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Effects of Dietary Supplementation of Cottonseed and Soybean meal on Reproductive Histology of Olive Flounder, *Paralichthys olivaceus*

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The gossypol existed in cottonseed meal is a well known antispermatogenic agent which can impair reproductive performances of male fish as well as mammals. Two feeding experiments were conducted to examine a toxic effect of dietary supplementation of cottonseed meal on reproduction in juvenile olive flounder (the first experiment) for 19 weeks and growing olive flounder (the second experiment) for 26 weeks. After each feeding study, females and males were sampled for histological examination in gonads and liver to verify any negative effects by the dietary supplementation of cottonseed and soybean meal on reproduction. After two feeding trial, the gonad somatic index (GSI) of male and female (from the first feeding trial) were not significantly different among all the dietary treatments. The GSI values of female (from the second feeding trial) were not significantly different among all the dietary treatments. However, males fed cottonseed and soybean meal containing diets exhibited significantly lower GSI than that fed the control diet after the second feeding trial. Histological examination of gonads and liver of fish fed cottonseed and soybean meal did not show any negative effects compared to those of fish fed the control diet. Hepatosomatic index of fish in the first and second feeding trials were not significantly different among all the dietary treatments. The findings in this study suggest that dietary supplementation of cottonseed and soybean meal up to 40% fish meal replacement might not deteriorate the gametogenesis of juvenile and growing olive flounder. However, the supplementation in diets over 30% fish meal replacement might reduce GSI of male in growing olive flounder.

Keywords: Olive flounder, Cottonseed meal, Soybean meal, Histology, GSI

Introduction

Fish meal (FM) replacement studies have extensively conducted focusing on the use of plant protein sources (Lee et al., 2001, 2002). FM has been a major ingredient in fish diets because of its high protein quality and palatability. Substituting less expensive protein sources for high-price FM in fish feeds is one way to lower production costs (Lee et al., 2001). For this reason, many studies have been conducted to replace or reduce its inclusion in fish diets by various cheaper alternative animal and vegetable protein sources; however, each candidate has characteristics that make it inferior in some respect to high-quality FM (Hardy, 1996).

Among plant origin protein sources, soybean meal has been considered as a promising protein source that can completely or partially replace fish meal in aquatic animal diets. A number of studies have shown that soybean meal alone or in combination with other protein sources can replace fish meal from 20% up to 90% in diets for many fish species (Pham et al., 2005). The use of soybean meal in fish feeds is still limited because of the presence of some antinutritional factors, such as protease inhibitors, phytates, lectins, saponins, non-starch polysaccharide and high fiber content (NRC, 1993; Storebakken et al., 2000; Hendricks, 2002). In addition, the deficiency of some essential amino acids in soybean meal, such as methionine and lysine, also reduces the inclusion level of this material in fish feeds (NRC, 1993).

One of cottonseed byproducts, cottonseed meal (CM) has been used in diets for both terrestrial animals (Colin-Negrete et al., 1996) and fish (Hendricks et al., 1980) because of its high protein content. CM has been examined in diets for fish, such as channel catfish (Dorsa et al., 1982; Robinson and Li, 1994; Robinson and Tiersch, 1995), rainbow trout (Hendricks et al., 1980; Dabrowski et al., 2000; Lee et al., 2001; Lee and Dabrowski, 2002; Rinchard et al., 2003), tilapia (El-Sayed 1990; Robinson et al., 1984; Mbahinzireki et al., 2001; Rinchard et al., 2002), and olive flounder (Pham et al., 2005). Despite its high nutritional value, cottonseed contains gossy-

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pol, polyphenolic compound, which is toxic to fish (Herman 1970; Rinchard et al., 2000) and terrestrial animals (Colin-Negrete et al., 1996; Makinde et al., 1997). The gossypol is a well known antispermatogenic agent which impairs reproductive performances of male fish (Ciereszko and Dabrowski, 2000) as well as mammals (Randell et al., 1992). In bulls, the feeding of cottonseed containing a high concentration of gossypol caused reduced sperm production and motility, and increased sperm abnormalities and testicular damage (Chase et al., 1994; Chenoweth et al., 1994; Randell et al., 1996). Anti-spermatogenic effects, such as a decrease in sperm motility and structural changes, were also exhibited in the testis of rats fed 15 ppm of gossypol per day for 3 weeks (Wang et al., 1988). Pathological and histopathological symptoms were exhibited in broiler chicks (Henry et al., 2001). However, in a long-term feeding study of fish (Robinson and Tiersch, 1995), CM inclusion over 37% did not depress the reproductive performance, such as testis weight, gonadosomatic index and sperm motility in male channel catfish reared in a pond.

