Research Article

Study on histophysiological effects of dietary soy extract on gonads in Yellowfin Seabream, *Acanthopargus latus*

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Abstract

The present study has performed to investigate the effects of different levels of soy phytoestrogens on gonad histological structure and E_2 and P_4 sex hormone levels in Yellowfin Seabream (*Acanthopagrus Latus)* . A total of 60 Yellowfin Seabream with an average weight of 73.70 ± 9.53 kg were divided into four groups: one control group that were feeding with standard diet without any extract and three treatment groups that received different doses of diet containing soy extract (2.5, 5 and 7.5 percent) in three replicates. Fish were fed at the rate of 3% of body weight twice a day for 14 days. Samples were taken in days of 0, 7, 10 and 14. Gonad sampling was done for histological studies and for evaluation of E_2 and P_4 levels, blood samples were collected from tail vein. The results showed that different doses of soy extract have various effects on gonads and in total, considering that *A. latus* is a protandrous hermaphroditic fish, by increasing the dose and days of exposure to soybean extract, the ovary returned to the previtellogenic state and also the appearance of testicular tissue in gonad was observed. Also, the measurement of E_2 and P_4 hormone levels showed that generally, decrease in plasma E2 levels and increase in plasma P4 levels was seen after feeding meal diets containing 2.5%, 5% and 7.5% soy extract in yellowfin seabream ($P>0.05$), which supported the findings that soybean phytoestrogens lead to endocrine disruption by different mechanisms, as shown in previous studies.

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Introduction

Phytoestrogens are plant derived compounds found in wide variety of foods. soybean contains high amounts of phenolic compounds which are known as estrogenic compounds or isoflavonoid phytoestrogens. Genistein and daidzein are known as the most abundant isoflavones in soybeans (Cleveland *et al.*, 2015). Isoflavones structure are similar to estrogen and have been shown to exert their estrogenic effects by binding to estrogen receptors (Ng., 2006) actually these phytoestrogens can impact on reproductive health due to their structural similarity to estradiol (Pool *et al*., 2022). Estrogens, such as estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol, phenols, such as bisphenol A, and alkylphenols, such as 4-octylphenol and 4-nonylphenol, and phytoestrogens, such as daidzein, genistein and biochanin A, have different origins and physicochemical properties but all of them may cause endocrine disruption in fish (Ribeiro *et al.*, 2009). It has been shown that exposure to phenols and alkylphenol ethoxylates (APEs) results in vitellogenesis, an estrogenic-response, gonadal atrophy and hermaphroditism in male fish (Purdom *et al*., 1994). Bagheri et al 2013 showed that inclusion of soybean meal in the diet can lead to a sex hormones biosynthesis disruption, which ultimately leads to reproductive disorders in fish and decreased hatching rates in the offspring. They have stated that the effect on the sex steroid - binding protein in plasma can have an effect on steroidogenesis, and this is probably one of the ways of isoflavonoids action on the other hand, it has been shown that eqoul, a component of soy isoflavones, can be used as an additive to diets for inducing ovary development in Beluga sturgeon, Huso huso (Yousefi Jourdehi *et al*., 2014). Yellowfin seabream, *Acanthopagrus latus*, is a porgy of the sparidae family with high potential for aquaculture in the Indo-Pacific region due to its high market value, easy adaptation to captivity and availability of production technology (Hesp *et al*., 2004; Sà *et al*., 2006). Yellowfin seabream has been reported as an omnivorous species and protandrous hermaphrodite distributed widely in the Indian Ocean including Persian Gulf (Karimi *et al*., 2014). In this study, the effects of oral administration of diet containing different amounts of soybean extract on histological structure of gonads and different levels of estrogen and progesterone hormones in the blood of yellowfin seabream were investigated.

Materials and methods

Preparation of soya ethanol extract and experimental diets

Soybean meal (prepared by Sobhan company) was dried at 23 °C and pulverized with a blender and the powder (300 g) was dissolved with 750 ml of 96% ethanol and 250 ml of distilled water solution. Extraction

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process was done by shaking with frequent shaking and stirring for 72 hours at room temperature. and the obtain composition was passed once through one-layer filter paper and once through two-layer filter paper, by Buchner funnel and a vacuum pump, and in the next step it was placed in a rotary evaporator at 50 °C at 70 rpm, that up to 1/3 of initial volume concentrated. Petri dish containing the solution was placed in the oven at 40°C for 24 hours to a final concentration. Extraction was kept at −20°C freezer until use.

