A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

# Nutritional study on cottonseed and soybean meal in diets for marine fish

Se-Jin Lim

Department of Marine Life Science

GRADUATE SCHOOL

CHEJU NATIONAL UNIVERSITY

2007. 8.

# Nutritional study on cottonseed and soybean meal in diets for marine fish

**Se-Jin Lim**
**(Supervised by Professsor Kyeong-Jun Lee)**

**A thesis submitted in partial fulfillment of the requirement for the degree of**

**Master of Science**

**2007. 6.**

**This thesis has been examined and approved.**

**……..**
**Ki-Wan Lee, Prof. of Marine Life Science**

**……..**
**Young-Don Lee, Prof. of Marine Life Science**

**……...**
**Kyeong-Jun Lee, Assistant Prof. of Marine Life Science**

**Department of Marine Life Science**
**GRADUATE SCHOOL**
**CHEJU NATIONAL UNIVERSITY**

# Contents

**Part Ⅱ. Use of cottonseed and soybean meal in diet for parrot fish (*Oplegnsthus fasciatus)***



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

# 국문초록

양어사료에서 주 단백질원으로 사용되는 어분의 대체는 양식산업의 비약적 발전과 어획량 감소로 인해 양식산업에서 중요한 사안으로 부각되고 있다. 면실박과 대두박은 높은 단백질 함량과 비교적 우수한 아미노산조성으로 양어사료에서 어분을 대체할 수 있는 식물성 단백질원으로서 많은 연구가 수행되어졌다. 그러나 이러한 식물성 단백질원들은 어분과 비교하여 제한 아미노산인 lysine과 methionine이 부족할 뿐만 아니라 phytic acid 및 gossypol과 같은 항영양인자들을 포함하고 있어 양어사료에서의 이용이 매우 제한적이다. 이 연구는 우리나라 주요 양식 어종인 넙치와 최근 새로운 양식 어종으로 각광받는 돌돔을 대상으로 사료 내 면실박과 대두박의 어분대체 가능성 및 그 이용성을 알아보고자 수행되었다.

성장기 넙치를 대상으로 한 장기간의 사료공급 사육실험결과(Part I), 사료에 철과 인이 보충되었을 때 면실박-대두박 혼합물은 어분단백질을 40%까지 대체할 수 있었다. 돌돔을 대상으로 한 사료공급 사육실험(Part II)에서는 사료에 철과 phytase가 보충된다면 면실박-대두박 혼합물로 사료 내 어분단백질을 30%까지 대체할 수 있었다. 치어기와 성장기 돌돔 사료에 면실박-대두박 혼합물이 첨가된다면 어류의 혈장 중성지방 및 콜레스테롤 함량을 낮출 수 있을 것으로 나타났다. 사육 실험 후, 넙치 간에서의 gossypol 축적 농도는 사료 내 면실박-대두박 혼합물 첨가농도와 비례하였다. 돌돔 간에서의 gossypol 축적 농도는 넙치에 비해 유의적으로 낮은 값을 나타내었다. 철을 보충한 면실박-대두박 혼합물 사료를 섭취한 어류의 gossypol 축적농도는 철을 보충하지 않은 사료를 섭취한 어류에 비해 유의적으로 낮은 값을 보임으로써 사료 내 철 첨가는 gossypol 독성을 줄일 수 있을 것으로 판단되었다. 번식조직학 분석실험에서는(Part III) 사료 내 면실박-대두박 혼합물 첨가가 넙치 생식능력에 아무런 영향을 미치지 않는 것으로 조사되었으며, 간 조직에도 영향을 주지 않는 것으로 나타났다.

위 결과들을 종합해 볼 때, 해산 어류 배합사료에 철과 인 및 phytase를 첨가 보충한다면 넙치에서는 면실박-대두박 혼합물로 사료 내 어분단백질을 40%까

지, 돌돔에서는 30%까지 대체할 수 있으며, 사료의 gossypol 독성을 줄일 수 있을 것으로 판단되어진다. 또한, 사료 내 gossypol (3400ppm 이하)은 치어 및 성장기 해산 어류의 생식능력에 영향을 미치지 않을 것으로 보여진다. 식물성 단백질원인 면실박-대두박 혼합물의 사료 내 첨가는 어류에서 중성지방과 콜레스테롤 축적을 낮춤으로써 이 연구의 결과는 향후 기능성 어류 생산을 위한 기초자료로도 활용될 수 있을 것으로 판단된다.

# List of Figures

# List of Tables

# REFERENCE REVIEW

Dietary replacement of fish meal (FM) have been an important issue in aquaculture industry due to a limited supply of FM and its dramatic price increase in recent years (FAO, 2003). Feed costs account for over 50% total production costs in most marine fish species, because of the use of the expensive FM with a large dietary proportion (Coyle et al., 2004). 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 diets 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).

Soybean meal (SM) has been the most frequently studied dietary ingredient as a FM replacer in diets for many fish species because of its high protein content, relatively well-balanced amino acid profiles, reasonable price and steady supply. (Storebakken et al., 2000). The value of SM as a substitute for FM in formulated diets has been investigated for many fish species, such as Atlantic salmon (Refstie et al., 2001), Asian sea bass (Boonyaratpalrin et al., 1998), channel catfish (Bai and Gatlin, 1994), rainbow trout (Cho et al., 1974), grass carp (Dabrowski and Kosak, 1979) and common carp (Viola et al., 1983). Kikuchi (1999) reported that about 45% of FM protein could be replaced with SM in combination with other protein sources in the diet of olive flounder. However, the use of SM in fish feed 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 (Spinelli et al., 1983; Snyder and Kwon, 1987; Davies et al., 1990; Krogdahl et al., 1994; Krogdahl, 1995; Liu, 1997; Storebakken et al., 1998; Refstie et al., 1999; Storebakken et al., 2000; Hendricks, 2002). In addition, the deficiency of some essential amino acids in SM, such as methionine and lysine also reduces the inclusion level of this material in fish feeds (NRC, 1993; Krogdahl, 1995).

Cottonseed meal (CM), a by-product of cottonseed has long 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, availability and low cost. CM, an oil-seed meal product, is the third largest in world production after soybean and rapeseed meal (USDA, 2000). In United States, CM is the second largest plant protein source after soybean meal, and the cotton industry has been an important part of U.S. agriculture over the past two centuries (Lee, 2002). In fish, although there have been many fish species cultured, CM has been investigated in just few species, such as Pacific salmon and rainbow trout (Herman, 1970; Fowler, 1980; Dabrowski et al., 2000; Cheng and Hardy, 2002; Lee and Dabrowski, 2002), channel catfish (Robinson et al., 1984; Robinson and Daniels, 1987; Robinson and Brent, 1989; Robinson and Li, 1994; Robinson and Tiersch, 1995), tilapia (Jackson et al., 1982; Ofojekwu and Ejike, 1984; Robinson et al., 1984b; El- Sayed, 1990; Salaro et al., 1999; Mbahinzireki, 1999), largemouth bass (Kurten et al., 1999) and sunshine bass (Rawles and Gatlin, 2000).

CM contains gossypol, a yellow cottonseed gland pigment, which is toxic to fish (Herman, 1970; Rinchard et al., 2000) and terrestrial animals (Colin-Negrete et al., 1996; Makinde et al., 1997) leading to a restriction of its use as a feed ingredient. The gossypol is a well known antispermatogenic agent which can impair 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). Also, 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 in 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 brood-sized male channel catfish reared in the pond.

Iron, as ferrous sulphate, has been used to bind with the toxic free gossypol and thereby reduce the toxicity in feeds for monogastric animals (Jones, 1987; Martin, 1990) and fish (Sealey et al., 1997; Barros et al., 2002). Barros et al. (2002) successfully replaced 50% SM with ferrous sulfate in diets for channel catfish whereas Sealey et al. (1997) reported a negative effect of high level of supplemental iron, such as disease susceptibility, in the fish species.

One major obstacle in using plant protein sources, such as SM and CM, is the presence of phytic acid (NRC, 1993). Phytic acid (myo-inositol hexakisphosphate) is the major compound for phosphorus storage (over 70%) in the plant seeds and cannot be digested and absorbed by monogastric animals including fish (Barual et al., 2004). Many fish nutritionists have tried to supplement phosphorus itself as phosphate to compensate unavailable phosphorus in the plant seeds and/or phytase, an enzyme, to liberate free phosphorus from phytic acid. In Atlantic cod phosphorus supplementation in plant protein based diets could replace 50% dietary FM without growth impairment (Albrektsen et al., 2006). Also, SM was successfully replaced by CM with supplementation of phosphorus as dicalciumphosphate in channel catfish diets (Robinson and Brent, 1989; Robinson and Tiersch, 1995).

SM and CM are sources of important flavonoid, isoflavones, glycitein, genistein and daidzein that act as free radical scavengers and have shown beneficial health-promoting effects in diseases. (Andlauer et al., 1999; Fritz et al., 2003). Their beneficial effects have been described for diabetis mellitus, allergy, cancer, viral infections, headache, stomach and duodenal ulcer, parodentosis and inflammations (Pathak et al., 1991; Wagner, 1985; Havsteen, 1983; Kuehnau, 1976). Recent studies have shown that the SM and CM products have an ability to reduce serum cholesterol in animals. A complete replacement of corn oil by cottonseed oil was reported to reduce total serum cholesterol in growing rats (Edwards and Radcliffe, 1995; Nwoha and Aire, 1995; Radcliffe et al., 2001). Venou et al. (2006) reported that plasma cholesterol decreased with inclusion of soybean meal in diet for gilthead sea bream. The mechanism for the plant protein sources effect on lipid metabolism has not been clearly determined. Further study is needed on this issue.

# Part Ⅰ

# Supplemental iron and phosphorus increase dietary inclusion of cottonseed and soybean meal in olive flounder (*Paralichthys olivaceus)*

**Part 1**

# Supplemental iron and phosphorus increase dietary inclusion of cottonseed and soybean meal in olive flounder (*Paralichthys olivaceus)*

## ABSTRACT

A long-term feeding experiment was conducted to investigate the use of cottonseed and soybean meal with iron and phosphorus supplements in diets for olive flounder (*Paralichthys olivaceus)*. Olive flounder with an initial average size of 28.5±0.35g (mean±SD) were divided into 15 groups (three tanks per dietary treatment) and fed 48% crude protein diets in which each of five isonitrogenous diets was formulated to contain different levels of cottonseed/soybean meal (CS) to replace fish meal (FM) with iron and phosphorus supplementations. The five experimental diets were as follows: Diet1 (control), 0%CS; diet2, 20%CS; diet3, 30%CS; diet4, 30%CS+Fe&P; and diet5, 40%CS+Fe&P. After 26 weeks of feeding trial, no significant differences were observed in weight gain, feed utilization and survival among all the treatments. The total gossypol accumulation in liver of fish fed diets supplemented with iron was significantly lower than that of fish fed diets without supplementation of iron. The results indicate that the addition of iron in diets could prevent the absorption of free gossypol. The findings in this study suggest that dietary supplements of iron and phosphorus could increase the inclusion of cottonseed and soybean meal for FM replacement in diets for marine fish species.

## MATERIALS AND METHODS

### Experimental diets

Five experimental diets were formulated to replace FM protein by equal proportion (1:1, w:w) of cottonseed/soybean meal (CS) with supplements of iron and phosphorus. The experimental diets were as follow: Diet 1 (control), 0%CS; diet2, 20%CS; diet3, 30%CS; diet4, 30%CS+Fe&P; and diet5, 40%CS+Fe&P. A 40%CS diet was excluded in the present study because a significantly lower growth rate was observed in juvenile olive flounder fed the diet in our previous study (Pham et al., 2005). The CS containing diets were supplemented by L-methionine and L-lysine to meet their dietary requirement of fish (NRC, 1993). The dietary formulation and proximate composition are presented in Table 1-1. All the experimental diets were formulated to be isonitrogenous (48% crude protein) and isocaloric (16.4 MJ/kg diet). The solvent extracted cottonseed meal was provided from Southern Cotton Oil Co., Memphis, TN, USA. Its protein and fiber contents were 43.5% and less than 12% in dry matter basis, respectively. Total gossypol concentration in the cottonseed meal was 1.65%. All the dry materials were thoroughly mixed with 30% of double distilled water, extruded through a meat chopper machine (SMC-12, Korea) at 5 mm in diameter, freeze-dried at –40 $^{0}$C for 24 hours and stored at –20 $^{0}$C until use.

### Fish and feeding trial

Olive flounder juveniles were transported from a private hatchery (at Cheju Island, Korea) to Marine and Environmental Research Institute, Cheju National University, Jeju, Korea. The fish were fed with a commercial diet for 2 weeks to be acclimated to experimental condition. Total 225 fish (mean body weight, 28.5 g) were randomly distributed into fifteen 200 L polyvinyl tanks (15 fish tank$^{-1}$) in a flow through system supplied with sand filtered seawater at a flow rate of 7-8 l min$^{-1}$. The triplicate groups of fish were fed the experimental diets to apparent satiation (twice a day, 9:00 and 17:00 h) for 26 weeks. The growth of fish was measured every 2 week. Feeding was stopped 24 h prior to weighing.

