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Effects of Dietary Soy Isoflavones on Feed Intake, Growth Performance and Digestibility in Juvenile Japanese Flounder (*Paralichthys olivaceus*)

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Abstract An 8-week feeding trial was conducted to investigate the effects of dietary soy isoflavones on feeding intake, growth performance, and digestion of juvenile Japanese flounder (*Paralichthys olivaceus*). Four isonitrogenous (49% crude protein) and isoenergetic (20.1 MJ kg^{-1}) diets were formulated to contain four graded levels of soy isoflavones, namely, 0, 1, 4 and 8 g soy isoflavones in 1 kg of diet. Each diet was randomly fed to triplicate tanks of fish (Initial average weight: 2.58 g ± 0.01 g), and each tank was stocked with 35 fish. No significant difference was observed among diets with levels of 0, 1 and 4 g kg^{-1} soy isoflavones in feed intake, weight gain, feed efficiency ratio (FER), proximate composition of fish whole body and apparent digestibility coefficients (ADC) of nutrients and energy (P>0.05). However, high dietary soy isoflavones level (8 g kg^{-1}) significantly depressed weight gain, FER, whole-body crude lipid content of fish and ADC of nutrients (P<0.05). These results indicate that high level of dietary soy isoflavones (above 4 g kg^{-1}) significantly depresses growth responses and FER of Japanese flounder. However, as the content of soy isoflavones in soybean meal is around 1 to 3 g kg^{-1}, the adverse effects might be neglected when soybean products are used as a fish feed ingredient.

Key words soy isoflavones; feed intake; digestibility; growth; Japanese flounder

1 Introduction

Aquaculture has become one of the fastest growing food-producing sectors, supplying approximately 40% of the world's fish food (Cole *et al*., 2009). However, the shortage of fish meal supplies is limiting the development of aquaculture. Soybean meal is being investigated as a replacement to fishmeal as the principal alternative protein source in fish feed, which is regarded as an economical and nutritious alternative due to its relatively low cost, high crude protein content and reasonably balanced amino acid profile (Hernández *et al*., 2007; Lilleeng *et al*., 2007). However, high inclusion of soybean protein has been reported to reduce the feed intake and nutrient utilization, and eventually inhibit the growth performance of turbot (Burrells *et al*., 1999; Burel *et al*., 2000; Day and Plascencia-GonzÁlez, 2000), Atlantic salmon (Refstie *et al*., 1998; Krogdahl *et al*., 2003; Opstvedt *et al*., 2003) as well as Japanese flounder (Kikuchi *et al*., 1994; Kikuchi, 1999; Kim *et al*., 2000; Saitoh *et al*., 2003; Deng *et al*., 2006). The content of anti-nutritional factors is one of major impediments toward increased use of soybean products in diets for fish according to NRC (2011).

Phytoestrogens are estrogen hormone-like anti-nutriational factors found in plants (Kurzer and Xu, 1997). Soy isoflavones, as phenolic compounds, are the major phytoestrogens of soybean (Setchell, 1998), and the three most abundant isoflavones found in soybean are genistein, daidzein and glycitein. Soy isoflavones could have potential biological effects in animals, including humans (Barrett, 1996; Friendman and baron, 2001), farm animals (Woclawek-Potocka *et al*., 2005) and fish (Van den Ingh and Krogdahl, 1990). Up to date, most relevant researches have been focused on their estrogen/ anti-estro- gen effects and the role in disease prevention which have received widespread attention since the 1990s (Messina, 2010). In the livestock and poultry farming, soy isoflavones (like daidzein) are used widely as an additive to promote the growth and production performance. However, limited knowledge is available at present on the investigation of the effects of dietary soy isoflavones on aquatic animals, especially the effects on growth performance and feed utilization. Previous related reports on fish have been controversial (Ko and Malison, 1999; Pollack *et al*., 2003; Ye and Chen, 2008; Zhang, 2010).