In this study, a control group that received only original diet meal (prepared by 21 beyza feed mill) and three treatment groups that received three different doses of phytoestrogen extract combined with original diet meal (2.5, 5 and 7.5 percentage of food weight) (Huang *et al*., 2010) were studied. To prepare a dose of 2.5, 89 ml of soy extract and 975 g of original diet meal, for dose of 5, 178 ml of soy extract and 950 g of original diet meal and for dose of 7.5, 267 ml of soy extract and 925 g of original diet meal were combined. All ingredients were mixed for 45 min. Then, 30 ml of boiled and cooled water in temperature of 15 °C was added to the mix until uniform dough was formed. The dough was passed twice through a meat grinder to form strings. The strings were dried at room temperature (25 °C) and were split according to the size of the fish mouth. Made diet were kept in the refrigerator (4 °C) until use.

Fishes were acclimated for a week in wet lab in 300 L indoor tanks containing filtered aerated pond water and were fed daily with standard diet. After adaptation they were divided into four groups with three replications: one control group that were feeding with standard diet without any extract and three treatment groups that received different doses of diet containing soy extract (2.5, 5 and 7.5 percent). Fish were fed daily at a rate of 3% of body weight twice for 14 days.

Fish and sample collection

Sixty wild *Acanthopagrus latus* were caught (73.70 ± 9.53 gr) from the Naseri Pond (Khuzestan, Iran) and were transferred immediately in 300 L fiberglass tank, equipped with aeration to the wet laboratory of Khorramshahr University of Marine Science and Technology and distributed randomly among the 12 experimental tanks, fish were divided into four groups. Water temperature, salinity, dissolved oxygen and pH were recorded daily. Salinity ranged between 18 – 22 ppt and temperature was between 19 - 23 °C and during the experimental period, average values for dissolved oxygen and pH were 7.5 mg $l^{-1}$and 7.9 respectively. Water substitution was 48 hours, up to 90 % of the water in the tank to avoid ammonia.

Sampling was carried out in days of 0, 7, 10 and 14 (Hossain *et al*., 2022; Kakeshian *et al*., 2022). Anesthesia was performed by gillyflower extract at a rate of 1 ml/L. Total

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length and body weight were measured, Blood samples collected from the caudal vein by nonheparinized syringes. Blood was centrifuged and the separated plasma stored at -70°C for hormonal analysis. After dissection, the gonads were weighed to calculate the gonadosomatic index (GSI: gonad weight/ body weight × 100) (Spanò *et al*., 2004).

Histology

For histological studies the mid portion of the right gonads was fixed in formalin (10%) solution, dehydrated in ethanol series and were passaged in autotechnicon and were blocked by paraffin wax. 5µm sections were made by microtome and stained by hematoxylin and eosin for histological examination under light microscopy. Diameter of oocytes during the oocyte growth was measured by Dino-lite Capture 1. Ovaries were classified into different stages of development based on the relative abundance of different types of oocytes in different growth stages (Shinkafi *et al*., 2011).

Hormonal analysis

Plasma hormonal analysis to determine the levels of E_2 and P_4 hormones was down by ELISA method (Mimeault *et al*., 2005).

Statistical analysis

The results were analyzed by SPSS software version 16. All data are presented as (mean ± S.D). Data were analyzed by one-way ANOVA and Duncan tests. Differences were considered significant when P value was lower than 0.05.

Results

Histology

Histological study of control samples taken at day zero showed that all of the gonads were ovary and in all three cases 100% of follicles were in pre-vitellogenic stage (fig. 1).

