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Original article

Effect of dietary 17 β- estradiol on serum sex hormones' levels and gamete quality in goldfish (*Carassius auratus*)

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Key words

Carassius auratus, 17 β-estradiol, gamete quality, sex hormone.

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Background: The use of high quality gametes from fish brood stock has predominant importance for ensuring the production of viable larvae for aquaculture. Also, availability of the both sex in sufficient number is necessary for successful artificial propagation activity. Hormonal induction of spawning is a good strategy to shorten and synchronizes of gamete maturation in hatcheries.

Objective: Effect of dietary17 β-estradiol (17E) on serum sex hormones and gamete quality was investigated in goldfish (Carassius auratus).

Methods: Fish were fed diets containing 0, 10, 25 and 50 mg/kg diet of 17E over a 193-day period. At the end of the trial, some gamete characteristics as well as serum sex hormone levels were determined in both male and female fish.

Results: Results showed significant effect of 17E on both male and female gamete quality including increase in oocyte diameter, gonadosomatic index (GSI), functional fecundity as well as decrease in oocyte surface: volume, spermatocrit, sperm density, and motile sperm percentage and sperm motility duration. On the other hand, fertilization and hatching rate as well as zygote diameter were higher in 17E-treated fish compared to the control group. Serum levels of estradiol and 17-hydroxyprogestrone were high in 17E-treated fish compared to the control group. However, the lower serum testosterone levels were found in treated group with 10 mg/kg diet of 17E, followed by the control group.

Conclusion: Supplemented diets with 17E had positive effects on gamete quality and caused to some hormonal disturbance in goldfish. Moreover, hatching rate, functional fecundity and the levels of serum 17OH showed a dose – dependent variation due to using the supplemented diet with different values of 17E and their values were significantly increased coincident with increasing the 17E values in the diet.

Introduction

Availability of the both sex in sufficient number is necessary for successful artificial propagation activity. Deficiency in either male or female individuals leads to fail to reach the predicted eggs as well as larvae, resulting in economic loss. Due to this, many efforts have been conducted to facilitate and ensure the availability of the each sex. Fish sperm cryopreservation is a method for ensuring male gamete availability which has been developed in any fish species (Harvey *et al*., 1982; Magyary *et al*., 1996; Linhart *et al*., 2000; Suquet *et al*., 2000; Huang *et al*., 2004) including goldfish (Babiak *et al*., 2000). On the other hand, dietary sex steroids have been used to achieve mono-sex population in some fish species (Hishidaand Kawamoto, 1970; Owusu-Frimpong and Nijjhar, 1981; Park *et al*., 2004; Flynn and Benfey, 2007; Wang et al., 2008; Zhang and Jin, 2011).

Dietary 17β - estradiol (17E) has been found to feminize the fish population in some fish species (Park et al., 2004; Flynn and Benfey, 2007; Wang *et al*., 2008; Naji *et al*., 2009; Zhang and Jin, 2011). This method can ensure the availability of the female individuals which allows the culturists to program their egg production in a better way. However, it has been shown that 17E treatment affect gamete quality, as well (Lahnsteiner *et al*., 2006; Schubert *et al*., 2008). Goldfish, *Carassius auratus*, is one of the most common ornamental fish in Iran. Its importance for Iranians refers to its necessity for the ancient Nowruz Holyday. Due to this, high market demand for goldfish, and also its breeding and rearing is performed by many people in potential regions of Iran, and brings reasonable profit for employers who many of them are completely satisfied with this job (Hoseini and Tarkhani, 2012).

Goldfish is the most extensively studied species with respect to basic reproductive physiology and endocrine regulated behavior (Bjerselius *et al*., 2001). Therefore, the present work aimed to determine the effect of dietary 17E treatment on gamete quality and serum sex hormones' levels in goldfish.

Materials and methods

Diets

The control diet composition is presented in table 1.

To prepare the control diet, the ingredients were weighed and mixed carefully and moisturized by appropriate water amount to facilitate pellet forming. The obtained dough then passed through a metal mesh (1 mm in diameter) to form threads. Threads were air-dried and re-grounded into appropriate size and passed through a 0.5 mm mesh. To prepare the 17E- supplemented diets, 17E was purchased (estradiol valerate, Sigma, St. Louis, MO) and 10, 25 and 50 mg 17E were dissolved in 400 ml ethanol (96 %) and added to 1kg diet ingredients mixture (control diet) (Wang *et al*., 2008). The mixtures were remained at room temperature for ethanol evaporation. Thereafter, the mixtures were moisturized with appropriate water amount and processed similar to the control diet to obtain the 0.5 mm particles. All diets were frozen at -18ºC until used.

