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A tolerance and safety assessment of daidzein in a female fish (*Carassius auratus gibelio*)

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Abstract

Daidzein is widely used in farmed animals as a dietary additive. However, limited information is available about its use in aquaculture. The effects of daidzein inclusion in the diet of gibel carp was assessed in terms of growth performance, immune response, disease resistance, antioxidant activity, hormone levels, daidzein tissue residues, as well as intestinal and liver morphology. The dietary daidzein inclusion levels were 0, 40, 200 and 400 mg kg^{-1} and six replicates of 30 fish were used for each group. No mortality was observed during the 80-day feeding trial. The growth performance of experimental fish was not significantly affected by dietary daidzein supplementation. However, the non-specific immune responses, resistance to *Aeromonas hydrophila*, antioxidant activities, 17β-oestradiol level, vitellogenin concentration, gonadosomatic index (GSI) and intestinal morphology were significantly affected by dietary daidzein. A dietary dose of 400 mg kg^{-1} daidzein significantly decreased the GSI, increased 17β-oestradiol and vitellogenin concentrations, and impaired the intestinal structure. The daidzein residue in muscle of gibel carp was increased by the high level (400 mg kg^{-1}) of dietary daidzein. Equol was not detected in fish muscle among all treatments. The present study proved that 40 mg kg^{-1} daidzein was safe to be included in diets of gibel carp, and a safety margin of 5 folds of the use-level (40 mg kg^{-1}) was determined.

Keywords: daidzein, *Carassius auratus gibelio*, safety assessment, diet

Introduction

Soy isoflavones are structurally similar to oestradiol and are known to have both oestrogenic and antioestrogenic activities mostly depending on the intrinsic oestrogenic state of the eaters (Cassidy, Bingham & Setchell 1995; Miksicek 1995). During the past 20 years, a remarkable amount of research into the health-regulatory effects of soy isoflavones has been conducted since soy isoflavones might play a key role in preventing certain types of cancer, in reducing the risk of osteoporosis, and in lowering plasma cholesterol, and might act as antioxidant agents and immune enhancers in humans and other animals (Messina 2010). Daidzein is one of the three main soy isoflavones, which has become more and more popular as dietary supplements over recent years. It has been observed that daidzein could improve the laying performance after the peak laying period in laying hens (5–10 mg kg^{-1}) (Ni, Zhu, Zhou, Rossmann, Chen & Zhao 2007; Liu & Zhang 2008) and ducks (5 mg kg^{-1}) (Zhao, Wang, Zhou, Ni, Lu, Grossmann & Chen 2004; Zhao, Zhou, Ni, Lu, Tao, Chen & Chen 2005) probably via its oestrogen-like activity and potent antioxidant capacity. However, the side effects of daidzein came in parallel, e.g. changed egg compositions and lower hatchability.

Besides, isoflavones could also act as endocrine disruptors which could decrease the fertility of animals feeding on isoflavones-enriched plants, e.g. alfalfa and clover (Paganetto, Campi, Varani, Piffanell, Giovannini & Borea 2000; Wuttke, Jarry, Becker, Schultens, Christoffel, Gorkow & Seidlova-Wuttke 2003; Safe 2004; Messina 2010), though this was not always observed in animals. Therefore, the assessment of the safety levels of daidzein in animals is necessary for guaranteeing the growth and health of animals.

Aquaculture is one of the most rapidly growing food-producing sectors. Its average annual growth rate between 1970 and 2009 was 8.3% (FAO 2012). There is increasing interest in the use of soy isoflavones in aquafeed to regulate the fish performance including growth, immunity, antioxidant capacity and gonad development. However, limited knowledge is available about the effects of isoflavones on aquatic animals especially when it is used in the form of daidzein. As in terrestrial animal, the limited studies on daidzein in fish also showed contradictory results, i.e. both benefits and risks of daidzein were observed (Ko, Malison & Reed 1999; Bennetau-Pelissero, Breton, Bennetau, Corraze, Le Menn, Davail-Cuisset, Helou & Kaushik 2001; Pollack & Ottinger 2003; Ye & Chen 2008; Zhang 2010; Mai, Zhang, Chen, Xu, Ai & Zhang 2012). So far, however, a safety assessment study on daidzein as a dietary ingredient for fish is absent. Since soybean meal is an increasingly important protein source worldwide, it is critical to estimate the safety of dietary daidzen in cultured fish. Gibel carp (*Carassius auratus gibelio*), which is a member of the family *Cyprinidae* and consists of all females, is widely cultured in Asia and Europe. It, as well as other *Cyprinidae*, is a commercially important food source, especially in China. Therefore, gibel carp was chosen as the target animal to evaluate the tolerance and safety margin of dietary daidzein.