Japanese flounder is a carnivorous species that is widely cultured in East Asia because of its delicious meat

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and rapid growth. Dietary soybean meal incorporation in diets of Japanese flounder should, however, generally be limited since this fish is very sensitive to the soybean products (Kikuchi, 1999; Deng *et al*., 2006). In this study, considering the adverse effects of soy isoflavone may be neutralized by the fish meal-based palatable diet, a maximum supplementation of 8 g kg^{-1} soy isoflavones was added, which was much higher than the contents of soy isoflavones in soybean (1 to 3 g kg^{-1}). The purpose of the present study was to evaluate how soy isoflavones (ranging from 0 to 8 g kg^{-1}) in diets of Japanese flounder affected the feed intake, growth, feed efficiency ratio and digestibility of nutrients. For this purpose, purified soy isoflavones were used to avoid the influences of other unknown factors present in soybean meal.

2 Materials and Methods

2.1 Experimental Diets

The basal diet (Table 1) contained fish meal, fish oil and wheat flour (with no inherent content of soybean isoflavone) to meet the protein (49.1% crude protein) and energy (20.1 MJ kg^{-1}) requirements of Japanese flounder (Yigit *et al*., 2004). Four experimental diets were formulated to contain 0, 1, 4 and 8 g kg^{-1} of soy isoflavones (named as Diet 1 to 4) respectively by supplementing a soy isoflavones product (purity, 80%), which is supplied by Xinxin Chemistry Technology Co., Ltd., China, in the basic formulation. Wheat flour content was adjusted correspondingly to make the formulation sum 100%. All diets contained 500 mg kg^{-1} yttrium oxide (Y_2O_3, Fluka Chemicals®) as an inert marker for determining digestibility.

Table 1 Ingredients and chemical composition of the basal diet fed to juvenile Japanese flounder

Ingredient (g kg^{-1})	Weight
Fish meal[a]	680.0
Fish oil[a]	40.0
Wheat flour	244.5
Vitamin mixture[b]	20.0
Mineral mixture[c]	15.0
Yttrium oxide	0.5
Proximate composition (g kg^{-1})	
Crude protein	491.0
Crude lipid	79.0
Ash	163.2
Gross energy (kJ g^{-1})	20.1

Notes: [a] Supplied by Liuhe Feed Co., Ltd. (Shandong, China); fish meal, crude protein, 67.6%. [b] Vitamin premix (mg kg^{-1} diet): retinol acetate, 32.0; cholecalciferol, 12.9; alpha-tocopherol, 200.0; thiamin, 110.0; riboflavin, 360.0; pyridoxine HCl, 86.0; pantothenic acid, 359.0; niacin acid, 1026.0; biotin, 10.0; folic acid, 20.0; vitamin B12, 1.2; inositol, 4000.0; ascorbic acid, 500.0; choline chloride , 2000.0. [c] Mineral premix (mg kg^{-1} diet): $MgSO_4$•$7H_2O$, 5066.9; KCl, 3020.0; $KAl(SO_4)_2$, 12.7; $CoCl_2$, 40.0; $ZnSO_4$•$7H_2O$, 253.0; $CuSO_4$•$5H_2O$, 10.0; KI, 8.0; $MnSO_4$•$4H_2O$, 73.2; Na_2SeO_3, 2.5; $C_6H_5O_7Fe$•$5H_2O$, 1632.0; NaCl, 100.0; NaF, 4.0; KH_2PO_4, 10000.0.

Experimental ingredients were ground into a fine powder through 320-μm mesh. Soy isoflavones were first dissolved in deionized water, mixed with the ingredients thoroughly, and then mixed with fish oil and water to produce stiff dough. The dough was pelletted using a twin-screw extruder (F-26 (II), South China University of Technology, China) through a 1.5-mm die. The moist pellets were dried in a forced air oven at room temperature for about 12 h, and then stored at −20℃ until used.

2.2 Experimental Procedure

Juvenile Japanese flounder (*Paralichthys olivaceus*) were obtained from Rizhao Fisheries Research Institute (Rizhao, China). Prior to the experiment, fish were transported to the experiment station (Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao, China), and stocked into a flow- through system (Fiberglass circular tanks, 400-L capacity filled to 320-L) to acclimatize to new conditions for 2 weeks and during this period fish were fed twice daily with a commercial diet (Nisshin Flour Milling Co., Ltd., Japan) to satiation. Sea water, continuously pumped from the adjacent coast to the experiment station, passed through sand filters into each tank at approximately 1.5 L min^{-1}. All rearing tanks were provided with continuous aeration and maintained under natural photoperiod (14 h light/10 h dark at the end of August and 12 h light/12 h dark at the end of October).