Table 1: The composition of control diet ingredients

Ingredient	*g/ kg diet*
Fish meal	*205.5*
Soybean meal	*385*
Wheat meal	*101*
Corn meal	*61*
Rice bran	*187.5*
Fish oil	*5*
Vitamin mix[#]	*20*
Mineral mix[$]	*20*
Lysine	*7.5*
Methionine	*7.5*
Proximate composition	
Protein (%)	*39*
Lipid (%)	*10.8*
Moisture (%)	*6*
Gross energy kcal kg^{-1}	*4000*

[#] Vitamin mix was formulated to provide the following amounts for diet (mg kg^{-1} of diet): B_1, 0.7; B_2, 10; B_3, 40; B_5, 40, B_6, 9; B_9, 3; B_{12}, 0.01; biotin, 1.5; vitamin K, 4.5; vitamin A, 6000 IU and vitamin E, 150 IU. All vitamins were purchased from companies Adisseo (Antony, France) and BASF (Ludwigshafen, Germany).

[$] Mineral mix was formulated to provide the following amounts for diet (mg kg^{-1} of diet): Mg, 500; Fe, 150; Zn, 20; Mn, 13; Cu, 4.8; Co, 0.1. All minerals were purchased from Company of Divan Shimi (Tehran, Iran).

Fish

The study was conducted using goldfish larvae. To obtain demanded larvae, goldfish brood stocks (30 g) were injected by Ovaprim for artificial propagation.

Oocytes and milt were stripped and mixed together using a light feather. After adhesion removal (2 ppt NaCl solution), the obtained eggs were transferred into glass aquarium supplied with gentle aeration. After 48 h the eggs were hatched (at 23ºC) with the hatching rate of 95%. Exogenous feeding (dry milk, green water and *Artemia naupli*) was started at 72 h post hatch. The larvae were fed by the mentioned foods over a 4-month period. Survival and average weight were 20 % and 0.1 g at the end of this period, respectively.

Experimental design

A total of 180 larvae (0.12 ± 0.002 g) were randomly distributed into 4 groups with 3 replicate including control, and treated groups with different dose of 17 E (10, 25 and 50 mg/kg diet). Fish were fed with the control diet over 10 days for adaptation and thereafter each treatment received its corresponding diets over a 193 - day period. The 80% of water content of each tank was exchanged every other day. Water dissolved oxygen, pH, temperature and total hardness were 6.5 ± 0.7 mg/l, 7.2 ± 0.3, 25 ± 1.4ºC and 200 ± 15.2 mg/l, respectively and also photoperiod was natural (March - October, 2011). At the end of trial, 5 male and 5 female fish were sampled from each treatment to evaluate the gamete characteristics. In the male individuals, seminal pH, spermatocrit, sperm motility duration, motile sperm percentage, sperm density and collectable-milt volume were recorded, whereas, in the female ones, oocyte diameter, and oocyte's surface: volume ratio, functional fecundity and gonadosomatic index (GSI) were checked. Likewise, 5 females were randomly captured from each treatment and anesthetized using clove solution (3000 ppm) and blood - sampled by caudal severance. Blood samples were collected into the non-heparinized tubes and centrifuged (5000 rpm, 5 min) to obtain serum. Serum sample were analyzed for 17E, 17-Hydroxyprogesterone (17OH) and testoste-rone concentrations. Thereafter, 5 females (6-8 g) from each treatment were sampled and distributed into the five separate aquariums, as well as, 5 males (6 g) fish were captured from the control group and stocked in another aquarium. All aquariums containing male and female fish were subjected to a temperature treatment as follow: initial temperature was 20ºC which decreased to 10ºC over a 10-day period (1ºC per day). Fish were kept at 10ºC over another 10-day period and thereafter temperature was increased to 20ºC over a -10-day period (1ºC per day). After temperature treatment, Ovaprim (0.2 ml) twice injected intramuscular to the female fish with 8 hr interval and simultaneous with the second injection, the males were once injected (Ovaprim 0.2 ml). Seventeen hours after the second injections, gametes were attained by hand-stripping. Eggs obtained from all treatments were fertilized by the mixed milt collected from the males (5 fish). Oocytes and milt were stripped and mixed together using a light feather. After adhesion removal (2 ppt NaCl solution), the obtained eggs were transferred into glass aquarium supplied with gentle aeration. After 48 h the eggs were hatched (at 23ºC). Fertilization rates as well as hatching rates were determined in each treatment.