Table 1-1. Formulation, and proximate composition of experimental diets (% dry matter)

| 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 [1] | 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 |
| Yeast | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 |
| Mineral mix [2] | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Vitamin mix [3] | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 |
| Choline chloride | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| Squid liver oil | 7.3 | 7.9 | 8.2 | 8.2 | 8.5 |
| CMC | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Lysine [4] | 0.0 | 0.4 | 0.6 | 0.6 | 0.8 |
| Methionine[5] | 0.0 | 0.2 | 0.3 | 0.3 | 0.4 |
| Ferrous Sulfate-$7H_2O$[6] | 0.0 | 0.0 | 0.0 | 0.2 | 0.3 |
| Monocalciumphosphate[7] | 0.0 | 0.0 | 0.0 | 1.0 | 1.5 |
| Cellulose | 2.4 | 2.4 | 2.4 | 1.2 | 0.6 |
| *Chemical analyses (dry matter basis)* | | | | | |
| Protein, % DM | 48.6 | 48.0 | 48.6 | 47.9 | 47.9 |
| Lipid, % DM | 12.6 | 12.7 | 12.7 | 12.7 | 12.7 |
| Ash, % DM | 9.1 | 8.0 | 8.0 | 8.5 | 8.9 |
| Gross energy, MJ/kg DM | 16.4 | 16.4 | 16.4 | 16.4 | 16.4 |
| Total gossypol (ug/g)[8] | | | | | |
| Total | nd[9] | 1643 | 2586 | 2306 | 3393 |
| (+)-Enantiomer | nd | 944 | 1726 | 1638 | 1921 |
| (-)-Enantiomer | nd | 699 | 860 | 669 | 1472 |

[1] Cottonseed meal was purchased from Southern Cotton Oil Co., Memphis, Tennessee 38108, USA

[2] Mineral premix (g/kg of mixture) $MgSO_4.7H_2O$, 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.

[3] Vitamin premix (g/kg of mixture) 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.

[4] L-lysine mono-hydrochloride, Sigma, USA

[5] L- methionine, Sigma, USA

[6] Ferrous Sulfate $7H_2O$, Sigma, USA

[7] Monocalciumphosphate, Sigma, USA

[8] Total gossypol includes free and bound gossypol

[9] nd, not detected

**Sample collection and analyses**

At the end of feeding trial, all fish in each tank were weighed and counted to compute the weight gain, feed conversion ratio, specific growth rate, final mean body weight, feed intake, protein efficiency ratio and survival. Uneaten feeds were gently removed by siphoning and subtracted for more exact calculation of FCR, FI, and PER.

Three fish per tank (nine fish per treatment) were randomly selected and anaesthetized with MS-222 solution (200 mg $L^{-1}$) for blood analyses. The blood samples were taken from caudal vein with heparinised syringes. Hematocrit was determined by microhematocrit technique (Brown, 1980). The hemoglobin was determined by using the slightly modification hemoglobin method as the following description. The blood samples were diluted into modified hemoglobin solution (composed of 0.7 g $K_3Fe(CN)_6$ and 0.1 g KCN in 1L water). The absorbance of mixture was read by spectrophotometer (Genesys 10 UV, USA) at 540 nm.

Analyses of crude protein, moisture and ash in the experimental diets were performed by the standard procedures (AOAC, 1995). Dietary lipid was determined according to the method described by Folch et al. (1957).

Total gossypol concentration in liver (6 fish per treatment) and diets were determined by High Performance Liquid Chromatography (HPLC) according to the method described by Kim and Calhoun (1995) with some modifications (Lee and Dabrowski, 2002). The liver and dry diets were weighed and 5 – 10 volumes of complexing reagent were added to obtain the 2-amino-1-propanol derivatives of gossypol. The complexing reagent was composed of 2 ml 2-amino-1-propanol (Sigma Chemical, St. Louis, MO), 10 ml glacial acetic acid (Sigma Chemical) and 88 ml N, N-dimethylformamide (Sigma Chemical). The samples were homogenized in complexing reagent for 30 sec, heated at 95$^{o}$C for 30 min, cooled on ice and then centrifuged at 1500 x g for 5 min. After centrifugation, an aliquot of the supernatant was diluted with mobile phase to obtain a desirable concentration, centrifuged again at 1500 x g for 5 min and filtered through a syringe filter (0.45 μm, Whatman Inc., Clifton, NJ) before

injection to HPLC.

Plasma lysozyme activity was measured according to the turbidometric method described by Ross et al (2000), with some modification. The lysozyme substrate was a 0.2 mg $ml^{-1}$ freeze-dried *Micrococcus lysodeikticus* (Sigma) suspension in sodium phosphate buffer (0.05M $Na_2HPO_4$, pH 6.2). Plasma (25 μL) was added to 750 μL of the bacterial suspension and the initial and final (10 min incubation at $30^0$C) absorbance of the samples were measured at 450nm. One unit of lysozyme activity (U $ml^{-1}$) was defined as a reduction in absorbance 0.001 $min^{-1}$ during the 10 min incubation.

**Statistical analysis**

Data were subjected to one-way ANOVA in SPSS version 11.0. The significant differences between group means were compared using Duncan's multiple test. Data were presented as means ± standard deviations. The percentage data of weight gain and specific growth rate were arcsine transformed before the ANOVA analysis. Differences were considered significant at $P < 0.05$.

Table 1-2. Growth performance of juvenile olive flounder (IBW, 28.7 ± 0.17g) fed different experimental diets for 26 week s[1]

| Diets | CS0 | CS20 | CS30 | CS30+Fe&P | CS40+Fe&P |
|---|---|---|---|---|---|
| Initial body weight, g | 27.9 ± 0.3 | 28.7 ± 0.5 | 28.6 ± 0.6 | 28.5 ± 0.6 | 28.8 ± 0.5 |
| Final body weight, g | 220 ± 10.8 | 207 ± 12.5 | 201 ± 20.6 | 214.2 ± 21.5 | 196 ± 20.3 |
| Weight gain (WG)[2] | 661 ± 31.4 | 623 ± 47.4 | 603 ± 70.6 | 652 ± 53.2 | 578 ± 61.3 |
| Feed intake, g (FI)[3] | 218 ± 3.5 | 219 ± 4.2 | 603 ± 70.6 | 218 ± 10.6 | 212 ± 13.6 |
| Feed conversion ratio (FCR)[4] | 1.14 ± 0.05 | 1.23 ± 0.10 | 1.25 ± 0.09 | 1.20 ± 0.05 | 1.27 ± 0.08 |
| Specific growth rate (SGR)[5] | 1.61 ± 0.03 | 1.57 ± 0.05 | 1.54 ± 0.08 | 1.60 ± 0.06 | 1.52 ± 0.07 |
| Protein efficiency ratio (PER)[6] | 1.80 ± 0.07 | 1.70 ± 0.14 | 1.65 ± 0.12 | 1.73 ± 0.07 | 1.64 ± 0.11 |
| Survival (%) | 95.3 ± 4.0 | 95.3 ± 4.0 | 95.3 ± 4.0 | 95.3 ± 4.0 | 100.0 ± 0.0 |

[1] Means of triplicate groups, values are presented as mean ± SD. Values in the same row having different superscript letters are significantly different ($P < 0.05$).

[2] WG (%) = 100 x (final mean body weight - initial mean body weight)/initial mean body weight

[3] FI = dry feed consumed/fish

[4] FCR = dry feed fed/wet weight gain

[5] SGR (%) = [(loge final body weight - loge initial body weight)/days] x 100

[6] PER = wet weight gain/ total protein given

## RESULTS

At the end of 26 weeks of feeding trial, no significant differences were observed in final body weight, feed intake, specific growth rate, protein efficiency ratio, feed conversion ratio and survival among all the dietary treatments (Table 2). No visible difference in the diet acceptance between the control and CS containing diets was noticed during the feeding trial. The experimental fish grew well during the feeding trial and mortality was lower than 5% in all the dietary groups.

Groups of fish fed diet 5 (the highest incorporation of CS with iron and phosphorus) had significantly lower hematocrit values than groups of fish fed other diets (Table 3). Meanwhile, the hemoglobin concentration was not significantly different among all the fish groups.

Dietary gossypol concentrations were increased with increment of CM incorporation in the diets. At the end of the feeding trial, total gossypol and each (+)- and (–)-gossypol enantiomer concentration in the liver of fish were significantly increased as the dietary CM inclusion increased (Table 4; compare CS0, CS20, and CS30 diets). However, the gossypol concentrations (both total and each enantiomer) in the liver of fish fed diets (CS30+Fe&P and CS40+Fe&P) supplemented with iron were significantly lower than that of fish fed CS30 diet without supplementation of iron at the same inclusion level. This result indicates a binding capacity of iron to gossypol molecules resulting in the reduction of gossypol accumulation in fish tissues.

Plasma lysozyme activity of fish fed diet 5 (40%CS+Fe&P) was significantly increased than that of fish fed the FM-based control diet (Table 1-5).

Table 1-3. Hematocrit and hemoglobin values of juvenile olive flounder fed different experimental diets for 26 weeks[1]

| Diets | CS0 | CS20 | CS30 | CS30 Fe&P | CS40 Fe&P |
|---|---|---|---|---|---|
| Hematocrits (%) | $24.7 \pm 1.9^{a}$ | $23.6 \pm 0.4^{a}$ | $23.3 \pm 2.7^{a}$ | $23.9 \pm 2.0^{a}$ | $19.0 \pm 3.0^{b}$ |
| Hemoglobin (g/dL) | $3.6 \pm 0.3$ | $3.5 \pm 0.3$ | $3.6 \pm 0.2$ | $3.5 \pm 0.1$ | $3.6 \pm 0.1$ |

[1]Means of triplicate groups, values are presented as mean ± SD. Values in the same row having different superscript letters are significantly different ($P < 0.05$).

Table 1-4. Total (+ and -) gossypol enantiomer accumulation in liver of juvenile olive flounder fed different experimental diets for 26 weeks[1]

| | Gossypol content in liver (ug/g wet weight) | | |
|---|---|---|---|
| Diets | (+)-enantiomer | (-)-enantiomer | Total |
| CS0 | nd[2] | nd | nd |
| CS20 | 92 ± 62.3[a] | 89 ± 54.2[a] | 181 ± 116.5[a] |
| CS30 | 274 ± 104.0[b] | 207 ± 49.8[b] | 481 ± 153.8[b] |
| CS30 + Fe&P | 77 ± 30.2[a] | 51 ± 24.3[a] | 128 ± 54.5[a] |
| CS40 + Fe&P | 113 ± 26.2[a] | 85 ± 17.5[a] | 198 ± 43.7[a] |

[1]Means of triplicate groups, values are presented as mean ± SD. Values in the same row having different superscript letters are significantly different ($P < 0.05$).
[2] nd, not detected

## DISCUSSION

The present study demonstrated that the mixture of cottonseed and soybean meal with iron and phosphorus supplements could replace dietary FM protein up to 40% without negative effects on growth performances, feed utilizations and survival of juvenile olive flounder. The result of the present study is significant because, to our knowledge, it is the first cottonseed meal used dietary formulation for olive flounder with long-term feeding trial over 6 months.

In our previous studies, cottonseed and soybean meal with lysine and methionine supplementations could replace 30% FM protein in diets for juvenile olive flounder (0.7-10 g) without growth retardation (Pham et al., 2005). In Pham et al. (2005), however, groups of fish fed diets replacing 40% FM protein with CS protein had significantly lower growth performances than groups of fish fed a FM based control diet. In the present study, it seemed that dietary incorporation of CS with supplements of iron and phosphorus as well as methionine and lysine could replace up to 40% FM protein which did not affect the dietary palatability and thereby did not impair the growth of the fish.

A beneficial effect of phosphorus supplementation in diets on growth performance was not clearly demonstrated in the present study (compare diets CS30 and CS30+Fe&P in Table 1-2). In a feeding study with juvenile olive flounder (Wang et al. 2005), the optimum dietary phosphorus level was found to be ranged from 0.45 to 0.51% for maximum growth rate. Uyan et al. (2007) also reported that the optimum dietary phosphorus level should be ranged from 1.47 to 1.65% to obtain maximum growth rate in juvenile olive flounder. The dietary phosphorus levels of the experimental diets in the present study were calculated from 1.66 (the control diet) to 2.07% (CS40+Fe&P) indicating that the levels were enough to meet its requirement for the growing olive flounder. Albrektsen et al. (2006) reported that 50% dietary FM was successfully replaced by a mixture of SM and corn gluten meal with phosphorus supplementation in Atlantic cod. In gilthead sea bream, however, phosphorus supplementation into SM containing diets did not improve the growth performance (Robaina

et al. 1998). The supplemental effect of phosphorus on growth performance in fish cannot simply be compared because it could be different depending on fish species and dietary phosphorus content, and more specifically on dietary composition in each feeding study.

A significantly lower blood hematocrit was observed in diet 5 groups than in other dietary groups whereas the hemoglobin concentration was not significant in all the dietary groups (Table 1-3). Reduced blood hematocrit and hemoglobin in fish fed CM containing diets were found in previous studies (Yildirim et al. 2003; Blom et al. 2001; Herman 1970). This phenomenon could be explained by adverse effects of gossypol on iron absorption in the intestine (Braham and Bressani, 1975), the gossypol-iron complex in liver (Skutches et al., 1974), or increased erythrocyte fragility (Brocas et al., 1997). However, none of these mechanisms have been confirmed in fish because of some contradictory (Yildirim et al., 2004) and complicated (Barros et al., 2002) results on the hematological values.