Materials and methods

Experimental diets

The dose design of daidzein was determined according to 'Technical Guidance: Tolerance and efficacy studies in target animals' (EFSA Panel on Additives and Products or Substances used in Animal Feed 2011) and 'The Guidelines for Tolerance Test of Feeds and Feed Additives in Target Aquatic Animals' (Ministry of Agriculture of China, Department of Animal Production 2012). The use-level of dietary daidzein was set to be 40 mg kg^{-1} (1×), since 40 mg kg^{-1} was the highest recommended dose for dietary daidzein supplementation in fish feed (Ye & Chen 2008); additionally, two tolerance groups, 200 (5×) and 400 mg kg^{-1} (10×), were designed based on the use-level group. A basal diet (the control diet) with approximate 43% crude protein and 4.5% crude lipid was prepared, and daidzein (Sichuan Guanghan Feed Co. Ltd., Guanghan, China; purity, 98.5%) was supplemented into the basal diet to obtain treatment dietary daidzein levels, 40, 200 and 400 mg kg^{-1}.

Ingredients were ground into fine powder through a 180-μm mesh. All the ingredients were thoroughly mixed with fish oil, and water was added to produce stiff dough. The dough was then pelleted with an experimental feed mill [F-26 (II), South China University of Technology, Guangzhou, China] and dried for about 12 h in a ventilated oven at 45°C. After dried, the diets were kept in a freezer at −20°C until use.

Proximate composition of the diets were determined following the procedures of AOAC: dry matter content was determined by drying feed samples to a constant weight at 105°C; crude protein was determined by measuring nitrogen (N × 6.25) using the Kjeldahl method (FOSS Kjeltec TM 8400, Tecator, Hoganas, Sweden); crude lipid by ether extraction using Soxhlet method (Buchi B-801, Flawil, Switzerland); ash by combustion at 550°C; and energy by an adiabatic bomb calorimeter (PARR1281, Moline, IL, USA). The formulation and proximate composition of the basal diets is presented in Table 1.

Experimental procedure

Young of year gynogenetic gibel carps (*Carassius auratus gibelio*) (initial mean body weight ± SD, 15.27 ± 0.02 g), developed via parthenogenesis (Wang, Zhu, Wang, Jiang, Guo, Zhou & Gui 2011), were obtained from the Freshwater Fisheries Research Center of Chinese Academy of Fishery Science (Wuxi, China). After arriving, fish were randomly distributed into 24 tanks (300 L) and each tank was stocked with 30 fish. Prior to the start of the feeding trial, fish were fed the basal diet for 2 weeks to acclimate to the experimental conditions and diets. At the onset of the feeding trial, fish were fasted for 24 h (day 15) and weighed after being anaesthetized with eugenol (1:10 000) (Shanghai Reagent,

Table 1 Formulation and proximate composition of the basal diet (% dry matter)

Ingredient	Concentration
Fish meal*	12.0
Soybean meal	31.0
Cottonseed meal	10.0
Brewers' yeast	6.0
Wheat flour	33.8
Fish oil	3.0
Choline Chloride (50%)	0.2
$Ca(H_2PO_4)_2$	1.5
Zeolite powder	0.3
MHA-Ca†	0.2
Premix‡	2.0
Proximate composition	
Dry matter %	93.7 ± 1.6
Crude protein %	43.1 ± 0.2
Crude fat %	4.5 ± 0.2
Gross energy MJ kg^{-1}	18.9 ± 0.2
Ash %	7.6 ± 0.1

*Fish meal: white fish meal with 67.6% crude protein and 10% crude lipid of dry matter.

†MHA-Ca: Methionine hydroxy analog-Ca.

‡Vitamin premix (mg kg^{-1} diet): retinyl acetate (VA), 28; thiamin (VB_1), 12; riboflavin (VB_2), 12; pyridoxine hydrochloride (VB_6), 16; Vitamin B_{12}, 0.2; amine nicotinic, 80; L-ascorbic acid 2-phosphate, 600; calcium pantothenate, 100; biotin, 0.4; folic acid, 3; DL-α-tocopherol acetate (VE), 300; Vitamin K, 320; Vitamin D, 14; Corn gluten meal, 314.4.

Mineral premix (mg kg^{-1} diet): $MgSO_4 \cdot 5H_2O$, 500; $FeSO_4 \cdot H_2O$, 300; $ZnSO_4 \cdot H_2O$, 300; $MnSO_4 \cdot H_2O$, 100; KI (10%), 80; Na_2SeO_3, 67; $CoCl_2 \cdot 6H_2O$ (10%), 7.5; Zeolite powder, 150.5.