Determination of gonad characteristics

Female

Oocyte diameters were determined using a scaled loop with 40 sub-replications for each treatment replicate (tank). The average of the sub-replicates was considered as one replicate of each treatment. Oocytes surface and volume were calculated according to the diameter. To estimate the functional fecundity, the weights of the total obtained oocytes for each fish (five fish per treatment) were recorded. Three fractions (~ 0.2 g) were detached from each gonad and the numbers of the oocytes were determined under a loop. Accordingly, functional fecundity was determined as follow:

$$FF=MONF\times GW/MFW$$

Where, FF = functional fecundity, MONF = mean oocyte number of the 3 fraction of gonad, GW = gonad weight (gr), and MFW = mean weight of the 3 fraction of gonad (gr).

Gonadosomatic index (GSI) was calculated as follow:

GSI = gonad mass (gr) / body mass (gr)

Male

Seminal pH was determined using a semi-micro-electrode (SM102 pH Meter). Spermatocrit was measured for each milt sample by filling two capillary tubes with milt and centrifuging them for 10 min in a microhematocrit (12000 rpm) and expressed

as percentage. Collectable-milt volume was determined gravimetrically. Sperm density was calculated by multiplying the collectable - milt volume to spermatocrit value.

To assess the sperm motility characteristics, 5 µl of milt was taken in a clean pipette, added to 1.5 ml of one of the activating solutions (NaCl Solution) at 5ºC and shook vigorously. A drop of the diluted and activated sperm sample was immediately added to the chamber of a Neubauer haemocytometer and sperm activity was recorded by digital camera (Canon IXUS 70, www.canon.com). The sperm characteristics were determined using these records as follow:

- Sperm motility duration: to determine the maximum duration of motility of spermatozoa, the elapsed time was measured from the moment sperm were activated until none of the sperm in the viewing grid were moving forward.

- Motile sperm percentage: to determine the motile sperm, recorded videos were checked and 30 randomly selected sperm were checked for motility, after activation. Motile sperm percentage was obtained by dividing the number of motile sperm to the whole sperm number multiplied by 100.

Fertilization and hatching rates

After mixing of the oocytes with the sperm of control fish, 10 batches of each treatment's eggs (100 eggs) were transferred into the 10 petri dishes and the number of eyeing eggs was recorded after 24 h. The fertilization rates were calculated by dividing the number of eyeing eggs to 100 multiplied by 100, for each petri dish. The values were presented as the treatments' mean ± standard deviation (SD).

To determine hatching rates, aforementioned above procedure was conducted and the number of hatched eggs were recorded. Hatching rates were calculated by dividing of the number of hatched eggs to 100 multiplied by 100, for each petri dish. The values were presented as the treatments' mean ± SD.

Serum hormones analysis

Serum 17E and testosterone (Neogen Co., Lexington, KY, USA) as well as 17OH (IBL International, Hamburg, Germany) levels were measured by ELISA method.

Statistical analysis

Data were subjected to one way ANOVA and Duncan's test to determine significant difference at $P < 0.05$ level. All data are presented as mean ± SD.

Results

No mortality was observed during the trial. Since all fish treated with 25 and 50 mg/kg diet of 17E groups were found to be female, therefore male gamete quality was compared only between the control and treated with 10 mg/kg diet of 17E groups.

Results showed significant effect of dietary 17E on female gamete quality ($P < 0.05$). Oocyte diameter was higher in treated groups with 25 and 50 mg/kg diet of 17E compared to the other groups (Figure 1). Oocyte surface: volume ratio in studied fish which had received 25 and 50 mg/diet of 17E was significantly lower than the rest groups ($P < 0.05$) (Figure 2).

Functional fecundity significantly increased with increase of the treated dose of 17E ($P < 0.05$) (Figure 3).

