



The estrogenic activity of β -Sitosterol and Genistein on the female gonad of Caspian kutum (*Rutilus frisii*) during reproductive migration

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ABSTRACT

This study aimed to investigate the possible reproductive effects of Genistein and β -Sitosterol on Kutum (*Rutilus frisii*) broodstock during migration to rivers for spawning. The fish were exposed to 3 levels of Genistein and β -Sitosterol (500, 50, and 10 ng L⁻¹) separately. After 21 days, we measured levels of 17 β -estradiol (E2) and testosterone (T) and assessed ovarian status. The results showed that, compared to the control, E2 levels were lower in fish exposed to β -Sitosterol, while genistein increased E2 levels in fish exposed to 500 ng L⁻¹. Comparison of ovarian tissue across treatments showed that oocytes were mainly in stages two and three, and that oocyte diameter was affected by different levels of Genistein and β -sitosterol. Finally, this study showed that high concentrations of Genistein and β -Sitosterol during Caspian kutum migration could alter ovarian structure and oocyte development, which was associated with changes in sex steroid hormones, especially 17 β -estradiol.

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Introduction

Phytoestrogens are natural compounds found in plants, which are structurally or functionally similar to mammalian estrogens and their active metabolites (Dixon, 2004). They can alter endocrine function or structure, influencing factors such as the timing of maturity, the fertility of offspring, and specific sexual behavior. (Turker and Takemura, 2011). Most phytoestrogens act as endocrine disruptors and cause intersex in aquatic organisms (Ze-hua Liu *et al.*, 2010). Genistein is a phytoestrogenic compound with an estrogenic effect that can alter aromatase activity, sex hormone synthesis, and the induction of yolk production or increased yolk levels in aquatic animals (Cederroth *et al.*, 2012). Also, phytoestrogens can affect the production of sex steroid hormones such as testosterone, 11KT, and 17 β -estradiol (Stevenson *et al.*, 2011).

β -Sitosterol (the main plant sterol in pulp and paper wastewater) is another herbal compound that can disrupt reproduction (Orrego *et al.*, 2010). This compound in water can inhibit steroid synthesis and alter sex hormone levels in fish. Because of their structural similarity to steroids, these compounds can bind to estrogen and androgen receptors (ER/AR) (Orrego *et al.*, 2010). Studies have shown that β -sitosterol, by disturbing steroid synthesis, reduces biosynthesis capacity and disrupts endocrine function (Akwasi, 2013).

In many countries, phytoestrogen concentrations in various aquatic environments have been reported in the range of 3 ng ml⁻¹ to 143 μ g l⁻¹, whereas concentrations of 1 ng l⁻¹ could have adverse effects on the endocrine system (Kiparsis *et al.*, 2003; Kuster *et al.*, 2009). Because these compounds interfere with steroid hormone synthesis, measuring these hormones and their effects on ovarian development can indicate the effects of phytoestrogens on aquatic organisms. Similarly, measuring steroid hormone synthesis and its effects on ovarian growth and development can indicate the effects of phytoestrogens on aquatic organisms.

Given Iran's industrialization and the discharge of various types of industrial wastewater and runoff (including from the Mazandaran paper mill), agriculture, and human activities into the southern rivers of the Caspian Sea, especially the Tajan River, this river may contain various phytoestrogens. *Rutilus frisii* (Kutum) belongs to the family Leuciscidae and is a species native to the Caspian Sea (Eagderi *et al.*, 2022). During March and April, it migrates into rivers draining into the Caspian Sea to complete its reproductive cycle (Jafari *et al.*, 2009). During this period, broodstock are exposed to various compounds and pollutants, including phytoestrogens, which can negatively affect reproductive performance and alter population structure over the long term. This study examined how Genistein and beta-sitosterol affect sex hormones and ovarian development in Kutum.

