Reproductive effects of dietary soy phytoestrogens, genistein and equol on farmed female beluga, *Huso huso*

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Summary

In this study, 54 five-year-old farmed female beluga at stage II of sexual maturation were treated with 0 (control), 0.2, 0.4, 0.8 and 1.6 g of dietary soy equol (EQ) and genistein (GE) per each kilogram (kg) of diet during a year. Blood samples were collected and ovarian biopsy was performed quarterly. Results showed that 64.4% of the *Huso huso* sexually matured at EQ 0.4 g/kg and reached stage IV of sexual maturation. Oocytes diameters increased significantly at all concentrations of EQ and 0.2, 0.4 and 0.8 concentrations of GE and reached a maximum (3 ± 0.2 mm) at 0.4 g/kg EQ concentration at the end of experiment ($P<0.05$). Blood plasma testosterone (T) level was 0.3 ± 0.06 ng/ml at the beginning and reached a maximum (21.04 ± 1.91 ng/ml) at EQ 0.4 g/kg at the end of the experiment ($P<0.05$). 17β-estradiol (E_2) levels increased significantly at some concentrations of GE and EQ at the end as compared to the beginning, reaching a maximum (12.6 ± 1.04 ng/ml) at EQ 0.4 g/kg at the end of the experiment ($P<0.05$). 17α-hydroxy progesterone (17α-OHP) levels showed no significant difference ($P>0.05$). In conclusion, EQ at a 0.4 g/kg concentration showed more powerful positive reproductive effects than other concentrations of EQ and GE in farmed female *H. huso*. Comparatively, EQ showed more estrogenic effects on ovary development in comparison to GE concentrations. Its use is therefore suggested as an additive to diets to induce ovary development in *Huso huso*.

Key words: Phytoestrogens, Equol, Genistein, Reproduction, *Huso huso*

Introduction

Phytoestrogens are plant-derived substances that can activate estrogen receptors (ER) (Cassidy *et al*., 2000). Isoflavones are a group of phytoestrogens found in many plants, particularly the legume family, often associated with a reduced risk of breast, colon and prostate cancer in human beings (Setchell and Cassidy, 1999; Magee and Rowland, 2004). The major constituents of soy isoflavones, genistein (GE) and equol (EQ) interact with estrogen ER in several tissues including gonads. The estrogenic activity can be enhanced after metabolization to more active compounds such as genistein and daidzein by gut microorganisms (Zhengkang *et al*., 2007). Genistein and daidzein are two of the major metabolites from isoflavones that can be further metabolized by intestinal microorganisms (Schoefer *et al*., 2002; Wang *et al*., 2005). Daidzein can be metabolized to EQ (King and Bursill, 1998).

In fish, vitellogenin (VTG), the major constituent of yolk, is synthesized in the liver under estrogenic induction and transferred to the ovaries and oocytes by binding to specific ER associated with endocytotic vesicles (Le Menn and Nunez-Rodrigues, 1991). In fish, only reproductively active females normally synthesize VTG. Estradiol is the main inducer during the reproductive cycle (Fostier *et al*., 1983). Estrogenic effects of phytoestrogens are well documented in fish (Pelissero *et al*., 2001). Phytoestrogens can bind to steroid-binding proteins (Dechaud *et al*., 1999) and estrogen ER of target cells (Casanova *et al*., 1999). Moreover, they exhibit endocrine-disturbing activities that interfere with enzymatic reactions either on steroid metabolism, i.e., aromatization (Chen *et al*., 1997) or on the mechanism of action of estrogens, i.e., tyrosine kinase activity (Huang *et al*., 1999). Phytoestrogens are able to stimulate VTG synthesis *in vivo* in Siberian sturgeon (Pelissero *et al*., 1991) and *in vitro* in trout hepatocyte (Pelissero *et al*., 1993). Intraperitoneal injections of GE and EQ to yearling Siberian sturgeon (*Acipenser baerii*) induced vitellogenesis, a physiological response associated with estrogenic activity (Pelissero *et al*., 1991).