GSI values were significantly higher in treated groups with different dose of 17E compared to the control group ($P <0.05$) which indicated to increasing effect of 17E on gonad mass in all the studied fish (Figure 4). Our results also revealed that the oocyte diameter increased along with gonad development.

There was no significant difference in seminal pH and milt volume between the control and treated group with 10 mg/kg diet of 17E (Table 2). However, in the experiment using of 17 E at the 10 mg/kg diet had significantly decreasing effect on spermatocrit, motile sperm percentage, sperm density and motility duration compared to the control ($P < 0.05$) (Table 2).

The values of zygote diameter in all the studied groups are presented in Figure 6. According to the results, 17 E had increasing effect on zygote diameter compared to the control group and we observed significant differences between all the studied groups ($P < 0.05$), except to the treated groups with 25 and 50 mg/kg diet of 17E (Figure 5). Fertilization rate was higher in all the treated groups with 17E compared to the control groups ($P < 0.05$), but we found no a significant difference between the treated groups (Figure 5). In addition, we observed a dose dependent effect of 17E on hatching rate in gold fish,

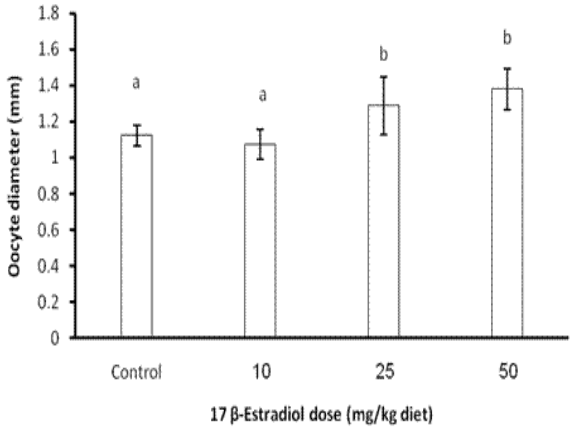
and with increase in dose of 17E resulted to significantly increase in hatching rate ($P < 0.05$) (Figure 7). The levels of serum 17E were significantly high in the treated groups with different levels of 17E compared to the control group ($P < 0.05$) (Figure 8) and the higher level of 17 E observed in treated group with 50 mg/kg diet of 17E (Figure 8). We found significant differences between all the studied groups ($P < 0.05$) and the lowest and highest values serum hatching testosterone observed in treated group with 10 and 50 mg/kg diet of 17E, respectively (Figure 9). Similar of rate and functional fecundity, the levels of serum 17OH showed a dose – dependent changes due to using the supplemented diet with different values of 17E and the values of serum 17 OH were significantly increased coincident with increasing the 17E values in the diet ($P < 0.05$) (Figure 10).

Table 2. Effect of dietary 17 β-estradiol on male gamete quality in goldfish (Mean ± SD).

	Control	*17 β-estradiol (10 mg/kg diet)*
Seminal pH	*8.28 ± 0.06* [a]	*8.32 ± 0.03* [a]
Spermatocrit (%)	*35.34 ± 0.37* [a]	*32.44 ± 0.21* [b]
Motile sperm percentage (%)	*88.62 ± 2.6* [a]	*85.36 ± 1.2* [b]
Sperm density (× 10^9 ml^{-1})	*6.12 ± 0.07* [a]	*5.78 ± 0.08* [b]
Motility duration (s)	*104 ± 4.3* [a]	*96 ± 1.87* [b]
Milt volume (ml)	*0.48 ± 0.11* [a]	*0.44 ± 0.10* [a]

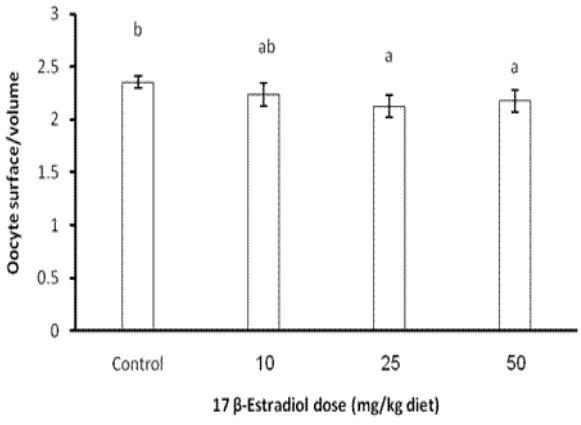
There is a significant difference between groups concerning the studied parameters with different superscript (a and b) ($P < 0.05$).

