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Effect of immersion treatment of soybean isoflavones extract on sex reversal in the Rainbow Trout (*Oncorhynchus mykiss*, Walbaum, 1792)

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Abstract. This study explores the possible utilization of soybean isoflavones extract as potent alternatives for induction of sex reversal in the rainbow trout (*Oncorhynchus mykiss*, Walbaum, 1792) with immersion treatment. Different concentrations (0, 0.10 and 0.20 g/L) of soybean isoflavones extract were tested for their effect on sex reversal in rainbow trout by immersing on newly hatched larvae twice for 1 months. The 14 days-old rainbow trout larvae (mean weight 0.123±0.03 g) were randomly removed from the hatching tank, and placed in 1000 L fiberglass tanks (200x100x75 cm). Each tanks comprised 1500 larvae and a total 4500 larvae were used for the experiment. When the fish reached four months, 50 randomly sampled fish from each group were sacrificed. For histological examination, the gonads were fixed in 10% neutral formalin and processed by routine dehydration and paraffin embedding procedures. At the end of experiment, the highest feminization (69.00±0.82%) was observed at 0.20 g/L soybean isoflavones extract treatment group. Morphological and histological examinations of the gonads in all groups revealed no intersex fish. Histological examination of fish treated with soy bean isoflavones extract revealed no damage to the testes or ovaries. In conclusion, these result indicate that the use of the higher doses of soybean isoflavones extract with immersion treatment can be effective for monosex rainbow trout population.

Key words: Rainbow trout, *Oncorhynchus mykiss*, sex reversal, Soybean isoflavones extract.

Introduction

Aquaculture has made an enormous contribution to the world food production, especially to the sustainable supply of animal proteins. The utility of diverse reproduction strategies in fish, such as the exploiting use of unisexual gynognesis, has created a typical case of fish genetic breeding. A number of fish species show substantial sexual dimorphism that is closely linked to multiple economic traits including growth rate and body size, and the efficient development of sex-linked genetic markers and sex control biotechnologies has provided significant approaches to increase the production and value for commercial purposes (Jie & Jian-Fang 2015). During early gonadal differentiation, exogenous steroid hormone treatment could effectively induce sex reversal in fish. Therefore, several hormones have been tested for enhancing sex reversal and for increasing fish culture productivity (Pandian & Sheela 1995, Makkar et al. 2007, Jie & Jian-Fang 2015). However, syntetic steroid treatments has most disadvantages such as expensivity, sterility at high dose and growth suppression in fish. Particularly, ingestion of synthetic steroid residues in sex reversed fish may be potentially hazardous to human consumers (Yilmaz 2013). The recent consumer demand for farmed fish has increasingly stressed quality and safety, and the absence of pollutants, antibiotics and carcinogens. All these reasons led investigators to study for natural alternative applications on sex reversal.

Phytoestrogens are plant-derived substances with estrogenic activity (Francis et al. 2001). 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 (Pelissero et al. 1991; Franke, 1994). Dry soybeans on average contain 1.107 milligrams of genistein per kilogram and 846 milligrams of daidzein per kilogram (Franke, 1994). Also, all soybean proteins and foods currently available for human consumption contain significant amounts of the isoflavones daidzein and genistein. For most of the soy flours and soy concentrates, isoflavone concentrations are relatively high (0.5–3.0 mg/g) (Setchell, 1998). These phytoestrogens may either have the same effects as estrogen or blocks estrogen's effects (Bennetau-Pelissero et al. 2001, Green & Kelly 2009), depending on the ratio of phytoestrogens to endogenous estrogens, aromatase activity, species and reproductive status, length of the exposure and method of administration in fish (Tsai et al. 2000, Trant et al. 2001, Andersen et al. 2003). The oestrogenic potency of these compounds on fish have been documented (Turan & Akyurt 2005, Turan 2006, Cek et al. 2007b, Chakraborty & Hancz 2011). The researchers (Tzchori et al. 2004, Ahmed et al. 2015, DiMaggio et al. 2016) found that genistein treated diets increased the proportion of females in juvenile eel (*Anguilla anguill*a), Southern flounder (*Paralichthys lethostigma*) and catfish (*Clarias gariepinus*) respectively. Similarly, Bennetau-Pelissero et al. (2001) reported that rainbow trout fed on a diet treated with genistein showed a decrease in 11- ketotestosterone (11-KT) and testosterone levels before and during spawning. In support, Zhang (2002) found that when Japanese rice fish *Oryzias latipes* was injected with genistein, testosterone production decreased, while estradiol production increased. The feminization effect of phytoestrogens on sex differentiation of African catfish (*Clarias gariepinus*) and Nile tilapia (*Oreochromis niloticus*) have also been reported by Yilmaz et al. (2009) and El-Sayed et al. (2012). Genistein, daidzein, and soybean-based diets also increased plasma vitellogenin concentrations in male and female rainbow trout (Bennetau-Pelissero et al. 2001).