Many studies have previously indicated that the amount of cottonseed and soybean meal that can be used in fish feeds depends mainly on the level of dietary free gossypol and available lysine content (Robinson and Li, 1994; Jones, 1987). The toxicity of gossypol has been extensively studied and reported in humans and animals including fish. The gossypol toxicity can be divided into two mechanisms. The first mechanism is a direct physiological toxicity, and the second is an indirect nutritional mechanism caused by reactions with proteins or iron that results in their decreased availabilities (Lee, 2002). Gossypol is easily bound to soluble proteins in the intestine, especially to the epsilon-amino group of lysine which results in a gossypol-lysine complex and is excreted through feces resulting in decreased lysine availability in fish. In the present study, it seemed that the indirect toxicity gossypol has in removing lysine availability was reduced by the dietary supplementation of surplus lysine. Also, reduced toxicity of free gossypol in fish body by dietary supplementation of ferrous sulfate was clearly demonstrated by liver gossypol concentration. Total and each (+) and (-) gossypol enantiomer concentration in liver of fish fed diets supplemented with iron were significantly lower than those in liver of fish fed diets without supplementation of iron (Table 1-4). Therefore, the result of the gossypol concentration in

the present study supports the notion that lysine and iron supplementation into CM containing diets reduces gossypol toxicities in fish (Lee et al., 2006; Dabrowski et al., 2000; Robinson and Li 1994; Robinson, 1991). It was reported that cottonseed meal could completely or partly replace soybean meal with supplemental lysine in the diet for channel catfish (Robinson and Li, 1994; Robinson, 1991;). In addition, Lee et al. (2006) showed that dietary cottonseed meal inclusion up to 44% (75% FM protein replacement) with corresponding supplement of lysine did not impair the growth and reproductive performance of broodstock rainbow trout in a long-term feeding study over 35 months.

Ferrous sulfate has been used to counteract the toxic effect of free gossypol for monogastric animals (Martin, 1990; Jones, 1987) and fish (El-Saidy and Gaber, 2004; Barros et al., 2002; Sealey et al., 1997). Barros et al. (2002) successfully replaced 50% dietary SM by CM with ferrous sulfate supplementation for channel catfish. El-Saidy and Gaber (2004) reported that supplemental iron as ferrous sulfate at 1:1 ratio of iron to free gossypol had no negative effects on dietary nutritional values. In terms of iron supplementation, there was a report (Sealey et al., 1997) that a negative effect of high level of supplemental iron as ferrous sulfate was found in channel catfish. Sealey et al. (1997) observed an increased susceptibility to *Edwardsiella ictaluri* infection in the fish fed high level of ferrous sulfate. However negative effects of supplemental iron was not observed in the present study. Significantly lower gossypol enantiomer concentration either (+)- or (-)-gossypol was found in liver of fish fed diets supplemented with iron than that of fish fed diets without iron supplementation (Table 1-4). The result in the present study clearly supports that supplemental iron reduces the toxicity of gossypol in fish body by forming a strong complex compound in the intestinal tract, thus preventing it from being absorbed (Wedegaertner, 1981).

Lysozyme, an enzyme produced by leucocytes, plays an important role in innate immunity by attacking the bacterial cell wall, thereby causing lysis and stimulation of phagocytosis for bacteria (Ellis, 1990). In the present study, plasma lysozyme activity of fish fed diet 5 (40%CS+Fe&P) was significantly increased than fish fed the FM-based control diet (Table 1-5). The increased lysozyme activity in the fish fed diet 5 might be attributed to

higher concentration of vitamin C in diet 5 compared to that in the control diet. The dietary ascorbate concentrations were 498, 598, 677, 654, and 728 mg $kg^{-1}$ diet in diets 1, 2, 3, 4, and 5 even though equal amount of ascorbic acid was supplemented into all the experimental diets by vitamin premix (data were not shown). Increased serum lysozyme activity in fish species by increased dietary ascorbic acid concentration was easily found in many previous studies (Ai et al., 2006; Lin and Shiau, 2005; Ai et al., 2004). A further study needs to be conducted on this issue.

Table 1-5. Plasma lysozyme activity of juvenile olive flounder fed different experimental diets for 26 weeks[1]

| Diets | Lysozyme activity (units/ml) |
|---|---|
| CS0 | $216 \pm 78.1^{a}$ |
| CS20 | $347 \pm 78.1^{ab}$ |
| CS30 | $340 \pm 33.8^{ab}$ |
| CS30 + Fe&P | $283 \pm 157.9^{ab}$ |
| CS40 + Fe&P | $396 \pm 121.0^{b}$ |

[1]Means of triplicate groups, values are presented as mean ± SD. Values in the same row having different superscript letters are significantly different ($P < 0.05$).

In conclusion, the mixture of cottonseed and soybean meal with iron and phosphorus supplementation can replace up to 40% FM protein in diets for juvenile olive flounder. However, based on the tendency in growth performances, 30% of FM protein replacement by CS with supplementation of iron and phosphorus might be a safe level for commercial use. The findings in this study suggest that dietary supplementation of iron and phosphorus could increase the inclusion of cottonseed and soybean meal for FM replacement in diets for marine fish species.

# Part Ⅱ

# Use of cottonseed and soybean meal in diet for parrot fish (*Oplegnsthus fasciatus)*

## Part Ⅱ

## Use of cottonseed and soybean meal in diet for parrot fish (*Oplegnsthus fasciatus)*


### ABSTRACT

Two consecutive experiments were conducted to determine a proper dietary inclusion level of cottonseed and soybean meal (CS) as fish meal (FM) replacers and to collect information on beneficial effects of FM replacement by CS on juvenile and growing parrot fish. In experiment Ⅰ, juvenile parrot fish at initial average size of 3.17±0.01g (mean ± SD) were divided into 18 groups (three tanks per dietary treatment) and fed one of six experimental diets for 12 weeks. Fish were fed isonitrogenous and isocaloric diets replacing 0, 10, 20, 30, 40 and 50% FM protein by equal proportion (1:1, w:w) of CS (designated by Control, CS10, CS20, CS30, CS40 and CS50, respectively). In experiment Ⅱ, growing parrot fish with an initial average size of 55±0.5g (mean ± SD) were divided into 15 groups (three tanks per dietary treatment) and fed one of five experimental diets for 9 weeks. Five isonitrogenous diets were formulated to contain different levels of CS to replace FM with iron and phytase supplementations. The five experimental diets were as follows: Diet 1 (control), 0%CS; diet2, 20%CS; diet3, 30%CS; diet4, 20%CS+Fe&P; and diet5, 30%CS+Fe&P. The fish readily accepted all experimental diets and no fish died during the two feeding trials. In experimental Ⅰ, negative effects on growth performance were obvious when 30% of FM protein was replaced by CS protein. However, significantly lower weight gain, feed intake, feed conversion ratio, protein efficiency ratio and specific growth rate were found when the replacement level for FM protein was increased from 20% to 30%. In experiment Ⅱ, no significant differences were observed in weight gain, feed utilization and survival among all the treatments. After the two consecutive feeding trials, total and each (+) and (-) gossypol enantiomer concentrations in the liver were increased as the CM inclusion

increased in the diets. However, the total gossypol concentration in liver of fish fed diets supplemented with iron was not detected. The findings in the present study indicate that the addition of iron in diets could prevent the absorption of free gossypol and its toxicity. Levels of plasma triglycerides and total cholesterol were lower in fish fed CS containing diet than those in fish fed FM based control diet. Therefore, 20% FM protein replacement by CS with supplementation of iron and phytase might be a safe level for commercial use in juvenile and growing parrot fish. The findings in this study suggest that dietary supplementation of CS might reduce levels of plasma triglycerides and total cholesterol and affect lipid metabolism in juvenile and growing parrot fish.

## MATERUALS AND METHODS

### EXP. Ⅰ.

### Experimental diets

Six experimental diets were formulated to replace FM protein by equal proportion (1:1, w:w) of cottonseed and soybean meal (CS). The experimental diets were as follow: Diet 1 (control), 0%CS; diet2, 10%CS; diet3, 20%CS; diet4, 30%CS; diet5, 40%CS and diet6, 50%. The CS containing diets were supplemented by L-methionine and L-lysine to meet their dietary requirements (NRC, 1993). The dietary formulation and proximate composition are presented in Table 2-1. All the experimental diets were formulated to be isonitrogenous (46% crude protein) and isocaloric (18MJ/kg diet). The solvent extracted cottonseed meal was provided from Southern Cotton Oil Co., Memphis, TN, USA. Its protein and fiber contents were 43.5% and less than 12% in dry matter basis, respectively. Total gossypol concentration in the cottonseed meal was 1.65%. All the dry materials were thoroughly mixed with 30% of double distilled water, extruded through a meat chopper machine (SMC-12, Korea) at 5 mm in diameter, freeze-dried at –40 $^{0}$C for 24 hours and stored at –20 $^{0}$C until use.

### Fish and feeding trial

Juvenile parrot fish were transported from a private hatchery (at Cheju Island, Korea) to Marine and Environmental Research Institute, Cheju National University, Jeju, Korea. The fish were fed with a commercial diet for 2 weeks to be acclimated to experimental conditions. Thirty fish (IBW, 3.17 ± 0.01g g/fish) were randomly distributed into eighteen 50 L polyvinyl circular tanks. The tanks were supplied with filtered seawater at flow of 2-3 l/min. The triplicate groups of fish were fed the experimental diets to apparent satiation (twice a day, 9:00 and 17:00 h) for 12 weeks. The growth of fish was measured every 3 week. Feeding was stopped 24 h prior to weighing.

Table 2-1. Composition of experimental diets for juvenile parrot fish in the first trial (% dry matter)

| Ingredients | Diets | | | | | |
|---|---|---|---|---|---|---|
| | CS0 | CS10 | CS20 | CS30 | CS40 | CS50 |
| White fish meal | 52.0 | 46.8 | 41.6 | 36.4 | 31.2 | 26.0 |
| Soybean meal | 0.0 | 3.8 | 7.7 | 11.5 | 15.3 | 19.2 |
| Cottonseed meal [1] | 0.0 | 4.0 | 8.1 | 12.1 | 16.1 | 20.2 |
| Corn gluten meal | 8.0 | 7.7 | 7.4 | 7.1 | 6.8 | 6.5 |
| Wheat flour | 6.5 | 6.5 | 6.5 | 6.5 | 6.5 | 6.5 |
| Starch | 16.0 | 13.7 | 11.4 | 9.1 | 6.8 | 4.5 |
| Yeast | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 |
| Mineral mix [2] | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Vitamin mix [3] | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Squid liver oil | 11.0 | 11.3 | 11.5 | 11.8 | 12.0 | 12.3 |
| Lysine [4] | 0.0 | 0.1 | 0.2 | 0.3 | 0.4 | 0.5 |
| Methionine [5] | 0.0 | 0.1 | 0.2 | 0.3 | 0.4 | 0.5 |
| Cellulose | 2.5 | 2.0 | 1.5 | 1.0 | 0.5 | 0.0 |
| *Proximate composition* | | | | | | |
| Protein, % DM | 46.3 | 46.1 | 46.3 | 46.4 | 46.5 | 46.8 |
| Lipid, % DM | 16.1 | 15.4 | 15.6 | 16.0 | 16.2 | 16.9 |
| Ash, % DM | 7.9 | 7.8 | 7.5 | 7.3 | 7.2 | 7.2 |
| Gross energy, MJ/kg DM[6] | 18.5 | 18.5 | 18.4 | 18.3 | 18.2 | 18.1 |
| Total gossypol (mg/kg)[7] | nd[8] | 316 | 507 | 858 | 1016 | 1274 |
| (+)-Enantiomer | nd | 192 | 317 | 528 | 623 | 783 |
| (-)-Enantiomer | nd | 124 | 190 | 330 | 393 | 491 |

[1]Cottonseed meal was purchased from Southern Cotton Oil Co., Memphis, Tennessee 38108, USA
[2]Mineral premix (g/kg of mixture) $MgSO_4.7H_2O$, 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.
[3]Vitamin premix (g/kg of mixture) 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.
[4]L-lysine mono-hydrochloride, Sigma, USA
[5]L- methionine, Sigma, USA
[6]Gross energy of experimental diets was calculated according to gross energy values 5.64 kcal/g protein, 4.11 kcal/g carbohydrate, and 9.44 kcal/g fat respectively (NRC, 1993).
[7] Total gossypol includes free and bound gossypol
[8] nd, not detected

**EXP. Ⅱ.**

**Experimental diets**

Five experimental diets were formulated to replace FM protein by equal proportion (1:1, w:w) of cottonseed and soybean meal (CS) with supplementations of iron and phytase. The experimental diets were as follow: Diet 1 (control), 0%CS; diet2, 20%CS; diet3, 30%CS; diet4, 20%CS+Fe&P; and diet5, 30%CS+Fe&P. The CS containing diets were supplemented by L-methionine and L-lysine to meet their dietary requirement (NRC, 1993). The dietary formulation and proximate composition are presented in Table 2-2. All the experimental diets were formulated to be isonitrogenous (46% crude protein) and isocaloric (18 MJ/kg diet).