Figure 1: Effect of dietary 17β-estradiol on oocyte diameter in goldfish.



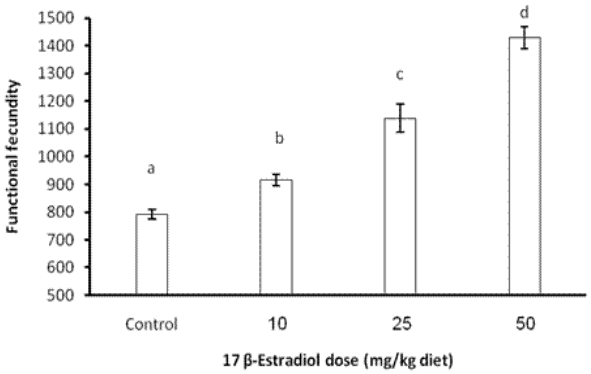
There are significant differences between groups concerning the studied parameters with different superscript (a and b) ($P < 0.05$).

Figure 2: Effect of dietary 17 β-estradiol on oocyte surface: volume in goldfish.



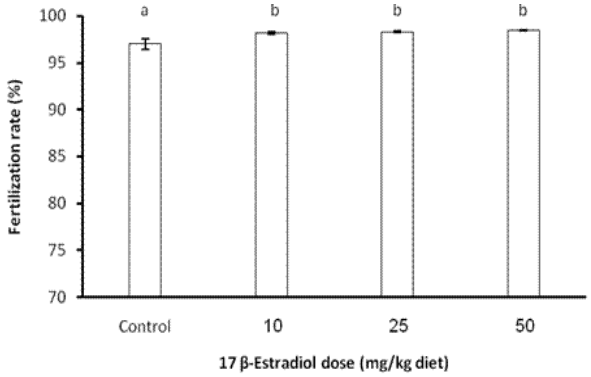
There is a significant difference between groups concerning the studied parameters with different superscript (a and b) ($P < 0.05$).

Figure 3: Effect of dietary 17 β-estradiol on functional fecundity in goldfish.



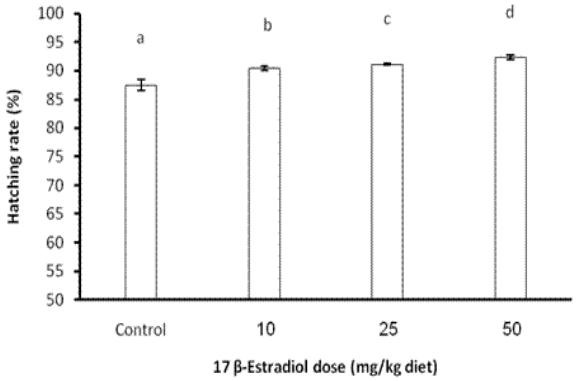
There were significant differences between groups with different letters (a, b, c and d) ($P < 0.05$).

Figure 5: Effect of dietary 17 β-estradiol on fertilization rate in goldfish.



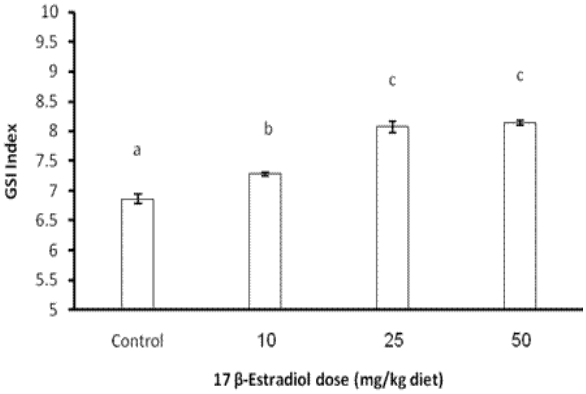
There is a significant difference between groups concerning the studied parameters with different superscript (a and b) ($P < 0.05$).

Figure 7: Effect of dietary 17 β-estradiol on hatching rate in goldfish.



