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RESEARCH ARTICLE

Effect of Total Isoflavones Found in Soybean on Vitellogenin Production in Common Carp

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Summary

Soybean meal is used most extensively as a replacement to fishmeal as the main protein source in fish diets. However, there is concern about the effect of soybean-based diets on reproductive development of the fish because of the phytoestrogenic properties of soybean. The objective of the present study was to determine whether a relationship exists between the concentration of soybean extract containing total isoflavones in diet and the production of vitellogenin protein in cyprinid fish. Five different doses (0, 250, 500, 1.000, and 10.000 mg/kg based on total isoflavones content) of commercial soybean extract were tested to determine the effect of pyhtoestrogens concentration on vitellogenin synthesis in female and male carp, *Cyprinius carpio* L. There were no significant differences in the growth of fish between any of the groups. All concentrations of soybean extract lowered male gonadosomatic index (GSI), but female GSI was increased in diets including up to 500 mg/kg soybean extract, and then decreased at higher doses. This was associated by an inhibition and induction, respectively, in plasma vitellogenin levels. Changes in GSI and vitellogenin appeared to be sensitive marker for detecting phytoestrogens exposure. Higher concentrations of isoflavones resulted in a higher amount of vitellogenin produced in both male and female fish. Our results clearly showed that exposure to phytoestrogens found in soybean extract significantly induces vitellogenin production in both males and females, in a dose-response manner. GSI and high vitellogenin production indicated that phytoestrogens found in soybean disrupt the endocrine system of fish. The endocrine disruptor effect of soybean products must be considered in fish meal preparation.

Keywords: *Common carp, Isoflavones, Soybean, Gonadosomatic index, Phytoestrogen, Vitellogenin*

Soya Fasulyesinde Bulunan Toplam İsoflavonların Sazan Balıklarında Vitellojenin Üretimine Etkisi

Özet

Balık diyetlerinde temel protein kaynağı olan balık ununun yerine soya fasulyesi yemi yaygın olarak kullanılmaktadır. Fakat soya fasulyesinin fitoestrojenik özellikleri balıklarda üremeyi etkileyeceğinden, diyetlerde soya fasulyesi bazlı yemlerin kullanılması endişe oluşturmaktadır. Bu çalışmanın amacı çeşitli konsantrasyonlarda hazırlanan toplam isoflavon içeren soya fasülyesi özütleri ile sazangil balıklarında vitellojenin proteini üretimi arasında bir ilişkinin olup olmadığını belirlemektir. Beş farklı dozda (içerdikleri toplam isofavon oranlarına göre 0, 250, 500, 1.000 ve 10.000 mg/kg) hazırlanan ticari soya fasulyesi özütleri fitoestrojen konsantrasyonlarının vitellojenin sentezine etkisini belirlemek için dişi ve erkek sazanda, *Cyprinius carpio* L., test edilmiştir. Bütün balık grupları içinde özütlerin büyümeye etkisinde herhangi bir farklılık bulunamamıştır. Soya özütlerinin bütün konsantrasyonları erkeklerde GSI değerini düşürmüş, fakat dişilerin GSI değerinde 500 mg/kg soya özütü konsantasyonuna kadar arttırmış daha sonra da yüksek dozlarda da düşürmüştür. Bu da plazma içindeki vitellojenin seviyesinin inhibisyonu ve indüklenmesi ile ilişkilendirilmiştir. GSI ve vitellojenin değerindeki değişikliklerin, fitoestrojenlere maruz kalan bireyleri belirleyebilecek hassas bir belirteç olduğunu ortaya çıkarmıştır. Daha yüksek dozlardaki isoflavonlar her iki cinsiyetteki balıklar için daha fazla vitellojenin üretmiştir. Bu çalışma sonucunda fitoestrojen içeren soya fasulyesi özütleriyle hazırlanan yemlerle beslenen her iki cinsiyetteki balıkların vitellojenin üretiminin indüklenmesi açıkça gösterilmiştir. GSI değerleri ve yüksek oranda vitellojenin üretimi, fitoestrojen içeren soya fasulyesinin balıkların endokrin sistemlerinde tahrip edici etkisi olduğunu işaret etmiştir. Balık yemlerinin hazırlanmasında, endokrin bozucu etkiye sahip olan soya fasulyesi ürünlerinin kullanımı dikkate alınması gereken bir husus olmalıdır.