Material and Methods

We conducted experiments on 489 female Kutum specimens collected from the Shirood River inlet (50.8002°N, 36.8559°E) in March and April 2015. The fish had an average body weight of 680 $\pm$ 15 g and were acclimated for 48 hours prior to the experiment. The fish were randomly stocked in seven 300-L fiberglass tanks, equipped with aeration systems and water exchange mechanisms to maintain Shirood River water levels. Each fish was considered an independent replicate, and stocking was performed based on biomass to ensure uniformity across treatment groups. The experimental design included three concentrations of β -sitosterol (Sigma, S1270: 10, 50, and 500 ng L⁻¹), three concentrations of Genistein (Sigma, G6649: 10, 50, and 500 ng

L⁻¹), and one control group, maintained for 21 days (Stevenson *et al.*, 2011). We renewed test solutions every two days.

Physicochemical parameters were monitored daily using an Oxi-pHmeter 40i (WTW, Germany). Anesthesia was induced using 150 ppm clove powder (Sharifpour *et al.*, 2003). Oocyte diameters were measured using a DINO digital lens equipped with a light microscope. For histological analysis, samples were fixed in Bouin's solution for 48 h, then dehydrated, paraffin-embedded, and mounted. Sections (6 μ m) were cut using a microtome (Leitz 1512, Germany), stained with Hematoxylin and Eosin (H&E), and examined under a light microscope (Mohammadrezaei *et al.*, 2012). Blood samples (5 mL) were collected from the caudal vein and centrifuged (Labofuge 200, Heraeus Sepatech, Germany) at 2500 rpm for 10 min. The resulting samples were stored at -20°C. Steroid hormone levels, specifically testosterone (T) and 17 α -estradiol (E2), were quantified via Enzyme-Linked Immunosorbent Assay (ELISA) using commercial kits (MyBioSource, USA; MBS933475 and MBS700179) at the Meher Laboratory (Tehran, Iran).

Data were subjected to normality tests and homogeneity of variance assessments. A one-way analysis of variance (ANOVA) was employed to test significant differences between the control and experimental groups, followed by Duncan's multiple range test for post-hoc mean comparisons. All statistical analyses were performed using SPSS version 17.

Results

Hormonal Responses (E2 and T): 17 β -estradiol (E2) concentrations showed divergent responses to the tested compounds. While β -sitosterol exposure did not significantly change E2 levels compared with the control group (Fig. 1), Genistein treatment significantly increased E2 ($P<0.05$). Notably, the group exposed to 500 ng L⁻¹ of genistein had the highest E2 concentration (Fig. 1).

Regarding testosterone (T), exposure to β -sitosterol elicited a dose-dependent increase, with the highest T levels observed at 500 ng L⁻¹; however, these changes were not statistically significant compared to the control ($P>0.05$) (Fig. 2). Conversely, Genistein treatment induced a contrasting trend: T levels significantly decreased in the 500 ng L⁻¹ Genistein group compared to the control ($P<0.05$) (Fig. 2).

Oocyte Morphometry and histological observations: Oocyte diameter remained unchanged across all β -sitosterol concentrations relative to the control. In contrast, Genistein treatment at 500 ng L⁻¹ significantly increased oocyte diameter, reaching a maximum of 46.1 ± 0.2 μ m ($P<0.05$) (Fig. 3).