**Fish and feeding trial**

Growing parrot fish used in this experiment was selected from fish used in the first experiment. After a 2 weeks conditioning period, fish with an initial body weight of 55 ± 0.5 g (mean ± S.D.) were distributed to each tank as groups of 20 fish per tank and fed one of the five experimental diets to apparent satiation (twice a day, 9:00 and 17:00 h) for 9 weeks. The tanks were supplied with filtered seawater at flow of 2-3 l/min and aeration was installed to maintain optimum dissolved oxygen level. The growth of fish was measured every 3 week. Feeding was stopped 24 h prior to weighing.

Table 2-2. Composition of experimental diets for growing parrot fish in the second trial (% dry matter)

| Ingredients | Diets | | | | |
|---|---|---|---|---|---|
| | CS0 | CS20 | CS30 | CS20 Fe&P | CS30 Fe&P |
| White fish meal | 52.0 | 41.6 | 36.4 | 41.6 | 36.4 |
| Soybean meal | 0.0 | 7.7 | 11.5 | 7.7 | 11.5 |
| Cottonseed meal [1] | 0.0 | 8.1 | 12.1 | 8.1 | 12.1 |
| Corn gluten meal | 8.5 | 7.9 | 7.6 | 7.9 | 7.6 |
| Wheat flour | 6.5 | 6.5 | 6.5 | 6.5 | 6.5 |
| Starch | 18.3 | 13.6 | 11.3 | 13.6 | 11.3 |
| Mineral mix [2] | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Vitamin mix [3] | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Squid liver oil | 11.0 | 11.5 | 11.8 | 11.5 | 11.8 |
| Lysine [4] | 0.0 | 0.2 | 0.3 | 0.2 | 0.3 |
| Methionine [5] | 0.0 | 0.2 | 0.3 | 0.2 | 0.3 |
| Ferrous Sulfate $7H_2O$ | 0.0 | 0.0 | 0.0 | 0.1 | 0.2 |
| Phytase | 0.0 | 0.0 | 0.0 | 0.01 | 0.01 |
| Cellulose | 1.7 | 0.7 | 0.2 | 0.6 | 0.0 |
| *Proximate composition* | | | | | |
| Protein, % DM | 46.0 | 45.7 | 45.8 | 45.5 | 46.1 |
| Lipid, % DM | 15.8 | 16.0 | 16.1 | 15.9 | 15.9 |
| Ash, % DM | 8.0 | 7.5 | 7.3 | 7.6 | 7.8 |
| Gross energy, MJ/kg DM[6] | 18.6 | 18.4 | 18.3 | 18.4 | 18.3 |
| Total gossypol (mg/kg)[7] | nd[8] | 461 | 661 | 439 | 695 |
| (+)-Enantiomer | nd | 319 | 461 | 303 | 477 |
| (-)-Enantiomer | nd | 142 | 200 | 136 | 218 |

[1]Cottonseed meal was purchased from Southern Cotton Oil Co., Memphis, Tennessee 38108, USA
[2]Mineral premix (g/kg of mixture) $MgSO_4.7H_2O$, 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.
[3]Vintamin premix (g/kg of mixture) 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.
[4]L-lysine mono-hydrochloride, Sigma, USA
[5]L- methionine, Sigma, USA
[6]Gross energy of experimental diets was calculated according to gross energy values 5.64 kcal/g protein, 4.11 kcal/g carbohydrate, and 9.44 kcal/g fat respectively (NRC, 1993).
[7] Total gossypol includes free and bound gossypol
[8] nd, not detected

**Sample collection and analysis**

At the end of feeding trial, all fish in each tank were weighed and counted to compute the weight gain, feed conversion ratio, specific growth rate, final mean body weight, feed intake, protein efficiency ratio and survival.

Three fish per tank (nine fish per treatment) were randomly selected and anaesthetized with MS-222 solution (200 mg/L) for blood analyses. The blood samples were collected from caudal vein with heparinised syringes. Hematocrit was determined by microhematocrit technique (Brown, 1980). The hemoglobin, tryglycerides and total cholesterol were determined by using the automated blood analyzer (Biochemistry analyzer ch 100 plus, Korea).

Analyses of crude protein, moisture and ash in the experimental diets were performed by the standard procedures (AOAC, 1995). Dietary lipid was determined by Soxhlet Extraction System (C-SH6, Korea).

Total gossypol concentration in diets and liver (6 fish per treatment) were determined by High Performance Liquid Chromatography (HPLC) according to the method described by Kim and Calhoun (1995) with some modifications (Lee and Dabrowski, 2002). The liver and dry diets were weighed and 3 – 10 volumes of complexing reagent were added to obtain the 2-amino-1-propanol derivatives of gossypol. The complexing reagent was composed of 2 ml 2-amino-1-propanol (Sigma Chemical, St. Louis, MO), 10 ml glacial acetic acid (Sigma Chemical) and 88 ml N, N-dimethylformamide (Sigma Chemical). The samples were homogenized in complexing reagent for 30 sec, heated at 95$^{o}$C for 30 min, cooled on ice and then centrifuged at 1500 x g for 5 min. After centrifugation, an aliquot of the supernatant was diluted with mobile phase to obtain a desirable concentration, centrifuged again at 1500 x g for 5 min and filtered through a syringe filter (0.45 μm, Whatman Inc., Clifton, NJ) before injection to HPLC.

**Statistical analysis**

Data were subjected to one-way ANOVA in SPSS version 11.0. The significant differences between group means were compared using Duncan's multiple test. Data were presented as means ± standard deviations. The percentage data of weight gain and specific growth rate were arcsine transformed before the ANOVA analysis. Differences were considered significant at $P < 0.05$.

## RESULTS

### EXP. Ⅰ.

The growth and feed utilization of juvenile parrot fish fed the experimental diets are presented in Table 2-3. Negative effects on growth performance were obvious when 30% of FM protein was replaced by CS protein. There were significant differences in fish weight gain, feed conversion ratio, protein efficiency ratio and specific growth rate when the replacement level for FM protein was increased from 20% to 30%. These results showed that 20% of FM protein could be replaced by CS protein in diets for juvenile parrot fish without any growth depression.

The results of blood parameters in juvenile parrot fish fed the experimental diets are illustrated in Table 2-5. Groups of fish fed diet 6 (CS 50) had significantly lower hematocrit values than groups of fish fed the control diets. Meanwhile the hemoglobin concentration was not significantly different among all the fish groups. Plasma triglycerides and plasma cholesterol levels were lower in fish fed CS containing diet than that of fish fed FM based control diet (Fig. 2-1).

The results of gossypol accumulation in the liver after 12 weeks of feeding trial are shown in Table 2-7. Total and each (+) and (-) gossypol enantiomer concentration in the liver increased as the CM inclusion increased in the diets. However, gossypol was not detected in the groups of fish fed diet 2 (CS 10).

### EXP. Ⅱ.

Result from the second experiment indicated that there were no significant differences in weight gain, specific growth rate, protein efficiency ratio, feed conversion ratio and survival among all the treatments (Table 2-4). The results indicate that CS can replace up to 30% FM protein in diets for growing parrot fish.

The results of blood parameters in growing parrot fish fed the experimental diets are shown in Table 2-6. Hemoglobin and hematocrit values were numerically lower in fish fed

diet 2 (CS20) and diet 3 (CS30) than that of fish fed the other diets. However, no significant differences were observed in hemoglobin and hematocrit values. The result of plasma triglycerides and cholesterol levels followed a similar trend to result of first experiment (Fig. 2-2).

Gossypol concentration in the liver after 9 weeks of feeding trial are shown in Table 2-8. Total and each (+) and (-) gossypol enantiomer concentration in liver increased as the CM inclusion increased in the diets. However, the total gossypol concentration in liver of fish fed diets supplemented with iron was not detected. The results indicate that the addition of iron in diets could prevent the absorption of free gossypol.

Table 2-3. Growth performance of juvenile parrot fish (IBW, 3.2 ± 0.01g) fed different experimental diets for 12 weeks[1]

| Diets | CS0 | CS10 | CS20 | CS30 | CS40 | CS50 |
|---|---|---|---|---|---|---|
| Weight gain (WG)[2] | 617 ± 32.7[a] | 616 ± 26.9[a] | 603 ± 21.9[a] | 529 ± 32.6[b] | 520 ± 26.7[b] | 465 ± 63.5[c] |
| Feed conversion ratio (FCR)[3] | 1.11 ± 0.06[a] | 1.15 ± 0.04[a] | 1.15 ± 0.02[ab] | 1.25 ± 0.04[bc] | 1.28 ± 0.09[cd] | 1.44 ± 0.12[d] |
| Specific growth rate (SGR)[4] | 1.96 ± 0.11[a] | 1.90 ± 0.07[ab] | 1.89 ± 0.03[ab] | 1.74 ± 0.06[bc] | 1.70 ± 0.11[c] | 1.51 ± 0.12[d] |
| Protein efficiency ratio (PER)[5] | 1.02 ± 0.02[a] | 1.02 ± 0.02[a] | 1.01 ± 0.02[a] | 0.95 ± 0.03[b] | 0.94 ± 0.02[b] | 0.89 ± 0.03[c] |
| Survival (%) | 100 | 100 | 100 | 100 | 100 | 100 |

[1] Means of triplicate groups, values are presented as mean ± SD. Values in the same row having different superscript letters are significantly different ($P < 0.05$).

[2] WG (%) = 100 x (final mean body weight - initial mean body weight)/initial mean body weight

[3] FCR = dry feed fed/wet weight gain

[4] SGR (%) = [(loge final body weight - loge initial body weight)/days] x 100

[5] PER = wet weight gain/ total protein given

Table 2-4. Growth performance of growing parrot fish (IBW, 55 ± 0.5g) fed different experimental diets for 9 weeks[1]

| Diets | CS0 | CS20 | CS30 | CS20+Fe&P | CS30+Fe&P |
|---|---|---|---|---|---|
| Weight gain (WG)[2] | 111 ± 10.8 | 104 ± 10.1 | 111 ± 7.2 | 121 ± 0.7 | 115 ± 9.2 |
| Feed conversion ratio (FCR)[3] | 1.72 ± 0.15 | 1.81 ± 0.23 | 1.72 ± 0.09 | 1.61 ± 0.03 | 1.69 ± 0.11 |
| Specific growth rate (SGR)[4] | 1.28 ± 0.09 | 1.22 ± 0.11 | 1.28 ± 0.07 | 1.33 ± 0.05 | 1.27 ± 0.09 |
| Protein efficiency ratio (PER)[5] | 1.30 ± 0.11 | 1.24 ± 0.15 | 1.29 ± 0.07 | 1.38 ± 0.03 | 1.32 ± 0.09 |
| Survival (%) | 100 | 100 | 100 | 100 | 100 |

[1] Means of triplicate groups, values are presented as mean ± SD. Values in the same row having different superscript letters are significantly different ($P < 0.05$).

[2] WG (%) = 100 x (final mean body weight - initial mean body weight)/initial mean body weight

[3] FCR = dry feed fed/wet weight gain

[4] SGR (%) = [(loge final body weight - loge initial body weight)/days] x 100

[5] PER = wet weight gain/ total protein given

Table 2-5. Blood parameters of juvenile parrot fish fed different experimental diets for 12 weeks[1]

| Diets | CS0 | CS10 | CS20 | CS30 | CS40 | CS50 |
|---|---|---|---|---|---|---|
| Hematocrits (%) | 45.6 ± 1.0$^{a}$ | 42.7 ± 3.5$^{ab}$ | 43.3 ± 3.0$^{ab}$ | 41.8 ± 0.2$^{ab}$ | 41.8 ± 2.8$^{ab}$ | 39.8 ± 1.6$^{b}$ |
| Hemoglobin (g/dL) | 10.0 ± 0.46 | 9.9 ± 0.53 | 9.8 ± 0.86 | 9.6 ± 0.71 | 9.5 ± 1.40 | 9.3 ± 1.74 |
| Triglyceride (mg/dl) | 108 ± 11$^{a}$ | 68 ± 15$^{b}$ | 75 ± 13$^{b}$ | 70 ± 22$^{b}$ | 68 ± 3$^{b}$ | 63 ± 5$^{b}$ |
| Cholesterol (mg/dl) | 274 ± 18$^{a}$ | 234 ± 0.3$^{ab}$ | 199 ± 10$^{b}$ | 191 ± 15$^{b}$ | 202 ± 17$^{b}$ | 198 ± 6.0$^{b}$ |

[1]Means of triplicate groups, values are presented as mean ± SD. Values in the same row having different superscript letters are significantly different ($P < 0.05$).