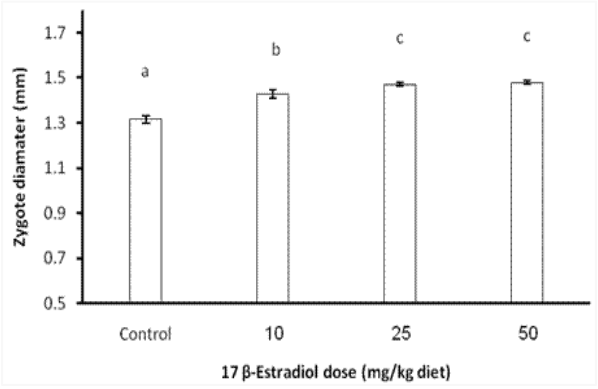
There are significant differences between groups concerning the studied parameters with different superscript (a, b, c and d) ($P < 0.05$).

Figure 4: Effect of dietary 17 β-estradiol on GSI in goldfish.



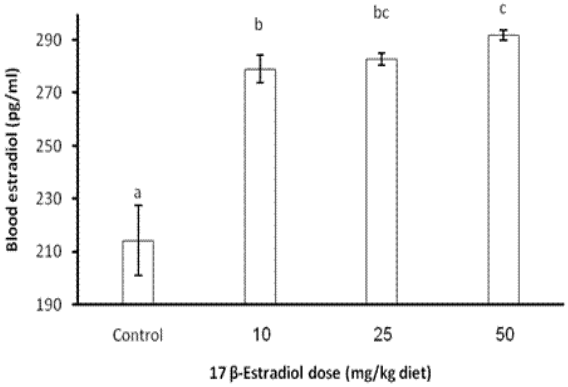
There are significant differences between groups concerning the studied parameters with different superscript (a, b and c) (P< 0.05).

Figure 6: Effect of dietary 17 β-estradiol on zygote diameter in goldfish.



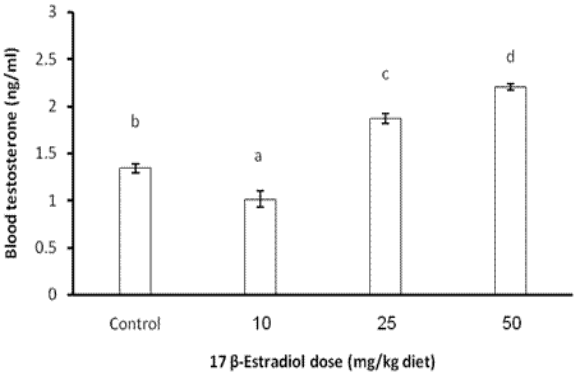
There are significant differences between groups concerning the studied parameters with different superscript (a, b and c) (P< 0.05).

Figure 8: Effect of dietary 17 β-estradiol on serum estradiol levels in goldfish.



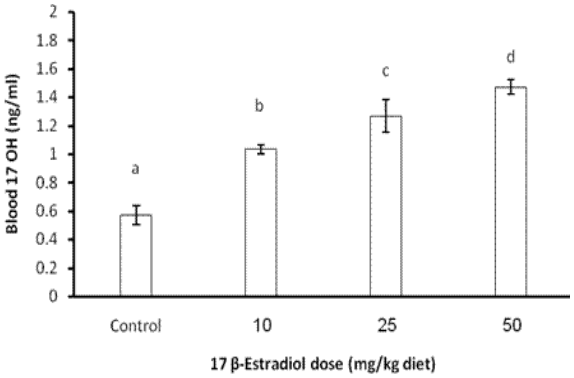
There are significant differences between groups concerning the studied parameters with different superscript (a, b and c) (P< 0.05).

Figure 9: Effect of dietary 17 β-estradiol on serum testosterone levels in goldfish.



There are significant differences between groups concerning the studied parameters with different superscript (a, b, c and d) (P<0.05).

Figure 10: Effect of dietary 17 β-estradiol on serum 17-hydroxyprogestrone levels in goldfish.



There are significant differences between groups concerning the studied parameters with different superscript (a, b, c and d) (P< 0.05).

Discussion

The present result showed the importance of exogenous 17E on gamete quality in fish. Gamete quality in turn, determines fertilization, hatching and larvae quality.