Due to its rapid growth and adaptation to nutrition regimes and farm conditions, beluga (*Huso huso*) is one of the most important sturgeon species in the world. As a result of the long length of the reproductive cycle of Caspian Sea beluga (18 years for females), many

countries including Iran are keen on farming this species in fresh waters. Considering the late maturity of *H. huso*, this study was carried out aiming at using dietary soy phytoestrogens GE and EQ to accelerate ovary development in female *H. huso* to obtain caviar in a shorter period of time.

Materials and Methods

Fishes and the experimental design

Experiments were carried out on 54 five-year-old female *H. huso,* farmed in terrestrial ponds of the Caspian Sea International Sturgeon Research Organization from summer 2011 to summer 2012. Gonads of all fishes were detected at sage II of sexual maturation by biopsy and laparoscopy methods at the start of the experiment. They had an average body weight of 13.25 ± 0.3 kg and a total length 140.3 ± 1.2 cm. Anesthesia was performed using 250 ppm Clove powder for 10 min. Fish were selected by biopsy and laparoscopy methods using a Stema Co. (Germany), model M-CAM1700, 30 degree telescope (4 mm in size, 17.5 cm in length and a 250 W cold light source halogen), and were stocked randomly in 18 concrete ponds ($3 \times 2.5 \times 1.2$ m^3) equipped with aeration systems and holes to mix in the Sepidroad River water. The fish were treated with different concentrations of GE and EQ. A commercial beluga diet was prepared with varying levels of purified GE and EQ (Xi, a Keen-Source Biotechnology Co., Ltd.). Genistein and EQ dissolved in 75 ml ethanol were added to the diet at 0, 0.2, 0.4, 0.8 and 1.6 g/kg concentrations. The feed was mixed in a mixer in 2000-g portions for 7-10 min with 15% water. The experimental diets were prepared with a standard pelleter with a 12 mm diameter, were mixed from low to high levels of GE to minimize overlap in GE and EQ concentrations, and were air-dried for 24 h. Each treatment included 6 fish in two replicates with 3 fish per pond. The diet which was 1% of their biomass, and consisted of 45% crude protein, 14% fat, 20% carbohydrate, 9% ash, less than 10% moisture and 19.5 Mj energy, was given to the fish twice a day. Physicochemical parameters were measured using an Oxi-pHmeter40i (WTW, Germany) daily. Oocyte diameter was measured using a scaled ruler and a stereomicroscope. Histological studies were carried out after fixating samples in Buin's solution for 48 h. After dehydrating, paraffinating and mounting, samples were sectioned (5-7 µm) by a Microtome set (Leitz 1512, Germany), stained using Haematoxilen and Eosin (H&E) methods and studied with a light microscope. Blood samples (5 ml for serum) were seasonally taken from a caudal vein.

Blood analysis

Blood plasma was separated using a centrifuge (Labofuge 200, Heraeus Sepatech, Germany) with 3000 rpm per 10 min. Samples were stored at -20°C (Pottinger and Carrick, 2001). Steroid hormone levels (including testosterone (T), 17α-hydroxy progesterone (17α-OHP) and 17α-estradiol levels) were measured using a radio immune assay (RIA), an Immunotech kit, and an I_{125} (France) and gamma counter LKB (Finland) based on ng/ml in the Dr. Fadaie Laboratory (Rasht, Iran).

Statistical analysis

A one-way ANOVA was used to compare the 2 groups and a Duncan's test was used for differential studies. Levene's test was used to test for equality of variance. An independent-sampled t-test was run to detect differences between hormonal indices. Excel and SPSS 17 software were used for data analysis. Data are presented as means ± SEM.