Table 2-6. Blood parameters of growing parrot fish fed different experimental diets for 9 weeks[1]

| Diets | CS0 | CS20 | CS30 | CS20+Fe&P | CS30+Fe+P |
|---|---|---|---|---|---|
| Hematocrits (%) | 45.4 ± 0.8 | 40.4 ± 1.7 | 40.1 ± 2.3 | 44.0 ± 1.9 | 43.8 ± 3.1 |
| Hemoglobin (g/dL) | 8.7 ± 0.8 | 7.5 ± 0.6 | 7.6 ± 0.5 | 7.9 ± 0.7 | 8.1 ± 0.6 |
| Triglyceride (mg/dl) | 51.9 ± 16.7$^{a}$ | 25.7 ± 8.6$^{b}$ | 27.6 ± 9.7$^{b}$ | 35.5 ± 5.4$^{ab}$ | 32.4 ± 1.6$^{b}$ |
| Cholesterol (mg/dl) | 140 ± 27$^{a}$ | 88 ± 32$^{b}$ | 78 ± 10$^{b}$ | 65 ± 11$^{b}$ | 89 ± 28$^{b}$ |

[1]Means of triplicate groups, values are presented as mean ± SD. Values in the same row having different superscript letters are significantly different ($P < 0.05$).

Table 2-7. Total (+ and -) gossypol enantiomer accumulation in liver of juvenile parrot fish fed different experimental diets for 12 weeks[1]

| | Gossypol content in liver (ug/g wet weight) | | |
|---|---|---|---|
| Diets | (+)-enantiomer | (-)-enantiomer | Total |
| CS0 | nd[2] | nd | nd |
| CS10 | nd | nd | nd |
| CS20 | $2.1 \pm 0.57^{a}$ | $0.8 \pm 0.15^{a}$ | $2.9 \pm 0.72^{a}$ |
| CS30 | $3.2 \pm 0.12^{b}$ | $1.2 \pm 0.04^{b}$ | $4.4 \pm 0.16^{b}$ |
| CS40 | $3.7 \pm 0.77^{b}$ | $1.5 \pm 0.11^{c}$ | $5.2 \pm 0.88^{b}$ |
| CS50 | $5.0 \pm 0.55^{c}$ | $2.3 \pm 0.13^{d}$ | $7.3 \pm 0.68^{c}$ |

[1]Means of triplicate groups, values are presented as mean ± SD. Values in the same row having different superscript letters are significantly different ($P < 0.05$).

Table 2-8. Total (+ and -) gossypol enantiomer accumulation in liver of growing parrot fish fed different experimental diets for 9 weeks[1]

| | Gossypol content in liver (ug/g wet weight) | | |
|---|---|---|---|
| Diets | (+)-enantiomer | (-)-enantiomer | Total |
| CS0 | nd[2] | nd | nd |
| CS20 | $2.2 \pm 0.44^{a}$ | $1.7 \pm 0.11^{a}$ | $3.9 \pm 0.55^{a}$ |
| CS30 | $6.6 \pm 1.11^{b}$ | $2.5 \pm 0.89^{b}$ | $9.1 \pm 2.0^{b}$ |
| CS20 + Fe&P | nd | nd | nd |
| CS30 + Fe&P | nd | nd | nd |

[1]Means of triplicate groups, values are presented as mean ± SD. Values in the same row having different superscript letters are significantly different ($P < 0.05$).

**DICCUSSION**

The present study demonstrated that the mixture of cottonseed and soybean meal could replace dietary FM protein up to 20% without negative effects on growth performances of juvenile (3-22g) parrot fish (Table 2-3). However, there were no significant differences in growth and feed utilization of the growing (55-120g) parrot fish fed up to CS30 diets compared to fish fed the control diet (Table 2-4). The reason might be the difference in fish size. Values reported in the literature suggest that smaller fish and/or juvenile stages of fish generally have higher feed efficiency, N retention efficiency and energy retention efficiency than larger and/or post-juvenile fish (Einen and Roem, 1997; Ronsholdt, 1995;). More research is needed in order to gain further insight into fish size effects on feed and nutrient utilization.

A beneficial effect of phytase supplementation in diets on growth performance was not clearly demonstrated in the present study (compare diets CS20 and CS20+Fe&P, and CS30 and CS30+Fe&P in Table 2-4). However, growth rate and protein efficiency ratio were numerically higher in fish fed diets supplemented with phytase than those of fish fed diets without phytase supplementation. Yoo et al. (2005) reported that 30% dietary FM was successfully replaced by SM with phytase supplementation in Korean rockfish. The supplemental effect of phytase on growth performance in fish cannot simply be compared because it could be different depending on fish species, size, dietary phytase content and experimental conditions.

A significantly or numerically lower blood hematocrits were observed in CS containing groups than in FM based control diet groups whereas the hemoglobin concentration was not significant in all the dietary groups (Table 2-5,6). Reduced blood hematocrit and hemoglobin in fish fed CM containing diets were found in previous studies (Yildirim et al., 2003; Blom et al., 2001; Herman, 1970). This phenomenon could be explained by adverse effects of gossypol on iron absorption in the intestine (Braham and Bressani, 1975), the gossypol-iron complex in liver (Skutches et al., 1974), or

increased erythrocyte fragility (Brocas et al., 1997). However, none of these mechanisms has been confirmed in fish because of some contradictory (Yildirim et al., 2004) and complicated (Barros et al., 2002) results on the hematological values.

The present study clearly demonstrated that dietary supplementation of CS reduces levels of plasma triglycerides and cholesterol (Fig 2-1, 2). One of the most serious modern diseases is coronary heart disease and is caused by atherosclerosis. High serum cholesterol and triglycerides are the major factors that contribute to atherosclerosis and coronary thrombosis, leading to the heart attack (Kannel et al., 1971; Albrink et al., 1961). Recent studies have shown that the cottonseed and soybean meal products have an ability to reduce serum cholesterol in animals. Shandilya and Clarkson (1982) found that gossypol decreased plasma cholesterol level, LDL, and VLDL in adult monkeys. A complete replacement of corn oil by cottonseed oil was reported to reduce total serum cholesterol in growing rats (Radcliffe et al., 2001; Nwoha and Aire, 1995; Edwards and Radcliffe, 1995). Venou et al. (2006) reported that plasma cholesterol decreased with inclusion of soybean meal in diet for gilthead sea bream. The mechanism how the cottonseed meal plays an important role on lipid metabolism has not been clearly determined. Further study is needed on this issue.

Gossypol has been known to have toxic effects on terrestrial animals, humans, and fish when ingested (Rinchard et al., 2000; Makinde et al., 1997; Colin-Negrete et al., 1996; Herman, 1970). Ferrous sulfate has been used to counteract the toxic effect of free gossypol for monogastric animals (Martin, 1990; Jones, 1987) and fish (El-Saidy and Gaber, 2004; Barros et al., 2002; Sealey et al., 1997). Barros et al. (2002) successfully replaced 50% dietary SM by CM with ferrous sulfate supplementation for channel catfish. El-Saidy and Gaber (2004) reported that supplemental iron as ferrous sulfate at 1:1 ratio of iron to free gossypol had no negative effects on dietary nutritional values. In terms of iron supplementation, there was a report (Sealey et al., 1997) that a negative effect of high level of supplemental iron as ferrous sulfate was found in channel catfish. In the present study, total and each (+) and (-) gossypol enantiomer concentration in the liver

were increased as the CM inclusion increased in the diets except the CS10 diet (Table 2-7). However, the total gossypol concentration in liver of fish fed diets supplemented with iron was not detected (Table 2-8). The result of the gossypol concentration in the present study supports the notion that iron supplementation into CM containing diets reduces gossypol toxicities in fish (Lee et al., 2006; Dabrowski et al., 2000; Robinson and Li, 1994; Robinson, 1991). Also, the total gossypol concentration in liver ranged from 2.9 ± 0.72 to 9.1 ± 2.0 ug/g in juvenile and growing parrot fish. The values in this study were lower than the results of the other studies. The finding on the gossypol accumulation in the present study indicate that a metabolic pathway of the gossypol molecules in parrot fish might be quite different from that in other fish species such as rainbow trout (Lee et al., 2002), channel catfish (Yildirim et al. 2004; Yildirim et al. 2003) and tilapia (Mbahinzireki et al., 2001). We do not clearly explain the reason for lower gossypol accumulation in tissues of the parrot fish, however it might be related to differences in fish species. Further studies are needed to investigate the pathway of gossypol metabolism in tissues on several other fish species.

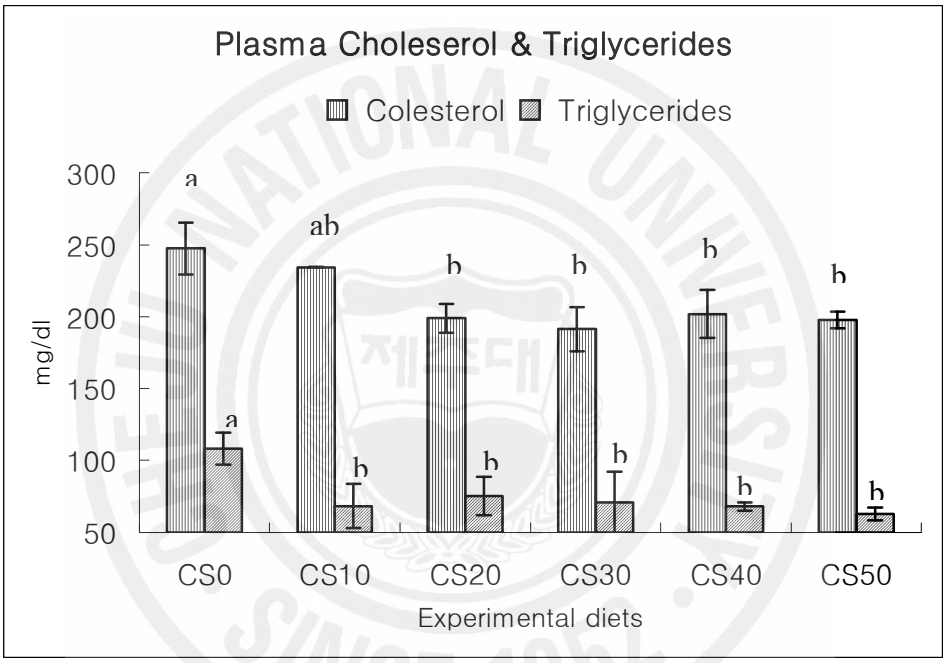


Figure. 2-1. Plasma cholesterol and triglycerides concentrations of juvenile parrot fish fed different experimental diets for 12 weeks

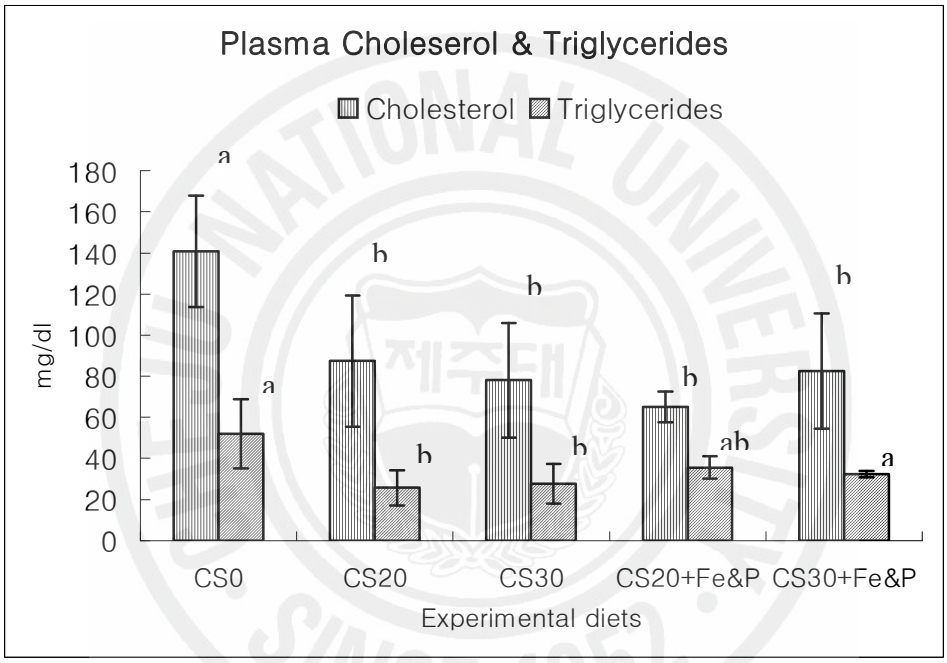


Figure. 2-2. Plasma cholesterol and triglycerides concentrations of growing parrot fish fed different experimental diets for 9 weeks

In conclusion, the mixture of cottonseed and soybean meal with iron and phytase supplementation can replace up to 30% FM protein in diets for growing parrot fish. However, 20% FM protein replacement by CS with supplementation of iron and phytase can be a safe level for commercial use in juvenile and growing parrot fish. The findings in this study suggest that dietary supplementation of iron and phytase could increase the inclusion of cottonseed and soybean meal for FM replacement and might reduce levels of plasma triglycerides and total cholesterol and affect lipid metabolism in marine fish species.

# Part Ⅲ

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

## Part Ⅲ

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


## ABSTRACT

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) values of male and female fed experimental diets (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 values of fish fed all the experimental diets both 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.