Anahtar sözcükler: *Sazan, Isoflavonlar, Soya fasulyesi, Gonadosomatik indeks, Fitoestrojen, Vitellojenin*

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INTRODUCTION

Phytoestrogens are a broad group of plant-derived compounds that are structurally and functionally similar to estrogen and have a range of estrogenic activity in animals [1]. Estrogenic activity has been reported to be present in soybeans, liquorice, linseed, clover, pomegranate, etc. Plant estrogens are mostly isoflavones that occur in the form of glycosides. Soybean phytoestrogens include several isoflavones (genistein, daidzein, and glycitein) and nonisoflavone (coumestrol) compounds with estrogenic activity. The soybean isoflavones are typically found in soybeans at a ratio of approximately 1.3:1.0:0.2 [2]. Dry soybeans on average contain 1.107 milligrams of genistein per kilogram and 846 milligrams of daidzein per kilogram [3].

The dietary estrogenic effects of phytoestrogenic compounds can have a wide range of consequences on various physiological processes in animals. The estrogenic activities of isoflavones have been assessed in biological assays such as the increase in the uterus weight of sheep [4], mouse [5] and rat [6] and by biochemical tests such as binding to estrogen receptors [7]. Phyto-estrogens are also a family of bioidentical hormones suspected to act as endocrine disruptors in human health [8]. There have been numerous studies on the utilization of soybean meal in the diet of various species of fish such as carp, tilapia, catfish, flounder and sea bream [9-13]. Soybeans are recommended to replace the amount of fish meal in some fish diet with these studies. The estrogenic potency of these compounds has also been researched in several species of fish. Dietary purified phytoestrogens produced large changes in the plasma vitellogenin levels of Siberian sturgeon, *Acipenser baer*i, [14] and rainbow trout, *Oncorhynchus mykiss,* [15], yellow perch, *Perca flavescens,* [16], striped bass, *Morone saxatilis,* [17] and European eel, *Anguilla anguilla,* [18].

Vitellogenin is one of the egg's main yolk precursors in many oviparous vertebrates, and is synthesized in the liver of females during the reproductive period [19]. Vitellogenin production can provide a valuable biomarker of reproductive disruption by estrogenic substances in fish [20-22]. Stimulation of vitellogenin in the blood of male and juvenile fish is indicative of exogenous exposure to estrogen.

Although the estrogenic potency of phytoestrogens has been demonstrated in fish, none of these compounds showed a dose-response effect on estrogen levels that could be verified separately in female and male fish by measuring plasma vitellogenin synthesis. Because soybean can be a component of a natural diet and is capable of acting as an estrogen mimic, the purpose of this study was to determine the dose-response effect of total isoflavones from a commercial soybean product on the synthesis of vitellogenin in common carp, *Cyprinius carpio* L.

MATERIAL and METHODS

Chemicals

Solgen 40®, commercial soybean extract, was obtained from Solbar Plant Extracts Ltd. (Ashdod, Israel). A rabbit polyclonal antibody against vitellogenin from carp was purchased from Biosense Laboratories (Bergen, Norway). The following chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA); acrylamide, bovine serum albumin, 2-amino-2(hydroxymethyl)-1.3-propandiol (Tris), phenazine methosulfate and sodium dodecyl sulphate (SDS). An alkaline phosphatase-conjugated goat anti-rabbit IgG, nitroblue tetrazolium, and 5-bromo-4-chloro-3-indolyl phosphate were obtained from CalbioChem, EMD Chemicals Inc. (Darmstadt, Germany). All other chemicals were obtained from commercial sources at the highest grade of purity available.