Shanghai, China) (day 16). Each diet was randomly assigned to six replicate tanks by drawing lots (day 17, the start of the feeding trial). During the feeding trial, fish were hand-fed the experimental diets to apparent satiation four times daily (08:00, 11:00, 14:00 and 17:00 hours). Feeds assigned to each tank were weighed and uneaten feeds were collected from the tank outlets and also weighed after dried for the calculation of feed intake. A circulating water system was used to rear the experimental fish and water was exchanged by 10% every day. Aeration was supplied to each tank continuously. During the feeding trial, the water temperature was 24 ± 2°C, pH 7.2–7.6, ammonia nitrogen was lower than 0.3 mg L^{-1}, and dissolved oxygen was higher than 7.0 mg L^{-1}. The feeding trial lasted 80 days.

Sample collection

At the end of the feeding trial, the fish were fasted for 24 h before harvest. Before handling or sacrificing, experimental fish were first anaesthetized with eugenol (1:10 000) (Shanghai Reagent) to ameliorate suffering. Fish was counted and weighed to determine the survival, weight gain (WG), specific growth rate (SGR), feed intake (FI) and feed conversion ratio (FCR). Three fish from each tank were randomly selected and the body weight, body length, and the weight of liver, viscera and ovaria were recorded individually to calculate the condition factor (CF), hepatosomatic index (HSI), viscerosomatic index (VSI) and gonadosomatic index (GSI). Liver samples and the middle section of the intermediate intestine (0.5 cm long) were sampled (Bonaldo, Parma, Mandrioli, Sirri, Fontanullas, Badiani & Gatta 2011). The gut contents were removed from the intestines and all samples were rinsed in saline (9 g L^{-1}). All the samples were fixed by immersion in Bouin's stationary liquid (saturated water solution of picric acid: 40% formol: acetic acid = 15:5:1). After 24 h, the samples were transferred to 70% ethanol (Ross & Pawlina 2006).

Blood samples of six fish from each tank were taken from the caudal vein using syringe (1 mL, equiped with 26 G needles), and allowed to clot at room temperature for 2 h and then at 4°C for 4–6 h. Then the samples were centrifuged (836 *g*, 10 min, 4°C) and the supernatant straw-coloured serum was collected and immediately stored at −80°C until analysis.

Head kidney macrophages from three fish from each tank were isolated as described by Secombes (1990) with some modifications. Briefly, the head kidney was excised, cut into small fragments and transferred to RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10 IU mL^{-1} heparin (Sigma, Saint Louis, MO, USA), 100 IU mL^{-1} penicillin (Amresco, Solon, OH, USA), 100 IU mL^{-1} streptomycin (Amresco) and 2% foetal calf serum (FCS) (Gibco, Carlsbad, CA, USA). Cell suspensions were prepared by forcing the head kidney through a 100 μm steel mesh. The resultant cell suspensions were enriched by centrifugation (836 *g* for 25 min at 4°C) on a 34%:51% Percoll (Pharmacia, Cambridge, England) density gradient. The cells were collected at the 34–51% interface and washed twice. Cell viability was determined by the trypan blue exclusion method (the cells stained with trypan blue were dead and should be excluded when the viable cells were counted) and the cell density was determined in a haemocytometer. Then additional RPMI 1640 medium was added to adjust the cell concentration (1×10^7 mL^{-1}) for analysis.

Muscle samples from three fish in each tank were obtained. Fish skin was peeled off gently from

the back muscle and the muscle was scraped from the fishbone on both sides. The muscles were frozen in liquid nitrogen and then freeze-dried (Christ Alpha 1-4, Osterode, Germany) for 12 h in a vacuum freeze drier for the subsequent determination of dadzein and equol contents.

Haematological, non-specific immune parameters and antioxidant activities

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), glucose (GLU), total protein (TPRO), creatinine (CRE) and triglyceride (TG) in serum were determined with a fully automatic biochemical analyser (HITACHI 7600, Tokyo, Japan). Respiratory burst activity of head kidney macrophages was evaluated using nitroblue tetrazolium (NBT) (Sigma, Saint Louis, MO, USA) reduction following the method of Secombes (1990) with some modifications. A 100 μL cell suspension was stained with 100 μL 0.3% NBT and 100 μL Phorbol 12-myristate 13-acetate (PMA) (1 mg mL^{-1}) (Sigma) for 40 min. Absolute methanol was added to terminate the staining. Each tube was washed three times with 70% methanol and air-dried. Then, 120 μL 2 M KOH and 140 μL dimethyl sulfoxide (DMSO, Sigma, USA) were added and the colour was subsequently measured at 630 nm with a spectrophotometer using KOH/DMSO as the blank.