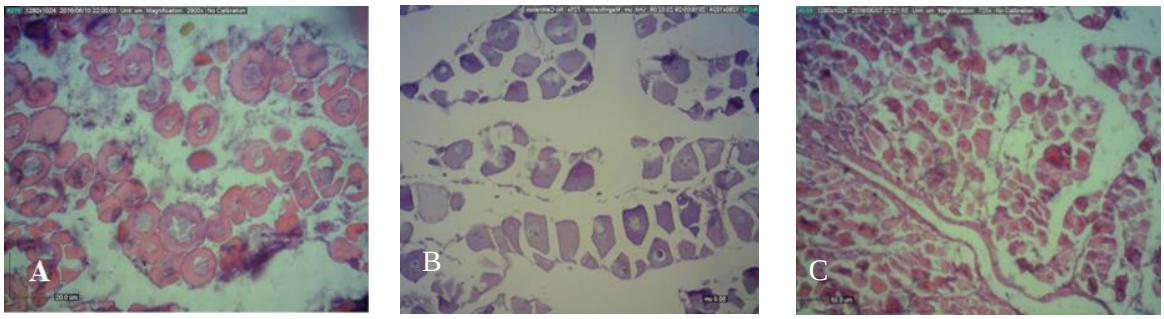


Figure1. Ovarian control samples on day zero. All ovaries were immature (H&E×2900).

Histological study of gonads after 7 days in all doses showed appearance of vitellogenic follicle, as in 2.5 dosage treatments in the first sample 49% of follicles were in vitellogenesis stage and 51% of follicles were in previtellogenesis stage. In the second sample, 41.4% of follicles were in vitellogenesis stage and 58.6% of follicles were in pre vitellogenesis stage and in the third sample, 100% of

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follicles were in pre-vitellogenic stage. In 5 dosage treatments in 7th day, in the first sample 32.4% of follicles were in vitellogenesis stage and 67.6% of follicles were in pre vitellogenesis stage. In the second sample, 31% of follicles were in vitellogenesis stage and 69% of follicles were in pre vitellogenesis stage and in the third sample, 15.5% of follicles were in vitellogenesis stage and 84.5% of follicles were in pre vitellogenesis stage. In 7.5 dosage treatments in 7th day, in the first sample 24% of follicles were in vitellogenesis stage and 76% of follicles were in pre vitellogenesis stage. In the second sample, 100% of follicles were in pre vitellogenesis stage and in the third sample, 19.5% of follicles were in vitellogenesis stage and 80.5% of follicles were in pre vitellogenesis stage. Histological study of gonads after 10 days showed that in dose 2.5, increasing in number of vitellogenic follicules was observed, as in 2.5 dosage treatments in the first sample 45.6% of follicles were in vitellogenesis stage and 54.5% of follicles were in pre vitellogenesis stage. In the second sample, 36.7% of follicles were in vitellogenesis stage and 63.3% of follicles were in pre vitellogenesis stage and in the third sample, 35.9% of follicles were vitellogenesis stage and 64.1 % of follicles were in pre-vitellogenic stage. Histological study of gonads on the 10th day in 5 and 7.5 treatments, of the three samples examined in each dose, in two samples only testicular tissue was detected and in a sample of both doses immature ovarian tissue (100% previtellogenic follicles) was observed. On 14th day samples, in dose 2.5, the first and the second samples, 100% of follicles were in previtellogenesis stage and in the third sample, 46% of follicles were in vitellogenesis stage and 54% of follicles were in pre vitellogenesis stage. In dose 5, all three gonad samples testis were reported. In dose 7.5, all three samples contained 100% small previtellogenic follicles along with testicular tissue (fig 2,3 and 4).

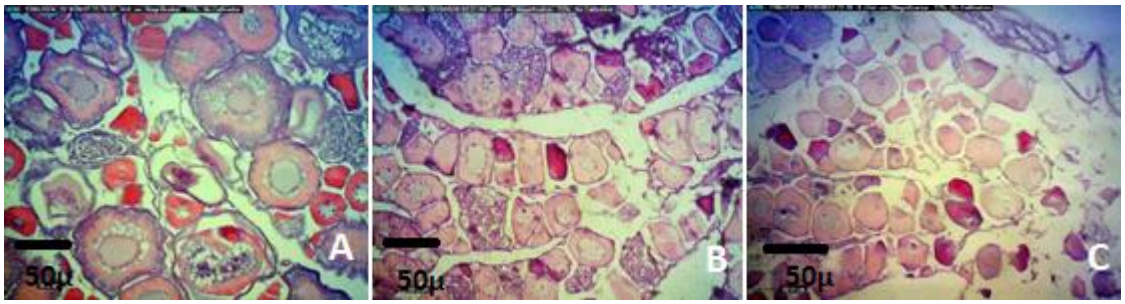


Figure 2. Microscopic samples of gonad tissue of fish receiving %2.5 dose of soybean extract, on the 7th day follicles in vitellogenic stage (A); on the 10th, follicles in vitellogenic and previtellogenic stage (B); on the 14th day most of follicles in previtellogenic stage (C) (H&E×2900).