Studies on the use of cottonseed meal for FM replacement in marine fish species are very rare (Pham et al., 2005), and no study was reported on the reproduction or histology of olive flounder. Therefore, the aim of this study was to investigate the effects of dietary supplementation of soybean and cottonseed meal on reproductive histology in juvenile and growing olive flounder.

Materials and Methods

Experimental design

There were two feeding experiments. The feeding experiments were conducted with juvenile olive flounder for 19 weeks (the first feeding study; Pham et al., 2005) and with growing olive flounder for 26 weeks (the second feeding trial; unpublished). After each feeding study, females and males were sampled for histological examination in gonads and liver to verify any effects by the dietary supplementation of soybean and cottonseed meal on reproduction.

Experimental diets and feeding

Two sets of the experimental diets were presented in Table 1. The experimental diets used in the first feeding trial were formulated to be isonitrogenous and isocaloric to replace 0, 10, 20, 30, and 40% of fish meal protein by equal proportion (1:1, w:w) of cottonseed and soybean meal (CS) (designated by Control, CS10, CS20, CS30, and CS40, respectively). The CS diets were supplemented by DL-methionine and L-lysine to meet their dietary requirements (NRC, 1993). The cottonseed meal was provided from Southern Cotton Oil Co.,

Table 1. Formulation of experimental diets (% dry matter)

EXP 1 Ingredients	Diets				
	CS0	CS10	CS20	CS30	CS40
White fish meal	60.0	54.0	48.0	42.0	36.0
Soybean meal	0.0	4.4	8.7	13.1	17.5
Cotton seed meal[a]	0.0	4.7	9.4	14.1	18.8
Corn gluten meal	8.0	8.3	8.7	9.0	9.3
Wheat flour	21.8	17.8	13.8	9.8	5.8
Squid liver oil	5.0	5.4	5.8	6.2	6.6
Lysine[b]	0.0	0.1	0.2	0.3	0.4
Methionine[c]	0.0	0.1	0.2	0.3	0.4
The others[d]	5.2	5.2	5.2	5.2	5.2
EXP 2 Ingredients	Diets				
	CS0	CS20	CS30	CS30+ Fe&P	CS40+ Fe&P
White fish meal	54.0	43.2	37.8	37.8	32.4
Soybean meal	0.0	7.9	11.8	11.8	15.7
Cotton seed meal	0.0	8.5	12.7	12.7	16.9
Corn gluten meal	6.6	7.0	7.2	7.2	7.4
Wheat flour	24.0	16.9	13.3	13.3	9.7
Squid liver oil	7.3	7.9	8.2	8.2	8.5
Lysine	0.0	0.4	0.6	0.6	0.8
Methionine	0.0	0.2	0.3	0.3	0.4
Ferrous Sulfate-$7H_2O$[e]	0.0	0.0	0.0	0.2	0.3
Monocalciumphosphate[f]	0.0	0.0	0.0	1.0	1.5
Cellulose	2.4	2.4	2.4	1.2	0.6
The others[g]	5.7	5.7	5.7	5.7	5.7