The lysozyme activity was determined as described by Ellis (1990). Results were expressed in units of lysozyme mL^{-1} serum. One unit is defined as the amount of sample causing a decrease in absorbance of 0.001 min^{-1} at 530 nm compared to the control (*Micrococcus lysodeikticus* suspension without serum).

Activities of superoxide dismutase (SOD) and catalase (CAT) were determined by commercial kits (Nanjing Jiancheng Biotechnique Co. Ltd., Nanjing, China).

Challenge test

The 72 h LD_{50} was determined by intraperitoneal injection of 15 fish with graded doses of *Aeromonas hydrophila* (BSK-10) (10^5, 10^6, 10^7, 10^8, 10^9 and 10^{10} cfu mL^{-1}, 0.2 mL $fish^{-1}$) at 25°C, and the result showed that the 72 h LD_{50} was 10^9 cfu mL^{-1}. Cultured for 24 h in broth medium at 25°C, the *A. hydrophila* was centrifuged (12 000 rpm, 10 min, 4°C), and re-suspended with cold sterile PBS to form the working concentration (10^9 cfu mL^{-1}) before use. Ten fish from each tank were injected intraperitoneally with 0.2 mL PBS containing 10^9 *A. hydrophila* cfu mL^{-1} and the cumulative mortality was recorded after 72 h.

The content of 17β-oestradiol (E_2) and vitellogenin (Vtg) in serum

Serum E_2 levels were measured by an ELISA kits (Roche Cobase 601). The Vtg concentration in serum was assayed by an ELISA kits for Crucian vitellogenin (Cusabio, Wuhan, China).

Liver and intestinal morphology

Following fixation, liver and intestine samples were routinely dehydrated in ethanol, equilibrated in xylene and embedded in paraffin wax according to the standard histological procedure (Ross & Pawlina 2006). The intermediate intestines were cut in 7 μm longitudinal sections following the axis of gut lumen with a Lecia Jung RM 2016 rotary microtome and stained with Haematoxylin-Eosin (H&E). Examination of slides was performed using Nikon eclipse Ti-S microscope.

The contents of daidzein and equol in muscle

The daidzein and equol contents in muscle (freeze-dried) were analysed with UHPLC-MS/MS (Waters Acquity™ Ultra preformance LC & XEVOTM TQ MS, Waters, USA).

Calculations and statistical analysis

$$\text{Weight gain (WG, \%)} = 100 \times [(W_t - W_i)/W_i]$$

$$\text{Mean metabolic body weight (MBW, g)} = [(\text{FMW}/1000)^{0.75} + (\text{IMW}/1000)^{0.75}]/2$$

$$\text{Daidzein intake (DI, mg kg MBW}^{-1}) = \text{FI} \times \text{C}$$

$$\text{Feed conversion ratio (FCR)} = \text{dry weight of feed consumed (g)}/\text{fish body weight gained (g)}$$

$$\text{Gonadosomatic index (GSI, \%)} = 100 \times (\text{gonad weight/body weight})$$

where W_t and W_i is the final and initial average weight, respectively; IMW and FMW is the initial and final mean body weight (g), respectively; C is the daidzein contents in diets (mg kg^{-1}). The

calculations of the survival, feed conversion ratio (FCR), feed intake (FI), condition factor (CF), viscerasomatic index (VSI) and hepatosomatic index (HSI) were according to Liu, Mai, Zhang, Chen and Leng (2013) and Men, Ai, Mai, Xu, Zhang and Zhou (2014).

SPSS 16.0 for windows was used for all statistical evaluations. All data were subjected to one-way analysis of variance (ANOVA) followed by Ducan's test. Differences were regarded as significant when $P < 0.05$. Two-tailed Pearson correlation analysis was used where necessary.

Results

No mortality and treatment-related pathology signs were observed during the feeding trial.

Growth performance

No significant differences ($P > 0.05$) in WG, SGR, FI, and FCR were observed among all experimental groups (Table 2).

Body indices

Dietary supplementation of 400 mg kg^{-1} dadzein significantly ($P < 0.05$) decreased the CF of gibel carp (Table 3). Fish fed 200 mg kg^{-1} dietary daidzein showed the highest VSI, HSI and GSI among all treatments ($P < 0.05$). The GSI of experimental fish was significantly ($P < 0.05$) increased by dietary supplementation of 40 and 200 mg kg^{-1} dadzein, but significantly ($P < 0.05$) decreased by the supplementation of 400 mg kg^{-1} dietary dadzein.