Results

Ovary laparoscopic and histological observations

Ovaries at stage II

Oocytes were not round but orthogonal. Yolk resembled a narrow strip. Protoplasmic growth and increase in oocyte diameter were detectable at this stage. Nucleoli were completely attached to the membrane of the nucleus. A chromatin mass was observed in the center of the nucleus. The number of nucleolus close to the nucleus membrane was raised and vacuoles were observed around the nucleus inside the cytoplasm. The animal pole was not differentiated from the vegetal pole. The appearance of pigments in lateral layers of the oocyte cytoplasm was the most obvious index of transition from stage II (Figs. 1A, D).

Ovaries at stage III

In this stage the growth of oocytes was associated with a reduction in fat layer. Along with the thickening of oocytes, pigments and fat tissue were observed in the middle and lateral parts of the gonads; however, they did not completely fill up the body cavity. Pigments were dark gray in color and formed beneath the cell membrane. Oocytes firmly adhered to the fat layer of the ovary. As a result, they did not pass through special caviar processing sieves. The ovary was fully grown in volume and oocytes were visible to the naked eye, yet indistinguishable from each other. Blood vessels were clearly dispersed. A thick layer of fat was present in the middle part of ovary while the oocyte was found in its lateral area. In comparison to the previous stage, oocyte abundance and volume was higher and fat was reduced. Oocytes passed through the sieve in this stage (Figs. 1B, E).