[a]Cottonseed meal was purchased from Southern Cotton Oil Co., Memphis, Tennessee 38108, USA
[b]L-lysine mono-hydrochloride, Sigma, USA
[c]L-methionine, Sigma, USA
[d]Mineral mix, 1% ($MgSO_4.7H_2O$, 80.0; $NaH_2PO_4.2H_2O$, 370.0; KCl, 130.0; Ferric citrate, 40.0; $ZnSO_4.7H_2O$, 20.0; Ca-lactate, 356.5; CuCl, 0.2; $AlCl_3$. $6H_2O$, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4.H_2O$, 2.0; $CoCl_2.6H_2O$, 1.0.); vitamin mix, 1% (L-ascorbic acid, 121.2; DL-α tocopheryl acetate, 18.8; thiamin hydrochloride, 2.7; riboflavin, 9.1; pyridoxine hydrochloride, 1.8; niacin, 36.4; Ca-D-pantothenate, 12.7; myo-inositol, 181.8; D-biotin, 0.27; folic acid, 0.68; p-aminobezoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalficerol, 0.003; cyanocobalamin, 0.003); yeast, 2%; CMC, 1%; and choline chloride, 0.2%
[e]Ferrous Sulfate $7H_2O$, Sigma, USA
[f]Monocalciumphosphate, Sigma, USA
[g]Mineral mix, 0.5% ($MgSO_4.7H_2O$, 80.0; $NaH_2PO_4.2H_2O$, 370.0; KCl, 130.0; Ferric citrate, 40.0; $ZnSO_4.7H_2O$, 20.0; Ca-lactate, 356.5; CuCl, 0.2; $AlCl_3$. $6H_2O$, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4.H_2O$, 2.0; $CoCl_2.6H_2O$, 1.0.); vitamin mix, 2% (L-ascorbic acid, 121.2; DL-α tocopheryl acetate, 18.8; thiamin hydrochloride, 2.7; riboflavin, 9.1; pyridoxine hydrochloride, 1.8; niacin, 36.4; Ca-D-pantothenate, 12.7; myo-inositol, 181.8; D-biotin, 0.27; folic acid, 0.68; p-aminobezoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalficerol, 0.003; cyanocobalamin, 0.003); yeast, 2%; CMC, 1%; and choline chloride, 0.2%.

Memphis, TN, USA, and its protein content was 43.5% in dry mater basis. The experimental diets used in the second feeding trial (Table 1) were formulated to be isonitrogenous and isocaloric to replace 0, 20, 30, and 40% of fish meal protein by equal proportion (1:1, w:w) of CS with supplementation of iron and phosphorus (designated by Control, CS20, CS20, CS30, CS30+Fe&P, and CS40+Fe&P, respectively). The experimental diets were manufactured and fed to the experimental fish by a general feeding method (Pham et al., 2005).

For the first feeding experiment, total 900 fish at the early juvenile stage were randomly distributed into 15 (35 L) plastic circular tanks at a density of 60 fish/tank (initial body weight 0.74±0.11 g). Each experimental diet was fed to triplicate groups of fish with the feeding rates ranging from 5% of fish weight at the beginning to 3% at the end of feeding trial. The fish were fed twice (9:00 and 16:00) a day, 7 days a week, for 19 weeks. The feeding trial was conducted in a flow through system supplied with sand filtered seawater at a flow rate of 2~3 L/min. Supplemental aeration was also provided to maintain dissolved oxygen levels near the saturation. For the second feeding experiment, total 225 fish (initial wt. 28.7±0.17 g) were randomly distributed into 15 plastic circular tanks (capacity 200 L). The tanks were supplied with filtered sea water at a flow of 7~8 L/min. The experimental diets were fed ad libitum to triplicate fish groups twice a day (9:00 and 16:00), 7 days a week, for 26 weeks. The feeding trials were conducted in Marine and Environmental Research Institute, Cheju National University.

Fish sample collection

At the end of two feeding trials, males and females were randomly selected from each tank (3 tanks per dietary treatment), dissected for liver and gonads, weighed for HSI (hepatosomatic index; liver wt.x100/fish wt.) and GSI (gonadosomatic index; gonad wt.x100/fish wt.), and preserved in 10% formalin solution until histological examination of liver and gonads. The sampled fish weight were 65±17.3 g and 200±15.4 g after the first and second feeding trial, respectively.

Histological examination

The sampled fish were dissected for gonads and liver. The gonads and liver were fixed in Bouin's solution, dehydrated in the series of ethanol, embedded in paraffin and then cut in 5-7 μm. Slides were stained with Hansen's hematoxylin and 0.5% eosin for histological observations.

