Aquaculture 489 (2018) 36–45

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Evaluation of genetically-improved (glandless) and genetically-modified low-gossypol cottonseed meal as alternative protein sources in the diet of juvenile southern flounder *Paralichthys lethostigma* reared in a recirculating aquaculture system

M.S. Alam[a], W.O. Watanabe[a,*], P.M. Carroll[a], J.E. Gabel[a], M.A. Corum[a], P. Seaton[a], T.C. Wedegaertner[b], K.S. Rathore[c], M.K. Dowd[d]

[a] University of North Carolina Wilmington, Center for Marine Science, Aquaculture Program, 601 S. College Road, Wilmington, NC 28403-5927, USA
[b] Cotton Incorporated, 6399 Weston Parkway, Cary, NC 27513, USA
[c] Institute for Plant Genomics & Biotechnology, Texas A&M University, College Station, TX, USA
[d] Agricultural Research Service-United States Department of Agriculture (ARS-USDA), New Orleans, LA, USA

ARTICLE INFO

Keywords:
Cottonseed meal
Low-gossypol
Alternative protein sources
Fish meal
Southern flounder
Paralichthys lethostigma

ABSTRACT

Cottonseed meal (CSM) proteins from genetically-improved (glandless) low-gossypol seed (GI-CSM, 52.1% crude protein, CP), genetically-modified low-gossypol seed (GMO-CSM, 56.0% CP) and from an untreated regular (glanded) seed (R-CSM 49.9% CP) were evaluated to replace fish meal (FM) protein (59.5% CP) in juvenile southern flounder *Paralichthys lethostigma* diets. Eight isonitrogenous (45% CP) and isolipidic (16% crude lipid, CL) diets were formulated. A control diet (0% CSM) contained 40% fishmeal (FM) and other practical protein sources. Six diets replaced 50, 75 and 100% FM protein with GI-CSM or GMO-CSM protein. One diet replaced 100% FM protein by R-CSM protein. Fifteen fish (mean = 1.81 g) were stocked in each of twenty-four 75-L tanks (N = 3 per treatment) and were fed the treatment diets for eight weeks. Final percent weight gain was not significantly ($P > 0.05$) different in fish fed the GI- and GMO- and R-CSM protein diets (898–1405%) compared with the control diet (0% CSM) (1242%), but percent weight gain was greater ($P < 0.05$) in fish fed the 50% GI-CSM diet (1405%) versus the 100% GI-CSM diet (898%). Feed conversion ratio was excellent for all treatments (FCR = 0.70–1.00), with no treatment differences ($P < 0.05$). Protein efficiency ratios (PER) were also not significantly different among the treatments (2.26–3.24), although the lowest value was for 100% R-CSM diets. After 8 weeks of feeding, survival of fish ranged from 80 to 91%, with no treatment differences. Apparent protein digestibility of diets was significantly higher for the fish fed 75% and 100% GI-CSM and 100% GMO-CSM protein diets (83.5, 83.5 and 86.5%, respectively) compared with the control diet (79.4%). After 8 weeks, no significant ($P > 0.05$) interactive effects between CSM sources and FM replacement levels on final weight, FCR and PER were observed. Arginine levels in the diets increased as CSM was increased, consistent with the high arginine concentrations found in CSM. Liver gossypol was only detectable in fish fed the 100% R-CSM diet (37 μg/g). Replacing up to 75% FM protein by GI- or GMO-CSM protein did not affect on whole body omega-3 PUFAs, or liver gossypol. The results suggest that up to 75% of fish meal protein may be replaced by GI- or GMO-CSM protein in the diet of juvenile flounder without adverse effects on growth performance and body composition.

Statement of relevance: Cottonseed meal (CSM) is a potentially cost-effective alternative plant protein source for use in aquafeeds. The results suggest that a 75% of fishmeal protein could be replaced by genetically-improved and genetically modified (GMO) low gossypol based cottonseed flour protein in the diet of southern flounder. These ultra-low gossypol cottonseed flour proteins could be inexpensive protein sources for the commercial culture of southern flounder and other finfish species.

* Corresponding author at: University of North Carolina Wilmington, Center for Marine Science, Aquaculture Program, 601 South College Road, Wilmington, NC 28403-5927, USA.
E-mail address: watanabew@uncw.edu (W.O. Watanabe).

https://doi.org/10.1016/j.aquaculture.2018.02.006
Received 1 November 2017; Received in revised form 1 February 2018; Accepted 2 February 2018
Available online 03 February 2018
0044-8486/

1. Introduction

To reduce the amount and cost of wild-caught fish used as a source of protein in aquafeeds and the nutrient levels in effluent waste, the identification of effective alternative protein sources to FM is imperative (Trushenski et al., 2006). However, there are species-specific limits to how much FM can be replaced by alternative plant protein sources without negatively affecting fish growth, feed efficiency and body composition (Gatlin III et al., 2007).

1.1. Cottonseed meal

Cottonseed meal (CSM) is a potentially cost-effective alternative plant protein source for use in aquafeeds (Anderson et al., 2016; Cook et al., 2016). CSM is produced by a several step process that removes the hulls and separates the oil. Seed is first passed through a series of knives and shakers to separate the hull from the kernels. The kernels are flaked and cooked and then the oil is expressed either by pressing or solvent extraction with hexane. The recovered oil is the most valuable by-product of the seed and it is primarily used in cooking and food production (Lin et al., 2015). After removing the oil from the kernels, the remaining defatted kernel tissue is known as CSM. CSM is much cheaper per unit of protein than FM and other FM replacement protein sources, due to the large global production of cotton and cottonseed by-products. However, high levels of the antinutrient gossypol in regular CSM (R-CSM) limit the incorporation of this ingredient in aquafeeds. Gossypol is a terpene-based secondary metabolite that has a role in the cotton plant's defense against pests and possibly diseases (Romano and Scheffler, 2008). Gossypol is produced and deposited in “glands” in the stems, leaves and green bolls of the plant, and in the seed (Lusas and Jividen, 1987). Anti-nutritional and anti-fertility effects have been reported in warm-blooded animals and fish fed cottonseed products containing high levels of gossypol (Blom et al., 2001; Henry et al., 2001; Romano and Scheffler, 2008). Furthermore, gossypol is known to bind with lysine, rendering this essential amino acid less available in fish feed (Wilson et al., 1981).

1.2. Low-gossypol CSM as a FM replacement

The utilization of CSM as an ingredient in the feeds of animals and fish has been improved by reducing or eliminating gossypol. In late 1950's McMichael discovered the existence of mutant cotton plants without the lysigenous glands that contain gossypol (McMichael, 1959; Siccardi et al., 2012), rendering the seed largely gossypol-free and less toxic to non-ruminant animals. Low-gossypol seed has also obtained by seed-specific, RNAi-mediated silencing of a delta-cadinene synthase gene (Sunilkumar et al., 2006). This genetically modified (GMO) genotype has ultra-low levels of gossypol in the seed but, unlike glandless plants, it maintains normal levels of gossypol in the foliage, roots and floral tissues to protect the plant against pests and diseases (Rathore et al., 2012; Palle et al., 2013).

Regular high-gossypol CSM (R-CSM) protein has been used to replace FM protein at maximum levels of 35% in grass carp *Ctenopharyngodon idellus* (Zheng et al., 2012) and 30% in parrotfish *Oplegnathus fasciatus* (Lim and Lee, 2009). In comparison, low-gossypol CSM protein has been used to replace FM protein at levels ranging from 20 to 100% for hybrid striped bass *Morone saxatilis* ♀ × *Morone chrysops* ♂ (Sullivan and Reigh, 1995), channel catfish *Ictalurus punctatus* (Robinson and Rawles, 1983; Li et al., 2008; Dorsa et al., 1982), rainbow trout *Oncorhynchus mykiss* (Lee et al., 2006), Florida pompano *Trachinotus carolinus* (Riche and Williams, 2010; Cook et al., 2016), white shrimp *Litopenaeus vannamei* (Siccardi et al., 2012; Richardson et al., 2016) and black sea bass *Centropristis striata* (Anderson et al., 2016).