Haematological parameters

No significant differences in haematological parameters were observed between the control group and the groups supplemented with daidzein although significant differences in TPRO and CRE were observed among groups supplemented with daidzein (Table 4).

Non-specific immune responses, the mortality after challenge test and antioxidant activities

The respiratory burst activity of head kidney macrophages in fish fed diets with 200 and 400 mg kg^{-1} daidzein was significantly ($P < 0.05$) higher compared with the other two treatments. The lysozyme activity increased significantly ($P < 0.05$) with the dietary daidzein level increasing from 0 to 40 mg kg^{-1} and then declined again significantly ($P < 0.05$) with further increase in dietary daidzein (from 200 to 400 mg kg^{-1}) (Table 5).

The cumulative mortality of fish challenged with *Aeromonas hydrophila* was significantly ($P < 0.05$) affected by dietary daidzein supplementation. The lowest mortality after 72 h ($32 \pm 6\%$) was observed in the treatment with 40 mg kg^{-1} daidzein ($P < 0.05$), significantly lower compared with the other three treatments, while there was no significant ($P > 0.05$) difference among the other three treatments ($56 \pm 10\%$, $45 \pm 5\%$, and $47 \pm 3\%$ in fish fed 0, 200 and 400 mg kg^{-1} dietary daidzein respectively).

Dietary supplementation of daidzein significantly ($P < 0.05$) increased the activities of SOD and CAT with no differences observed among daidzein-supplemented groups (Table 5).

Table 2 Effects of dietary daidzein on growth performance of gibel carp

	Dietary daidzein supplementation (mg kg^{-1})			
Growth	**0**	**40**	**200**	**400**
IMW (g)	15.3 ± 0.05	15.3 ± 0.04	15.3 ± 0.03	15.2 ± 0.04
FMW (g)	31.1 ± 1.09	30.2 ± 0.80	31.2 ± 1.17	31.4 ± 1.06
WG (%)	101.85 ± 7.61	95.44 ± 4.20	102.20 ± 6.21	97.26 ± 5.93
SGR (% day^{-1})	0.88 ± 0.04	0.85 ± 0.03	0.89 ± 0.05	0.90 ± 0.04
FCR	2.22 ± 0.15	2.38 ± 0.12	2.18 ± 0.13	2.30 ± 0.13
FI (g kg MBW^{-1})	7.29 ± 0.12	7.40 ± 0.11	7.12 ± 0.11	7.21 ± 0.14
DI (mg kg MBW^{-1})	0.38 ± 0.01[a]	0.61 ± 0.01[b]	1.68 ± 0.03[c]	3.18 ± 0.06[d]

Data are reported as mean ± SE from $n = 6$ replicates. Values in the same row with different letters are significantly different determined by Duncan's test ($P < 0.05$).
IMW, initial mean weight; FWM, final mean weight; WG, weight gain; SGR, specific growth rate; FCR, feed conversion ratio; FI, feed intake; DI, daidzein intake; MBW, mean body weight.

Table 3 Effects of dietary daidzein on body indices of gibel carp

	Dietary daidzein (mg kg^{-1})			
Body indices	**0**	**40**	**200**	**400**
CF	3.11 ± 0.06^{b}	3.13 ± 0.05^{b}	3.09 ± 0.03^{b}	2.97 ± 0.03^{a}
VSI %	10.1 ± 0.54^{ab}	10.6 ± 0.44^{ab}	11.5 ± 0.47^{b}	9.60 ± 0.51^{a}
HSI %	3.07 ± 0.21^{a}	3.20 ± 0.12^{a}	4.19 ± 0.22^{b}	3.04 ± 0.25^{a}
GSI %	3.34 ± 0.23^{b}	4.01 ± 0.13^{c}	4.32 ± 0.17^{c}	2.46 ± 0.32^{a}

Data are reported as mean ± SE from $n = 6$ replicates. Values in the same row with different letters are significantly different determined by Duncan's test ($P < 0.05$).
CF, condition factor; HSI, hepatosomatic index; VSI, viscerosomatic index; GSI, gonadosomatic index.