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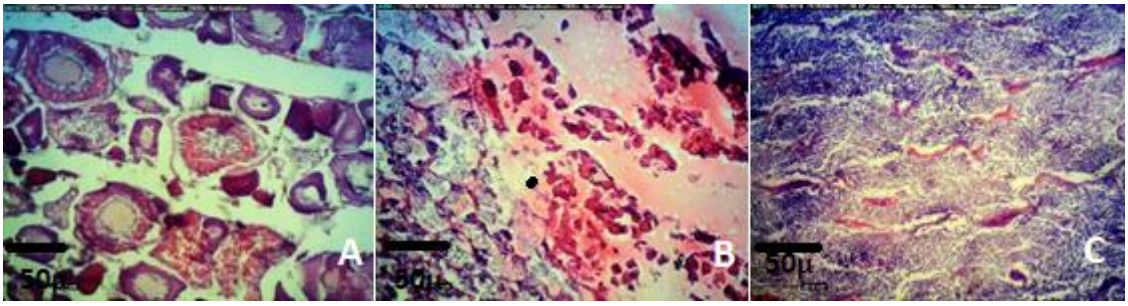


Figure 3. Microscopic samples of gonad tissue of fish receiving %5 dose of soybean extract, on the 7th day follicles in vitellogenic stage (A); on the 10th, primary ovary with testicular tissue (B); on the 14th day testicular tissue (C) (H&E×2900).

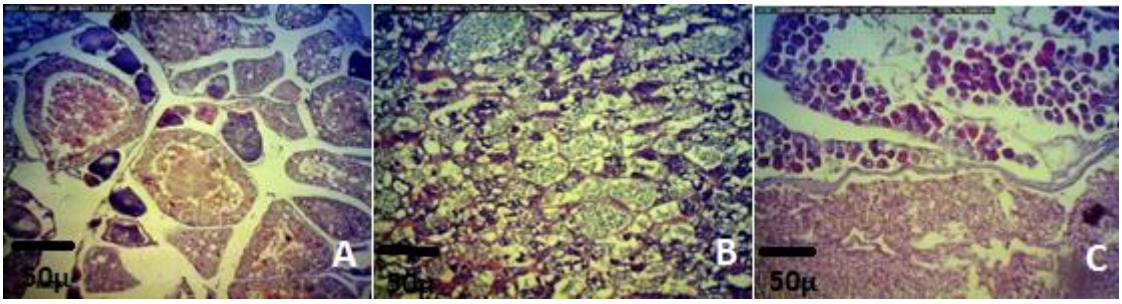
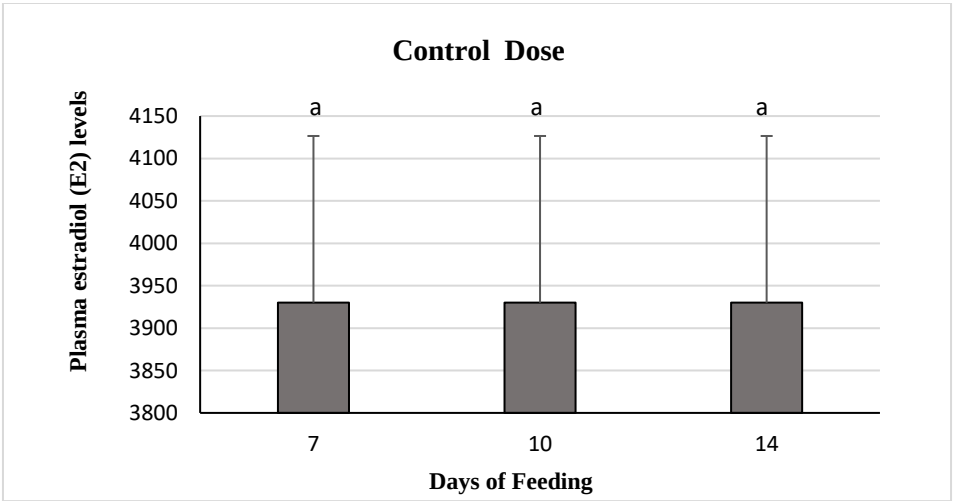