1.3. Southern flounder aquaculture

The southern flounder *Paralichthys lethostigma* is a flatfish in the family Paralichthyidae. It can be found in coastal waters from Albemarle Sound, North Carolina, through the South Atlantic states with the exception of South Florida. Southern flounder landings have declined, leading to more stringent fishery regulations and interest in culturing native flatfishes for stock enhancement or food fish production. The development of intensive culture methods for southern flounder in the southeastern United States is of great interest because of its euryhaline character and its status as a highly desirable food and recreational species and potential for commercial culture. Hatchery methodology for spawning and larval rearing is well investigated (Daniels and Watanabe, 2003; Watanabe et al., 2006). Dietary protein and lipid requirements of juvenile southern flounder have been established (Alam et al., 2009, 2011), and a number of studies have determined the substitution limits of alternative plant (i.e., soybean meal) (Alam et al., 201) and animal (i.e., poultry by-product meal) (Dawson, 2012) for FM protein in the diet of juvenile southern flounder. To date, no studies have been conducted to determine the efficacy of low-gossypol CSM protein as a replacement for FM protein in the diet of southern flounder. The objectives of this study were to determine, under controlled laboratory conditions, the effects of different levels of substitution of FM protein with low-gossypol CSM protein from genetically-improved (glandless) and genetically-modified (GMO) plants on growth performance, feed utilization, dietary protein digestibility, and body composition of southern flounder.

2. Materials and methods

2.1. Experimental fish

Juvenile southern flounder (approximately 90 days post-hatching) were cultured from eggs produced by photothermally conditioned captive broodstock held at the University of North Carolina Wilmington Center for Marine Science (UNCW-CMS) Aquaculture Facility (Wrightsville Beach, NC). Broodstock was induced to spawn using luteinizing hormone-releasing hormone analogue (LHRHa) implants (Watanabe et al., 2001; Watanabe et al., 2006). Eggs were hatched and larvae reared through juvenile stages using methods established at UNCW (Watanabe et al., 2001). Juveniles were reared in 500-L tanks on a commercial diet (Skretting Vancouver, British Columbia, 50% crude protein and 15% lipid) until they were stocked in the experimental tanks.

2.2. Experimental system

The experimental system consisted of twenty-four 75-L rectangular tanks located in an indoor climate–controlled laboratory supported by a recirculating aquaculture system (RAS). The RAS included a Kaldness moving bed (Anox Kaldness, Inc., Providence, Rhode Island) biofilter, a bead filter (Aquaculture Systems Technologies, LLC, New Orleans, Louisiana) to remove solids, a protein skimmer to remove small particulate and dissolved materials, and an ultraviolet sterilizer for disinfection. Each tank was supplied with diffused air supplemented with pure oxygen, and the water temperature was controlled by a heat pump. Total ammonia and nitrate in water were measured weekly using a portable data-logging spectrometer (HACH DR/2010 SPEC). Water temperature, dissolved oxygen, salinity, and pH were measured using a multi-parameter probe (YSI 556 MPS, GEO Scientific, Ltd., Vancouver, British Columbia).

2.3. Cottonseed meals

Three CSMs were prepared at Cotton Inc. (Cary, NC, USA) (Anderson et al., 2016). A high-gossypol CSM was prepared from

Table 1
Proximate composition, essential amino acids and gossypol levels of cottonseed meal[a] (%).

Parameters	Glandless	GMO[c]	Regular
Gossypol[b]	0.017%	0.090%	0.773%
Crude protein	52.1%	56.0%	49.9%
Crude lipid	11.5%	13.5%	10.1%
Ash (%)	7.84%	5.30%	6.26%
Moisture	5.74%	5.87%	5.26%
Crude fiber	2.36%	2.67%	2.32%
Essential amino acid profile (% sample)			
Methionine	0.82	0.74	0.78
Cystine	0.95	1.03	0.82
Lysine	2.14	2.59	1.94
Phenylalanine	2.75	3.16	2.42
Leucine	2.82	3.32	2.66
Isoleucine	1.48	1.78	1.41
Threonine	1.58	1.74	1.47
Valine	3.00	2.54	2.47
Histidine	1.40	1.65	1.26
Arginine	5.97	5.62	5.28
Tryptophan	0.53	–	0.52

[a] Supplied by Cotton Incorporated, Cary, NC and analyzed at New Jersey Feed Lab.
[b] Analyzed at USDA-ARS (New Orleans, LA, USA).
[c] Genetically modified.

regular (glanded) cottonseed (R-CSM). A low-gossypol CSM was prepared from genetically-improved (glandless) cottonseed (GI-CSM), and another low-gossypol CSM was prepared from genetically-modified cottonseed (GMO-CSM). All three meal samples were prepared from dehulled kernels by extracting the bulk of the oil with an Ag-Oil laboratory-scale screw press (Eau Claire, WI, USA). The proximate and amino acid compositions of all three CSMs were analyzed at New Jersey Feed Lab (Table 1). Gossypol levels were analyzed at an ARS-USDA Laboratory by a slightly modified AOCS Official Method (AOCS, 1998) (Table 1).

2.4. Experimental diets and design

Three isonitrogenous and isolipidic test diets were formulated to replace 50, 75, and 100% of FM protein by either GI-CSM or GMO-CSM protein (Table 2). A control diet was formulated with a high level of FM and other practical protein sources, including soybean meal and poultry by-product meal. Another control diet was formulated to replace 100% of FM protein by R-CSM protein that contained typical meal levels of gossypol. All other nutrients were added based on recent nutritional studies on southern flounder and other marine finfish (Alam et al., 2009, 2011; Anderson et al., 2016). Diets were prepared at UNCW-CMS using a Kitchen Aid mixer, meat grinder and a drying oven and stored in a freezer (−20 °C).

2.5. Feeding trial and experimental conditions

Twenty early juveniles (mean weight = 1.81 g) were stocked in each of twenty-four 75-L tanks in a controlled-environment laboratory. The fish were acclimatized in the tanks with a commercial diet (50% CP, 15% CL) for one week. To start the experiment, the test diets were fed to triplicate groups of fish twice per day (0900 and 1600 h) to apparent satiation (i.e., as much as they consumed during a 20-min period without wastage) and the amount of diet consumed was recorded. A 14:10 h light/dark photoperiod was maintained. Temperature was maintained between 22 and 24 °C, salinity between 33 and 35 g/L, dissolved oxygen between 6.5 and 8.0 mg/L, and pH between 7.5 and 8.5. Ammonia and nitrite levels were maintained at < 0.3 mg/L and < 0.2 mg/L, respectively (Watanabe et al., 2006). The feeding trial was conducted for 8 weeks. At the end of the trial, three fish from each tank were sampled, euthanized by immersion in a bath of seawater and ice and then stored at −85 °C for whole body proximate composition and other biochemical analysis.

2.6. Apparent protein digestibility of diets (APDD)

After collecting fish for biochemical analyses, the remaining fish (approximately 10–12 per tank) were fed their respective diets treated with chromic oxide as a marker, and feces were collected to determine the APDD. Each diet was reformulated to include 0.5% chromic oxide, which was offset by a reduction of cellulose. These diets were fed twice daily, and the tanks were cleaned following the last feeding. The next morning, fecal material produced overnight was collected, rinsed with deionized water, and stored in a freezer (−20 °C) for chromic oxide and protein analyses. Frozen fecal samples were freeze-dried to remove all moisture. Chromic oxide content in feces and diets were determined using a spectrophotometer (Thermo Fisher Scientific, Two Rivers, Wisconsin, USA) through a modified Furukawa and Tsukahara (1966) method. The APDD was calculated using the formula:

$$\text{APDD}\ (\%) = 100 \times [1 - (F_{\text{protein}}/D_{\text{protein}} \times D_{\text{chromic}}/F_{\text{chromic}})]$$

where F is the percent of nutrient or chromic oxide in the fecal matter and D is the percent of protein or chromic oxide in the diet.

2.7. Proximate composition

Experimental diets and whole body tissue proximate compositions were analyzed at the UNCW Aquaculture Facility. Crude protein was analyzed with a Labconco Kjeltec System (Rapid Digestor, Distilling Unit-Rapid Still II and Titration Unit, Labconco Corporation, Kansas City, MO, USA) using boric acid to trap ammonia by the Kjeldahl method. Crude lipid (Soxhlet extraction with acetone), ash (BARNSTAD Thermolyne Muffle Furnace, IA, USA) and moisture (Fisher Scientific Isotemp Oven, Pittsburgh, PA, USA) contents in the diets were determined using standard methods (AOAC, 2000). Fish moisture content was determined by drying in a freeze dryer (Labconco Freeze Dryer, Kansas City, MO, USA).