Table 4 Effects of dietary daidzein on haematological parameters of gibel carp

	Dietary daidzein supplementation (mg kg^{-1})			
Haematological parameters	**0**	**40**	**200**	**400**
ALT (U L^{-1})	88.7 ± 13.1	90.8 ± 8.41	97.8 ± 9.60	101.0 ± 6.35
AST (U L^{-1})	457 ± 73.9	491 ± 49.6	527 ± 61.1	515 ± 37.7
GLU (mmol L^{-1})	4.85 ± 0.45	4.75 ± 0.48	4.76 ± 0.37	5.81 ± 0.81
TPRO (g L^{-1})	36.1 ± 1.55^{ab}	32.4 ± 1.37^{a}	38.6 ± 1.52^{b}	34.6 ± 1.32^{ab}
CRE (μmol L^{-1})	25.2 ± 0.40^{ab}	21.8 ± 1.16^{a}	24.8 ± 1.42^{ab}	27.7 ± 1.17^{b}
TG (mmol L^{-1})	2.26 ± 0.23	2.09 ± 0.16	2.23 ± 0.18	1.89 ± 0.32

Data are reported as mean ± SE from $n = 6$ replicates. Values in the same row with different letters are significantly different determined by Duncan's test ($P < 0.05$).
ALT, alanine aminotransferase; AST, aspartate aminotransferase; GLU, glucose; TPRO, total protein; CRE, creatinine; TG, triglyceride.

Table 5 Effects of dietary daidzein on non-specific immune response and antioxidant activity of gibel carp

	Dietary daidzein supplementation (mg kg^{-1})			
Immune response and antioxidant activity	**0**	**40**	**200**	**400**
Respiratory burst (OD/10^{9} cell)	9.44 ± 2.02^{a}	9.17 ± 0.48^{a}	18.9 ± 0.85^{b}	20.5 ± 4.57^{b}
Lysozyme (U mL^{-1})	103 ± 10.3^{a}	235 ± 27.4^{b}	158 ± 11.9^{a}	154 ± 5.50^{a}
CAT (U mL^{-1})	2.25 ± 0.30^{a}	4.58 ± 0.18^{b}	3.86 ± 0.30^{b}	4.29 ± 0.71^{b}
SOD (U mL^{-1})	181 ± 8.39^{a}	216 ± 9.27^{b}	208 ± 2.90^{b}	214 ± 8.80^{b}

Data are reported as mean ± SE from $n = 6$ replicates. Values in the same row with different letters are significantly different determined by Duncan's test ($P < 0.05$).
CAT, catalase; SOD, superoxide dismutase.

Hormone levels in serum

The concentrations of E_2 and Vtg in serum increased with increasing dietary daidzein levels. The highest contents of serum E_2 and Vtg were observed in the group with 400 mg kg^{-1} dietary daidzein, which was significantly ($P < 0.05$) higher compared to the control (Table 6).

The contents of daidzein and equol in fish muscle

Dietary supplementation of 400 mg kg^{-1} daidzein significantly ($P < 0.05$) increased the daidzein residue in muscle (261 ± 12.4 ng g^{-1}) compared with the other three groups, however, no significant differences were observed among groups with 0, 40 and 200 mg kg^{-1} dietary daidzein (133 ± 21.6, 128 ± 21.0 and 146 ± 11.9 ng g^{-1} respectively). No equol was detected in muscle of fish fed any level of dietary daidzein.

Histology

The histological examination of intermediate intestines from the same treatment showed similar results and representative pictures of each

Table 6 Effects of dietary daidzein on hormone levels in serum and ovary of gibel carp

	Dietary daidzein supplementation (mg kg^{-1})			
Hormone	**0**	**40**	**200**	**400**
E_2 (pmol L^{-1})	161 ± 7.14[a]	219 ± 39.5[a]	240 ± 45.4[a]	464 ± 26.3[b]
Vtg (ng mL^{-1})	157 ± 2.86[a]	200 ± 9.22[ab]	207 ± 30.00[ab]	242 ± 5.14[b]

Data are reported as mean ± SE from $n = 6$ replicates. Values in the same row with different letters are significantly different determined by Duncan's test ($P < 0.05$).
E_2, oestradiol-17β; Vtg, vitellogenin.

treatment are presented in Fig. 1. No noticeable difference in intestines was observed among fish fed diets with 0, 40 and 200 mg kg^{-1} daidzein. All intestinal sections from these treatments showed integrated histological structures with intact mucosa, unbroken villus folds and enterocytes with regular basal nucleus. However, the intestinal sections from fish fed the diet with 400 mg kg^{-1} daidzein showed impaired integrity of histological structure, such as damage and retraction of intestine villi in intestine mucosa.

No obvious pathological changes in liver were observed in all groups (Data not shown).