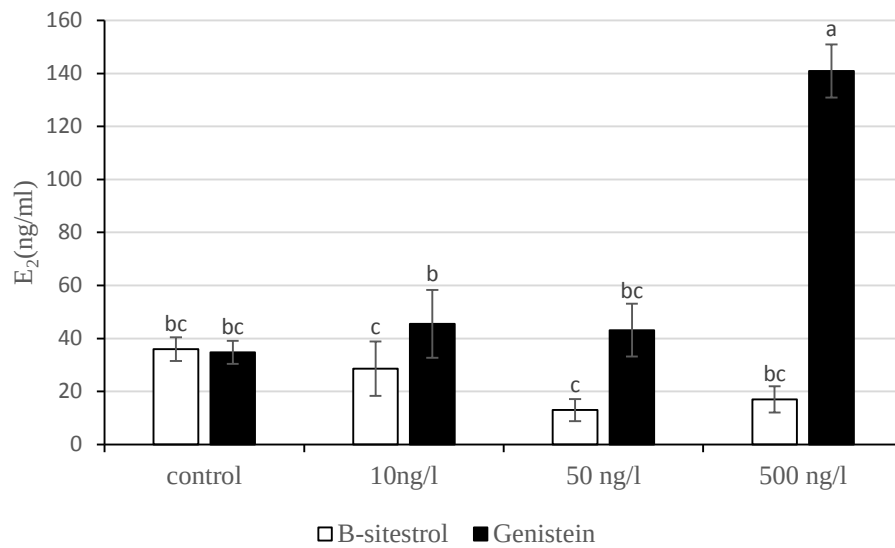


Figure 1. Effects of graded concentrations of Genistein and β -sitosterol (ng L^{-1}) on plasma 17β -estradiol (E_2) levels. Values are presented as mean $\pm$ SEM. Different letters indicate significant differences ($P<0.05$) compared with the control.

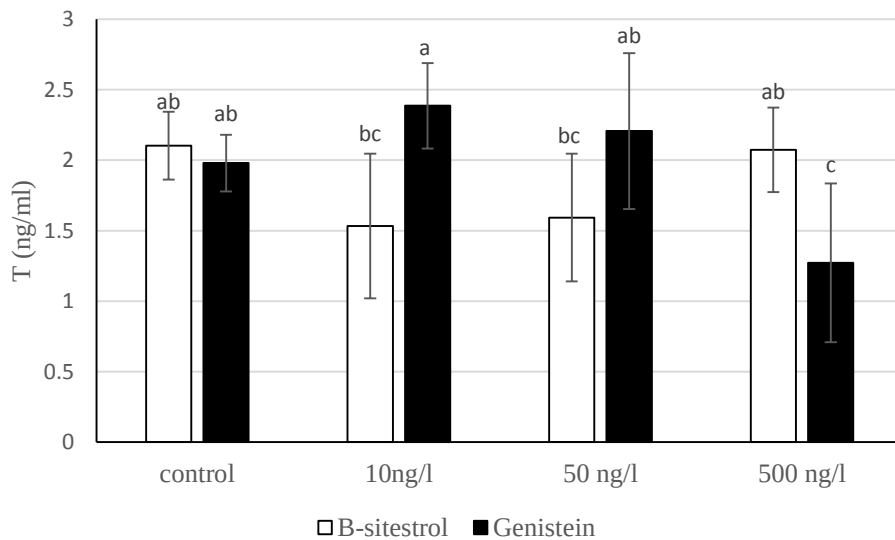


Figure 2. Effects of graded concentrations of Genistein and β -sitosterol (ng L^{-1}) on plasma testosterone (T) levels. Significant differences ($P<0.05$) are indicated by different letters. Note the inverse relationship between Genistein concentration and T levels.

At the start of the experiment, histological analysis confirmed that all specimens were in the final maturation stage, characterized by nuclear migration toward the animal pole. Post-exposure (21 days), histological examination revealed that both phytoestrogens accelerated oocyte atresia compared with the control group (Fig. 1, 4). Furthermore, ovarian development was disrupted; while control group ovaries maintained standard developmental morphology, ovaries in treated fish were predominantly in stages II and III.