2.8. Amino acid compositions

Amino acid composition of the diets and whole fish bodies was analyzed by AAA Service Laboratory (Damascus, OR, USA). Samples were hydrolyzed in 6 N HCl at 110 °C for 22 h. Nor-leucine was used as internal standard. After hydrolysis, samples were analyzed using post-column derivatization on Hitachi L8900 analyzers (Hitachi High Technologies America, Inc., USA).

2.9. Fatty acid composition

Fatty acid composition of the diets and whole fish bodies was determined by first extracting total lipids into chloroform–methanol (Folch et al., 1957) as described by Alam et al. (2011) and Anderson et al. (2016). Nonadecanoic acid (19:0) was added to each sample as an internal standard (1 mL of a 0.001 g mL^{-1} solution). Lipid fatty acids were converted to their methyl esters (FAMEs) for GC analysis as described by Rezek et al. (2010). GC analysis was performed on a HP-6890 Gas Chromatograph using a 30 m length × 0.25 µm film thickness × 0.25 mm internal diameter (Department of Chemistry and Biochemistry, UNCW).

2.10. Gossypol analyses

The concentration of gossypol in the CSM meals and liver samples of fish was determined at USDA-ARS, New Orleans, LA by AOCS Method Ba 8a-99 (AOCS, 1998) with slight modifications (Anderson et al., 2016). In brief, each meal was derivatized with *R*-(-)-2-amino-1-propanol, and the resulting complex was separated by reverse-phase HPLC

Table 2
Composition of diets (g/100 g) replacing FM protein with CSM protein of different sources: GI = genetically improved (glandless), GMO = genetically modified (glandless), and R = regular (glanded). A control FM-based diet (0% CSM) was also tested.

Diets	1	2	3	4	5	6	7	8
Replacement levels[a]	(0%)	50%	75%	100%	50%	75%	100%	100%
Ingredients	Control	GI-CSM	GI-CSM	GI-CSM	GMO-CSM	GMO-CSM	GMO-CSM	R-CSM
Menhaden fish meal[b]	40.00	20.00	10.00	0.00	20.00	10.00	0.00	0.00
Cotton seed flour[c]	0.00	19.00	28.51	38.02	21.06	31.60	42.13	47.29
Soybean meal[d]	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Poultry meal[e]	15.00	15.00	15.00	15.00	15.00	15.00	15.00	15.00
Soy protein concentrate[f]	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Wheat starch	11.00	12.00	12.00	12.00	10.00	10.00	9.75	3.73
Wheat gluten[g]	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Menhaden fish oil[h]	6.00	6.50	6.50	6.50	5.80	5.50	5.20	6.10
Soybean lecithin[i]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Vitamin premix[j]	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50
Mineral premix[j]	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Stay C (Vit C)	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Calcium phosphate	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Methionine	0.76	0.93	1.01	1.10	0.93	1.01	1.09	1.02
Lysine	0.00	0.45	0.67	0.90	0.36	0.54	0.73	0.76
Taurine	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Cellulose	1.64	1.02	0.71	0.88	1.25	0.75	0.50	0.50
Total	100	100	100	100	100	100	100	100
Protein % (analyzed)	45.5	44.7	44.6	44.6	45.5	44.1	44.1	45.1
Digestible protein[k]	36.1	35.2	37.2	37.2	35.6	37.0	38.1	38.9
Lipid % (analyzed)	15.9	16.1	16.0	16.8	16.5	16.4	16.0	16.1
Energy (kj/g diet)[l]	18.11	18.57	18.62	18.67	18.19	18.24	18.24	17.20
Gossypol (mg/kg diet)	0	32.3	48.5	64.6	189.5	284.4	379.2	3466.4

[a] Percent FM protein replacement with CSM protein.
[b] Omega protein, Houston, TX, USA (crude protein 59%, lipid 11%).
[c] Cotton Incorporated, Cary, NC. GI- CSM 52.1% protein, 11.5% lipid, GMO-CSM 56.0% protein, 13.5% lipid, Regular CSM 49.9% protein, 10.1% lipid.
[d] Southern States, Wallace, NC, USA (solvent extracted, crude protein 47.5%).
[e] Malick Aquafeed, Pensylvania, USA (crude protein 65%, lipid 12%).
[f] Soy Protein Concentrate, Solae LLC, St. Louis, MO, USA (crude protein 66%).
[g] VWR International, Radnor, PA, USA (crude protein 78%).
[h] Virginia Prime Silver, Omega Protein, Hammond, LA, USA.
[i] ADM, IL,USA.
[j] Kokhin Chemical Company, Kagoshima, Japan (As Alam et al., 2011).
[k] calculated as per apparent digestibility of protein in the diets (as per Table 3).
[l] Calculated based on carbohydrates, proteins and lipids are 17.2, 23.6, and 39.5 kJ g^{-1}, respectively (Blaxter, 1989).

and detected by UV absorbance at 254 nm. Quantification was done by a standard curve generated from an authentic sample of gossypol–acetic acid (1:1). At the end of the feeding trial, three fish from each tank were dissected, and the livers were frozen at −80 °C, then freeze-dried and ground, and liver tissue was analyzed for gossypol by the same method used for the CSM meals.

2.11. Statistical analysis

Statistical analysis was performed using the JMP 11.0.0 statistical software (SAS Institute Inc., Cary, North Carolina). Treatment means were compared using a one-way ANOVA. Two way ANOVA was also used to determine interactive effects between CSM sources (glandless and GMO) and replacement levels (50, 75 and 100%) on fish performance. Significant differences between the means were further analyzed using Tukey-Kramer HSD (Kramer, 1956). $P < 0.05$ was considered significant.

3. Results

3.1. Growth performance, feed efficiency and survival

The growth performance, feed utilization and survival after the 8-week feeding trial are presented in Table 3. No significant ($P > 0.05$) differences were observed in final weights (18.5–26.8 g) among fish fed diets containing the three types of CSM protein (GI, GMO, or R) or their levels of substitution compared with fish fed the control FM protein-based diet (24.3 g). However, fish fed the 50% GI-CSM protein diet showed a significantly higher final weight (26.8 g) than did fish fed 100% GI-CSM protein diet (18.5 g). The percent weight gain after the 8-week feeding trial also showed a similar trend (Fig. 1), with no differences between the control FM protein-based diet (1242%) and the diets containing CSM protein (898–1405%). However, the percent body weight gain of fish fed the 50% GI-CSM diet (1405%) was higher ($P < 0.05$) than the weight gain in fish fed the 100% GI-CSM diet (898%) (Fig. 1).

There were no significant differences among treatments in feed intake (FI = 0.25–0.32 g/fish/d). Feed conversion ratio was excellent for all treatments (FCR = 0.70–1.00), with no treatment differences. Protein efficiency ratios (PER) were also not significantly different among the treatments (2.26–3.24), however, the lowest value was for 100% R-CSM. After 8 weeks of feeding, survival of fish ranged from 80 to 91%, with no treatment differences (Table 3). After 8 weeks, no significant ($P > 0.05$) interactive effect between CSM sources and FM replacement levels on final weight, FI, FCR, PER and survival were observed (Table 3).

3.2. Apparent protein digestibility of diets (APDD)

There were no significant differences in APDD among the control (79.4%), 50% GI-CSM (78.7%) and 50% GMO-CSM (78.2%) protein-based diets (Table 3). However, APDD of dietary protein was significantly higher for the fish fed 75% and 100% GI-CSM and 100% GMO-CSM protein diets (83.5, 83.5 and 86.5%, respectively) compared with the control diet (79.4%). After 8 weeks, a significant ($P < 0.05$) interactive effect between CSM sources (Glandless and GMO) and

Table 3
Growth performance, feed utilization and apparent protein digestibility of diets (APDD) after 56 days feeding trial. Fish were fed diets replacing FM protein with CSM protein of different sources: GI = genetically improved (glandless), GMO = genetically modified (glandless), and R = regular (glanded). A control FM-based diet (0% CSM) was also tested. Values are means ± SEM (N = 3). Means with different letters in the same column differ significantly ($P < 0.05$). Initial fish weight was 1.81 ± 0.08 g (means ± SEM).