However, recent evidence in mammals and fish indicate that one such alternative mechanism by which estrogenic compounds may act as inhibiting the activity of peripheral estogen-metabolizing enzymes. Ng et al. (2006) reported that genistein, an isoflavone found in high concentrations in soybeans, inhibits hepatic and renal estrogen metabolism in salmonid fish. Also, they stated that high doses (1 and 10

µM) of genistein inhibited estrogen metabolism in the liver and kidney of Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhyncus mykiss*), and in the liver of lake trout (*Salvelinus namaycush*).

Considering these aspects, the present study was focused to investigate the effects of Soybean isoflavones extract on sex reversal in rainbow trout (*O. mykiss*).

Material and methods

The experiment was carried out in Kahramanmaras trout farm of Kilic Holding Co. in Turkey and newly hatched four thousand five hundred larvae used in the sex reversal trials were obtained from the commercial production. The 14 days-old rainbow trout larvae (mean weight 0.123±0.03 g) were randomly removed from the hatching tank, and placed in 1000 L fiberglass tanks (200x100x75 cm) at a density of 1500 larvae per tank. Soybean isoflavones extract was purchased from Solgar® (U.S.A). The aqueous extracts of Soybean isoflavones extract were prepared by boiling in 250 ml distilled water for 20 minutes and then filtering it through a Whatman paper filter twice (Gauthaman & Adaikan 2005, Cek et al. 2007a). The solutions were freshly prepared in each treatment time. Different concentrations (0, 0.10 and 0.20 g/L) of Soybean isoflavones extracts were administered by immersing (twice for 1 months) newly hatched offspring with 2 hour treatment each. The Soybean isoflavones extract immersions were carried out in 100 L buckets in which the plastic chambers were floated. Each bucket was well oxygenated with an airstone (Hunter et al. 1986, Feist et al. 1995). The one remaining group served as control and was immersed only without soybean isoflavones extract.

Fish were fed with pellets with the appropriate size (Aqua K, Kılıc A.Ş. Turkey) five times a day using standard trout aquaculture procedure until sex ratios could be determined through histological observations. During 4 months, gonads developed sufficiently, for histological observation and hormonal effects as demonstrated by (Johnstone et al. 1989). When the fish reached four months, 50 randomly sampled fish from each group were weighed and sacrificed using a lethal dose of anesthetic (2-phenoxy ethanol) (Park et al. 1988). For histological examination, the gonads were fixed in 10% neutral formalin and processed by routine dehydration and paraffin embedding procedures. Cross-sections (4-6 µ thick) were stained with Mayer's hematoxylin and eosin phloxine B solution, examined, and microphotography (Cek et al. 2001). Growth and survival rate were monitored to determine the growth in each treatment groups at the end of experiment. Each fish was individually weighed and measured (total length) to the nearest 0.0001 g and 0.01 cm, respectively. A Chi-square (χ^2) test was used to determine whether observed sex ratios are different from an expected 1:1. Also, differences in growth were assessed by one-way ANOVA test and Duncan test was used to analyze which dosage groups cause the difference (Norusis 1993).