At the start of the experiment, the fish were fastened for 24 h before weighing. Fish of similar sizes (initial weight 2.58 g ± 0.01 g, mean ± S.E.M.) were distributed into 12 tanks with 35 juveniles per tank. Each diet was randomly assigned to triplicate tanks. Fish were hand-fed to apparent satiation twice (08:30 and 16:30) a day for 8 weeks. During the experimental period, the temperature ranged from 18.0 to 26.0℃, the salinity 30–33, and pH 7.7–7.9.

2.3 Sample Collection and Chemical Analysis

The fish were weighed in bulk at the end of the feeding trial and the fish were fastened for 24 h before weighted. Total number and mean body weight of fish in each tank were measured to calculate the feed efficiency ratio (FER) and survival rate. Thirty fish at the initiation of feeding trial and five fish per tank at the termination were randomly collected and stored frozen (−70℃) for proximate analysis. During days 49 to 52, all fish were feces-stripped as described by Kaushik *et al*. (2004). The fecal samples were pooled per tank and frozen until analysis. Analysis of dry matter (105℃, 24 h), crude protein (Kjeldahl nitrogen×6.25), crude lipid (ether extraction by Soxhlet method) and ash (550℃, 18 h) in diets, freeze-dried feces and whole-body samples were performed following standard laboratory procedures (AOAC, 2000). Gross energy in diets and feces was determined by an adiabatic bomb calorimeter (Parr 1281, USA). Yttrium concentrations in diets and fecal samples were determined with an inductively coupled plasma-atomic emission spectrophotometer (ICP-OES; VISTA-MPX, VARIAN)

after perchloric acid digestion.

2.4 Calculations and Statistical Analysis

Weight was calculated by the mean weight at the termination of the feeding trial. The following variables were calculated:

$$\text{Feed efficiency ratio (FER)} = \frac{\text{Wet weight gain (g)}}{\text{Dry feed fed (g)}},$$

$$\text{Survival rate} = 100 \times \frac{N_t}{N_0},$$

$$\text{Apparent digestibility coefficient of nutrients and energy (\%)} = \left(1 - \frac{\text{dietary } Y_2O_3}{\text{fecal } Y_2O_3} \times \frac{\text{fecal nutrient or energy}}{\text{dietary nutrient or energy}}\right) \times 100,$$

where N_t and N_0 are final and initial number of fish, respectively, t is duration of experimental days.

All percentage data were arcsine transformed before analysis. Data were subjected to one-way analysis of variance (ANOVA) to determine if significant differences occurred in fish fed the different diets. If a significant difference was identified, differences among means were compared by Tukey's multiple range tests. Results were considered significantly different at the level of $P<0.05$. Statistical analysis was performed using the SPSS 13.0 for Windows.

3 Results

3.1 Survival and Growth Performance

Data on survival rate, feed intake, weight gain, and FER of Japanese flounder are presented in Table 2. The cumulative survival rate ranged from 98.1% to 99% at day 56, and was independent of dietary treatments ($P>0.05$). No significant difference was observed on feed intake of fish fed different levels of soy isoflavones. As soy isoflavone levels increased from 0 to 8 g kg^{-1}, weight gain and FER decreased significantly ($P<0.05$); however, there was no significant difference among fish fed diet 1, diet 2 and diet 3.