Discussion

In this study, no mortality and abnormal behaviour were observed among fish fed any level of dietary daidzein during the experimental period. The growth performances of the fish, as assessed by SGR, WG, FI and FCR, were not significantly affected by dietary daidzein supplementation. Up to date, the results of previous studies regarding the effects of soy isoflavones on fish growth performance are contradictory. Dietary soy isoflavones including genistein and daidzein were found not to affect the growth of rainbow trout (Bennetau-Pelissero *et al.* 2001), striped bass (Pollack & Ottinger 2003) or American eel (Ye & Chen 2008). However, affected growth performances of fish by dietary soy isoflavones were observed in other reports (Ko *et al.* 1999; Zhang 2010; Mai *et al.* 2012). Among these reports, it is noteworthy that in a previous study in gibel carp a higher level of isoflavones inclusion of 0.72% (containing approximately 0.24% daidzein) significantly impaired the growth performance of fish, whereas lower doses of isoflavones inclusion (0.045–0.36%, containing approximately 0.015–0.12% daidzein) exerted no influence. This result indicated that the effect of

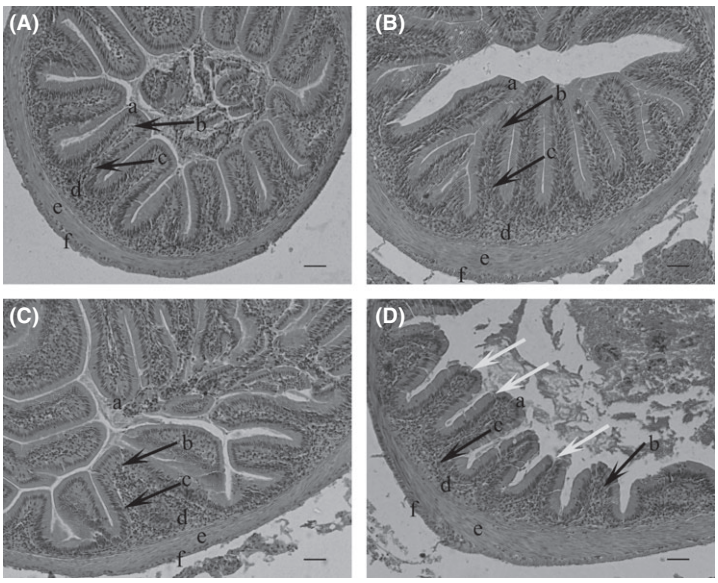

Figure 1 Histological sections of intermediate intestines from treated groups with 0, 40, 200 and 400 mg kg^{-1} dietary daidzein (A, B, C and D respectively) (40× magnification). Note: the normal intermediate intestine (A, B and C) with well-developed intestine villi and lamina propria differs from intestines with retraction or damage of intestine villi (arrows in lighter colour, D). (a) Intestine villi (folds); (b) Lamina propria; (c) Muscularis mucosa; (d) Submucosa; (e) Intestinal muscle; (f) Intestinal membrane. Stainings: H & E. Scale bar = 50 μm.

daidzein on the growth performance of gibel carp could be related to the daidzein doses used.

Despite of the absence of influence of dietary daidzein on growth of gibel carp in this study, lower levels of dietary daidzein (40 or 200 mg kg^{-1}) enhanced the non-specific immune responses of fish, whereas the higher level (400 mg kg^{-1}) did not show further enhancement. This result was supported (e.g. the correlation coefficient was −0.999 between the serum lysozyme activity and the mortality in challenge test, $P = 0.000$) by the challenge test which showed that the mortality of experimental fish challenged with *Aeromonas hydrophila* was the lowest in the group supplied of 40 mg kg^{-1} dietary daidzein. Up to date, the effects of daidzein on fish immunity have not been reported in other fish although the effects of soy isoflavones on immunity have been extensively studied in mouse, including both positive and negative immune modulation (Sakai & Kogiso 2008).

Consistent with the immunity results, the activities of CAT and SOD, which play critical roles in controlling the balance of release and clearance of reactive oxygen species and thus are useful indicators for antioxidant activity, were also affected by dietary daidzein in the present study, with the activities improved in all groups supplemented with daidzein. The antioxidant activity of daidzein was also observed in broilers and cows, although the effective doses of daidzein were different from this study (Liu, Gu, Wang, Ding & Chen 2008; Ni, Wu, Tong, Huang, Lu, Grossmann & Zhao 2012; Liu, He, Jin, Liu, Tang, Li & Zhong 2013). Up to date, the mechanism involved in the antioxidant activity of daidzein is not very clear. Previous studies indicated that the antioxidant effects of dietary isoflavonone might be caused indirectly by up-regulation of the antioxidant enzyme catalase or by formation of the antioxidant metabolites 6-OH-daidzein and 3′-OH-daidzein which have strong antioxidant effects (Kampkötter, Chovolou, Kulawik, Röhrdanz, Weber, Proksch & Wätjen 2008; Liu, He *et al.* 2013). Besides, according to Dwiecki, Neunert, Polewski and Polewski (2009), the mechanism most likely to account for the inhibition of lipid oxidation by flavones was scavenging of lipid peroxyl radicals. In addition, the location and binding of daidzein in the membrane interface region which locally affected the rigidity/fluidity of the lipid bilayer may facilitate the inhibition of peroxy radical formation and decrease the rate of lipid peroxidation.