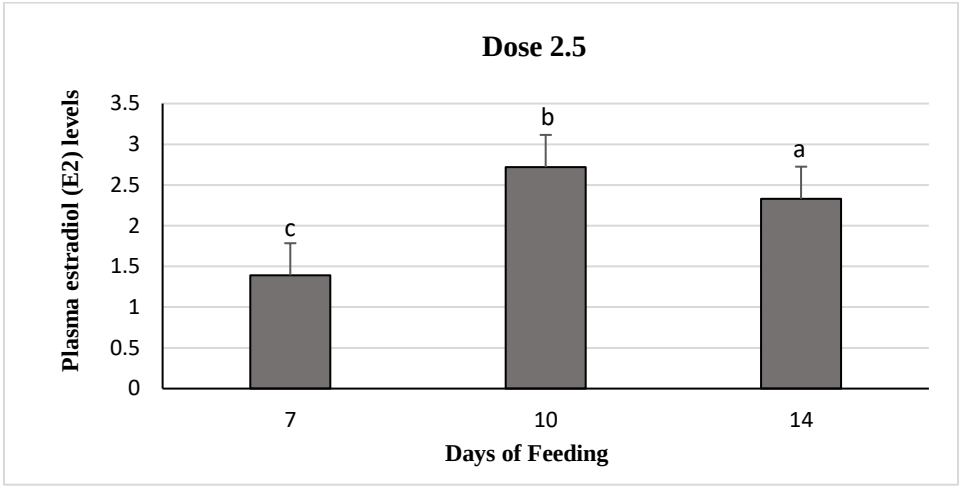
Figure 4. Microscopic samples of gonad tissue of fish receiving %7 dose of soybean extract, on the 7th day, vitellogenic stage (A); on the 10th, testicular tissue (B); on the 14th day primary ovary with testicular tissue (C) (H&E×2900).

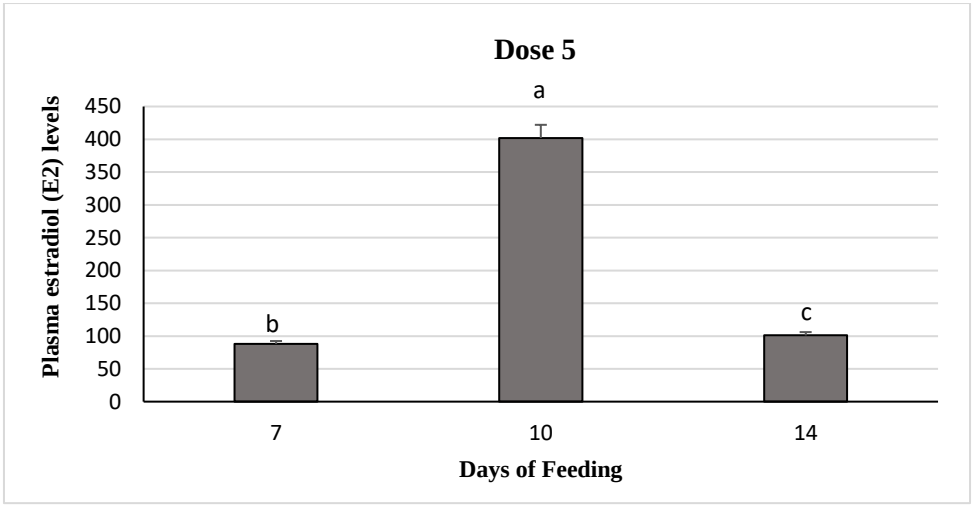
Plasma sex hormones (E2 & P4)

In the present study, generally, decrease in plasma E2 levels and increase in plasma P4 levels was seen after feeding meal diets containing 2.5%, 5% and 7.5% soy extract in yellowfin seabream (fig 5 and 6).