Increase in oocyte diameter, GSI and fecundity as well as decrease in oocyte surface: volume ration in 17E groups are believed to be related to estradiol. It has been previously found that estradiol has an important effect on ovary development in fish (Nagahama, 1994; Campbell *et al.*, 2006). De-Vlaming *et al*. (1984) found correlation between serum estradiol levels and oocyte size and GSI in *Leptocottus armatus*. Likewise, Specker and Sullivan (1994) reported that estradiol have modulating effect on synthesis and secretion of the precursor of yolk proteins, vitellogenin by liver. Yolk size is one of the factors affecting oocyte diameter. Thus it seems that the higher oocyte size in 17E-treated fish is attributed to higher vitellogenin synthesis by liver as well as higher yolk size in the oocytes.

17E is reported to play a central role in the hypothalamus-pituitary-gonadal neuroendocrine axis regulating fecundity and oocyte development in fish (Nagahama, 1994; Campbell *et al*., 2006); although, its exact mechanisms are relatively unknown (Tyler and Sumpter, 996; Jalabert *et al*., 2000). Estrogens have been regarded as activator on oogonial proliferation (Jalabert *et al*., 2000). Higher functional fecundity in the present study in 17E-treated fish seems to be due to increment in ovulation rate. Ovulation is affected by maturation - inducing hormone, 17α, 20β- Dihydroxy- 4- pregnen- 3 - one (Nagahama 1997; Delvin and Nagahama, 2002). This hormone is synthesized from 17OH by the action of the enzyme 20β- hydroxysteroid dehydrogenase (Nagahama 1997; Delvin and Nagahama, 2002). Since higher levels of 17OH were observed in 17E-treated fish, it might that the rate of the 17α, 20β-Dihydroxy- 4- pregnen- 3- one synthesis increases which in turn extols the number of the ovulated oocytes. Previous studies on salmonids (Lahnsteiner *et al*., 2006) and zebra fish (Brion *et al*., 2004) showed increase in the number of ovulated fish following estradiol therapy. Likewise, Jobling *et al*. (2003) showed increase in the mean number of spawned eggs as a result of exposure to low concentrations of estradiol in fathead minnow *Pimephales promelas*.

The investigated semen quality showed decrease in 17E-treated fish. It is suggested that estradiol administration to male fish leads to the regression of testicular tissue and to the development of secondary ovaries (sex reversal) (Devlin and Nagahama, 2002). Complete sex reversal was observed in the treated groups with 25 and 50 mg/diet of 17E as no male individuals was present in these groups. Decrease in semen quality was reported in male rainbow trout *Oncorhynchus mykiss* exposed to 17β- ethinyl-estradiol (Schultz *et al*., 2003). Lahnsteiner *et al*. (2006) reported decrease in semen volume, sperm density and semen fertility in rainbow trout, *O.mykiss,* exposed to 17E over 50 days. Decrease in the secondary sex characteristics was observed in male fathead minnows, *P. promelas,* after 17E

exposure, including reduced nuptial breeding tubercles number as well as diameter (Miles-Richardson *et al*., 1999).

In the present study, fish were sampled approximately at the end of the vitellogenesis stage for hormone assay. According to Hyllner *et al*. (1994) 17E is responsible for vitellogenine synthesis in the liver, thus the levels 17E is assumed to be elevated during the vitellogenesis stage. However, the higher levels of serum estradiol in 17E-treated fish seem to be due to exogenous estradiol intake, because the 17 OH levels were high in these groups and elevation in this hormone shows initiation of final oocyte maturation and ovulation (Peter and Yu, 1997). Likewise, the higher levels of 17OH might suggested that the fish treated group with 17E have more developed oocyte than control group, since this hormone levels increase after vitellogenesis stage termination.

However, testosterone pattern is hard to interpreting between the treatments. Testosterone has been suggested to be elevated during vitellogenesis, but decrease during final maturation (Kime, 1993; Linard *et al*., 1995). Such pattern might be due to endocrine disruption effect of exogenous estradiol. Endocrine disruption effect of estradiol has been reported in previous studies (Segner *et al*., 2003; Jobling *et al*., 2003; Schultz *et al*., 2003).

Conclusion

In conclusion, dietary 17E increases oocyte diameter, oocyte surface: volume ration, GSI, functional fecundity in female and decreases semen quality in male goldfish. Likewise, egg diameter, fertilization and hatching rates improve as a result of 17E treatment. However, it may cause some hormonal disturbance, as well. Moreover, hatching rate, functional fecundity and the levels of serum 17OH showed a dose - dependent variation due to using the supplemented diet with different values of 17E and their values were significantly increased coincident with increasing the 17E values in the diet.

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