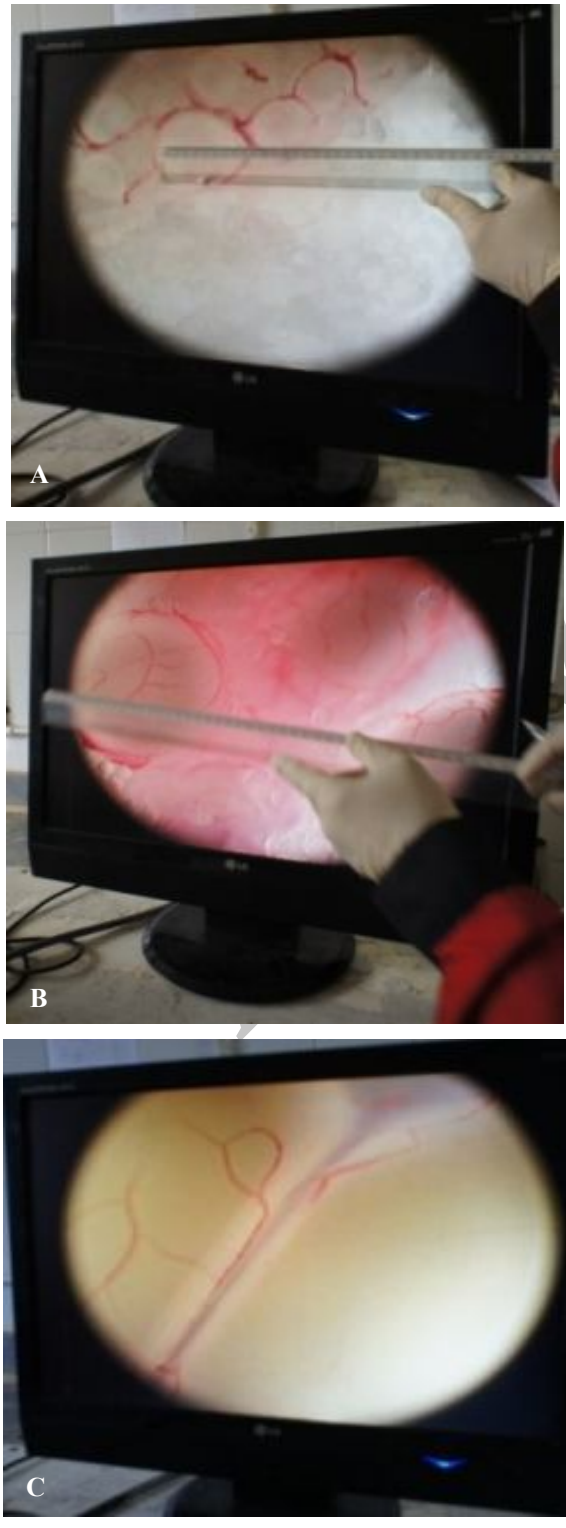
Ovaries at stage IV

Yolk granules almost filled the whole space around nucleus and only a tiny cytoplasm was spread out near the nucleolus and oocyte membranes. Small yolk granules and nuclei were located in the animal pole while larger yolk granules and fat droplets were in the vegetal pole. The nucleus was positioned from the cell center toward the animal pole at the micropyle site. Nucleoli were mostly situated at the center although few were

distributed in different areas of the cell. Based on the histological study on the ovaries of *H. huso,* 9 major layers (from outside to inside) are discernible at stage IV of maturity: the follicle epithelial layer, the jelly coat, the external and internal zona radiata, the fat layer, pigments, cytoplasm, nucleus, nucleoli and micropyle (Figs. 1C, F).

Oocyte diameter

Oocyte diameter showed no significant difference at different concentrations of GE at the end of the experiment, but increased significantly at EQ 0.4 g/kg and reached a maximum of 3.1 ± 0.2 mm. Significant differences were also observed at all concentrations except for the GE 1.6 g/kg and control between the beginning and end of the experiments ($P<0.05$) (Fig. 2).



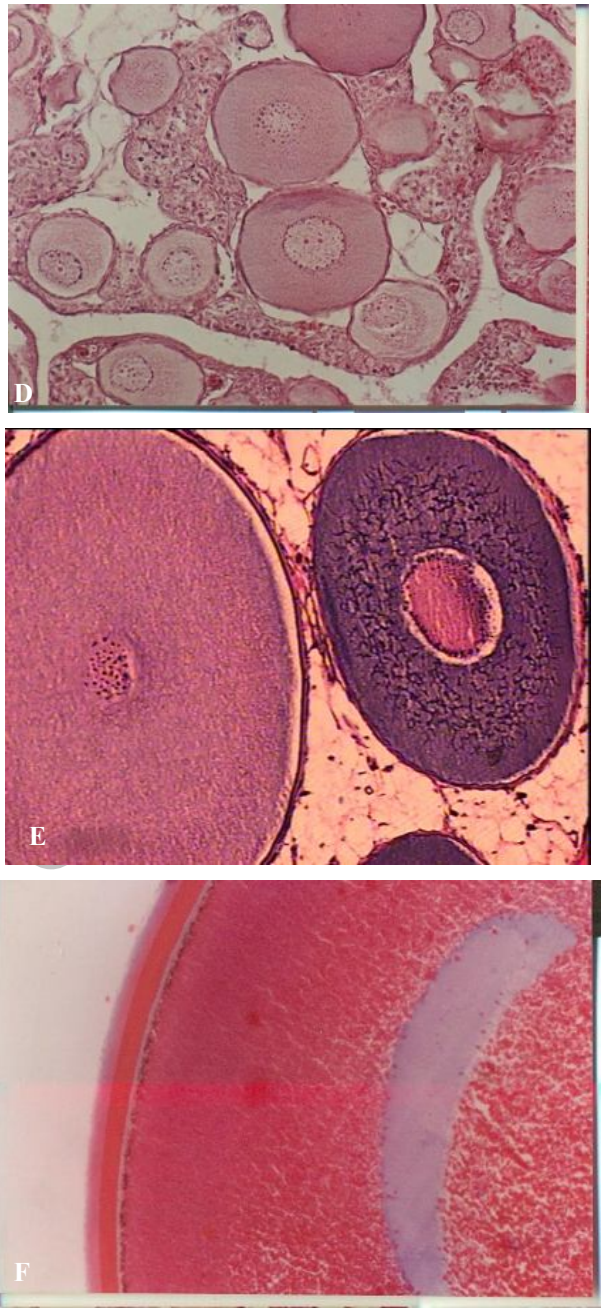


Fig. 1: (A, D): Oocytes at stage II, (B, E): Oocytes at stage III, and (C, F): Oocyte at stage IV, (H&E, 40×)