In vertebrates, sexual differentiation or organization of the reproductive axis relies on many endogenous factors, including steroid hormones. Oestradiol-17β (E_2) is one of the dominant sex steroids during oogenesis in female teleosts (Frantzen, Arnesen, Damsgård, Tveiten & Johnsen 2004; Skjæraasen, Salvanes, Karlsen, Dahle, Nilsen & Norberg 2004) and it can induce the production of vitellogenin (Vtg), a precursor to yolk protein (Mommsen & Walsh 1988). In the present study, 400 mg kg^{-1} dietary daidzein increased both E_2 and Vtg contents in serum of experimental fish. Similar results were found in *in vitro* studies on Siberian sturgeon (Pelissero, Bennetau, Babin, Le Menn & Dunogues 1991; Pelissero, Le Menn & Kaushick 1991) and rainbow trout (Pelissero, Foucher, Bennetau, Dunogues, Flouriot & Sumpter 1991). However, in the present study the gonadosomatic index (GSI) of fish was depressed by 400 mg kg^{-1} dietary daidzein. This may be due to higher levels of E_2 having a feedback regulation to the hypothalamic-hypophyseal axis, which finally depressed the ovarian development, since it is well known that Vtg uptake by the ovary is a gonadotropin-dependent process (Nath & Sundararaj 1981). It may also be due to the higher amount of Vtg induced by E_2 costing too much energy to the fish, which otherwise could be used in the ovarian development (Lange, Hutchinson, Croudase, Siegmund, Schweinfurth, Hampe, Panter & Sumpter 2001).

Gonad was not the only organ affected by dietary daidzein supplementation. The intestinal structure was also impaired by dietary supplementation of 400 mg kg^{-1} daidzein. A previous study on gibel carp clearly showed that 0.72% soy isoflavones (containing approximately 0.24% daidzein) changed the intestinal structure (Zhang 2010). Similar results were also reported in Japanese flounder fed 0.8% soy isoflavones (containing approximately 0.27% daidzein) (Chen 2009). Further studies are needed to elucidate the mechanism of the adverse effects of dietary soy isoflavones or daidzein on fish.

The daidzein residue in the fish muscle was significantly increased by feeding 400 mg kg^{-1} dietary daidzein. The daidzein concentrations in muscle of experimental fish ranged from 128 to 261 ng kg^{-1}. These levels are negligible compared with those of foods daily consumed by people in

China and Japan, such as tofu (173–299 μg g^{-1} isoflavonas, of which daidzein accounts for 30–35%, Chen 2009) and soymilk (77–117 μg g^{-1} isoflavonas, USDA 2004). It was suggested that an average daily intake of 25–50 mg isoflavones might be efficacious in preventing breast and prostate cancer and a reasonable upper limit for isoflavones intake was approximately 100 mg day^{-1} (Messina, Nagata & Wu 2006). Despite of the daidzein residue in fish muscle, no equol was detected in muscle, which is a known metabolite of formonetin and daidzein produced by the gut microflora of farm animals (Burnison, Neheli, Nuttley, Hartmann, McInnis, Jurkovic, Terry, Ternes, Lee, Peart & Servos 2000). This may indicate that the gibel carp lacks the bacteria that can convert daidzein into equol. This is an interesting aspect which is worth further study.

In conclusion, lower levels of dietary daidzein (40 or 200 mg kg^{-1}) improved certain non-specific immune responses, resistance to *Aeromonas hydrophila*, antioxidant activity, and ovarian development of gibel carp, but a dietary supplementation of 400 mg kg^{-1} daidzein depressed the GSI significantly and also impaired the intestinal morphology. The daidzein residue in muscle of experimental fish was increased by a high level (400 mg kg^{-1}) of dietary daidzein though the muscle daidzein concentrations were acceptable. The present study proved that the use-level of daidzein (40 mg kg^{-1}) in diet of *Carassius auratus gibelio* was safe and the safety margin of daidzein in diets of gibel carp was fivefold of the use-level, 200 mg kg^{-1}.

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