Fish

Since vitellogenin standard from common carp was available commercially, it was used as a model fish in this study. Juvenile common carp were provided from the DSI Golkoy Fish Production Station, Bolu, Turkey. The fish were acclimated to laboratory conditions in a 500 l indoor recirculating tank system and fed with commercial trout diet (Blueaq trout feed, Abalıoğlu Yem A.Ş., Denizli, Turkey) for 10 days during the acclimation period. Ten juveniles, averaging 1.5 g/fish, were randomly placed into each of ten 50 l fiberglass tanks with two replicates. Tanks were supplied with a continuous water exchange from a closed floating-bead recirculation system at the Fish Biology Laboratory.

Fish treatment and sampling

The concentrations of phytoestrogen in the diets of fish were adjusted according to total isoflavones amount. The percentage of total isoflavones was 45% based on dry weight in the Solgen 40®extract (certificate of analysis by HPLC, Solbar Plant Extracts

Ltd.). 0, 250, 500, 1.000 and 10.000 mg/kg soybean extract containing total isoflavones dissolved in 100 ml ethanol and added into the diet. The experimental diets were mixed in a mixer, prepared with a pelleter and dried under a hood at room temperature then stored in a freezer. Each of the 10 tanks was randomly assigned one of five experimental diets in duplicate and fed once daily to satiation for 180 days.

The fish were anaesthetized in a 100 mg/L concentration of benzocaine (Sigma Chemical Company) to allow blood samples to be collected via cardiac puncture into heparinized 1 ml syringe fitted with a 22 gauge needle (4 mg/ml, Sigma Chemical Company). These were centrifuged at 3.000 rpm for 15 min at 4°C, then the plasma was collected and stored at -80°C prior to protein and Western blot analyses. Hemolyzed blood samples were not used for the analyses. The fish were then weighed and dissected to remove the gonads for determining the gonadosomatic index (GSI = gonadal weight/total weight x 100).

Protein measurement and Western blotting

Protein concentrations of plasma were determined according to the method of Lowry [23] using bovine serum albumin as a standard. Western blot analysis was conducted essentially as described by Towbin et al.[24]. Details of the procedure were given in Arinç and Sen [25]. Polyclonal anti-carp vitellogenin was used as a primary antibody. Each plasma sample (50 µg) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed on 4% stacking gel and 7.5% separating gel in a discontinuous buffer system as described by Laemmli [26]. Separated proteins were then transferred from gel to a nitrocellulose membrane using a trans-blot electrophoretic transfer cell (Bio-Rad Laboratories, Hercules, CA, USA) containing Tris-glycine buffer, pH 8.3, and methanol, at 90 V (400 mA). Transfer was carried out at 0-4°C for 90 min. Immunochemical staining of the separated proteins on the nitrocellulose sheet was done by incubating the nitrocellulose sheet in 1/500 diluted anti-carp vitellogenin in Tris-buffered saline plus 0.05% Tween 20 (TBST) containing 5% non-fat dried milk at room temperature for 2 h with shaking. After three washes with TBST, the blot was further incubated with a secondary antibody, alkaline phosphatase conjugated goat anti-rabbit IgG (diluted 1/15.000 with TBST) for 1 h and then washed three times with TBST. Alkaline phosphatase activity was detected as described by Ey and Ashman [27]. The final images of the blots were photographed using a computer-based gel imaging instrument (Infinity 3000-CN-3000 darkroom, Vilber Lourmat, Marne-la-Vallee Cedex 1, France), and relative peak area (RPA) of the blots was analyzed using the Scion Image software for Windows (Version 4.0.2, Scion Corporation, Maryland, USA) as a quantitative tool to determine vitellogenin levels.

Statistical analysis

Results are expressed as mean ± standard error of the mean (S.E.M.). All data were analyzed by analysis of variance (ANOVA) and differences among treatments were compared using Duncan's Multiple Range Test at significance levels of 0.05.