Diets	Final wt. (g)	FI (g/F/d)	FCR	APDD (%)	PER	Survival (%)
0%	24.3 ± 1.2ab	0.31 ± 0.01	0.78 ± 0.05	79.4 ± 1.01b	2.83 ± 0.18	85
50% GI-CSM	26.8 ± 0.61a	0.34 ± 0.02	0.76 ± 0.03	78.7 ± 0.52b	2.93 ± 0.09	91
75% GI-CSM	24.6 ± 0.19ab	0.28 ± 0.03	0.70 ± 0.06	83.5 ± 0.15a	3.24 ± 0.30	87
100% GI-CSM	18.5 ± 0.67b	0.25 ± 0.03	0.84 ± 0.12	83.5 ± 0.30a	2.81 ± 0.47	80
50% GMO-CSM	23.8 ± 3.9ab	0.30 ± 0.03	0.79 ± 0.06	78.2 ± 1.06b	2.83 ± 0.23	89
75% GMO-CSM	22.2 ± 1.6ab	0.31 ± 0.02	0.86 ± 0.04	83.9 ± 0.90a	2.63 ± 0.11	82
100% GMO-CSM	20.8 ± 1.3ab	0.30 ± 0.02	0.89 ± 0.04	86.5 ± 0.65a	2.58 ± 0.11	80
100% R-CSM	19.4 ± 2.1ab	0.32 ± 0.04	1.00 ± 0.11	86.4 ± 0.55a	2.26 ± 0.24	91
Two way ANOVA (P value)						
CSM (GI and GMO)	0.5124	0.5080	0.1713	0.0573	0.1612	0.6272
Level (50, 75 and 100%) 0.0295	0.2384	0.3515	0.0000	0.6201	0.2753	
CSM × level	0.3290	0.2556	0.5436	0.0272	0.6029	0.9175

FI (feed intake) = g/fish/day; FCR (Feed conversion ratio) = feed intake (g)/wet weight gain (g); PER (protein efficiency ratio) = wet weight gain (g)/protein intake (g).

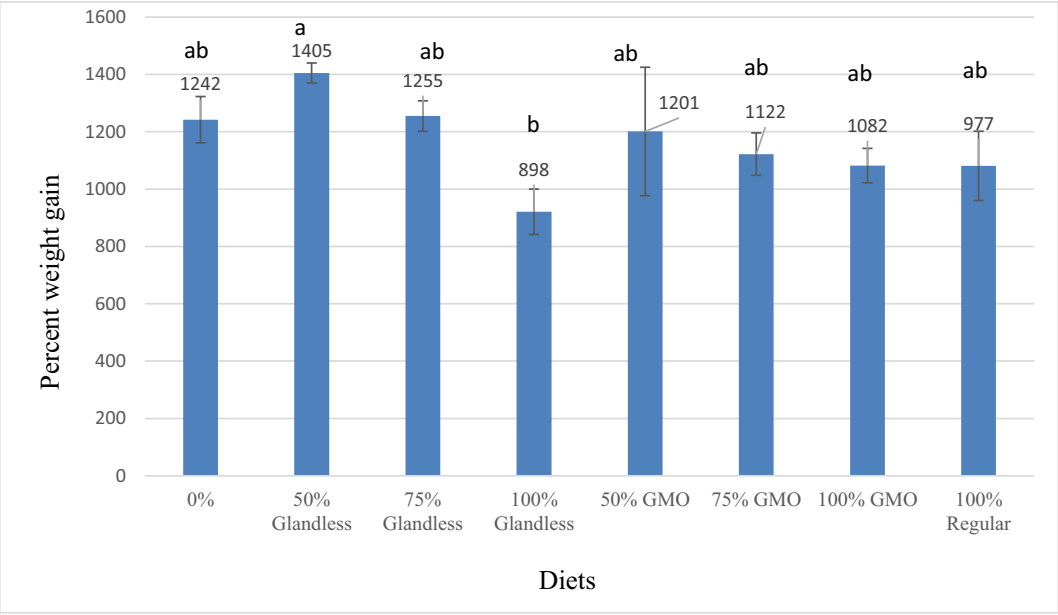

Fig. 1. Effects of replacing FM protein with CSM protein of different sources: GI = genetically improved (glandless), GMO = genetically modified and R = regular (glanded) on percent weight gain on juvenile southern flounder. A control FM-based diet (0% CSM) was also tested. Bars represent percent body weight gain of juvenile flounder after 8 weeks of feeding. Values are mean ± SEM of triplicate tanks. Means with different letters differ significantly.

replacement levels (50, 75 and 100%) on APDD was observed (Table 3). At the 50 and 75% replacement levels, APDD values were closely similar for GI- and GMO- CSM diets; however, at the 100% replacement level, APDD was higher in the GMO-CSM diet.

3.3. Whole body proximate composition and liver gossypol level

After 8 weeks of feeding, fish whole body moisture (74.5–75.5%) and crude protein (15.5–16.5%) contents were not significantly different among the treatments (Table 4). The whole body lipid levels in fish fed 75% and 100% GI-CSM and GMO-CSM protein (5.86–6.56%) and 100% R-CSM protein diets (6.21%) were significantly higher than were the lipid levels in fish fed the control FM protein-based diet (4.72%). Ash contents in fish fed 50% GI-CSM (2.98%) and 50% GMO-CSM protein diets (3.04%) were not significantly different from the control FM protein-based diet (3.33%); however, ash content in fish fed 75 and 100% GI- or GMO-CSM or R-CSM protein diets (2.36–2.98%) were significantly lower than the ash level of the fish fed the control diet (3.33%). Liver gossypol (37 μg/g) was only detectable in fish fed the 100% R-CSM protein-based diet (Table 4).

3.4. Diet and whole body fatty acid profiles

3.4.1. Diets

The fatty acid compositions of the diets are presented in Table 5. Total saturated fatty acids (SFAs) varied within a narrow range of 21.1 to 24.5 mg/g dry wt., with palmitic acid (16:0) representing the major component of SFAs. Total monounsaturated fatty acids (MUFAs) also varied within a narrow range of 17.2–21.7 mg/g dry wt., with palmitoleic (16:1n-7) and oleic (18:1n-9) acids as the major components. Total n-3 PUFAs generally decreased in the diets as the GI-, GMO- and R-CSM protein were increased in replacement of FM protein. The n-3 PUFAs EPA (8.36–4.02 mg/g dry wt.) and DHA levels (7.61–3.19 mg/g dry wt.) likewise decreased as FM protein was replaced by CSM protein, irrespective of source (Table 5). In contrast, total n-6 PUFAs in the diets (9.65–28.2 mg/g dry wt.) clearly increased as CSM protein was increased in the diets, also irrespective of source. This was primarily related to an increase in the level of linoleic acid (18:2n-6), the most prevalent fatty acid in cottonseed lipids.

3.4.2. Fish whole bodies

In general, final whole body fatty acid profiles (Table 6) reflected

Table 4
Whole body proximate composition (% wet basis) and liver gossypol content of fish fed diets replacing FM protein with CSM protein of different sources: GI = genetically improved (glandless), GMO = genetically modified (glandless), and R = regular (glanded). A control FM-based diet (0% CSM) was also tested. Values are means ± SEM (N = 3). Means with different letters in the same column differ significantly ($P < 0.05$).

Diets	Moisture	Crude protein	Lipid	Ash	Liver gossypol
	%	%	%	%	μg/g
0%	75.5 ± 0.17	16.5 ± 0.09	4.72 ± 0.05b	3.33 ± 0.12a	Below detectable
50% GI-CSM	74.6 ± 0.41	15.9 ± 0.14	5.75 ± 0.24ab	2.98 ± 0.11abc	Below detectable
75% GI-CSM	74.5 ± 0.70	16.2 ± 0.28	6.08 ± 1.0a	2.76 ± 0.08bcd	Below detectable
100% GI-CSM	74.8 ± 0.15	15.7 ± 0.09	6.41 ± 0.19a	2.39 ± 0.06d	Below detectable
50% GMO-CSM	74.9 ± 0.91	15.8 ± 0.34	5.70 ± 0.06ab	3.04 ± 0.0.02ab	Below detectable
75% GMO-CSM	75.1 ± 0.26	16.0 ± 0.39	5.86 ± 0.43a	2.79 ± 0.11bcd	Below detectable
100% GMO-CSM	74.9 ± 0.19	16.2 ± 0.27	6.56 ± 0.08a	2.36 ± 0.11d	Below detectable
100% R-CSM	74.9 ± 0.51	15.5 ± 0.17	6.21 ± 0.35a	2.58 ± 0.04 cd	37

Table 5
Fatty acid composition of diets (mg/g dry wt.) replacing FM protein with CSM protein of different sources: GI = genetically improved (glandless), GMO = genetically modified (glandless), and R = regular (glanded). A control FM-based diet (0% CSM) was also tested. Values are means ± SEM (N = 3).