Steroid hormones levels

Compared to the beginning, testosterone levels were higher at the end of experiment. EQ concentrations also showed significant differences between the beginning and end of the experiments and reached a maximum at EQ 0.4 g/kg ($P<0.05$) (Fig. 3). 17α-hydroxy progesterone

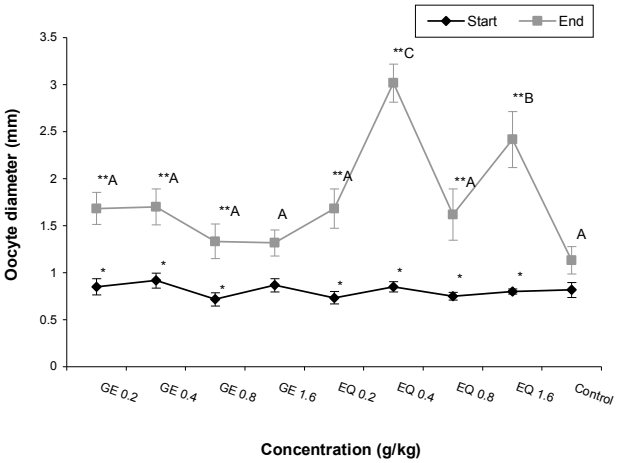


Fig. 2: Oocyte diameter changes at different concentrations of GE and EQ. A and B indicate significant comparisions between treatments at the end of experiment. * indicates significant comparisions between the beginning and end of experiment

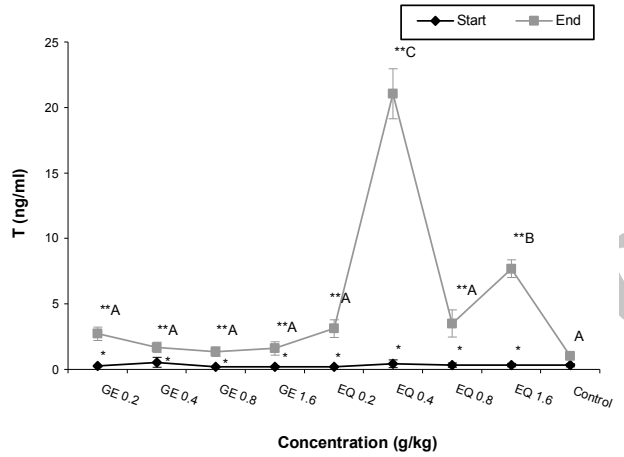


Fig. 3: Testosterone level changes at different concentrattions of GE and EQ. A and B indicate significant differences between treatments at the end of experiment. * indicates significant differences between the beginning and end of the experiment

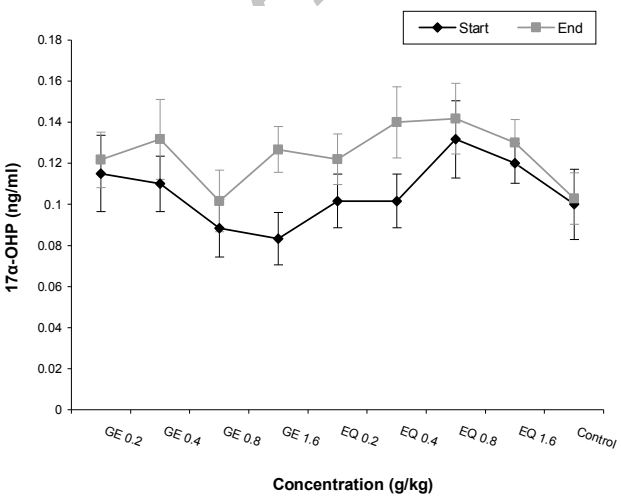


Fig. 4: 17α-hydroxy progesterone level changes at different concentrattions of GE and EQ