RESULTS

The final weights of the control (0 mg/kg), 250 mg/kg, 500 mg/kg and 1.000 mg/kg soybean extract containing total isoflavones treated female carp were not significantly different from each other *(Fig 1A)*. Female carp also fed the 10.000 mg/kg diet had a similar final weight as the fish fed diets of 0 mg/kg and 500 mg/kg soybean extract. No significant differences in final body weight were found between the controls or any of soybean extract treatment groups in male carp *(Fig 1B)*. The GSIs of the female carp in the 500 mg/kg group were higher than those in the 10.000 mg/kg and control diet groups, but not different from the 250 mg/kg and 1.000 mg/kg diet groups *(Fig 2A)*. Male carp in both the control and 250 mg/kg diet groups had significantly higher GSI than those in the 500, 1.000 and 10.000 mg/kg diet groups which did not differ from one another *(Fig 2B)*.

For either sex, the fish given the control diet showed no detectable vitellogenin protein bands with anti-carp vitellogenin. However, the high level of plasma vitellogenin in both sexes and rate of vitellogenin production coincided with significantly elevated concentrations of soybean phytoestrogen in the experimental diets. Vitellogenin level of carp increased in both sexes treated with 0 mg/kg to 10.000 mg/kg soybean extract concentrations in the diet for 180 days *(Fig 3)*.

The percentage of vitellogenin (proportion of individuals with detectable vitellogenin) produced by female fish increased gradually with increasing soybean extract in the diet *(Fig 4)*. Concentrations of 250, 500, 1.000 and 10.000 mg/kg soybean extract diets corresponded to 62%, 67%, 75% and 100% of the

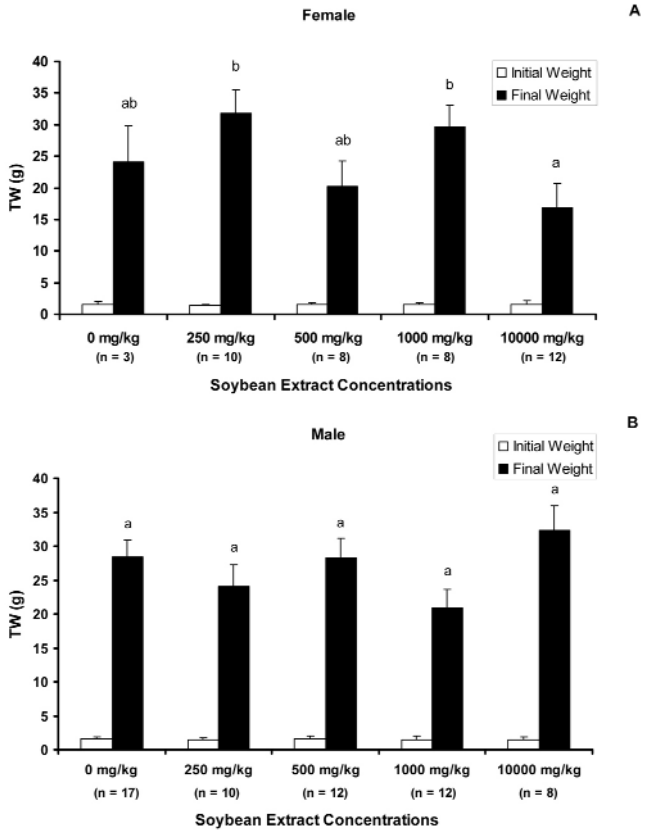


Fig 1. Initial and final body weights (mean±SE) of female and male carp fed diets containing different concentrations of soybean extract for 180 days. Mean values with the same letters are not significantly different from each other within each sex (P>0.05)

Şekil 1. 180 gün boyunca farklı konsantrasyonlarda soya fasulyesi özütü içeren diyetlerle beslenen dişi ve erkek sazan balıklarının başlangıç ve son vücut ağırlıkları (ortalama±SH). Aynı cinsiyet içinde aynı harflere sahip olan ortalamalar birbirinden anlamlı olarak farklı değildir (P>0.05)