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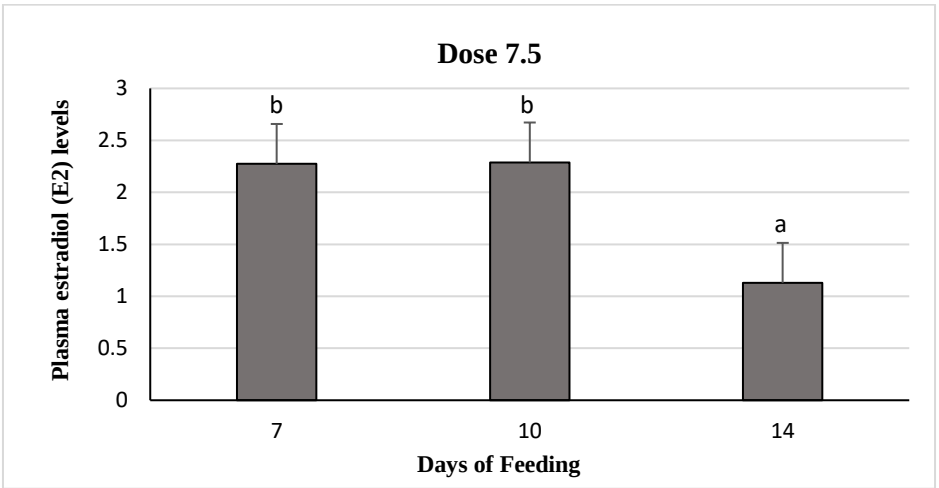
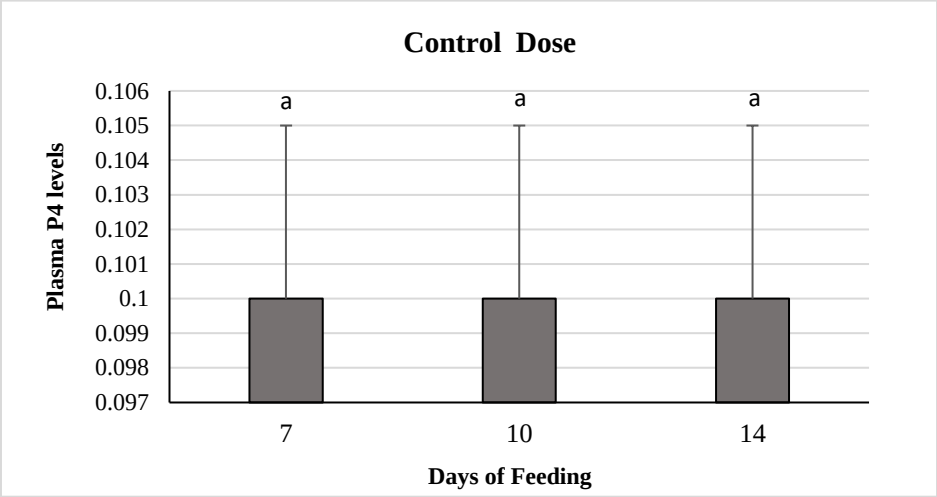
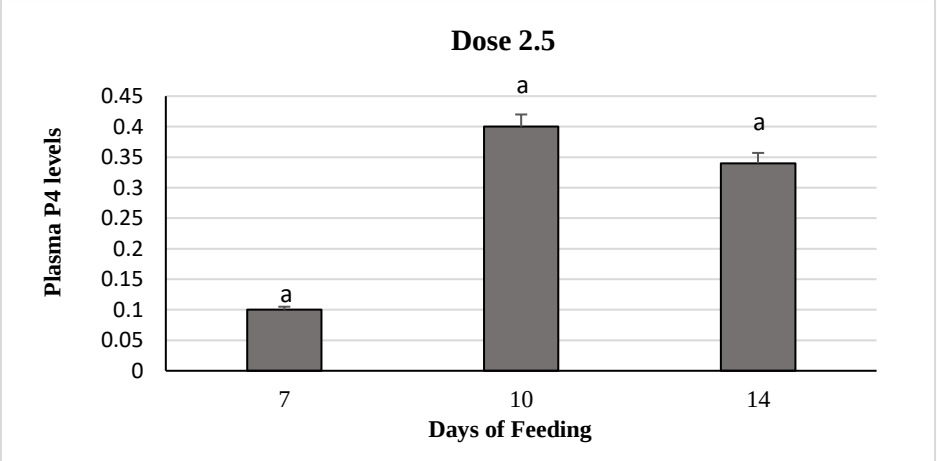
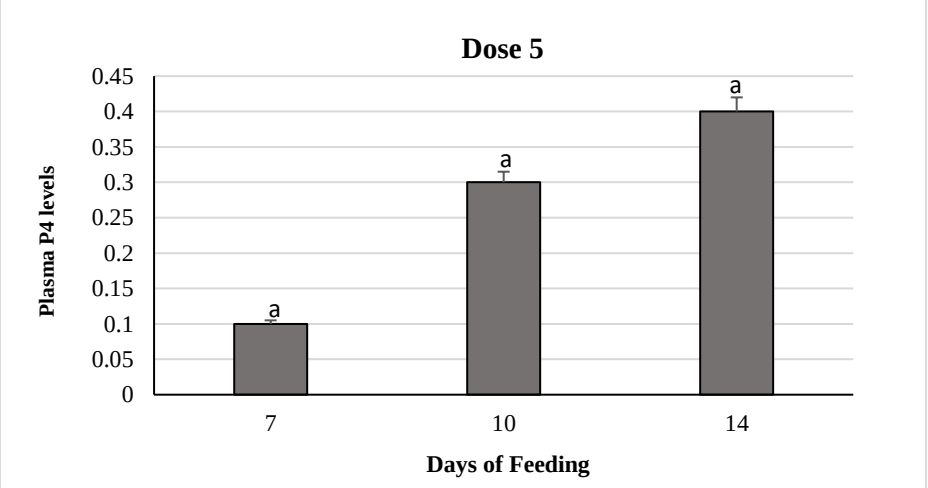