Statistical analysis

For the GSI and HSI comparison, data were subjected to one-way ANOVA in SPSS version 11.0. Significant differences between group means were compared using Duncan's multiple test. Data presented are means±S.D. The percentage data were arcsine transformed before the ANOVA analysis. Differences were considered significant at $P<0.05$.

Results and Discussion

The growth and feed utilization of fish fed the experimental diets in the first experiment (Pham et al., 2005) and the second experiment (unpublished) are presented elsewhere. The GSI of male and female after the first feeding trial was not significantly different among all the dietary treatments (Fig. 1). However, males fed CS containing diets exhibited significantly lower GSI than that fed the control diet except

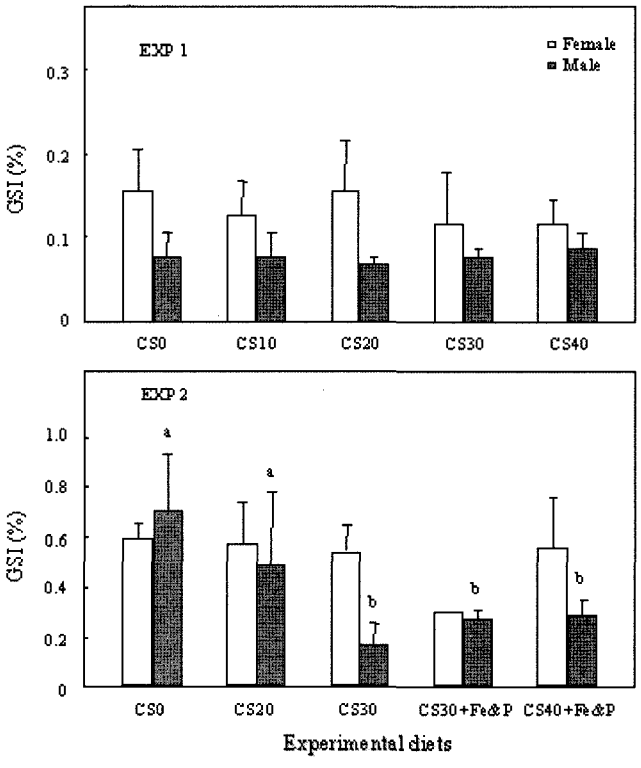

Fig. 1. Gonadosomatic index (GSI) of olive flounder fed diets containing cottonseed and soybean meal for 19 and 26 weeks (Experiment 1 and 2, respectively). EXP 1; CS0, CS10, CS20, CS30 and CS40 are experimental diets in which 0%, 10%, 20%, 30%, and 40% fish meal protein were respectively replaced by mixture of cottonseed and soybean meal (1:1, w:w). EXP2; CS0, CS20, CS30, CS30+Fe&P and CS40+Fe&P are experimental diets in which 0%, 20%, 30%, 30% and 40% fish meal protein was respectively replaced by mixture of cottonseed and soybean meal (1:1, w:w) with/without supplementation of iron and phosphorous.

for the CS20 dietary groups (Fig. 1), even though GSI of female after the second feeding trial was not significantly different among all the dietary treatments.

In monogastric animals, the toxicity of gossypol in cottonseed meal has been extensively studied and reported. The most commonly reported toxicity symptom was a reproductive impairment by decreased sperm counts and motility. The mechanism of the action as a male contraceptive agent has been thought to inhibit cellular energy metabolism (Coyle et al., 1994). The gossypol molecule inhibits glycolysis by inhibiting specific testis lactate dehydrogenase isoenzyme (Giridharan et al., 1982), mitochondrial oxidative phosphorylation and electron transport (Reyes et al., 1988). Yuan and Shi (2000) found that gossypol limited the fertilizing ability of spermatozoa in hamster *in vivo*, and they concluded that it can be attributed to the inhibition of acrosomal enzyme activity, such as acrosin and arylsulfatase. Anti-spermatogenic effects, such as decrease in sperm motility and structural changes, were also exhibited in the testis of rats fed 15 ppm of gossypol per day for 3 weeks (Wang et al., 1988). In studies conducted with human males, it was reported that gossypol is a very strong contraceptive (National Coordinating Group, 1978; Frick and Danner, 1985). As mentioned above, the reproductive impairment by gossypol in cottonseed meal was mainly reported in males than females of animal. In the present study, we found that in juvenile olive flounder the GSI was not affected by CS supplementation up to 20% in diets. Interestingly, however, the males were significantly affected by the CS supplementation over 30% fish meal replacement showing decreased GSI. The GSI in the present study showed that male would be more easily affected by the gossypol in cottonseed meal than female.