Result

In the present investigation, 0.20 g/L groups of soybean isoflavones extract– treated fish exhibited more female number than male number comparing to the control group and the highest feminization (69.00±0.82%) was observed at this group ($p<0.05$; Table 1). The sex ratio observed for the rainbow trout (*Oncorhyncus mykiss*) was nearly the expected ratio of 1:1 (female:male) in control group. (Table 1).

Table 2 shows the survival rate and growth rates in body weight of the rainbow trout. Total survival rates and growth rate in all treatments and controls were uniformly high ranging from 93.75% to 94.58% and 15.33 to 17.42 g respectively ($p>0.05$). This indicates that soybean isoflavones extract has no negative effect on survival and growth rate of rainbow trout at the tested concentrations (Table 2).

Morphological and histological examination of the gonads in all treatment groups revealed that no intersex fish were identified in rainbow trout. Histological examination of fish treated with soybean isoflavones extract revealed no damage to the testes or ovaries. Despite differences in sex ratio, no marked differences in the ovaries structure were found between control and soybean isoflavones extract-treated groups.

Table 1. Effects of treatment with soy extract on sex reversal of the rainbow trout *Oncorhynchus mykiss*.

	Soy extract treatments (g/L)		
Sex ratio	0	0.10	0.20
Female (n)	72	87	103
Male (n)	78	63	47
Female (%)'	48.66±1.69	58.00±0.82	69.00±0.82
Male (%)'	51.33±1.69	42.00±0.82	31.00±0.82
χ^2	-	3.011	13.179***
Asymp Sig.	-	0.083	0.000
df	-	1	1

χ^2 values are for comparisons of sex ratios (n: a number of fish) with the control (*,P<0.05; **, P<0.01; ***, P<0.001). Sex ratio significantly different from expected 1 M:1 F (n: 150 sample for per groups) 'Values (mean±S.D. of triplicate) for sex ratio (%).

Table 2. Effects of treatment with soy extract on survival and growth of the rainbow trout.

	Dose (g /L)	Survival Rate (%)	Initial weight (g)	Final weight (g)
Soy extract	0	94.58±2.60[a]	0.125±0.01[a]	17.42±5.21 [a]
	0.10	93.75±3.31[a]	0.120±0.01[a]	15.39±5.38 [a]
	0.20	94.17±2.60[a]	0.124±0.01[a]	15.33±6.25[a]

*Values (mean±S.D. of triplicate) with same superscripts in each column indicate insignificant differences (P>0.05).

Discussion

There has been growing demand in recent years for the culture of monosex fish populations in aquaculture sector. In trout culture conditions, female rainbow trout are more resistant against diseases and have better meat quality (Feist et al., 1995). Likewise, hormone treatment may accelerate growth in juveniles, and accelerated growth of juvenile stage may be an advantage in escaping from larger predators but with the penalty of suppressed growth of adults. These perceptions may clarify a portion of the conflicting reports on development of the hormone treated fish. From the accessible data on development of similarly matured or estimated treated people, summed up development patterns watched for hormonally sex switched fish having a place with salmonids (*O. kisutch*) and rainbow trout (*O. mykiss*) (Goetz et al., 1979; Feist et al., 1995). Initial examinations about sex reversal in fish culture were performed by Hendry et al. (2003), Pandian and Sheela (1995) and Yamamoto (1969). Common techniques have been modified for sex reversal by

synthetic steroids (Hunter et al., 1986; Atar et al., 2009). But, there are limited studies for the effect of isoflavones on sex reversal in rainbow trout.

To our knowledge, this is the first report regarding the potential of low concentrations of soybean isoflavones extract with immersion treatment as a feminization agent in rainbow trout. The 0.20 g\L soybean isoflavones extract group was the most potent dose in this study, for feminization. Although the treatment of the newly-born progenies using soybean isoflavones extract significantly increased the percentage of females to 69.00%, we cannot conclude that this potency was caused by an increase of estrogen since we did not measure plasma estrogen levels. Most authors have reported similar observation for other fish species, treated with various phytoestogens (Rodriguez Montes de Oca 2005, Turan & Akyurt 2005, Turan 2006, Cek et al. 2007b, Chakraborty & Hancz 2011). Also, Bennetau-Pelissero et al. (2001) reported that rainbow trout fed on a diet treated with genistein showed a decrease in 11- ketotestosterone (11-KT) and testosterone levels before and during spawning. Genistein, daidzein, and soybean-based diets also increased plasma vitellogenin concentrations in male and female rainbow trout (Bennetau-Pelissero et al. 2001).

