ($P < 0.05$). However, 11-DOC was found to be the most effective hormone in GVBD at a dose of 200 ng/ml (70% GVBD). In conclusion, 11-DOC synthesized during final oocyte maturation in *C. tarichi* was found to be a potent inducer of GVBD, which shows that 11-DOC may be described as an oocyte maturation steroid in this species.

Keywords *Chalcalburnus tarichi* · hCG · MIS · Oocyte maturation

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Introduction

Oocyte development in fish is divided into the phases of oocyte growth and oocyte maturation. Vitellogenesis plays an important role in oocyte growth. Vitellogenesis and oocyte maturation are regulated by gonadotropins via steroid hormones secreted by the follicle cells that surround the oocyte. Among these steroids, 17β-estradiol stimulates the liver to synthesize vitellogenin, which is a precursor of the specific yolk proteins accumulated in the oocytes. The process of oocyte maturation is characterized by the initiation

of meiosis, migration, and breakdown of the germinal vesicle, coalescence of lipid droplets and yolk globules in the cytoplasm, a rapid size increase of the oocyte caused by hydration that is accompanied by an overall increase in oocyte translucency, and the release of the first polar body (Goetz 1983; Nagahama et al. 1983; Takasima and Hibiya 1995).

It was reported that maturation-inducing steroid (MIS) synthesis increased in various fish in the presence of some hypothalamic and hypophysis hormones (Kagawa et al. 1994; Greenwood et al. 2001; Modesto and Canario 2002). Certain progestagens in fish are described as MISs due to the fact that they induce final oocyte maturation (FOM). Of these, 17,20β-P and/or 20β-S are known as steroids that induce oocyte maturation in most fish (Canario and Scott 1990; Garcia-Alonso et al. 2004; Matsuyama et al. 1998; Modesta and Canario 2002; Ohta and Matsuyama 2002; Yueh and Chang 2002; Webb et al. 2002). However, in other fish species, different steroids may be effective in inducing oocyte maturation and ovulation. During oocyte maturation, little or no increase was observed in the plasma concentration of one or both of these steroids in some fish (Scott and Canario 1987, 1990; Rahman et al. 2001), whereas certain differences were reported between in vivo plasma levels and in vitro effects in some other fish during FOM. For instance, it was reported that in plaice, *Pleuronectes platessa*, these steroids had a significant effect on the induction of FOM in vitro (Canario and Scott 1990). Nevertheless, the plasma level of 17α,20β,21-P did not change after injection with hCG, whereas a slight increase was observed in the level of 17,20β (Scott and Canario 1990). Similarly, in Korean spotted sea bass, *Lateolabrax maculatus*, serum 17,20β-P was also at a low level during maturation of oocytes in vitro (Lee and Yang 2002). Kime (1993) suggested that derivative steroids from progestagens or progesterone could also be effective for FOM in some fish. Webb et al. (2002) asserted that in white sturgeon, *Acipenser transmontanus*, C19 and C21 steroids were synthesized by the follicle cells of oocytes in vitro during germinal vesicle breakdown (GVBD), which occurred following injection with hypophysis homogenate and gonadotropin releasing hormone analogue (GnRHa). In black porgy, *Acanthopagrus schlegeli*, 20β-S and 17,20β-P are very effective steroids in the process of in vitro maturation of oocytes taken from fish injected with luteinizing hormone releasing hormone analogue (LHRH-A). However, 17α-hydroxyprogesterone, 11-dehydroxycortisol, and 20,21-dihydroxy are also effective in oocyte maturation (Yueh and Chang 2002).

More in vitro studies revealed that various steroids were effective in GVBD (King et al. 1995; Lee and Young 2002; Matsuyama et al. 1998; Rahman et al. 2001; Yueh and Chang 2002). In mummichog, *Fundulus heteroclitus*, two 20β-dihydroprogestin steroids are very effective MISs with a 50% GVBD effect, and they have been found to have a physiological role in the initiation of meiotic maturation of oocytes (Greeley et al. 1986).

Chalcalburnus tarichi is an endemic cyprinid species living in the Lake Van basin, in the eastern Anatolia region of Turkey. Lake Van is the largest soda lake on Earth, containing abundant amounts of carbonate and bicarbonate. The lake has a pH of 9.8 that makes its alkaline, brackish waters unsuitable for either drinking or irrigation. Very few fish can survive in it, but *C. tarichi* has adapted to this saline environment. This is an anadromous fish that migrates once a year in the spring months to freshwater rivers inlets the lake for spawning, and then returns to the lake. The aim of the present study was to determine the hormone/hormones inducing oocyte maturation in *C. tarichi* utilizing in vivo and in vitro experiments.