## MATERIALS AND METHODS

### Experimental design

There were two feeding experiments. The feeding experiments were conducted with juvenile olive flounder for 19 weeks (unpublished) and with growing olive flounder for 26 weeks (unpublished), for the first and second feeding study, respectively. 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 3-1. 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., Memphis, TN, USA, and its protein content was 43.5% in dry mater basis. The experimental diets used in the second feeding trial (Table 3-1) 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 cottonseed and soybean meal (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 per day (9:00 and 16:00) 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; total liver wt. x 100/total fish wt.) and GSI (gonadosomatic index; total gonad wt. x 100/total 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 ㎛. Slides were stained with Hansen's hematoxylin and 0.5% eosin (HE) for histological observations.

**Statistical analysis**

For the GSI and HSI comparison, data were subjected to one-way ANOVA in SPSS version 11.0. The 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 $P < 0.05$.

Table 3-1. Formulation of experimental diets (% dry matter)

| EXP Ⅰ | Diets | | | | |
|---|---|---|---|---|---|
| Ingredients | 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 Ⅱ | Diets | | | | |
|---|---|---|---|---|---|
| Ingredients | 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; choline chloride, 0.2

Continued from Table 3-1

[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; choline chloride, 0.2

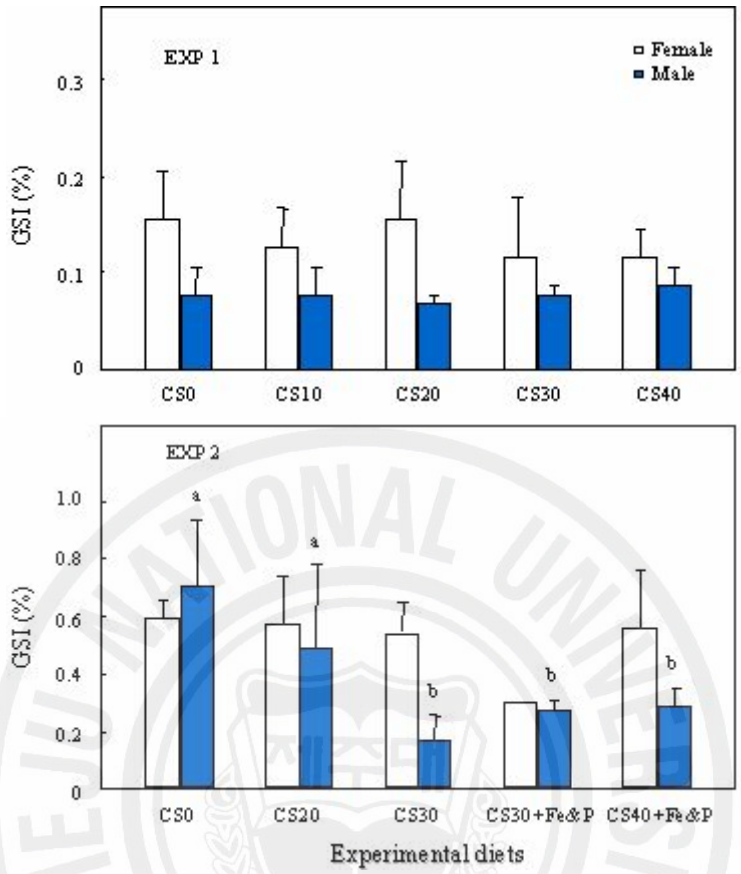


Figure. 3-1. Gonadosomatic index (GSI) of olive flounder fed diets containing cottonseed and soybean meal for 19 and 26 weeks (Experiment 1 and 2, respectively). EXP 1, CS0 is control and CS10, CS20, CS30 and CS40 are experimental diets in which 10%, 20%, 30%, and 40% fish meal protein was respectively replaced by mixture of cottonseed and soybean meal (1:1, w:w). EXP2, CS0 is control and CS20, CS30, CS30+Fe&P and CS40+Fe&P are experimental diets in which 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.

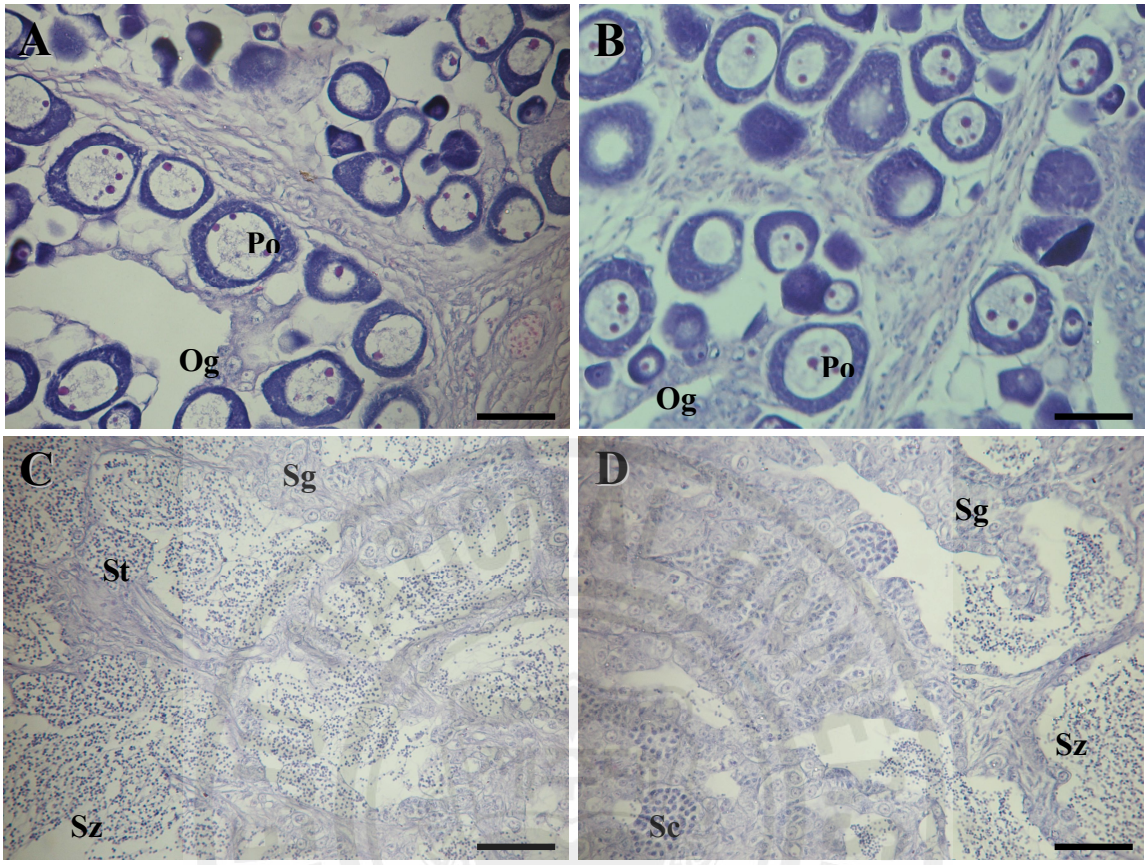


Figure. 3-2. Histological observation of gonads in olive flounder. A , Ovary of control female; B, Ovary of female fed CS40+P&Fe diet; C, Testis of control male; D, Testis of male fed CS40+P&Fe diet. Og, oogonium; Po, peri-nucleolus oocyte; Sc, spermatocyte, Sg, spermatogonia; St, spermatid; Sz, spermatozoa, Bar A, B, C and D = 50 ㎛

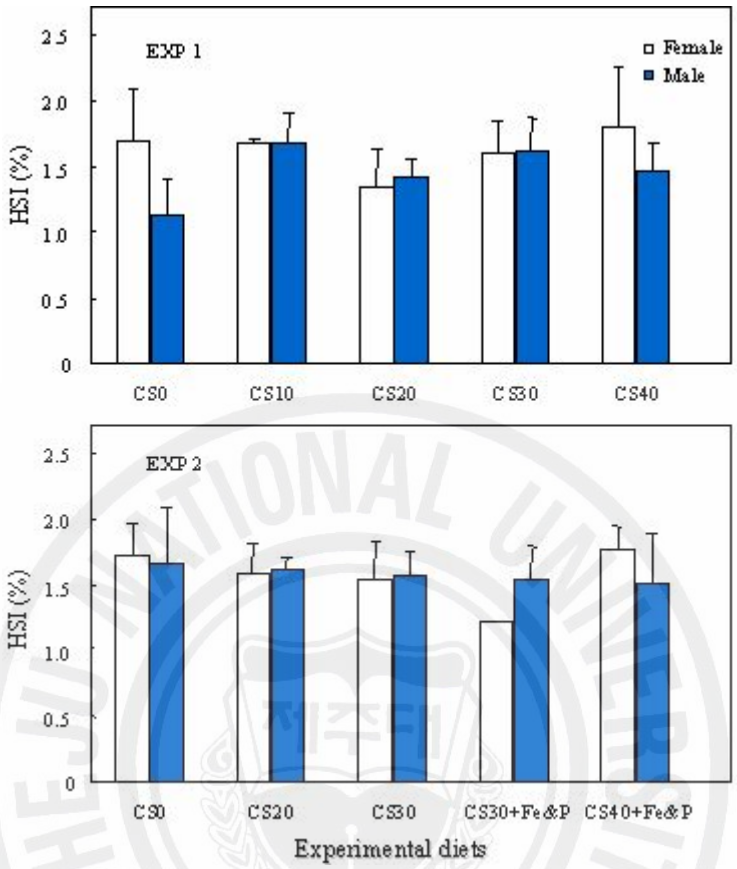


Figure. 3-3. Hapatosomatic index (HSI) of olive flounder fed experimental diets for 19 and 26 weeks (Experiment 1 and 2, respectively). EXP 1, CS0 is control and CS10, CS20, CS30 and CS40 are experimental diets in which 10%, 20%, 30%, and 40% fish meal protein was respectively replaced by mixture of cottonseed and soybean meal (1:1, w:w). EXP2, CS0 is control and CS20, CS30, CS30+Fe&P and CS40+Fe&P are experimental diets in which 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.

## 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 values of male and female fed experimental diets (from the first feeding trial) were not significantly different among all the dietary treatments (Fig 3-1). The GSI values of female fed all the experimental diets (from the second feeding trial) were not significantly different among all the dietary treatments. However, males fed CS containing diets exhibited significantly lower GSI than that fed the control diet except for the CS20 dietary groups (Fig 3-1).

In monogastric animals, the toxicity of gossypol in cottonseed meal has been extensively studied and reported. The most common toxicity symptoms have been in reproductive impairment through 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 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). In studies conducted with human males, it was reported that gossypol is a very strong contraceptive (Frick and Danner, 1985; National Coordinating Group, 1978). 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 values 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 3-2). 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 (Ciereszko and Dabrowski, 2000; Randel et al., 1992; Ikeda, 1990). 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 the pond through a long-term feeding study. However, Nile tilapia fed dietary cottonseed meal up to 24% inclusion revealed an impaired testis activity for 120 days 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 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 3-2). 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.

The HSI values of fish fed all the experimental diets both in the first and second feeding trials were not significantly different among all the dietary treatments (Fig 3-3). The histological examination of liver also did not show any differences among all the dietary treatment (data not presented). However, in the previous 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 values were significantly higher in cottonseed meal fed fish than in the 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 histopathology of liver of olive flounder might be examined after relatively longer period of feeding trial to verify the toxic effects of gossypol in cottonseed meal.

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, the supplementation in diets over 30% fish meal replacement might reduce gonadosomatic index in growing 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.

# SUMMARY

Dietary replacement of fish meal (FM) have been an important issue in aquaculture industry due to a limited supply of FM and its dramatic price increase in recent years. Feed costs account for over 50% of total production costs in most marine fish species, because of the use of the expensive FM with a large dietary proportion. Plant origin byproducts have been promising candidates for the FM replacement and successfully used in many fish species. Cottonseed and soybean meal (CS) have received considerable attention in the replacement of FM in fish feeds because of its balanced amino acid profile, consistent composition, worldwide availability and lower price. However, when compared to FM, they are characterized by a lower composition of essential amino acids, mainly methionine and lysine, and contain some antinutritional factors such as phytic acid and gossypol. The gossypol existed in cottonseed meal is a well known antispermatogenic agent which can impair reproductive performances of male fish as well as mammals. Therefore, the purposes of this study is to evaluate CS as a fish meal replacer with/without iron, phosphorus and phytase supplements and to get the information on the beneficial effects of FM replacement by CS in diet for olive flounder (*Paralichthys olivaceus)* and parrot fish (*Oplegnsthus fasciatus)*.

The results indicated that CS with iron and phosphorus supplementations could replace dietary FM protein up to 40% without negative effects on growth performances of growing olive flounder (Part I). In parrot fish experiments, 30% FM protein could be replaced by CS protein with iron and phytase supplementations in diets for growing parrot fish (Part II). Total gossypol concentration in liver were increased as the CM inclusion increased in diet. The total gossypol concentration in liver of parrot fish were lower than the result of olive flounder. Significantly lower gossypol enantiomer concentration either (+)- or (-)-gossypol was found in liver of fish fed diets supplemented with iron than that of fish fed diets without iron supplementation. The results indicated that the addition of iron in diets could prevent the absorption of free gossypol and its

toxicity. Levels of plasma triglycerides and total cholesterol were lower in parrot fish fed CS containing diet than those in fish fed FM based control diet. The results indicated that, for reproductive histology of olive flounder, dietary supplementation of CS up to 40% FM replacement might not deteriorate the gametogenesis of juvenile and growing olive flounder (Part III).