Fatty Acid	0%	50%	75%	100%	50%	75%	100%	100%
	Control	GI-CSM	GI-CSM	GI-CSM	GMO-CSM	GMO-CSM	GMO-CSM	GMO-CSM
14:0	5.33	4.42	4.03	3.29	4.06	3.67	2.83	3.00
16:0	14.74	16.13	16.89	16.21	15.24	16.51	15.21	15.10
16:1n-7	8.94	7.61	7.02	5.83	6.98	6.44	5.03	5.25
18:0	3.58	3.52	3.39	3.12	3.29	3.31	2.87	2.85
18:1n-9	9.93	11.38	12.31	11.92	10.74	12.05	11.19	10.51
18:1n-11	2.17	1.77	1.57	1.27	1.50	1.43	1.17	1.12
18:2n-6	8.56	18.55	23.90	26.88	18.81	25.08	27.48	21.75
18:4n-3	2.05	1.68	1.51	1.23	1.53	1.38	1.01	1.07
20:0	0.16	0.19	0.20	0.19	0.18	0.20	0.17	0.18
20:1n-9	0.62	0.54	0.49	0.40	0.50	0.46	0.35	0.36
20:2n-6	0.21	0.19	0.17	0.14	0.17	0.16	0.12	0.12
20:4n-6 (ARA)0.88	0.82	0.79	0.66	0.78	0.76	0.59	0.57	
20:5n-3 (EPA)	8.36	6.68	5.96	4.74	6.15	5.40	4.02	4.34
22:5n-3	1.64	1.28	1.21	0.97	1.23	1.11	0.83	0.87
22:6n-3 (DHA)7.61	5.80	5.01	3.76	5.38	4.58	3.19	3.41	
Σ SFA	23.81	24.26	24.51	22.81	22.77	23.69	21.08	21.13
Σ MUFA	21.66	21.30	21.39	19.42	19.72	20.38	17.74	17.24
Σ n-3 PUFA	19.66	15.44	13.69	10.70	14.29	12.47	9.05	9.69
Σ n-6 PUFA	9.65	19.56	24.86	27.68	19.76	26.00	28.19	22.44
n-3/n-6 PUFA	2.04	0.79	0.55	0.39	0.72	0.48	0.32	0.43
DHA/EPA	0.91	0.87	0.84	0.79	0.87	0.85	0.79	0.79

dietary levels. Total SFAs ranged from 20.2 to 33.9 mg/g dry wt., with 16:0 representing the major component. Total MUFAs in whole bodies ranged from 21.1 to 31.3 mg/g dry wt., with 16:1n-7 and 18:1n-9, representing the major components. Total n-3 PUFAs ranged from 15.4 to 20.5 mg/g dry wt. and showed a trend of decrease with increasing CSM in the diet. Total n-3 fatty acids in fish whole bodies fed the control FM protein-based diet (20.50 mg/g) were comparable to fish fed the 50 to 75% GI- or GMO-CSM diets (18.3–20.7 mg/g sample), but were lower in fish fed 100% GI- and GMO-CSM diets (15.4 to 15.5 mg/g) (Table 6). EPA and DHA levels in fish fed the control FM protein-based diet were not significantly different from the diets substituting CSM protein. The n-6 fatty acids were much lower in fish fed the 0% CSM protein (control) diet (9.44 mg/g dry wt.) compared to the other diets (22.0–43.7 mg/g dry wt.), and these differences were primarily related to differences in 18:2n-6 levels (Table 6).

3.5. Amino acid composition of diets and whole bodies

Although there were some variations in the levels of certain amino acids among diets, these differences were relatively small (Table 7). Methionine levels appeared slightly lower in the control FM protein-based diet (0.93%) compared to other diets substituting CSM protein (1.12 to 1.30%). Arginine and glutamic acid levels also appeared to be slightly lower in the control FM protein-based diet compared with the CSM protein-based diets, irrespective of source.

The amino acid composition of whole bodies after the feeding trial is presented in Table 8. Methionine content in whole fish fed the control FM protein-based diet (1.62%) was significantly higher than its content in the whole fish fed the 100% CSM protein-based diets (1.40–1.43%), irrespective of source. The concentrations of some essential amino acids, such as lysine, leucine, isoleucine, histidine and arginine, in fish whole bodies were not significantly different among treatments. However, phenylalanine level in fish fed the control FM protein- and GI-CSM protein-based diets (2.27–2.47%) was significantly higher than the phenylalanine level of the fish fed the GMO- and R-CSM diets (1.89–2.02%). Likewise, threonine level was significantly greater in fish fed the control FM protein-based diet (2.35%) than it was for fish fed the 100% GI-, GMO- or R-CSM protein-based diets (2.09, 2.08%, and 2.09, respectively). Glutamic acid levels were also significantly lower in fish fed 100% GI-, GMO- and R-CSM protein-based diets (6.66, 6.37 and 6.39%, respectively) (Table 8) compared with the fish fed the FM protein-based control diet (7.39%).

4. Discussion

4.1. Survival, growth performance and feed utilization

In this study, survival of juvenile southern flounder was not

Table 6
Fatty acid composition of whole bodies (mg/g dry wt.) of fish fed diets replacing FM protein with CSM protein of different sources: GI = genetically improved (glandless), GMO = genetically modified (glandless), and R = regular (glanded). A control FM-based diet (0% CSM) was also tested. Values are means ± SEM (N = 3). Means with different letters in the same row differ significantly ($P < 0.05$).

Fatty acid	0%	50%	75%	100%	50%	75%	100%	100%
	Control	GI-CSM	GI-CSM	GI-CSM	GMO-CSM	GMO-CSM	GMO-CSM	R-CSM
14:0	4.22 ± 0.05	4.40 ± 0.29	5.29 ± 0.38	4.71 ± 0.28	5.16 ± 0.29	4.29 ± 0.15	4.15 ± 0.19	4.64 ± 0.36
16:0	13.08 ± 0.53c	17.17 ± 1.29bc	23.01 ± 1.70a	23.92 ± 1.11a	20.54 ± 0.87ab	19.56 ± 0.65ab	21.71 ± 0.83ab	23.99 ± 1.46a
16:1n-7	8.42 ± 0.21	8.67 ± 0.55	10.33 ± 0.71	9.34 ± 0.54	10.05 ± 0.54	8.61 ± 0.24	8.22 ± 0.29	9.25 ± 0.63
18:0	2.89 ± 0.07c	3.92 ± 0.26bc	5.02 ± 0.29ab	5.05 ± 0.28a	4.72 ± 0.24ab	4.43 ± 0.19ab	4.88 ± 0.29ab	2.89 ± 0.07c
18:1n-9	9.87 ± 0.27c	12.69 ± 0.79bc	17.50 ± 1.28a	18.45 ± 1.06a	15.28 ± 0.82ab	15.11 ± 0.23ab	17.23 ± 0.51a	18.31 ± 0.28c
18:1n-11	2.10 ± 0.15	2.22 ± 0.16	2.58 ± 0.25	2.27 ± 0.10	2.60 ± 0.12	2.13 ± 0.05	2.02 ± 0.06	2.30 ± 0.15
18:2n-6	7.82 ± 0.13e	20.23 ± 1.06d	33.33 ± 2.17b	38.83 ± 2.45ab	25.10 ± 1.36 cd	31.77 ± 1.19bc	41.47 ± 1.48a	7.82 ± 0.13e
20:0	0.06 ± 0.03b	0.13 ± 0.01ab	0.17 ± 0.01a	0.19 ± 0.01a	0.15 ± 0.01a	0.15 ± 0.01a	0.17 ± 0.01a	0.19 ± 0.02a
20:1n-9	0.68 ± 0.03	0.67 ± 0.03	0.85 ± 0.07	0.74 ± 0.02	0.79 ± 0.04	0.71 ± 0.05	0.72 ± 0.06	0.77 ± 0.08
20:2n-6	0.39 ± 0.01d	0.57 ± 0.03 cd	0.85 ± 0.06abc	0.90 ± 0.04ab	0.77 ± 0.04bc	0.84 ± 0.06abc	1.11 ± 0.10a	1.00 ± 0.07ab
20:4n-6 (ARA)1.23 ± 0.08	1.19 ± 0.09	1.35 ± 0.11	1.14 ± 0.09	1.40 ± 0.02	1.27 ± 0.03	1.14 ± 0.03	1.29 ± 0.08	
20:5n-3 (EPA)	5.96 ± 0.25	5.97 ± 0.38	6.46 ± 0.40	5.13 ± 0.54	6.51 ± 0.29	5.78 ± 0.23	4.93 ± 0.14	5.96 ± 0.56
22:5n-3	3.68 ± 0.17	3.00 ± 0.29	3.46 ± 0.43	2.58 ± 0.29	3.37 ± 0.10	2.93 ± 0.13	2.77 ± 0.13	3.21 ± 0.36
22:6n-3 (DHA)8.96 ± 0.51	8.00 ± 0.77	8.38 ± 0.92	5.99 ± 0.78	8.71 ± 0.18	7.64 ± 0.34	6.10 ± 0.13	7.20 ± 0.73	
Σ SFA	20.25	25.62	33.49	33.87	30.57	28.43	30.91	33.85
Σ MUFA	21.07	24.25	31.26	30.80	28.72	26.56	28.19	30.63
Σ n-3 PUFA	20.50	18.87	20.47	15.40	20.74	18.26	15.51	18.36
Σ n-6 PUFA	9.44	21.99	35.53	40.87	27.27	33.88	43.72	38.61
n-3/n-6 PUFA	2.17	0.86	0.58	0.38	0.76	0.54	0.35	0.48
DHA/EPA	1.50	1.34	1.30	1.17	1.34	1.32	1.24	1.21