The histological examination for reproduction showed that fish were not affected by cottonseed and soybean meal (CS) with respect to gonads compared to that of fish fed the control diet (Fig. 2). Both males and females showed a normal gametogenesis after the feeding of CS containing diets.

Toxic effects of gossypol in cottonseed meal on male gametes were reported to impair spermatogenesis and mature spermatozoa (Ikeda, 1990; Randel et al., 1992; Ciereszko and Dabrowski, 2000). In fish, reproductive impairment by dietary cottonseed meal has been inconclusive. Robinson and Tiersch (1995) reported that cottonseed meal inclusion over 37% did not depress the reproductive performance, such as testis weight, GSI, and sperm motility in brood-sized male channel catfish reared in a pond through a long-term feeding study. However, Nile tilapia fed dietary cottonseed meal up to 24% inclusion revealed an impaired testis activity after 120 days

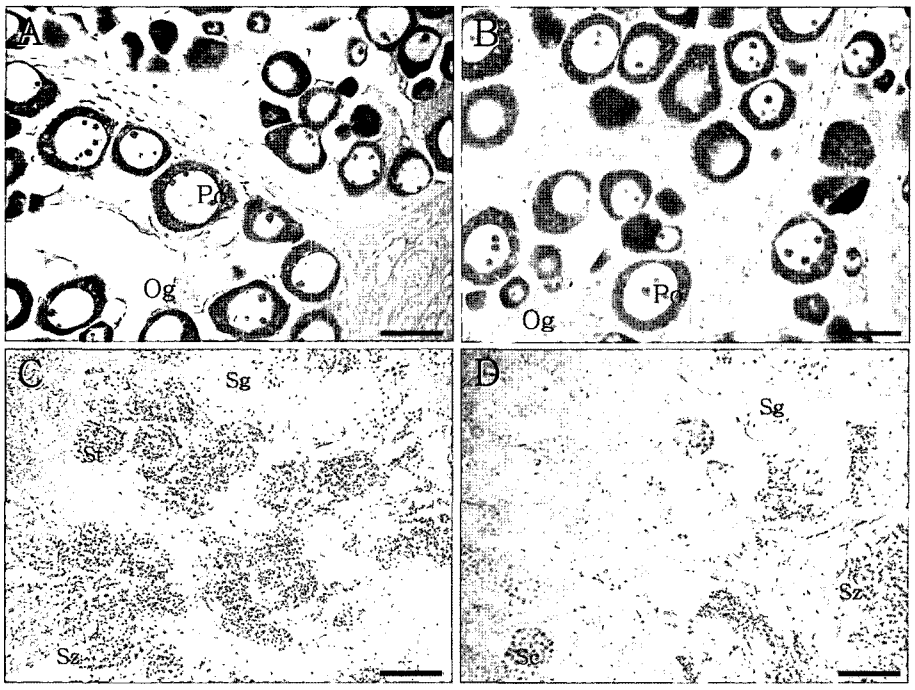

Fig. 2. Histological observation of gonads in olive flounder. A, Ovary of control female; B, Ovary of female fed CS40+P&Fe diet; C, Testis of control male; D, Testis of male fed CS40+P&Fe diet. Og, oogonium; Po, peri-nucleolus oocyte; Sc, spermatocyte; Sg, spermatogonia; St, spermatid; Sz, spermatozoa, Bar A, B, C and D=50 μm.

of feeding even though the fish were not adversely affected with respect to weight gain (Salaro et al., 1999). Ciereszko and Dabrowski (2000) also showed through an *in vitro* test that spermatozoa of yellow perch were negatively affected by gossypol acetate in terms of mobilization and fertilizing ability. In male sea lamprey (Rinchard et al., 2000), the sperm motility was significantly lowered by gossypol injection even though sperm concentration was not affected. In the present study, therefore, the gametogenesis was examined to verify the effects of dietary supplementation of CS on reproduction in olive flounder. The result of the histological examination of ovary and testis in fish fed CS containing diets in the present study did not show any impairment in gametogenesis of juvenile (data not presented) and growing olive flounder (Fig. 2). The result from this study is very significant because to our best knowledge no study has been reported for the effects of dietary supplementation on histological examination of gonads in olive flounder. Rinchard et al. (2000) also reported no differences in histological examination of gonads of male sea lamprey by gossypol injection, even though the sperm motility was impaired.