Therefore, this study concluded that dietary supplementations of iron, phosphorus and phytase could increase the inclusion of CS for FM replacement and might reduce levels of plasma triglycerides and total cholesterol and affect lipid metabolism in marine fish species.

## REFERENCES


Ai, Q., Mai, K., Tan, B., Xu, W., Zhang, W., Ma, H. and Liufu, Z. 2004. Effects of dietary vitamin C on growth and immunity response of Japanese seabass, *Lateolabrax japonicus*. Aquaculture, 242, 489-500.

Ai, Q., Mai, K., Tan, B., Xu, W., Zhang, W., Ma, H. and Liufu, Z. 2006. Effects of dietary vitamin C on survival, growth, and immunity of large yellow croaker, *Pseudosciaena crocea*. Aquaculture, 261, 327-336.

Albrektsen, S., Mundheim, H. and Aksnes, A. 2006. Growth, feed efficiency, digestibility and nutrient distribution in Atlantic cod (*Gadus morhua*) fed two different fish meal qualities at three dietary levels of vegetable protein sources. Aquaculture, 261, 626-640.

Albrink, M.J., Meige, J.W. and Man, E.B. 1961. Serum lipids, hypertension and coronary artery disease. Am. J. Med., 31, 4-23.

Andlauer, W., Martena, M.J. and Furst, P. 1999. Determination of selected phytochemicals by reversed-phase high-performance liquid chromatography combined with ultraviolet and mass spectrometric detection. Journal of Chromatography A, 849, 341-348.

AOAC (Association of Official Analytical Chemists). 1995. Official Methods of Analysis of AOAC International, 16th ed., Arlington, VA.

Bai, S.C. and Gatlin, D.M. 1994. Effects of L-lysine supplementation of diets with different protein levels and sources on channel catfish *Ictalurus punctatus* (Rafinesque). Aquac. Fish Manage., 25, 465–474.

Barros, M.M., Lim, C. and Klesius, P.H. 2002. Effect of soybean meal replacement by cottonseed meal and iron supplementation on growth, immune response and resistance of channel catfish (*Ictalurus punctatus*) to *Edwardsiella ictaluri* challenge. Aquaculture, 207, 263-279.

Barual, K., Sahu, N.P., Pal, A.K. and Debnath, D. 2004. Dietary phytase: An ideal

approach for a cost effective and low-polluting aquafeed. NAGA, World Fish Center Quarterly, 27, 15-19.

Blom, J.H., Lee, K.J., Rinchard, J., Dabrowski, K. and Ottobre, J. 2001. Reproductive efficiency and maternal offspring transfer of gossypol in rainbow trout (*Oncorhynchus mykiss*) fed diets containing cottonseed meal. J. Anim. Sci., 79, 1533pp.

Boonyaratpalrin, M., Suraneiranat, P. and Tunpibai, T. 1998. Replacement of fish meal with various types of soybean products in diets for Asian sea bass (*Lates calcarifer*). Aquaculture 161, 67–78.

Braham, J.E. and Bressani, R. 1975. Effect of different levels of gossypol on transaminase activity, on nonessential to essential amino acid ratio, and on iron and nitrogen retention in rat. J. Nutr., 105, 348-355.

Brocas, C., Rivera, R.M., Paula-Lopes, F.F., McDowell, L.R., Calhoun, M.C., Staples, C.R., Wilkinson, N.S., Boning, A.J., Chenoweth, P.J. and Hansen, P.J. 1997. Deleterious actions of gossypol on bovine spermatozoa, oocytes and embryos. Biol. Reprod., 57, 901-907.

Brown, B.A. 1980. Routine hematology procedures. In: Brown B. A. (editor), Hematology, Principles and Procedures. Lea and Febiger, Philadelphia, pp. 71-112.

Chase, C.C., Bastidas, J.L., Ruttle, C.R. and Randel, R.D. 1994. Growth and reproductive development in Brahman bulls fed diets containing gossypol. J. Anim. Sci., 72, 445-452.

Cheng, Z.J. and Hardy, W.R. 2002. Apparent digestibility coefficients and nutritional value of cottonseed meal for rainbow trout (*Oncorhynchus mykiss*). Aquaculture, 212, 361-372.

Chenoweth, P. J., C. A. Risco, R. E. Larsen, R. E. Larsen, J. Velez, T. Tran and C. C. Chase, 1994. Effects of dietary gossypol on aspects of semen quality, sperm morphology and sperm production in young Brahman bulls. Theriogenology, 42,

1-13.

Cho, C.Y., Bayley, H.S. and Slinger, S.J. 1974. Partial replacement of herring meal with soybean meal and other changes in a diet for rainbow trout (*Salmo gairdneri*). J. Fish. Res. Board Can., 31, 1523–1528.

Ciereszko, A. and Dabrowski, K. 2000. In vitro effect of gossypol acetate on yellow perch (*Perca lavescens*) spermatozoa. Aquat. Toxicol., 49, 181-187.

Colin-Negrete, J., Kiesling, H.E., Ross, T.T. and Smith, J.F. 1996. Effect of whole cottonseed on serum constituents, fragility of erythrocyte cells, and reproduction of growing Holstein heifers. J. Dairy Sci., 79, 2016-2023

Coyle, S.D., Mengel, G.J., Tidwell, J.H. and Webster, D.C. 2004. Evaluation of growth, feed utilization, and economics of hybrid tilapia, *Oreochromis niloticus* x *Oreochromis aureus*, fed diets containing different protein resources in combination with distillers dried grains with solubles. Aquacu. Res., 35, 365-370.

Coyle, T., Levante, S., Shetler, M. and Winfield, J. 1994. In vitro and in vivo cytotoxicity of gossypol against central nervous system tumor cell lines. J. Neuro-Oncol., 19, 25-35.

Dabrowski, K. and Kosac, B. K. 1979. The use of fish meal and soybean meal as a protein source in the diet of grass carp fry. Aquaculture, 18, 107–114.

Dabrowski, K., Rinchard, J., Lee, K.J., Blom, J.H., Ciereszko, A. and Ottobre, J. 2000. Effects of diets containing gossypol on reproductive capacity of rainbow trout (*Oncorhynchus mykiss*). Biol. Reprod., 62, 227-234.

Davies, S.J., McConnell, S. and Bateson, R.I. 1990. Potential of rapeseed meal as an alternative protein source in complete diets for tilapia (*Oreochromis mossambicus* Peters). Aquaculture, 87, 145-154.

Edwards, M.S. and Radcliffe, J.D. 1995. A comparison of the effect of cottonseed oil and corn oil on lipid status in the rat. Biochem, Arch., 11, 103-109.

Einen, O., Roem, A.J. 1997. Dietary protein/energy ratios for Atlantic salmon in relation

to fish size: growth, feed utilization and slaughter quality. Aquac. Nutr., 3, 115–126.

El-Saidy, D.M.S.D. and Gaber, M.M.A. 2004. Use of cottonseed meal supplemented with iron for detoxification of gossypol as a total replacement of fish meal in Nile tilapia, *Oreochromis niloticus* (L.) diets. Aquac. Res., 35, 859-865.

El-sayed, A.F.M. 1990. Long-term evaluation of cottonseed meal as a protein source for Nile tilapia, Oreocbromis niloticus(Linn.). Aquaculture, 84, 315-320.

Ellis, A.E. 1990. Lysozyme assays. In: Techniques in fish Immunology (Stolen, J. S., Fletcher, T. C., Anderson, B. S. and Van Muiswinkel, W. B. eds), pp. 101-103. SOS Publications, Fair Haven, NJ, USA.

FAO (Food and Agriculture Organization). 2003. Year Book of Fishery Statistics Summary Tables. Sales and Marketing Group, Information Division, FAO, Rome, Italy.

Folch, J., Lee, M. and Sloane-Stanley, G.H. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem., 226, 497-509.

Fowler, L.G. 1980. Substitution of soybean and cottonseed products for fish meal in diets fed to chinook and coho salmon. Prog. Fish-Cult., 42, 87-91.

Frick, J. and Danner, C. 1985. Effects of gossypol on human testicular function. Evaluation of seminal and hormonal parameters. In: Segal SJ, ed. Gossypol. A potintial contraceptive for men. New York: Plenum Perss, 17-24.

Fritz, K.L., Seppanen, C.M., Kurzer, M.S. and Csallany, A.S. 2003. The in vivo antioxidant activity of soybean iso.avones in human subjects. Nutrition Research, 23, 479-487.

Giridharan, N., Bamji, M.S. and Sankaram, A.V.B. 1982. Inhibition of rat testis LDH-X activitiy by gossypol. Contraception, 26, 607-615.

Hardy, R.W. 1996. Alternate protein sources for salmon and trout diets. Anim. Feed. Sci. Tech., 59, 71-80.

Havsteen, B. 1983. Flavonoids, A class of natural products of high pharmacological

potency. Biochem. Pharmacol., 32, 1141–1148.

Hendricks, J.D. 2002. Adventitious toxins. In: Halver, J.E., and Hardy, R.W. (Eds.), Fish Nutrition, 3rd ed. Academic Press, San Diego, CA, USA, pp 601-649.

Hendricks, J.D., Sinnhuber, R.O., Loveland, P.M., Pawlowski, N.E. and Nixon, J.E. 1980. Hepatocarcinogenicity of glandless cottonseed and cottonseed oil to rainbow trout. Science, 208, 309-310.

Henry, M.H., Pesti, G.M., Bakalli, R., Lee, J., Toledo, R.T., Eitenmiller, R.R. and Phillips, R.D. 2001. The performance of broiler chicks fed containing extruded cottonseed meal supplemented with lysine. Poult. Sci., 80, 762-768.

Herman, R.L. 1970. Effects of gossypol on rainbow trout Salmo gairdneri Richardson. J. Fish Biol., 2, 293-303.

Ikeda, M. 1990. Inhibition kinetic of NAD-linked enzymes by gossypol acetic acid. Andrologia, 22, 133-136

Jackson, A.J., Capper, B.S. and Matty, A.J. 1982. Evaluation of some plant proteins in complete diets for the tilapia *S. mossambicus*. Aquaculture, 27, 97-109.

Jones, L.A. 1987. Recent advances in using cottonseed products. Proceedings of the Florida Nutrition Conference, 12– 13 March 1987, Daytona Beach, FL, 119-138.

Kannel, W.B., Castelli, W.P., Gordon, T. and McNamara, P.M. 1971. Serum cholesterol, lipoprotein and the risk of heart disease. The Framingham study. Ann lntern Med., 74, 1-12

Kikuchi, K. 1999. Use of defatted soybean meal as a substitute for fish meal in diets of Japanese flounder (*Paralichthys olivaceus*). Aquaculture, 179, 3-11.

Kim, H.L. and Calhoun, M.C. 1995. Determination of gossypol in plasma and tissues of animals. Proceedings of 86th AOCS Annual Meeting, San Antonio, Texas, Abstract B, 487.

Krogdahl, A. 1995. Soybean products may cause intestinal problems (Abstract). In: Produksjon av laksefisk (PAL) programmets rapportsammendrag. Oslo, Norwegian Research Council, 23-24.

Krogdahl, A., Lea, T.B. and Olli, J.J. 1994. Soybean protein are inhibitors affect intestinal trypsin activities and amino acid digestibility in rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Phys., 107A, 215-219.

Kuehnau, J. 1976. The flavonoids, A class of semi-essential food components: their role in human nutrition. World Rev. Nutr. Diet, 24, 117–191.

Kurten, G., Hall, L. and Thompson, N. 1999. Evaluation of cottonseed meal supplementation of inorganically fertilized Florida largemouth bass spawning ponds. North Am. J. Aquacult., 61, 115-125.

Lee, K-J., Dabrowski, K., Blom, J.H., Baizand, S.C. and Stromberg, P.C. 2002. A mixture of cottonseed meal, soybean meal and animal byproduct mixture as a fish meal substitute: growth and tissue gossypol enantiomer in juvenile rainbow trout (*Oncorhynchus mykiss*). J. Anim. Physiol.. Anim. Nutr., 86, 201–213.

Lee, K.J. 2002. Nutritional and physiological studies of rainbow trout following utilization of cottonseed meal-based diets. Dissertation for the degree of doctor scientiarum. Ohio State University. Columbus, Ohio, U.S.A.

Lee, K.J. and Dabrowski, K. 2002. Tissue gossypol and gossypolone isomers in rainbow trout fed low and high levels of dietary cottonseed meal. J. Agri. Food Chem., 50, 3056-3061.

Lee, K.J., Dabrowski, K., Blom, J.H. and Bai, S.C. 2001. Replacement of fish meal by a mixture of animal by-products in juvenile rainbow trout, *Oncorhynchus mykiss*. North American Journal of Aquaculture, 63, 109-117.

Lee, K.J., Rinchard, J., Dabrowski, K., Babiak, I., Ottobre, J.S. and Christensen, J.E. 2006. Long-term effects of dietary cottonseed meal on growth and reproductive performance of rainbow trout: Three-year study. Anim. Feed Sci. Tech., 126, 93-106.