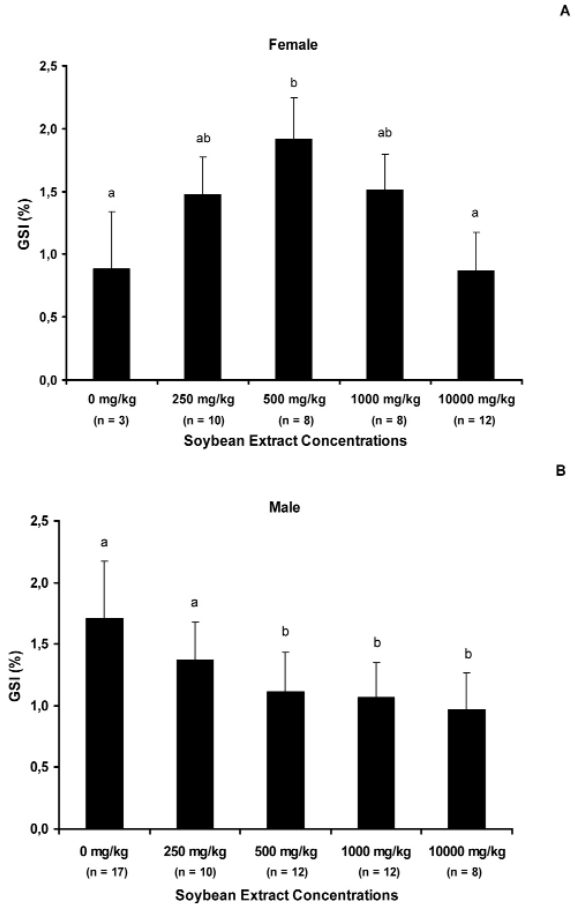
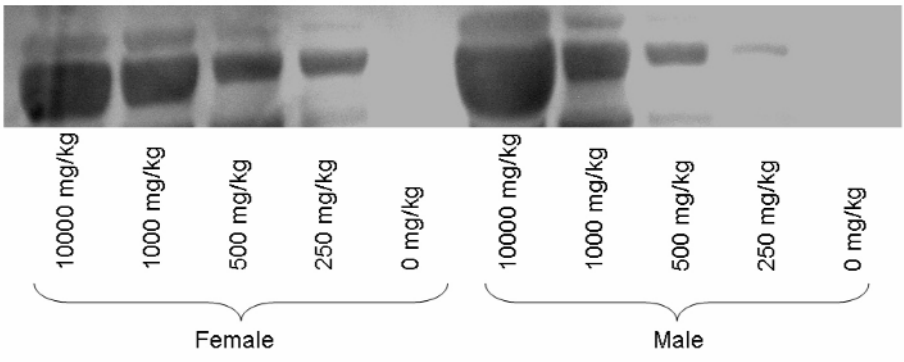


Fig 2. Gonadosomatic index (GSI = gonadal weight / total weight x 100) values (mean±SE) of female and male carp fed with different concentrations of soybean extract for 180 days. Mean values with the same letters are not significantly different from each other within each sex (P>0.05)

Şekil 2. 180 gün boyunca farklı konsantrasyonlarda soya fasulyesi özütü içeren diyetlerle beslenen dişi ve erkek sazan balıklarının Gonadosomatik indeksleri (GSI= gonad ağırlığı / toplam ağırlık x 100) değerleri (ortalama±SH). Aynı cinsiyet içinde aynı harflere sahip olan ortalamalar birbirinden anlamlı olarak farklı değildir (P>0.05)

Fig 3. Comparison of vitellogenin protein in groups of female and male carp fed diets containing different concentrations of soybean extract. Wells contained 50 µg protein for both sexes, with the exception of the well for females fed 10.000 mg/kg (25 µg)

Şekil 3. Farklı konsantrasyonlarda soya fasulyesi özütü içeren diyetlerle beslenen dişi ve erkek sazan grupları içinde vitellojenin proteinlerinin karşılaştırılması. Kuyular 10.000 mg/kg beslenen dişiler hariç (25 µg), diğer konsantrasyonlarda her iki cinsiyet içinde 50 µg protein içerir



vitellogenin produced by the female carp. The relative peak area (RPA) of the vitellogenin protein giving cross-reaction with anti-carp vitellogenin in the 10.000 mg/kg soybean extract diet was significantly higher than for any of the other diets in female carp *(Fig 5)*. The band intensity for the vitellogenin in the 10.000 mg/kg diet was double the intensity seen from the other soy isoflavones diets for female carp.