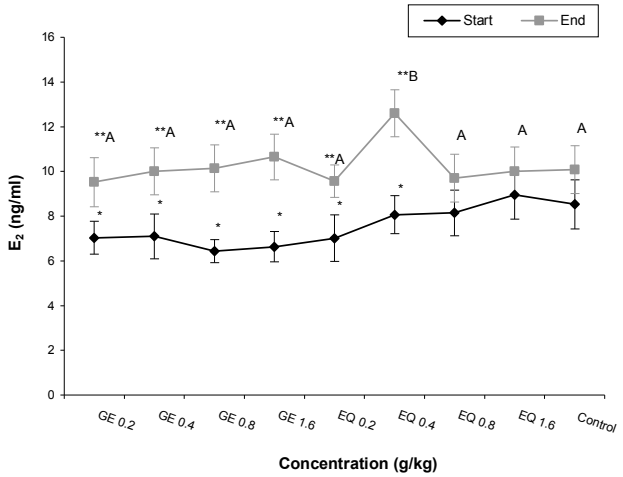


Fig. 5: 17β-estradiol level changes at different concentrattions of GE and EQ. A and B indicate significant differences between treatments at the end of experiment. * indicates significant differences between the beginning and end of the experiment

levels were higher at the end of the experiment; however, no significant differences were found between GE and EQ concentrations at the beginning and end of the experiment (P>0.05) (Fig. 4). 17β-estradiol (E_2) levels were found to be higher at all treatments at the end of the experiment and showed significant increase at EQ 0.4 g/kg (Fig. 5).

Discussion

This study demonstrated that isoflavonic phytoestrogen EQ could improve ovary performance of farmed *H. huso*. The results showed that diets supplemented with EQ at 0.4 g/kg induced significant ovary development as compared to GE. The diameter of oocytes increased significantly at an EQ concentration of 0.4 as compared to the others because it induced more vitellogenesis development. Previous studies have reported phytoestrogens to be able to stimulate VTG synthesis *in vivo* in Siberian sturgeon and *in vitro* in trout hepatocyte (Pelissero *et al*., 1991, 1993). Estrogenic potency of the isoflavones has also been reported to range differently between the two species in the following order: biochanin A < daidzein = formononetin < GE < EQ in trout, and biochanin A < GE < daidzein < formononetin < EQ in Siberian sturgeon (*Acipenser baerii*) (Latonelle *et al*., 2002). The levels of GE utilized in the striped bass (*Morone saxatilis*) affect hepatic, gonadal or normal somatic growth (Pollack *et al*., 2003).

In the present study, some doses of GE and EQ induced significant effects in plasma steroid hormone levels. Testosterone levels were elevated significantly by EQ 0.4 and 1.6 g/kg as compared to other treatments. Compared to the control and other groups, *H. huso* 17β-estradiol levels significantly increased at EQ 0.4 g/kg. In Japanese medaka (*Oryzias latipes*) exposed intraperitoneally to GE, VTG was not induced in males or females; nevertheless, plasma E_2 increased in exposed females, and plasma T levels reduced in exposed males

(Zhang *et al*., 2002). Testosterone and estradiol levels increased at the final stages of sex maturity in different species of teleost fish and sturgeon due to decreases in aromatase enzyme activity (Frederiek *et al*., 2007; Bahmani *et al*., 2009). Testosterone in theca cells of the ovary can incorporate granulosa cells and induce aromatase enzyme (P_{450}) gene expression which converts T to estradiol to promote ova and lead to a decrease in T and increase in estradiol hormones during gametogenesis caused by increasing aromatase activities and temporal changes (Zhang *et al*., 2011). In immature fish, sex steroid hormone levels were low but increased with sex development. Testosterone and progesterone are precursors of E_2 which is the most important female hormone (Barannikova *et al*., 2004). Continued and positive relationships exist between these hormones (Barannikova *et al*., 2006; Nazari and Ghomi, 2010). In fact, T is the main hormone controlling the reproductive process in sturgeon under natural and cultural conditions (Bukovskaya, 1997). In this case, cholesterol converts to pregnenolone, progesterone and androstandion and is then converted to E_2 after changing with T in the granulosa layer (Nagahama, 1994). Therefore, E_2 production increases at vitellogenesis with increasing gonad development.