Liu, K. 1997. In: Soybeans: Chemistry, Technology and Utilization. Chapman and Hall, International Thomsom Publishing, Singapore, 532 pp.

Makinde, M.O., Akingbemi, B.T. and Aire, T.A. 1997. Gossypol-induced erythrocyte

fragility in the rat is ameliorated by ethanol intake. South. Afri. J. Sci., 93, 141-143.

Martin, S.D. 1990. Gossypol effects in animal feeding can be controlled. Feedstuffs, 62, 14-17.

Mbahinzireki, G.B. 1999. Tilapia, *Oreochromis* Sp. (L): Sex reversal and performance on alternative sources of protein. Dissertation for the degree of doctor scientiarum. Ohio State University. Columbus, Ohio, U.S.A. pp. 118.

Mbahinzireki, G.B., Dabrowski, K., Lee, K.J., El-Saidy, D. and Wisner, E.R. 2001. Growth, feed utilization and body composition of tilapia (*Oreochromis sp.*) fed with cottonseed meal-based diets in a re-circulating system. Aquacult. Nutr., 7, 189-200.

National Coordinating Group on Male Antifertility Agents, 1978. Gossypol-A new antifertility agent for males. Chinese Med. J-Peking., 92, 417-418.

NRC (National Research Council). 1993. Nutritional Requirements of Fish, 144pp. National Academy Press, Washington, DC, USA.

Nwoha, P.U. and Aire, T.A. 1995 Reduced level of serum cholesterol in low protein fed Wistar rats. Contraception, 52, 261-265.

Ofojekwu, P.C. and Ejike, C. 1984. Growth response and food utilization in the tropical cichlid, *Oreochromis niloticus* (Linn) fed on cottonseed-based diets. Aquaculture, 42, 27-36.

Pathak, D., Pathak, K. and Singla, A.K. 1991. Flavonoids as medicinal agents-Recent advances. Fitoterapia LXII, 371-389.

Pham, M.A., Lee, K.J., Lim, S.J., Lee, B.J., Kim, S.S., Park Y.J. and Lee, S.M. 2005. Fish meal replacement by cottonseed and soybean meal in diets for juvenile olive flounder, *paralichthys olivaceus*. J. Aquacult., 18, 215-221.

Radcliffe, J.D., King, C.C., C-N, D.M. and Imrhan, V. 2001. Serum and liver lipids in rats fed diets containing corn oil, cottonseed oil, or a mixture of corn and cottonseed oils. Plant Food. Human Nutr., 56, 51-60.

Randel, R.D., Chase, C.C. and Wyse, S.J. 1992. Effects of gossypol and cottonseed products on reproduction of mammals. J. Anim. Sci., 70, 1628-1638.

Randel, R. D., S. T. Willard, S. J. Wyse and L. N. French, 1996. Effects of diets containing free gossypol on follicular development embryo recovery and corpus luteum function in Brangus heifers treated with bFSH. Theriogenology, 45, 911-922.

Rawles, S.D. and Gatlin, D.M. 2000. Nutrient digestibility of common feedstuffs in extruded diets for sunshine bass, *Morone chrysops* female × *M. sasatilis* male. J. World Aquacult. Soc., 31, 570-579.

Refstie, S., Storebakken, T., Baeverfjord, G. and Roem, A.J. 2001. Long-term protein and lipid growth of Atlantic salmon (*Salmo salar*) fed diets with partial replacement of fish meal by soy protein products at medium or high lipid level. Aquaculture, 193, 91-106.

Refstie, S., Svihus, B., Shearer, K.D. and Storebakken, T. 1999. Nutrient digestibility in Atlantic salmon and broiler chickens related to viscosity and non-starch polysaccharides content in different soybean products. Aquaculture, 79, 331-345.

Reyes, J., Borriero, L. and Benos, D.J. 1988. A bioenergetic medel of gossypol action: effects of gossypol on adult rat spermatogenic cells. Am. J. Physiol., 254, C564-C570.

Rinchard, J., Ciereszko, A., Dabrowski, K. and Ottobre, J. 2000. Effects of gossypol on sperm viability and plasma sex steroid hormones in male sea lamprey, Petromyzon marinus. Toxico. Lett., 111, 189-198.

Robaina, L., Izquierdo, M.S., Moyano, F.J., Socorro, J., Vergara, J.M. and Montero, D. 1998. Increase of the dietary *n*-3 / *n*-6 fatty acid ratio and addition of phosphorus improves liver histological alterations induced by gilthead seabream, *Sparus aurata*. Aquaculture, 161, 281-293.

Robinson, E.H. 1991. Improvement of cottonseed meal protein with supplemental lysine in feeds for channel catfish. J. Appl. Aquacult., 1, 1-14.

Robinson, E.H. and Brent, J.R. 1989. Use of cottonseed meal in channel catfish feeds. J. World Aquacult. Soc., 20, 250-255.

Robinson, E.H. and Daniels, W.H. 1987. Substitution of soybean meal with cottonseed meal in pond feeds for channel catfish reared at low densities. J. World Aquacult. Soc., 18, 101-106.

Robinson, E.H. and Li, M.H. 1994. Use of cottonseed meal in channel catfish feeds: replacement of soybean meal with cottonseed meal and replacement of fish meal with soybean meal and cottonseed meal. J. World Aquacult. Soc., 25, 271-276.

Robinson, E.H. and Tiersch, T.R. 1995. Effects of long-term feeding of cottonseed meal on growth, testis development, and sperm motility of male channel catfish *Ictalurus punctatus* broodfish. J. World Aquac. Soc., 26, 426-431.

Robinson, E.H., Rawles. S.D. and Stickney, R.T. 1984. Evaluation of glanded and glandless cottonseed products in catfish diets. Prog. Fish-Cult., 46, 92-97.

Ronsholdt, B. 1995. Effect of size/age and feed composition on body composition and phosphorus content of rainbow trout, *Oncorhynchus mykiss*. Water Sci. Technol., 31 (10), 175- 183.

Ross, N.W., Firth, K.J., Wang, A., Burka, J.F. and Johnson, S.C. 2000. Changes in hydrolutic enzyme activities of naïve Atlantic salmon (*Salmo salar*) skin mucus due to infection with the salmon louse (*Lepeophtheirus salmonis*) and cortisol implantation. Dis. Aquat. Org., 41, 43-51.

Salaro, A.L., Pezzato, L.E., Barros, M.M. and Vicentini, C.A. 1999. Desempenho E espermatogenese de alevinos de tilapia alimentados com farelo ou farinha de semente de algodao. Pesqui. Agropecu. Bras., 34, 449-457.

Sealey, W.M., Lim, C. and Klesius, P.H. 1997. Influence of the dietary level of iron from iron methionine and iron sulfate on immune response and resistance of channel catfish to *Edwardsiella ictaluri.* J.World Aquacult. Soc., 28, 142-149.

Shandilya, L.N. and Clarkson, T.B. 1982. Hypolipidemic effects of gossypol in cynomolgus monkeys (Macaca Fascicularis). 17, 285-290.

Skutches, C.L., Herman, D.L. and Smith. F.H. 1974. Effect of dietary free gossypol on blood components and tissue iron in swine and rat. J. Nutr., 104, 415-422.

Snyder, H.E. and Kwon, T.W. 1987. Soybean Utilization, Van Nostrand-Reinhold, New York, NY, USA, 346 pp.

Spinelli, H.E., Houle, C.R. and Wekell, J.C. 1983. The effects of phytate on the growth of rainbow trout (*Salmor gairdneri*) fed purified diets containing varies quantities of calcium and magnesium. Aquaculture, 30, 71-83.

Storebakken, T., Refstie, S. and Ruyter, B. 2000. Soy products as fat and protein sources in fish diets for intensive aquaculture. In: Drackley, J.K. (Editor), Soy in Animal Nutrition. Fed. Anim. Sci. Soc., Savoy, IL, pp.127-170.

Storebakken, T., Shearer, K.D. and Reom, A.J. 1998. Availability of protein, phosphorus and other elements in fish meal, soy-protein concentrate and phytate treated soy-protein-concentrate-based diets to Atlantic salmon (*Salmon salar*). Aquaculture, 161, 365-379.

USDA (United States Department of Agriculture). 2000. Counselor and attaché reports, Official Statistics, USDA estimates.

Uyan, O., Koshio, S., Ishikawa, M., Uyan, S., Ren, T., Yokoyama, S., Komilus, C.F. and Michael, F.R. 2007. Effects of dietary phosphorus and phospholipid level on growth, and phosphorus deficiency signs in juvenile Japanese flounder, *Paralichthys olivaceus*. Aquaculture, 267, 44-54.

Venou, B., Alexis, M.N., Fountoulaki, E. and Haralabous, J. 2006 Effects of extrusion and inclusion level of soybean meal on diet digestibility, performance and nutrient utilization of gilthead sea bream (*Sparus aurata*). Aquaculture, 261, 343-356.

Viola, S., Mokady, S. and Arieli, Y. 1983. Effects of soybean processing methods on the growth of carp (*Cyprinus carpio*). Aquaculture, 32, 27-38.

Wagner, H. 1985. New plant phenolics of pharmaceutical interest. In: Annual Proceedings of the Phytochemical Society of Europe, Vol. 25. Eds. Van Sumere, C. F. and Lea, P. J. pp. 409-425.

Wang, J., Qiu, J., Wu, X., Zhang, Z., Shao, Y. and Cao, R. 1988. The correlation between the gossypol contens in blood plasma, rete testis fluid, and caudal epididymal fluid following chronic treatment with gossypol in rats. J. Androl., 9, 397-402.

Wang, X., Choi, S., Park, S., Yoo, G., Kim, K., Kang, J. and Bai, S.B. 2005. Optimum dietary phosphorus level of juvenile Japanese flounder *Paralichthys olivaceus* reared in the recirculating system. Fish. Sci., 71, 168-173.

Wedegaertner, T.C. 1981. Making the Most of cottonseed Meal. National Cottonseed Products Association, Memphis, TN, USA.

Yildirim, M., Lim, C., Wan, P. and Klesius, P.H. 2003. Growth performance and immune response of channel catfish (*Ictalurus punctatus*) fed diets containing graded levels of gossypol-acetic acid. Aquaculture, 219, 751-768.

Yildirim, M., Lim, C., Wan, P. and Klesius, P.H., 2004. Effect of natural free gossypol and gossypol-acetic acid on growth performance and resistance of channel catfish (*Ictalurus punctatus*) to *Edwardsiella ictaluri* challenge. Aquacult. Nutr., 10, 153-165.

Yoo, G.Y., Wang, X., Choi, S., Han, K., Kang, J.C., and Bai, S.C. 2005. Dietary microbial phytase increased the phosphorus digestibility in juvenile Korean rockfish Sebastes schlegeli fed diets containing soybean meal. Aquaculture, 243; 315-322.

Yuan, Y.Y. and Shi, Q.X. 2000. Inhibition of hamster sperm acrosomal enzyme by gossypol is closely associated with the decrease in fertilization capacity. Contraception, 62, 203-209.

# ACKNOWLEDGEMENT

한없이 부족한 저에게 학문과 인생의 길이 무엇인지를 깨우쳐 주신 이경준 교수님의 노고와 은혜에 머리 숙여 감사드리며, 아울러 바쁘신 와중에도 세심한 조언으로 논문을 다듬어 주신 이기환 교수님과 이영돈 교수님께 진심으로 감사 말씀을 드립니다. 그리고 학부생 시절부터 지금까지 많은 가르침을 주신 노섬 교수님, 정상철 교수님, 최광식 교수님, 송춘복 교수님, 전유진 교수님, 이제희 교수님, 여인규 교수님, 김기영 교수님에게도 깊은 감사 말씀을 드립니다.

그리고 사육실험에 많은 도움을 주신 해양과환경연구소 어류생리생태연구실원 께도 감사 말씀을 드리고, 처음부터 지금까지 늘 함께 였던 나의 동기이자 영원한 친구인 이봉주에게 진심으로 감사의 마음을 전합니다. 또한, 대학원 생활을 하면서 항상 옆에서 버팀목이 되어주시고, 관심을 아끼지 않았던 박영준 선배님과 인생의 선배로서 많은 격려와 조언을 해주신 장계환 선배님에게도 진심으로 감사 말씀을 드립니다. 그리고 실험실 생활에 있어서 나의 모든 불만과 짜증을 너그럽게 받아준 지훈, 성삼, 지웅, 경용, 대한, 진이에게 이 글을 빌어 진심으로 감사의 마음을 전합니다.

정말로 많은 분들의 도움으로 제가 이 자리에 올 수 있었던 것 같습니다. 알게 모르게 많은 도움을 주신 모든 분들에게 이 글을 빌어 감사 말씀을 드립니다.

끝으로 오늘의 제가 있기까지 저를 헌신적으로 사랑해주시고, 믿어주신 사랑하는 어머니, 아버지, 형, 누나 그리고 믿음으로 저를 성원해준 친구들과 제가 항상 학업에 전념할 수 있게 도와주는 사랑하는 지은이에게 감사의 마음을 전합니다.