Table 2 Growth, feed intake, feed efficiency ratio (FER) and survival rate of Japanese flounder fed experimental diets containing graded levels of soy isoflavones for 8 weeks†

Experimental diets	Soy isoflavones level (g kg^{-1})	Growth performance				
		Initial weight (g)	Final weight (g)	Feed intake (%)	FER	Survival rate (%)
1	0.0	2.58±0.00	11.38±0.24^{a}	2.16±0.01	0.67±0.01^{a}	98.10±1.65
2	1.0	2.58±0.02	10.76±0.39^{a}	2.20±0.08	0.64±0.04^{a}	99.00±1.65
3	4.0	2.58±0.02	10.24±1.11^{ab}	2.24±0.12	0.63±0.10^{ab}	98.10±1.65
4	8.0	2.58±0.01	8.35±0.96^{b}	2.16±0.13	0.57±0.08^{b}	99.00±1.65
ANOVA††						
F value		0.130	8.660	0.570	7.940	0.330
P value		0.942	0.007	0.648	0.009	0.800

Notes: † Values are presented as means of triples. Means in the same column with different superscripts are significantly different from each other determined by Tukey's test ($P<0.05$). †† ANOVA: one-way analysis of variance.

3.2 Whole Body Composition

The whole body composition of Japanese flounder at the end of growth trial is presented in Table 3. The whole-body crude lipid content of fish decreased signify cantly from 3.62% to 2.47% with increasing dietary soy isoflavones ($P<0.05$). However, no significant differences were found in the whole-body moisture, crude protein and ash content among all dietary treatments ($P>0.05$).

Table 3 Proximate composition (% wet weight) of the whole body of Japanese flounder fed experimental diets containing graded levels of soy isoflavones for 8 weeks†

Experimental diets	Soy isoflavones level (g kg^{-1})	Moisture	Crude protein	Crude lipid	Ash
1	0.0	76.86±1.01	16.07±0.72	3.62±0.41^{a}	3.86±0.17
2	1.0	76.52±0.21	16.60±0.15	3.38±0.11^{ab}	4.23±0.02
3	4.0	76.89±0.54	16.84±0.15	2.81±0.31^{ab}	4.19±0.18
4	8.0	78.15±1.15	15.45±1.51	2.47±0.56^{b}	4.27±0.34
ANOVA††					
F value		2.33	1.57	5.49	2.40
P value		0.15	0.27	0.024	0.14

Notes: Same as those of Table 2.

3.3 Nutrient Digestibility

Apparent digestibility coefficients (ADC) of crude protein, dry matter and gross energy at diet 4 (8 g kg^{-1}, the highest level of soy isoflavones supplementation) significantly lower compared to the control treatment ($P<0.05$); however, no significant differences were found among diet1, diet 2 and diet 3 groups (Table 4).

Table 4 Apparent digestibility coefficients (%) of dry matter, crude protein and gross energy in Japanese flounder fed experimental diets containing graded levels of soy isoflavones for 8 weeks†

Experimental diets	Soy isoflavones level ($g\,kg^{-1}$)	Dry matter	Crude protein	Gross energy
1	0.0	67.8 ± 0.59^{a}	83.2 ± 0.12^{a}	75.9 ± 0.77^{a}
2	1.0	67.7 ± 1.41^{a}	82.8 ± 1.71^{ab}	75.2 ± 1.11^{a}
3	4.0	68.0 ± 1.01^{a}	83.4 ± 0.41^{a}	76.0 ± 0.95^{a}
4	8.0	65.4 ± 1.37^{b}	81.5 ± 0.59^{b}	72.2 ± 1.22^{b}
ANOVA††				
F value		10.85	5.86	16.54
P value		0.03	0.02	0.001

Notes: Same as those of Table 2.

4 Discussion

The present study indicated that the lower level of soy isoflavones supplementation in diet (1 and $4\,g\,kg^{-1}$) did not decrease the growth of Japanese flounder significantly. Similar results had been observed in silver prussian carp (*Carassius auratus gibelo*) (Zhang, 2010). Also, the soy isoflavones daidzein and geinstein had been reported to have similar effects on rainbow trout (Catherine *et al*., 2001), striped bass (*Morone chrysops*) (Pollack *et al*., 2003) and American eel (*Anguilla rostrata*) (Ye and Chen, 2008). However, compared to the diet with higher level ($7.5\,mg\,kg^{-1}$) of soy isoflavones genistein supplementation, the diet with lower level ($0.75\,mg\,kg^{-1}$) significantly promoted the growth performance of yellow perch (*Perca flavescens*) (Ko and Malison, 1999). The differences may due to the species, form and level of soy isoflavones supplementation used in the experiment. In the present study, fish meal (68%) was used as the main protein source to formulate the experimental diets, which may alleviate the negative effects because of the attractant factors in fish meal, such as essential amino acids (Zhou *et al*., 2004) and taurine (Gaylord *et al*., 2006; Takagi *et al*., 2008). As is known, the levels of soy isoflavones contained in soybean are around 0.1 to 0.3% (Kudou *et al*., 1991), which is lower than the level of $4\,g\,kg^{-1}$ (0.4%) soy isoflavones used in this study. Thus, the present results indicated that soy isoflavones may not work or not work alone as are not anti-nutritional factors on Japanese flounder when soybean products are used as a fish feed ingredient.