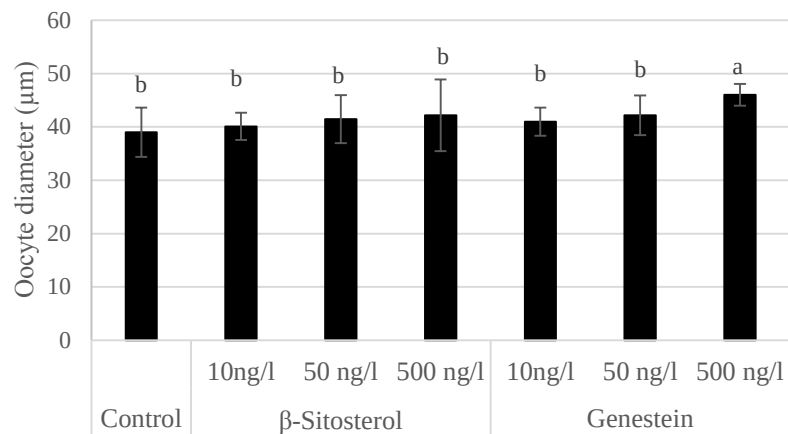


Figure 3. Impact of different concentrations of Genistein and β -sitosterol (ng L⁻¹) on oocyte diameter (μm). Significant differences compared to the control are marked with different letters ($P < 0.05$).

Morphological deviations included loss of oocyte circularity, restricted yolk accumulation (appearing as a narrow strip), and an increased number of nucleoli near the nuclear membrane. Additionally, cytoplasmic vacuolization was frequently observed in the perinuclear region. Unlike the control, treated fish lacked differentiation between animal and vegetal poles. Notably, β -sitosterol exposure caused severe structural disintegration, whereas oocytes in the Genistein-treated groups maintained relatively higher structural regularity, despite the observed larger oocyte size at 500 ng L⁻¹, which was associated with the initiation of early vitellogenesis.

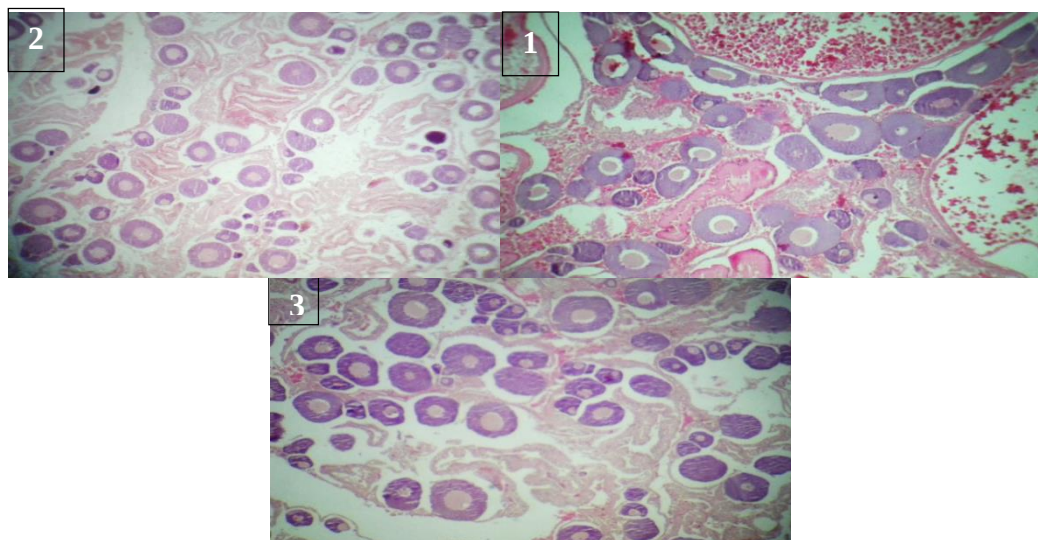


Figure 4. Comparative histology of *Rutilus frisii* oocytes (H&E staining). 1) Control group: Normal architecture with well-defined polarity. 2) β -sitosterol treatment: Signs of severe structural disintegration and accelerated atresia. 3) Genistein treatment (500 ng L⁻¹): Oocytes showing enlarged diameter and early vitellogenesis features, including cytoplasmic vacuolization and perinuclear nucleolar migration, yet exhibiting higher structural integrity compared to the β -sitosterol group.