Figure 5. Plasma estradiol (E2) levels in yellowfin seabream in control groups and after feeding with diets containing (2.5%, 5% and 7.5%) soy extract for 2 weeks. Superscript letters indicate statistically significant differences between means at $P \geq 0.05$.

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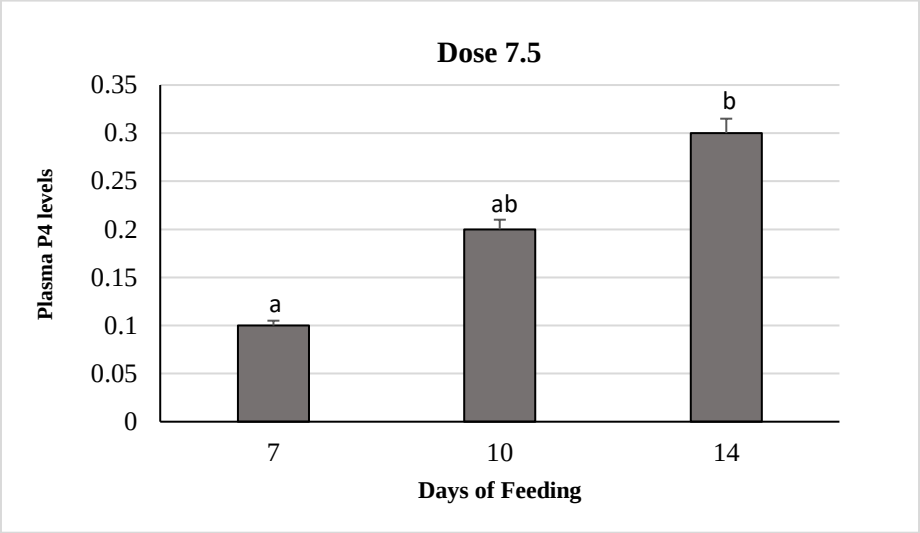


Figure 6. Plasma P4 levels in yellowfin seabream in control groups and after feeding with diets containing (2.5%, 5%, and 7.5%) soy extract for 2 weeks. Superscript letters indicate statistically significant differences between means at P ≥ 0.05.