In the present study, the higher dietary level of soy isoflavones ($8\,g\,kg^{-1}$) greatly depressed the growth performance of Japanese flounder by reducing weigh gain, FER, whole-body crude lipid content, ADC of dry matter, crude protein and gross energy. The similar result was observed in silver prussian carp (*Carassius auratus gibelio*), showing that higher levels ($7.2\,g\,kg^{-1}$) of soy isoflavones significantly depressed the growth performance of fish (Zhang, 2010). Higher level of isoflavone genistein ($7.5\,mg\,kg^{-1}$) also depressed the growth performance of yellow perch significantly compared with lower level ($0.75\,mg\,kg^{-1}$) (Ko and Malison, 1999). These results indicate that the higher level of soy isoflavones exceeds the tolerance dose of fish though the fish are fed with fish-meal-based diet. However, no significant differences have been observed among the growth variables in striped bass (*Morone chrysops*) fed with higher level of isoflavone genistein diets ($8\,g\,kg^{-1}$) (Pollack *et al*., 2003) and in American eel (*Anguilla rostrata*) fed with higher level of isoflavone daidzein ($40\,mg\,kg^{-1}$) (Ye and Chen, 2008). The controversial effects among different studies may due to the differences of fish species, developmental stages, sex, form and level of soy isoflavones supplementation and dietary protein source.

In this study, no positive effect was observed on the growth of Japanese flounder fed both higher and lower levels of soy isoflavones, which is consistent with some previous studies on fish. However, isoflavones daidzein significantly promoted the growth of male blue tilapia (*Oreochromis aureus*), though no obvious increase of growth in female fish was observed (Yu *et al*., 2006). The results of the study on blue tilapia is consistent with the studies on rats, broiler chickens, piglets and ruminants, which proves that isoflavones daidzein could increase body weight gain and feed efficiency, and elevate the levels of growth hormone and insulin-like growth factor-I (IGF-I) in male animals and birds significantly, while no change of growth and metabolic hormone status in female has been observed (Han, 1999). It has been suggested that soy isoflavones could function as the regulator of growth hormone (GH) and insulin-like growth factors because of the estrogen-like activity, which finally could stimulate the growth of animals (Han, 1999; Guo and Zhao, 2005). The mechanism of these effects of dietary soy isoflavones in fish is still not clear. However, some studies on fish revealed the neuroendocrine interaction between the gonadotropic axis and the somatotropic axis (Lin, 2000), which may provide some details on the mechanism of soy isoflavones involved in the growth of fish. Most studies on fish have been conducted without sex separation because of the related limitation of fish and their living conditions; further investigations are needed in this research field.

In the present study, the whole-body crude lipid content of fish decreased significantly with increasing dietary soy isoflavones ($P<0.05$). Up to date, the mechanism of soy isoflavones involved in the lipid metabolism is not fully understood. However, increasing evidences from animal studies have suggested that soy isoflavones may regulate lipid metabolism by modulating the activities of key transcription factors, and thereby changing the downstream gene expression involved in lipogenesis or lipolysis (Xiao *et al*., 2008; Cao *et al*., 2012). Further studies need to be carried out in this interesting field.

5 Conclusions

The present study suggests that the detrimental effects of soy isoflavones in the soybean products may be neglected when soybean products are used as alternative

fish feed ingredients with the content of soy isoflavones in soybean being around 0.1%–0.3%, which is lower than 0.4% soy isoflavones used in this study. The mechanisms of effects of dietary soy isoflavones on fish need further studies, especially those involving the estrogen effect of soy isoflavones on fish with separate sex.

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