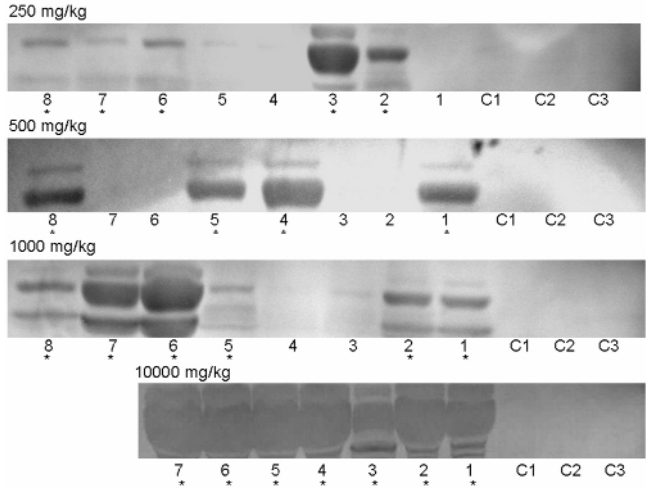


Fig 4. Comparison of vitellogenin protein in groups of female carp fed diets containing different concentrations of soybean extract. Wells contained 50 µg protein, except for the 10.000 mg/kg group (25 µg). The hemolyzed blood samples were not included. The numbers are used for the individual plasma sample, * for vitellogenin protein produced in females and C for control (0 mg/kg) in each concentration below each band

Şekil 4. Farklı konsantrasyonlarda soya fasulyesi özütü içeren diyetlerle beslenen dişi sazan grupları içinde vitellojenin proteinlerinin karşılaştırılması. Kuyular 10.000 mg/kg beslenenler hariç (25 µg), diğer konsantrasyonlarda 50 µg protein içerir. Hemolize olmuş kan örnekleri dahil edilmemiştir. Her bir konsantrasyon altındaki numaralar her bir plazma örneği, * vitellojenin üreten dişi balığı ve C kontrol (0 mg/kg) grubu için kullanılmıştır

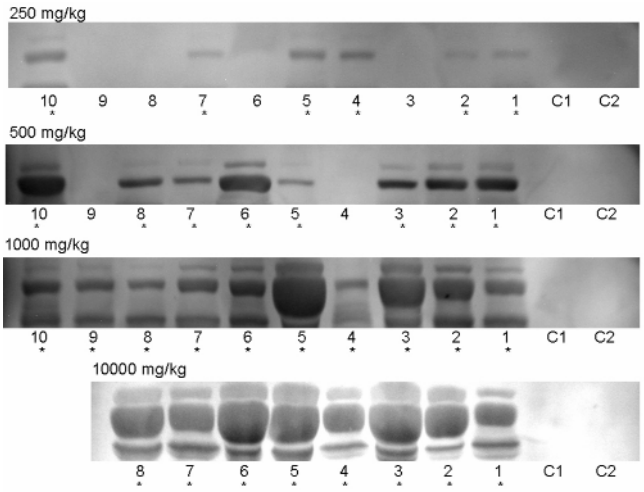


Fig 6. Comparison of vitellogenin protein in groups of male carp fed diets containing different concentrations of soybean extract. Wells contained 50 µg protein. The hemolyzed blood samples were not included. The numbers are used for the individual plasma sample, * for vitellogenin protein produced by male and C for control (0 mg/kg) in each concentration below each band

Şekil 6. Farklı konsantrasyonlarda soya fasulyesi özütü içeren diyetlerle beslenen erkek sazan grupları içinde vitellojenin proteinlerinin karşılaştırılması. Kuyular 50 µg protein içerir. Hemolize olmuş kan örnekleri dahil edilmemiştir. Her bir konsantrasyon altındaki numaralar her bir plazma örneği, * vitellojenin üreten erkek balığı ve C kontrol (0 mg/kg) grubu için kullanılmıştır