Materials and methods

Chemicals

hCG and steroid hormones (P, 17α-HOP, 17,20β-P, 20β-S, and 11-DOC) were obtained from Sigma (Sigma-Aldrich Co., St. Louis, MO, USA). hCG was dissolved in physiological solution before injection. Hormones used for the oocyte culture were dissolved in absolute alcohol and stored at −20°C.

Fish

Mature female *C. tarichi* that were 5–6 years of age were used in the study. These fish migrate to freshwater for spawning during May and June. The fish were caught from Lake Van at the beginning of the oocyte maturation phase (1–6 May 2003) and from the Karasu River during maturation (20 June 2003), and were transferred to the laboratory. They

were stored in fiberglass tanks (300 l) at 10–15°C. The fish from Lake Van and the Karasu River were kept in tanks containing lake water and freshwater, respectively. Fish caught from the lake were used for the hormone measurement, hCG injection, and oocyte culture, while the fish from the river were used only for the hormone measurement.

Hormone application

After the fish were anesthetized using 3-aminobenzoic acid ethyl ester (MS222), their lengths and weights were measured. Fish that were approximately of the same length (20 cm) and the same weight (90–100 g) were used for injection of hCG. Doses of hCG (1,000 and 1,500 IU/kg) were determined in similar ranges according to the work of Lee and Yang (2002). The fish were placed in aquaria (40 l) where the water temperature was adjusted to an average 15°C. hCG was dissolved in Ringer's solution for the freshwater fish. The experiment was completed after 24 h. Three fish were used for each dose.

Blood samples

The fish were anesthetized with MS222 and blood samples were taken from the caudal vessels using heparinized syringes. After centrifugation, the plasma was stored at −80°C until steroid analysis.

In vitro oocyte culture

Hormones and their doses used in the oocyte culture (17α-HOP, 17,20β-P, 20β-S, and 11-DOC) were prepared according to the procedure described by Lee and Yang (2002). The fish caught from the lake were killed by decapitation. Their ovaries were removed and placed in ice-cold Leibovitz's-15 (L-15) culture medium (Sigma). The pH was adjusted to 7.4, and then gentamicin sulfate (200 mg/ml) was added. The oocytes were carefully separated from one another under a binocular stereomicroscope (Nikon SMZ 800). One-half milliliter of culture medium containing 50, 200, and 1,000 ng/ml, respectively, of the hormones was placed in multiwell Petri dishes. In preliminary trials, oocytes were incubated for 24 h at 19°C and 21°C, and only incubation at 21°C was found to be effective. Groups of 20 oocytes of approximately the same size were placed in a Petri dish well containing culture medium plus hormone, and then incubated for 24 h at 21°C. After the incubation period, the medium was replaced with a cleaning solution of ethanol, formalin, and glacial acetic acid (6:3:1). Digital images of the oocytes were taken using a Nikon TE 300 inverted microscope, recorded on a computer by using software (Image J), and were inspected for GVBD.

High-performance liquid chromatography (HPLC)

Plasma levels of P, 17α-HOP, 17,20β-P, 20β-S, and 11-DOC were measured by HPLC in a procedure modified from Inbaraj et al. (1997). Firstly, P, 17α-HOP, 17,20β-P, 20β-S, and 11-DOC standards were injected and appropriate peaks and retentions times of these hormones were obtained (Table 1). Then, the HPLC system was calibrated with a mixture of these standards. The plasma samples (500 µl) were passed through Sep Pak C_{18} (Waters) cartridges which had been preconditioned with 5 ml methanol followed by 5 ml distilled water. Steroids absorbed on the cartridges were eluted with 5 ml of 80% methanol, and the eluate was injected into the chromatograph (Shimadzu, LC-10 AD). P, 17α-HOP, and 17,20β-P were measured by the chromatographic system under isocratic conditions at a flow rate of 1.5 ml/min; a µBondapak column with acetonitrile:water (52:48) was used as the mobile phase. The pressure and wavelength were adjusted to 2.8 psi and 254 nm, respectively. All injections were performed in triplicate.