Discussion

This study shows that both Genistein and β -sitosterol significantly affect the reproductive performance and steroidogenesis of female *R. frisii*. Our results show that both compounds

modulate ovarian development, with Genistein (500 ng L⁻¹) significantly increasing oocyte diameter, likely by stimulating vitellogenesis. This observation aligns with previous findings where phytoestrogens were shown to stimulate vitellogenin (VTG) synthesis in vivo in various species, including Siberian sturgeon and rainbow trout (Pelissero *et al.*, 1991; 2001), *Huso huso* (Yousefi Jourdehi *et al.*, 2014), and *Trichogaster trichopterus* (Sepehr *et al.*, 2015). However, unlike in some other cyprinids where such exposure leads to developmental arrest, our results suggest that in *R. frisii*, these compounds initiate an early, albeit potentially abnormal, vitellogenic pathway.

Regarding endocrine disruption, Genistein (500 ng L⁻¹) significantly elevated 17 β -estradiol (E2) levels while concurrently depressing testosterone (T) concentrations. This inverse relationship is consistent with findings in Japanese medaka (*Oryzias latipes*) exposed to Genistein (Zhang *et al.*, 2002). Genistein acts as a potent estrogen receptor (ER) agonist, mimicking endogenous 17 β -estradiol and triggering functional responses by binding to intracellular ERs (Whitehead and Rice, 2006). This estrogenic activity likely suppresses the hypothalamic-pituitary-gonadal (HPG) axis, leading to a feedback-mediated reduction in T production.

Conversely, the β -sitosterol treatment groups showed a different endocrine profile. The accumulation of testosterone in β -sitosterol-exposed fish, relative to other treatments, suggests inhibition of aromatase (the enzyme that converts androgens to estrogens). This mechanism has been described in other fish models, where β -sitosterol disrupts the steroid biosynthetic pathway, preventing the conversion of T to E2 (Hinfray *et al.*, 2006; Cheshenko *et al.*, 2008). Under normal physiological conditions, granulosa cells convert testosterone in theca cells to E2 to promote oocyte maturation; aromatase (P450) gene expression strictly regulates this process (Barannikova *et al.*, 2004; Zhang *et al.*, 2011). Our data suggest that while β -sitosterol-exposed fish attempt this transition, the resulting aromatase inhibition prevents the successful conversion of T to E2, a phenomenon also observed in *Carassius auratus* under phytoestrogen stress.

Histological analysis further confirmed that both phytoestrogens accelerated oocyte atresia. While atresia is a normal component of the reproductive cycle in teleosts, the premature occurrence observed here indicates reproductive impairment. This finding mirrors reports in *Trichogaster trichopterus* (Sepehr *et al.*, 2015), suggesting that *R. frisii*, like gourami, is highly sensitive to the atretic effects of exogenous estrogenic compounds. The observed increase in oocyte diameter in the high-concentration treatment groups likely reflects expanded cytoplasmic space, indicating an abnormal initiation of endogenous vitellogenesis (Kausch *et al.*, 2008).

Previous studies on species such as *Huso huso*, guppy, and goldfish have also highlighted the susceptibility of immature fish to phytoestrogenic ovarian development (Bagheri *et al.*, 2013; Yousefi Jourdehi *et al.*, 2014; Sepehr *et al.*, 2015; Roozbehani and Nazari, 2015). However, our results emphasize that the magnitude and structural consequences of this disruption—specifically oocyte deformation and atresia in *R. frisii*—differ from those in other models, potentially reflecting species-specific sensitivity to exposure dosage and duration.

In conclusion, Genistein and β -sitosterol significantly alter the reproductive physiology of female *R. frisii* by disrupting steroid hormone balance and accelerating ovarian atresia. These

effects depend strongly on dosage and oocyte developmental stage. Future studies should focus on the transcriptional regulation of ER genes in *R. frisi* to further elucidate the molecular pathways underlying these phenotypic observations.

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