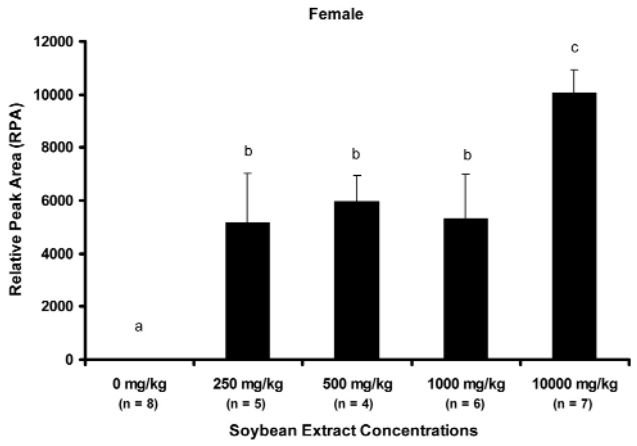


Fig 5. The relative peak areas (RPA) of vitellogenin protein (mean ± SE) produced by female carp fed diets containing different concentrations of soybean extract, as determined from Figure 4 using Scion Image software. n indicates the number of female carp producing vitellogenin protein. Mean values with the same letters are not significantly different from each other within each concentration (P>0.05)

Şekil 5. Farklı konsantrasyonlarda soya fasulyesi özütü içeren diyetlerle beslenen dişi sazan balıklarınca üretilen vitellojenin proteininin (ortalama±SH) göreceli tepe alanlarının Scion Image programı kullanılarak Şekil 4 üzerinden belirlenmesi. Her sütun altında vitellojenin proteini üreten dişi balıkların sayısı (n). Her bir konsantrasyon içinde aynı harflere sahip olan ortalamalar birbirinden anlamlı olarak farklı değildir (P>0.05)

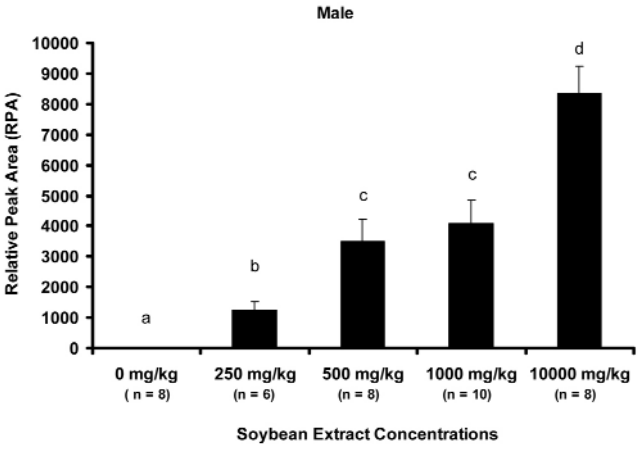


Fig 7. The relative peak areas (RPA) of vitellogenin protein (mean±SE) produced by male carp fed diets containing different concentrations of soybean extract, as determined from Figure 6 using Scion Image software. n indicates the number of male carp producing vitellogenin protein. Mean values with the same letters are not significantly different from each other within each concentration (P>0.05)

Şekil 7. Farklı konsantrasyonlarda soya fasulyesi özütü içeren diyetlerle beslenen erkek sazan balıklarınca üretilen vitellojenin proteininin (ortalama±SH) göreceli tepe alanlarının Scion Image programı kullanılarak Şekil 6 üzerinden belirlenmesi. Her sütun altında vitellojenin proteini üreten erkek balıkların sayısı (n). Her bir konsantrasyon içinde aynı harflere sahip olan ortalamalar birbirinden anlamlı olarak farklı değildir (P>0.05)

Soybean phytoestrogen supplemented feeds also increased the percentage of vitellogenin produced by male fish *(Fig 6)*. 60%, 80%, 100% and 100% of male fish produced vitellogenin were achieved at 250, 500, 1.000 and 10.000 mg/kg soybean extract concentrations, respectively. The significantly greatest band intensity for vitellogenin protein was found in the 10.000 mg/kg diet compared with the 250, 500 and 1.000 mg/kg diets *(Fig 7)*.