Statistics

Comparisons among binary groups were carried out by means of the unpaired Student *t* test, while for comparisons among multiple groups, analysis of variance (ANOVA) followed by the Dunnett test were used. Data are expressed as the mean ± standard error of the mean (SEM), and statistical significance was inferred at $P < 0.05$.

Results

Plasma hormone levels in fish caught from Lake Van and the Karasu River

It appeared that the germinal vesicle had not migrated during the oocyte maturation phase of the fish caught

Table 1 Steroid standards for high-performance liquid chromatography and their relative retention times

Nomenclature	Common name	Abbreviation	Retention times
4-Pregnene-17α,21-diol-3,20-dione	11-Deoxycortisol	11-DOC	2.165
4-Pregnene-17α,20β-diol-one	17α-Hydroxy-20β-dihydroprogesterone	17,20β-P	3.707
17α,20β,21-Trihydroxy-4-pregnen-3-one 20β,21-diacetate	17α,20β,21-Trihydroxyprogesterone	20β-S	4.268
4-Pregnen-17α-ol-3,20-dione	17α-Hydroxyprogesterone	17α-HOP	4.357
4-Pregnene-3,20-dione	Progesterone	P	7.823

Stationary phase: μBondapak column; mobile phase: acetonitrile:water (52:48); flow rate: 1.5 ml/min; pressure: 2.8 psi; detection: UV (254 nm)

Table 2 The hormone changes of *C. tarichi* caught in Lake Van and the Karasu River

Steroids	*n*	Concentrations (ng/ml)	
		Lake	River
20β-S	5	0.119 ± 0.043	0.263 ± 0.092
P	6	0.036 ± 0.013	0.029 ± 0.012
17β-HOP	5	0.018 ± 0.001	0.189 ± 0.006*
11-DOC	6	3.874 ± 0.703	5.435 ± 1.510
17,20β-P	6	–	–

20β-S: 17α,20β,21-trihydroxyprogesterone, P: progesterone, 17α-HOP: 17α-hydroxyprogesterone; 11-DOC: 11-deoxycortisol; 17,20β-P: 17α-hydroxy-20β-dihydroprogesterone. 17,20β-P could not be detected in any samples. * Indicates statistically significant differences ($P < 0.05$). Values are expressed as mean ± SEM; *n*: number of sample

from Lake Van, and that germinal vesicle breakdown had initiated in most oocytes in the fish caught from the Karasu River. The plasma steroid hormone levels of the fish caught from Lake Van and the Karasu River are shown in Table 2. 17α-HOP was at the lowest level (0.018 ± 0.001 ng/ml) while 11-DOC was at the highest level (3.874 ± 0.703 ng/ml) before the migration of the germinal vesicle in fish caught from Lake Van. No important changes in 20β-S, P, or 11-DOC levels were observed, while a significant increase in the amount of 17α-HOP ($P < 0.05$) was found during the breakdown of the germinal vesicle in fish caught from the river. However, 17,20β-P was not detectable in any sample.

The effect of hCG on plasma hormone levels in vivo is shown in Table 3. While 17,20β-P levels could not be detected after either dose of the hCG injection, other hormone levels significantly increased after a 1,000 IU/kg dose of hCG by injection when compared to the control ($P < 0.05$). At a dose of 1,500 IU/kg, a significant decrease was observed in the P level as compared with both the 1,000 IU/kg dose and the control ($P < 0.05$). The level of other hormones increased also at the 1,500 IU/kg dose. This increase was found to be significant when compared with the 1,000 IU/kg dose and control ($P < 0.05$). The highest increase in production was measured for 11-DOC, 17α-HOP, and 20β-S, respectively.

Table 3 The hormone changes of *C. tarichi* injected with human chorionic hormone (hCG)

Steroids	*n*	Concentrations (ng/ml)		
		Control	1,000 IU/kg	1,500 IU/kg
20β-S	3	0.163 ± 0.060	0.362 ± 0.012*	0.842 ± 0.023*
P	3	0.060 ± 0.016	0.173 ± 0.005*	0.021 ± 0.002*
17α-HOP	3	0.018 ± 0.001	2.036 ± 0.071*	3.556 ± 0.163*
11-DOC	3	5.107 ± 0.540	20.803 ± 0.058*	26.422 ± 0.723*
17,20β-P	3	–	–	–