significantly affected by the level of FM protein replaced by CSM protein in the diet, irrespective of source (GI-, GMO-, or R-CSM) suggesting that juvenile southern flounder were able to tolerate a relatively high level of dietary gossypol, an antinutrient present in much higher concentrations in the 100% R-CSM diet (3466 mg/kg diet) than in all the other diets (32.3–379 mg/kg diet) (Table 2). However, the apparent trends toward lower growth performance with increasing levels of CSM protein in the diet suggest that treatment effects may become more distinct over time. Dietary substitution of R-CSM protein for soybean meal protein at a level of 100% (647 mg gossypol/kg diet) did not affect survival in the common carp *Cyprinus carpio* (Wang et al., 2014), and replacement of FM protein with R-CSM protein up to 100% (9160 mg gossypol/kg diet) did not affect survival in juvenile tilapia *Oreochromis* sp. (Mbahinzireki et al., 2000). In black sea bass, replacement of FM protein by GI-CSM protein (108.6 mg gossypol/kg diet) did not affect survival (Anderson et al., 2016). Li and Robinson (2006) noted that there was no toxic effects of gossypol on the survival of a number of aquatic animals such as channel catfish *Ictalurus punctatus*, Nile tilapia *Oreochromis niloticus*, rainbow trout, and Pacific white shrimp *Litopenaeus vannamei*.

Similar to survival, growth performance (final weight and percent weight gain) of southern flounder juveniles fed diets replacing from 50 to 100% FM protein with GI-, GMO-, or R-CSM protein was not different from fish fed a control FM protein-based diet (Table 3, Fig. 1). However, in the present study, replacing FM by 100% GI, GMO or R-CSM appeared to lower growth performance (albeit not significantly), suggesting that longer-term studies are needed. Adult rainbow trout fed diets replacing 100% of FM protein by R-CSM protein showed normal growth performance and survival after a 6-month grow-out trial (Blom et al., 2001).

With supplemental methionine and lysine, up to 40% FM protein in the diet can be replaced by R-CSM protein (3200 mg gossypol/kg diet) in Japanese flounder without negative effects on growth (Pham et al., 2007). Cook et al. (2016) reported that the inclusion of 19.6% GI- CSM does not affect performance of Florida pompano if the diet is balanced for lysine. In the present study, crystalline lysine was added to the diets in order to mitigate the deficiency in the CSM protein and to fulfill the lysine requirement of southern flounder. In black sea bass juveniles, replacing FM protein by 100% GI-CSM protein did not alter growth performance or survival when diets were supplemented with lysine (Anderson et al., 2016), results similar to what was found in southern flounder in the present study. Therefore, CSM can be an effective alternative protein ingredient in diet of southern flounder when supplemented with lysine and in combination with other alternative protein sources such as poultry meal (Dawson, 2012) and soybean meal (Alam et al., 2011) used as the main protein sources. GI-CSM was included in the diet of white shrimp *Litopenaeus vannamei* at 67% (Siccardi et al., 2012) and of channel catfish at 30% (Li et al., 2008), without negative effects on survival and growth. Without supplemental lysine, 50% of soybean meal protein can be replaced by solvent-extracted CSM protein in channel catfish, whereas 100% of soybean meal protein can be replaced by solvent-extracted CSM protein with supplemental lysine (Robinson, 1991).

In the present study, southern flounder were able to tolerate significant quantities of gossypol in the GI- and GMO-CSM protein-based diets (32.2–380 mg/kg diet) (Table 2), without measurable accumulation in their livers (Table 4). Liver gossypol was detectable (37 μg/g) only in flounder fed the 100% R-CSM diet (3466 mg gossypol/kg diet) as previously reported in black sea bass (Anderson et al., 2016) and in Florida pompano (Cook et al., 2016). However, whereas elevated liver gossypol was associated with reduced growth in black sea bass (25.9 μg/g) (Anderson et al., 2016) and Florida pompano (79.7 μg/g) (Cook et al., 2016) fed 100% R-CSM, growth of southern flounder fed 100% R-CSM was not inhibited even though liver gossypol concentration was elevated (37 μg/g), indicating a relatively high tolerance to gossypol. Longer-term studies are needed to confirm these findings. R-

Table 7
Amino acid composition of diets replacing FM protein with CSM protein of different sources: GI = genetically improved (glandless), GMO = genetically modified (glandless), and R = regular (glanded). A control FM-based diet (0% CSM) was also tested. (% dry wt.). Values are means ± SEM (N = 2).

Amino acids	0%	50%	75%	100%	50%	75%	100%	100%
	Control	GI-CSM	GI-CSM	GI-CSM	GMO-CSM	GMO-CSM	GMO-CSM	R-CSM
Met	0.93	1.12	1.23	1.22	1.28	1.29	1.26	1.30
Lys	3.27	3.24	3.25	3.10	3.11	3.10	3.15	3.11
Phy	1.98	2.15	2.16	2.24	2.17	2.17	2.32	2.36
Leu	3.44	3.25	3.05	2.96	3.33	3.01	3.00	3.07
Ileu	2.01	1.97	1.68	1.66	1.92	1.60	1.62	1.78
Thr	2.02	1.80	1.59	1.64	1.74	1.73	1.83	1.77
Val	2.34	2.30	2.05	2.01	2.25	2.01	2.07	2.24
His	1.30	1.32	1.28	1.36	1.33	1.30	1.28	1.33
Arg	3.27	3.90	4.20	4.60	4.11	4.09	4.48	4.57
Cys	0.55	0.67	0.74	0.80	0.66	0.72	0.77	0.82
Gly	3.60	2.97	2.72	2.46	3.12	2.68	2.51	2.55
Asp	4.24	4.01	4.14	4.10	4.35	4.44	4.65	4.46
Ser	2.18	2.14	2.05	2.15	2.33	2.18	2.29	2.26
Glu	6.77	7.31	8.05	8.41	8.11	7.82	8.43	8.45
Pro	3.25	2.85	2.91	2.81	3.08	2.63	2.56	2.54
Hypro	1.01	0.67	0.57	0.51	0.68	0.56	0.50	0.46
Ala	2.99	2.59	2.06	1.93	2.38	2.19	2.09	2.18
Tyr	1.57	1.57	1.48	1.47	1.55	1.44	1.49	1.51

Table 8
Amino acid composition of southern flounder whole bodies (g/100 g dry basis) after 8 weeks of feeding diets replacing FM protein with CSM protein of different sources: GI = genetically improved (glandless), GMO = genetically modified (glandless), and R = regular (glanded). A control FM-based diet (0% CSM) was also tested. Values are means ± SEM (N = 3). Means with different letters in the same row differ significantly ($P < 0.05$).