Decrease of E_2 after vitellogenesis at the final stages of sexual maturity occurred because aromatse activity ceased as a result of egg development and a sharp reduction of feedback in steroid hormones (Shafiei Sabet *et al*., 2010). Progesterone levels decreased significantly in teleost female brood stocks at different stages of sexual maturity (Najafipoor, 2005). Higher levels of progesterone in mature female breeders, compared with immature breeders, was because of the effect of progestin (especially progesterone), which reaches a maximum at spawning time, on promoting oocytes in adult teleost fish.

In conclusion, isoflavonic phytoestrogens can exhibit estrogenic activity in reproduction, exert female ovary development with proper dosage, and improve reproductive performances of female *H. huso*. These effects are usually coupled with their influence on steroid hormones especially at the T level. Thus, the observed effects of isoflavonic phytoestrogens may be mediated by their modulation of endocrine.

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مقاله کامل:

اثرات تولید مثلی فیتواستروژن‌های جنیستین و اکوال موجود در سویا بر فیل‌ماهی ماده پرورشی

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در این تحقیق، 54 قطعه فیل‌ماهی پرورشی 5 ساله در مرحله II رسیدگی جنسی با غلظت‌های صفر (شاهد)، 0/2، 0/4، 0/8 و 1/6 گرم از فیتواستروژن‌های جنیستین و اکوال در هر کیلوگرم جیره به مدت یک سال تغذیه شدند. خونگیری و نمونه‌برداری از گناد به صورت فصلی انجام شد. نتایج نشان داد که 64٪ از فیل ماهیان ماده در تیمار اکوال با غلظت 0/4 گرم در کیلوگرم به مرحله IV رسیدگی جنسی رسیدند و قطر اووسیت‌ها به طور معنی‌داری در همه غلظت‌های اکوال و در غلظت‌های 0/2، 0/4و 0/8 گرم جنیستین در انتهای دوره افزایش یافت و در تیمار اکوال با غلظت 0/4 گرم در کیلوگرم به حداکثر (0/2 ± 3 میلی‌متر) رسید ($P<0.05$). سطوح هورمون تستوسترون از 0/06 ± 0/3 نانوگرم در میلی‌لیتر در ابتدای دوره به حداکثر میزان 1/91 ± 21/04 نانوگرم در میلی‌لیتر در تیمار اکوال با غلظت 0/4 گرم در کیلوگرم در انتهای دوره رسید ($P<0.05$). سطوح هورمون 17 بتا-استرادیول (E_2) در برخی از غلظت‌های جنیستین و اکوال در انتهای دوره نسبت به ابتدای دوره به طور معنی‌داری افزایش یافت و به حداکثر میزان 1/04 ± 12/6 نانوگرم در میلی‌لیتر در تیمار اکوال با غلظت 0/4 گرم در کیلوگرم در انتهای دوره رسید ($P<0.05$). سطوح هورمون 17 آلفا-هیدروکسی پروژسترون اختلاف معنی‌داری در غلظت‌های مختلف جنیستین و اکوال نشان نداد ($P>0.05$). در مقایسه، اکوال اثرات استرونیک قوی‌تری بر رشد تخمدان در مقایسه با جنیستین القاء نمود. بنابراین، استفاده از اکوال در جیره غذایی به منظور القای رشد تخمدان در گونه فیل‌ماهی توصیه می‌گردد.

واژه‌های کلیدی: فیتواستروژن، اکوال، جنیستین، تولیدمثل، فیل‌ماهی