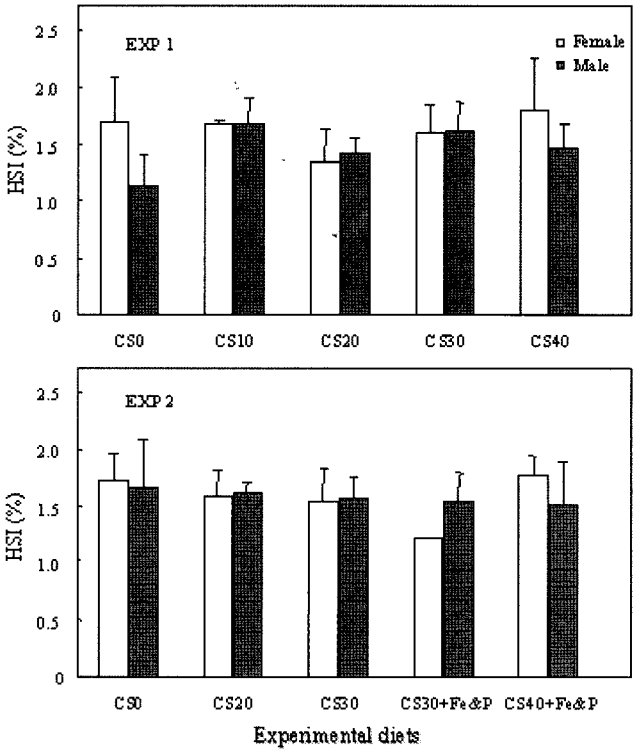

Fig. 3. Hapatosomatic index (HSI) of olive flounder fed experimental diets for 19 and 26 weeks (Experiment 1 and 2, respectively). EXP 1; CS0, CS10, CS20, CS30 and CS40 are experimental diets in which 0%, 10%, 20%, 30%, and 40% fish meal protein were respectively replaced by mixture of cottonseed and soybean meal (1:1, w:w). EXP2; CS0, CS20, CS30, CS30+Fe&P and CS40+Fe&P are experimental diets in which 0%, 20%, 30%, 30% and 40% fish meal protein was respectively replaced by mixture of cottonseed and soybean meal (1:1, w:w) with/without supplementation of iron and phosphorous.

The HSI of fish in the first and second feeding trials was not significantly different among all the dietary treatments (Fig. 3). The histological examination of liver also did not show any differences among all the dietary treatment (data not presented). However, in a study with juvenile rainbow trout feeding a diet containing 25% cottonseed meal for a long time (12 months) impaired liver tissue (Hendricks et al., 1980). In the study (Hendricks et al., 1980), HSI was significantly higher in cottonseed meal fed fish than a control diet fed fish. Also, the fish fed cottonseed meal exhibited a tumor in their liver tissue. The histology of the liver was characterized by broad trabeculae of deeply basophilic cells, numerous mitotic figures, and various degrees of hyperplastic bile duct. The histological examination of liver in the present study was based on 6 months of feeding trial. Therefore, the liver histopathology of olive flounder needs to be examined after relatively longer period of feeding trial to verify the toxic effects of gossypol.

In conclusion, dietary supplementation of cottonseed and soybean meal up to 40% fish meal replacement might not deteriorate the gametogenesis of juvenile and growing olive flounder. However, their supplementation in diets over 30% fish meal replacement might reduce gonadosomatic index in male olive flounder. Further study needs to be focused on the reproductive events, such as sperm motility and concentration, fertilization rate, and egg counts in olive flounder broodstocks fed cottonseed containing diets.

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