DISCUSSION

In this study, the effect of soybean extract on growth rate, gonad somatic index (GSI) and vitellogenesis were investigated in vivo on juvenile common carp. The concentrations of total isoflavones used in this study did not affect the somatic growth of the carp. However, our results clearly showed that isoflavones had inhibitory effects on gonad growth for carp fed a diet from 0 to 10.000 mg/kg soybean extract in males, or 500 and 10.000 mg/kg in females. Bimodal effect of isoflavones was observed in female carp fed a diet containing up to 1.000 mg/kg soybean extract had higher GSIs, while soybean extract levels higher than 1.000 mg/kg in the diet lowered GSIs. The highest level of vitellogenin occurred in females fed a diet of 10.000 mg/kg soybean extract, which resulted in decreased GSIs. This result corroborated the finding of the earlier reports that high level of vitellogenesis can be indicative of decreased GSI [28-30]. This may have resulted from lower isoflavone levels having a positive influence and higher levels having a negative feedback effect on the release of gonadotropin from the pituitary in female carp.

Treatments with phytoestrogen resulted in 100% of the vitellogenin produced fish at 1.000 and 10.000 mg/kg concentrations in males and at 10.000 mg/kg concentration in females in our study. Vitellogenin is normally produced in sexually maturing females as a response to endogenous estrogens circulating in the plasma. In male fish, there is normally no expression of the gene responsible for vitellogenin production [19]. If fish are exposed to exogenous sources of estrogens, male fish are able to synthesize vitellogenin. High vitellogenin concentration in the plasma is evidence of endocrine disruption on normal reproductive functions in male and immature female fish [22]. Exposure of fish at this stage to phytoestrogens could affect sexual differentiation as seen by vitellogenin production. Sex differentiation has been already established by exogenous steroid treatment. Estradiol treatments increased gonadal aromatase activity in differentiating gonads of common carp *(C. carpio)*, grey mullet (Mugil cephalus) and medaka (Oryzias latipes) [31-33]. Tzchori et al.[18] also demonstrated that phytoestrogen additives in eel diets could affect sex differentiation and increase percentage of females under the culture conditions. Devlin and Nagahama [34] implied that exogenous steroids change the expression of steroidogenic enzyme genes involved in sex differentiation.

The dose-response effect of soybean extract on vitellogenin levels in both sexes was potentially stimulatory depending on the concentration of soybean extract in the diets. The control group (0 mg/kg soybean extract diet), however, did not produce any vitellogenin and gave no cross-reactivity with anti-carp vitellogenin in both sexes of carp. A direct stimulatory effect of soybean extract on vitellogenin production was observed in both sexes as isoflavones were increased from 0 mg/kg to 10.000 mg/kg in the diet. The clear dose response effect in this study suggests that soybean phytoestrogens are acting as an estrogen mimic resulting in vitellogenin production in males and immature females. Although stimulatory effects of soybean isoflavones at all concentrations were observed in males, the level of vitellogenin stayed constant from 250 to 1.000 mg/kg soybean extract in the diet for females. The most effective concentration of soybean extract for increasing vitellogenin protein level may be somewhat higher than the 1.000 mg/kg treatment in female carp. Female fish fed the 10.000 mg/kg soybean extract diet had higher vitellogenin production than that of male fish. Estrogens can act on the hepatic receptors to induce vitellogenin in both male and female carp, but only the livers of female fish will normally be exposed to estrogens [22]. The total isoflavones-treated females may already have had vitellogenic oocytes in their ovaries. Therefore, higher vitellogenin production at the highest concentration of soybean extract was probably a result of the increased estrogen from these oocytes.

In conclusion, the results of this study clearly indicated that feeding carp with protein rich soybean extract diet for 180 days in the laboratory did not have any positive effect on body weight in either sex. However, vitellogenin protein production was stimulated both in male and female fish after in vivo soybean phytoestrogen exposure, indicating the presence of endocrine distruption. In addition, the

secretion of vitellogenin in response to soybean extract is dose-dependent, and therefore can be used to assess the potential estrogenic activity of isoflavones found in soybean. Although soybean seems to be good and cheap protein source, phytoestrogen compounds in this source may be very dramatic for the wild populations in view of their effect on the reproductive development.

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