Amino acid	0% (control)	50% GI-CSM	75% GI-CSM	100% GI-CSM	50% GMO-CSM	75% GMO-CSM	100% GMO-CSM	100% R-CSM
Methionine	1.62 ± 0.02a	1.49 ± 0.06ab	1.51 ± 0.04 ac	1.40 ± 0.03b	1.49 ± 0.08ab	1.51 ± 0.02ab	1.42 ± 0.03b	1.43 ± 0.04b
Lysine	4.36 ± 0.09	4.01 ± 0.12	4.07 ± 0.06	3.83 ± 0.16	3.92 ± 0.21	4.02 ± 0.11	3.77 ± 0.12	3.87 ± 0.07
Phenylalanine	2.47 ± 0.05a	2.40 ± 0.07a	2.44 ± 0.03a	2.27 ± 0.06ab	1.98 ± 0.10c	2.02 ± 0.03bc	1.89 ± 0.05c	1.91 ± 0.04c
Leucine	3.90 ± 0.07	3.57 ± 0.12	3.62 ± 0.08	3.37 ± 0.16	3.51 ± 0.19	3.61 ± 0.09	3.36 ± 0.16	2.42 ± 0.06
Isoleucine	2.09 ± 0.03	1.93 ± 0.06	1.96 ± 0.04	1.80 ± 0.08	1.92 ± 0.11	1.95 ± 0.06	1.82 ± 0.07	1.88 ± 0.04
Threonine	2.35 ± 0.04a	2.20 ± 0.04ab	2.24 ± 0.02ab	2.09 ± 0.05b	2.14 ± 0.10ab	2.21 ± 0.02ab	2.08 ± 0.05b	2.09 ± 0.04b
Valine	2.35 ± 0.03ab	2.19 ± 0.05ab	2.24 ± 0.03ab	2.05 ± 0.06b	2.40 ± 0.15a	2.45 ± 0.06a	2.31 ± 0.06ab	2.31 ± 0.06ab
Histidine	1.10 ± 0.02	1.02 ± 0.02	1.05 ± 0.01	0.97 ± 0.03	1.00 ± 0.06	1.03 ± 0.01	0.97 ± 0.04	0.99 ± 0.04
Arginine	3.47 ± 0.07	3.38 ± 0.03	3.46 ± 0.04	3.17 ± 0.11	3.26 ± 0.21	3.38 ± 0.04	3.20 ± 0.01	3.11 ± 0.11
Glycine	4.03 ± 0.22	4.22 ± 0.16	4.35 ± 0.11	3.82 ± 0.67	3.64 ± 0.52	3.92 ± 0.35	3.79 ± 0.21	3.27 ± 0.25
Aspartic acid	5.20 ± 0.10	4.92 ± 0.07	4.93 ± 0.04	4.71 ± 0.12	4.88 ± 0.22	5.00 ± 0.07	4.72 ± 0.13	4.76 ± 0.08
Serine	2.30 ± 0.06a	2.20 ± 0.02abc	2.24 ± 0.02ab	2.11 ± 0.02abc	2.07 ± 0.08bc	2.18 ± 0.03abc	2.08 ± 0.03bc	2.03 ± 0.05c
Glutamic acid	7.39 ± 0.19a	6.93 ± 0.13ab	6.95 ± 0.08ab	6.66 ± 0.17b	6.43 ± 0.21b	6.74 ± 0.03ab	6.37 ± 0.16b	6.39 ± 0.08b
Proline	2.38 ± 0.14	2.45 ± 0.07	2.53 ± 0.05	2.24 ± 0.32	2.48 ± 0.28	2.59 ± 0.15	2.50 ± 0.10	2.25 ± 0.16
Alanine	3.53 ± 0.10	3.47 ± 0.02	3.53 ± 0.05	3.25 ± 0.17	3.25 ± 0.20	3.42 ± 0.08	3.23 ± 0.03	3.08 ± 0.11
Tyrosine	1.70 ± 0.03	1.54 ± 0.05	1.59 ± 0.03	1.49 ± 0.08	1.62 ± 0.09	1.66 ± 0.05	1.54 ± 0.06	1.59 ± 0.03
Hydroxyproline	0.90 ± 0.01	0.87 ± 0.09	0.98 ± 0.05	0.82 ± 0.25	0.82 ± 0.18	0.93 ± 0.16	0.91 ± 0.10	0.68 ± 0.08

CSM protein has been used to replace FM protein at a level of 35% in grass carp *Ctenopharyngodon idellus* (Zheng et al., 2012) and 30% in parrotfish *Oplegnathus fasciatus* (Lim and Lee, 2009) without negative effects on growth.

The FI, FCR and PER of southern flounder fed diets replacing 50–100% FM protein with both GI- and GMO-CSM protein were not different from those of a control FM protein-based diet. This suggests that these CSM proteins were utilized as efficiently as FM protein by juvenile southern flounder. Similar findings were recently reported in black sea bass (Anderson et al., 2016) and Florida pompano (Cook et al., 2016). Compared to the control FM protein-based diet, feed FI and FCR were also not reduced by the high-gossypol R-CSM diet, consistent with a relatively high tolerance to gossypol in the southern flounder. However, the detection of liver gossypol only in those fish fed the R-CSM diet suggests that more studies are needed to assess longer term effects.

4.2. Fish whole body proximate composition

In accord with growth performance data (Table 3), there were no significant differences in whole body protein content of fish fed the control FM protein- and the CSM protein-based diets (Table 4). Whole body lipid content, however, was significantly higher in fish fed the 75 and 100% CSM protein-based diets (regardless of source) compared with the control FM protein-based diet (Table 4). The higher lipid contents in whole bodies could be due to impaired liver function, or indicate less use of lipid for energy in fish fed high CSM protein-based diets, possibly due to their lower levels of saturated and mono-unsaturated fatty acids (Table 5), which are known to be preferentially used as energy sources in marine fish (Sargent, 1995). In contrast, black sea bass juveniles fed a 100% R-CSM protein-based diet showed slower growth and a lower whole body lipid level than fish fed a control FM protein-based diet (Anderson et al., 2016), suggesting higher utilization of lipid from CSM in this species. Ash contents in whole bodies decreased with increasing CSM in the diets (Table 4). African catfish *Clarias gariepinus* fed diets containing up to 60% CSM showed no differences in whole body ash (Toko et al., 2008).

4.3. Amino acid profiles of diets and whole bodies

All of the essential amino acids in the test diets were within the reported requirement values of essential amino acids for marine finfish

(Alam et al., 2005). Methionine and lysine were supplemented to the diets containing CSM to attain concentrations similar to the control FM protein-based diet (0% CSM) (Table 2) as soybean meal and CSM are deficient in these two essential amino acids. Except for methionine and arginine, essential amino acids were similar among the experimental diets. Arginine levels in the diets increased as CSM was increased, consistent with the high arginine concentrations found in CSM (Anderson et al., 2016). The supplementation of methionine and lysine likely improved growth of juvenile southern flounder fed different levels of CSM protein in this study. The concentration of methionine in the fish whole bodies were similar to or higher than the diet levels, indicating that methionine supplementation was effective. Whole body lysine concentrations did not differ among diet treatments (Table 8), also suggesting that dietary lysine was sufficient under all treatments and that the bioavailability of lysine was not reduced by binding with gossypol. This is in accord with a previous study on black sea bass fed high CSM protein-based diets supplemented with methionine and lysine (Anderson et al., 2016). Without supplemental lysine, 50% of soybean meal protein can be replaced by solvent-extracted CSM protein in channel catfish, whereas 100% of soybean meal protein can be replaced by solvent-extracted CSM protein with supplemental lysine (Robinson, 1991).

4.4. Fatty acid profile of diets and whole bodies

MUFA concentrations were slightly lower in the diets replacing FM protein with CSM protein (Table 5), but the opposite pattern was seen in the fish whole bodies, which showed higher MUFA concentrations as dietary CSM increased (Table 6) due to high levels of 18:1n-9 in CSM (Table 5). This is similar to what was reported in black sea bass fed with high CSM-based diets (Anderson et al., 2016) and was related to the reduction of fish oil with increased CSM in the diet.