Discussion

In the present study in total, by increasing the dose and days of exposure to soybean extract, the ovary returned to the previtellogenic state and the appearance of testicular tissue in gonad was observed. In some studies, after exposure to phytoestrogens have been observed results such as reduced gonad size altered secondary sexual characteristics impairment of the biosynthesis of sex steroid hormones and increases in ovarian apoptosis, or follicle death (van den Heuvel *et al.*, 2002; Tempfer *et al.*, 2009). Bagheri *et al* 2013 showed that soybean meal in the diet of goldfish can lead to disturbances in the biosynthesis of sex hormones and reproductive disorders. Also, Yousefi Jourdehi *et al* 2014 has been shown that eqoul, a component of soy isoflavones, can be used as an additive to diets for inducing ovary development in Beluga sturgeon, *Huso huso*. On the other hand in a study on the effects of garlic extract (as phytoestrogens) on Yellowfin seabream (*Acanthopagrus latus*), it has been shown that by increasing the extract, at first an increase in the number of vitellogenic follicles and also an increase in ovarian maturation was observed, but using higher doses and also increasing the time of taking the extract led to the opposite results (Kakeshian et al., 2022). Phytoestrogens and its derivatives (Genistein, daidzein, Coumestrol and the École) can impair reproductive function. May bind to estrogen receptor agonists to the estrogen receptor, and stimulates expression of the genes related to estrogen or may as antagonists binding endogenous estrogen to its receptor disrupt and have physiological antiestrogenic effect (Inudo *et al.*, 2004). In

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sturgeon led to a decrease plasma estrogen level and moreover hyperandrogenism plasma (Patel *et al*., 2016; Pelissero *et al.*, 1996).

Several mechanisms of genistein action on P4 and E2 production were indicated. In the inhibition of the tyrosine kinase – dependent signaling system phytoestrogens are barrier repression. Genistein was characterized as a potent and relatively specific inhibitor of protein tyrosine kinases (Nynca *et al*., 2013). In males, exposure to environmental estrogens may be responsible for the reported decline in human sperm counts and the apparent increase in the incidence of cryptorchid testes, testicular cancer and hypospadias. Some results indicate that genistein inhibits growth of antral follicles. Additionally, it has been shown that genistein increases progesterone, testosterone and dehydroepiandrosterone levels, but decreases estrone and estradiol levels (Patel *et al*., 2016). These findings are in agreement with the results of the present study that decrease in plasma E2 and increase in plasma P4 levels was seen after feeding meal diets containing increasing percent of soy extract. Depending on the tissue and the concentration of circulating endogenous estrogens phytoestrogens may have both estrogenic and anti-estrogenic effects. The underlying mechanisms are different (Bagheri *et al*., 2013). Also it has been shown that phytoestrogens can changes molecular pathways by changing the expression profile of sex-related genes, which can ultimately lead to sexual differentiation disorders in sturgeon (Fajkowska, 2021).

Although the response patterns for the steroids and phytoestrogen is not well known, the interspecies differences in the effects of phytoestrogens that have been investigated suggest that vitellogenin production varies depending on the species and also on different life stages of individuals (Van den Belt *et al*., 2003; Holbech *et al*., 2013). There is no known mechanism to phytoestrogens and estrogen (Holbech *et al*., 2013). Estrogen can increase the concentration of endogenous estrogen or cut its production by creating disorder in the form of metabolites. May contain isoflavones bind to estrogen circulating in the target tissue (Ng *et al*., 2006). Researchers believe that some effects of soy genestein is due to similarities between the structure of genestein and estrogen. This structural similarity, allows genestein to displace estrogen from cellular receptors thus blocking their hormonal activity (Sharifi – Rad, 2021).

The highest number of vitellogenic follicles was observed in dose 2.5% on the 10^{th} day. But overall the present study showed that feeding with diets containing soybean extract meal significantly disrupted plasma E2 and P4 levels in yellowfin seabream, which supported the findings that soybean phytoestrogens disrupt endocrine

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homeostasis in previous studies in vitro and in vivo.

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Conflict of interest

The authors have no conflict of interest in this work.

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