20β-S: 17α,20β,21-trihydroxyprogesterone; P: progesterone; 17α-HOP: 17α-hydroxyprogesterone; 11-DOC: 11-deoxycortisol; 17,20β-P: 17α-hydroxy-20β-dihydroprogesterone. 17,20β-P could not be detected in any samples. * Indicates statistically significant differences ($P < 0.05$). Three fish were used for each injection. Values are expressed as mean ± SEM; *n*: number of sample

Effect of various hormones on oocyte maturation in vitro

Results from preliminary trials indicated that in vitro experiments were most successful when carried out at 21°C for 24 h. Relative effects of 20β-S, 17,20β-P, 17α-HOP, and 11-DOC at different concentrations on germinal vesicle breakdown in vitro are shown in Fig. 1. Among those hormones, 11-DOC was found to have the greatest effect on GVBD. It was also determined that the effect of 11-DOC on GVBD at the lowest dose was almost the same as the effect of 20β-S at the highest dose (47% GVBD). While the most effective dose of 11-DOC on GVBD was found to be 200 ng/ml (70.416 $\pm$ 6.137), at a dose of 1,000 ng/ml it caused a decrease in GVBD (60.256 $\pm$ 4.083). 17α-HOP, 20β-P, and 20β-S were found to have the same effect on GVBD at a dose of 1,000 ng/ml.

Discussion

Chalcalburnus tarichi has a group-synchronized-type ovary and spawns in freshwater annually. The fish start to migrate from the lake into freshwater rivers after completing vitellogenesis, during the maturation phase (Ünal et al. 1999). Oocyte maturation continues during the migration, which continues for about 1–2 months, depending on the temperature of the water. In the fish caught from Lake Van, it was observed that the germinal vesicle of the oocytes had not yet started to migrate. The migration of the germinal vesicle was already initiated in the fish caught from the river. In *C. tarichi*, various ovarian hormone levels have been determined during vitellogenesis (Ünal et al. 2005), however, there are no known studies related to hormones that control maturation.

The germinal vesicle disappears during the FOM. Therefore, maturation is characterized by GVBD. In this study, 11-DOC levels were the highest (3.874 $\pm$ 0.703 ng/ml) at the beginning of the maturation, followed by 20β-S (0.119 $\pm$ 0.043 ng/ml), P (0.036 $\pm$ 0.013 ng/ml), and 17α-HOP (0.018 $\pm$ 0.001 ng/ml), respectively. In the river samples, 20β-S, P and 11-DOC levels showed no difference when compared to fish caught from the lake. An increase was found to be significant only for 17α-HOP ($P < 0.05$). The results of in vivo and in vitro experiments in *C. tarichi* revealed that 11-DOC, 17α-HOP, and 20β-S were effective in oocyte maturation, but that 11-DOC was the most effective hormone. 17,20β-P and 20β-S are generally known as steroids that induce oocyte maturation. These steroids are effective either together or separately. It was determined that both 17,20β-P and 20β-S were effective for GVBD in some species such as white perch, *Morone americana*, white bass *Morone chrysops* (King et al. 1995), and bambooleaf wrasse, *Pseudolabrus sieboldi* (Ohta and Matsuyama 2002). In Lusitanian toadfish, *Halobatrachus didactylus*, it was reported that 17,20β-P and 20β-S were effective for GVBD in vivo, whereas 20β-S was more effective than 17,20β-P in fish treated with pituitary extract in vitro (Modesto and Canario 2002). However, it was also reported that 20β-S was never synthesized in the ovarian culture of Japanese yellowtail, *Seriola quinqueradiata*, during FOM, and that 17,20β-P was the maturation-inducing hormone (Rahman et al. 2001). In female plaice, *P. platessa*, 20β-S could not be determined after hCG hormone injection (Scott and Canario 1990), whereas in carp, *Cyprinus carpio*, 17,20β-P could be determined during the migration of the germinal vesicle (Levavi-Zermonsky and Yaron