Survival rates and trout growth at the termination of the present experiment were found similar to all soybean isoflavones extract-treated groups, and were almost 94%. Similarly, Rodriguez Montes de Oca (2005) and Akinwande et al. (2011) reported that genistein did not affect tilapia growth and survival. Aromatase inhibitor did not also affect the survival of bluegill sunfish *Lepomis macrochirus* (Gao 2010). However, the effect of phytoestrogen on fish survival is controversial. For example, El-Sayed et al. (2012) reported that survival rate decreased with increasing dietary genistein and daidzein in nile tilapia (*Oreochromis niloticus*). In a way that supports, Ingham et al. (2004) found that exposing fathead minnows to genistein decreased fish survival. Due to controversial results, more work is needed to resolve this conflict.

In summary, this study demonstrated that sex differentiation of rainbow trout was diverted toward the female gender with treatment of low concentrations of soybean isoflavones extract. The highest feminization (69.00%) was obtained at 0.20 g\L soybean isoflavones extract group in this study. However, further studies will be required to determine the optimum treatment regime for induction of 100 % sex reversal with this extract in rainbow trout.

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OF RAINBOW TROUT (OncOrhynchus mykiss WALBAUM). And production of all-female populations. H.H. Atar, S. Bekcan and L. Dogankaya.Â Sex-reversal studies from the aquaculture literature provide insight in to the evolvability of determinants of sexual phenotype. Additionally, induced sex reversal can provide information about the evolution of sex chromosomes and sex-linked traits. Recently, naturally occurring ESR has been implicated as a mechanism contributing to the evolution of sex chromosomes. Cryopreservation of rainbow trout Oncorhynchus mykiss spermatozoa: Effects of extender supplemented with different antioxidants on sperm motility, velocity and fertility. Filiz Kutluyer et al. Cryobiology, 2014.Â The in vitro effect of Lambda-cyhalothrin on quality and antioxidant responses of rainbow trout Oncorhynchus mykiss spermatozoa. Filiz Kutluyer et al. Environmental Toxicology and Pharmacology, 2015. Rainbow trout Oncorhynchus mykiss has a fusiform body shape which is blue to olive green, with a pink colouration along the lateral line (Figure 1). In freshwater, these fish are dark green, yellow-green or brown with dark spots on the body, dorsal fin and tail, and have a pink or red band extending along the body. In salt water or brackish water, O. mykiss is silvery with the top half of the fish darker and has dark spots above the lateral line.Â In the wild, rainbow trout only spawn once a year, usually between June and September in South Africa (Skelton 2001). In the wild, spawning takes place in rocky or gravel habitats in rivers or streams. The lack of gravel beds in dams is thought to be a limiting factor for the natural distribution of O. mykiss (S. Porter, Anchor Environmental, pers. obs.). Effect of solvent extracted soybean meal and full-fat soya on the protein and amino acid digestibility and body amino acid composition in rainbow trout (Oncorhynchus mykiss). Iranian Journal of Fisheries Sciences, 11(3), 504-517.Â Nutritional, physiological and haematological responses in rainbow trout (Oncorhynchus mykiss) juveniles fed detoxified Jatropha curcas kernel meal. Aquaculture Nutrition, 17(4), 451-467.Â Effects of different levels of soybean meal inclusion in replacement for fish meal on growth, digestive enzymes and transaminase activities in practical diets for juvenile tilapia, Oreochromis niloticusÃ— O. aureus. Animal Feed Science and Technology, 168(1-2), 80-87.Â Dietary isoflavones: biological effects and relevance to human health.