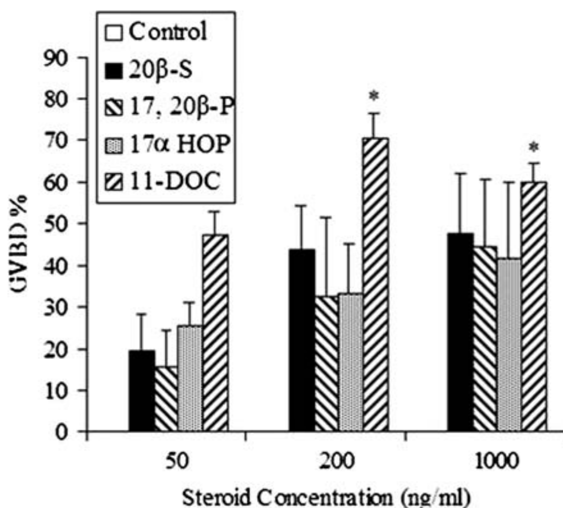

Fig. 1 The effects on germinal vesicle breakdown (GVBD) of four steroids in in vitro oocytes of *C. tarichi*. 20β-S: 17α,20β,21-trihydroxy-4-pregnen-3-one 20β,21-diacetate; 17, 20β-P: 17α,20β-dihydroxy-4-pregnen-3-one; 17α-HOP: 17α-hydroxyprogesterone; 11-DOC: 11-deoxycortisol. GVBD in the control is 0%. * Indicates statistically significant differences ($P < 0.05$). Three fish were used for each experiment. Values are expressed as mean $\pm$ SEM

1986). Nagahama et al. (1995) noted that 17,20β-P increased during maturation, causing GVBD. In the fish species ayu, *Plecoglossus altivelis*, amago salmon, *Oncorhynchus rhodurus*, rainbow trout, *Oncorhynchus mykiss*, and goldfish, *Carassius auratus auratus*, 17,20β-P was found to be an effective hormone in FOM when various pregnene derivations were applied to in vitro oocytes (Nagahama et al. 1983). Unlike these fish, 17,20β-P could not be detected in in vivo experiments in *C. tarichi* during oocyte maturation.

It was observed that there was a significant increase in hormone levels resulting from the hCG injection at a dose of 1,000 IU/kg ($P < 0.05$). At a dose of 1,500 IU/kg, it appeared that the P level significantly decreased, while there was a significant increase in the levels of other hormones. It is known that P is a precursor to other steroids (Scott et al. 1983). In *C. tarichi*, it may also be a precursor to other steroids. However, plasma 17,20β-P levels could not be determined from the hCG injection at either dose. According to Mugnier et al. (1997), 17,20β-P is not an MIS, and its presence could be interpreted as a local hormone that was produced during a brief synthesis span and is rapidly metabolized. However, in plaice, *P. platessa*, 17,20β-P was reported as a MIS that rapidly metabolizes (Scott et al. 1997). In some studies it was indicated that the level of enzymes that transform 17,20β-P into metabolites increases when large quantities of 17,20β-P are produced (Suzuki et al. 1981; Kime 1990; Kime et al. 1991, 1992).

Goswami and Sundararaj (1971) reported that the main hormones affecting maturation and ovulation in stinging catfish, *Heteropneustes fossilis*, were corticosteroids. In brook trout, *Salvelinus fontinalis*, and yellow perch, *Perca flavescens*, both progesterones and 11-dioxycorticosteroids induce GVBD in vitro (Geotz and Bergman 1978). In brook trout, *S. fontinalis*, not only 17,20β-P but also deoxycorticosterone was effective on GVBD, the effect being greater in vitro (Duffey and Goetz 1980). In goldfish, *C. auratus auratus*, it was reported that deoxycorticosterone could be a natural mediator of oocyte maturation (Jalabert et al. 1973). Accordingly, it can be interpreted that high levels of 11-DOC found in *C. tarichi* are effective in oocyte maturation. The inability to detect 17,20β-P in *C. tarichi* may suggest that this hormone is rapidly metabolized and transformed into 11-DOC, in vivo and after hCG injection.

In *C. tarichi*, all hormones applied to oocytes in vitro induced GVBD at three different doses as compared to the control (Fig. 1). Similarly, most steroids are known to induce GVBD in vitro (Garcia-Alonso et al. 2004; Greeley et al. 1986; Lee and Yang 2002; Mugnier et al. 1997). In *C. tarichi*, 11-DOC caused 70% GVBD at a dose of 200 ng/ml, whereas this rate was observed to drop to 60% at 1,000 ng/ml. Consequently, this shows that the maximum effect of 11-DOC on GVBD is dose dependent.

In conclusion, it can be stated that 11-DOC is the most effective hormone in vivo and in vitro during oocyte maturation of *C. tarichi*, followed by 20β-S in a natural environment, and 17α-HOP in hCG injection and in vitro applications.

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