Concentration of n-3 PUFAs were the highest in the FM-based control diet and decreased as FM was replaced by CSM (Table 5). Dietary n-3 PUFAs generally decrease when FM protein is replaced by terrestrial animal or plant protein sources, which are often deficient in n-3 PUFAs. This trend, however, was not fully reflected in the n-3 PUFA contents of fish whole bodies as n-3 PUFA contents in fish fed the control diets were only slightly higher than the fish fed the CSM protein-based diets (Table 6). Higher percentage of n-3 PUFA in CSM protein-based diets reflected that the requirements for n-3 PUFA in the CSM-based diets were met by addition of fish oil, or from FM and poultry meal in the diets. However, slightly lower n-3 PUFA in the whole bodies of fish fed 100% GI- and GMO-CSM protein-based diets were related to the lower levels found in these diets, as previously reported in black sea bass fed 100% GI-CSM protein-based diets (Anderson et al., 2016). A mixture of plant protein sources decreased DHA, EPA, and total n-3 PUFAs levels in whole body tissues of gilthead sea bream compared to the level of these acids in fish fed a 100% FM protein-based control diet (De Francesco et al., 2007).

In this study, n-6 PUFA concentrations (notably 18:2 n-6) in both diets (Table 5) and whole bodies (Table 6) of juvenile southern flounder increased with increasing level of dietary CSM protein. This is consistent with previous findings for other species fed plant-protein-based diets, including black sea bass fed high CSM protein-based diets (Anderson et al., 2016) and Atlantic cod *Gadus morhua* fed high soybean meal protein-based diets (Karalazos et al., 2007). The n-6 PUFA arachidonic acid (ARA, 20:4n-6) is essential for carnivorous marine fish (Koven et al., 2001). Although the ARA concentration in the control FM protein-based diet (0.88 mg/g) (Table 5) was slightly lower than in the CMS-protein-based diets, ARA concentration in the whole bodies of fish fed the test diets were not significantly different (Table 6). This indicates that ARA content in all of the diets was sufficient for southern flounder growth.

In southern flounder whole bodies, the ratio of DHA to EPA was relatively high under all diet treatments (1.17–1.50) (Table 6), irrespective of CSM protein sources and levels of supplementation. Although there are no published reports for the optimum dietary concentrations of DHA, EPA, or the ratio of DHA to EPA for southern flounder, DHA-to-EPA ratios in all diets (0.79–0.87) (Table 5) exceeded those required for good growth in gilthead sea bream (minimum DHA-to-EPA ratio = 0.5) (Ibeas et al., 1997) and were comparable to those producing maximum growth in grouper *Epinephelus malabaricus* (DHA-to-EPA ratio > 1) (Wu et al., 2002) and in black sea bass (0.81–0.91) (Anderson et al., 2016).

4.5. Apparent protein digestibility of diets (APDD)

The significant interaction between CMS sources and FM replacement levels on APDD was related to higher APDD in the GMO-CSM than in GI-CSM protein-based diets at the 100% replacement level, but not at the 50% and 75% levels. This suggests that, in combination with other alternative protein sources, GMO-CSM protein may be more digestible than GI-CSM protein when used in diets that fully replace fish meal proteins.

Irrespective of sources, the APDD was significantly higher for the 75 and 100% CSM protein-based diets (83.5–86.5%) compared with the APDD for the control FM protein-based diet (79.4%) (Table 3). These findings suggest that CSM protein, regardless of source, was more digestible than menhaden FM in juvenile southern flounder. The APDD for southern flounder (78.7–86.5%) was similar to those reported in black sea bass (75.4–85.7%) (Anderson et al., 2016) and in Florida pompano (72.2–86.1%) (Cook et al., 2016). This suggests that cottonseed products were effectively utilized by southern flounder as a protein source in practical diets. In Pacific white shrimp, relatively high protein digestibility of 72.3 to 94.1% was reported for both GI- and GMO-CSM by Siccardi et al. (2016), who also suggested that these low-gossypol CSM products may be cost-effective proteins source for shrimp diets. In red drum *Sciaenops ocellatus*, diets containing 30% high-gossypol R-CSM protein had only a slightly lower APDD (84.5%) than did diets based on 100% menhaden FM protein (87.9%) (Gaylord and Gatlin, 1996). In rainbow trout, APDD for R-CSM protein (81.6 to 87.9%) (Cheng and Hardy, 2002) was very close to the value obtained for southern flounder fed 100% R-CSM protein in the present study (86.4%). In Nile tilapia, diets containing 100% R-CSM protein also showed a similar APDD (87.8%) as a control FM protein-based diet (87.7%) (El-Saidy and Gaber, 2004), related to the high digestibility of R-, GI-, or GMO-CSM protein by southern flounder in the present study.

5. Conclusions

Results of the present study suggest that replacement of up to 75% or more FM protein by both GI- and GMO-CSM protein is possible in the diet of juvenile southern flounder, without a reduction in growth performance, survival, feed efficiency, body composition, or increase in liver gossypol.

Acknowledgements

This research was supported by Cotton Inc., Cary, NC (Agreement No. 13-806).

References

Alam, M.S., Teshima, S., Yaniharto, D., Sumule, O., Ishikawa, M., Koshio, S., 2005. Assessment of reference amino acid pattern for diet of juvenile red sea bream, *Pagrus major*. Aquac. Int. 13, 369–379.

Alam, M.S., Watanabe, W.O., Daniels, H.V., 2009. Effect of different dietary protein and lipid levels on growth performance and body composition of juvenile southern flounder (*Paralichthys lethostigma)* reared in recirculating aquaculture system. J. World Aquacult. Soc. 40 (4), 513–521.

Alam, M.S., Watanabe, W.O., Myers, A.R., Rezek, T.C., Carroll, P.M., Longfellow, S., 2011. Effects of replacement of menhaden fish meal protein by solvent-extracted soybean meal protein supplemented with or without L-methionine and L-lysine in the diet of

juvenile southern flounder. N. Am. J. Aquac. 73, 350–359.
Anderson, A.D., Alam, M.S., Watanabe, W.O., Carroll, P.M., Wedegaertner, T.C., Dowd, M.K., 2016. Full replacement of menhaden fish meal protein by low-gossypol cottonseed flour protein in the diet of juvenile black sea bass, *Centropristis striata*. Aquaculture 464, 618–628.
AOAC, 2000. Official Methods of Analysis of the Association of Official Analytical Chemists, 16th Ed. Association of Official Analytical Chemists, Arlington, VA, USA.
AOCS, 1998. Official Methods and Recommended Practices of the AOCS, 5th Ed. American Oil Chemists' Society, Champaign, IL, USA.
Blaxter, K., 1989. Energy Metabolism in Animals and Man. Cambridge University Press, Cambridge.
Blom, J.H., Lee, K.J., Rinchard, J., Dabrowski, K., Ottobre, J., 2001. Reproductiveefficiency and maternal-offspring transfer of gossypol in rainbow trout (*Oncorhynchus mykiss*) fed diets containing cottonseed meal. J. Anim. Sci. 79, 1533–1539.
Cheng, Z.J., Hardy, R.W., 2002. Apparent digestibility coefficients and nutritional value of cottonseed meal for rainbow trout (*Oncorhynchus mykiss*). Aquaculture 212, 361–372.
Cook, R.L., Zhou, Y., Rhodes, M.A., Davis, D.A., 2016. Evaluation of various cottonseed products on the growth and digestibility performance in Florida pompano *Trachinotus carolinus*. Aquaculture 453, 10–18.
Daniels, H.V., Watanabe, W.O., 2003. A Practical Hatchery Manual: Production of Southern Flounder Fingerlings. North Carolina Sea Grant Publication, UNC-SG-02-08, Raleigh.
Dawson, M.R., 2012. Evaluation of Alternative Protein Sources to Fish Meal in Practical Diets for Juvenile Black Sea Bass *Centropristis striata* and Southern Flounder *Paralichthys lethostigma*. Masters of Marine Science Thesis. Center for Marine Science, University of North Carolina Wilmington.
De Francesco, M., Parisi, G., Perez-Sanchez, J., Gomez-Requeni, P., Medale, F., Kaushik, S.J., Mecatti, M., Poli, B.M., 2007. Effect of high-level fish meal replacement by plant proteins in gilthead sea bream (*Sparus aurata*) on growth and body/fillet quality traits. Aquac. Nutr. 13, 361–372.
Dorsa, W.J., Robinette, H.R., Robinson, E.H., Poe, W.E., 1982. Effects of dietary cottonseed meal and gossypol on growth of young channel catfish. Trans. Am. Fish. Soc. 111, 651–655.
El-Saidy, D.M., Gaber, M.M., 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.
Folch, J., Lees, M., 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.
Furukawa, A., Tsukahara, H., 1966. On the acid digestion method for the determination of chromic oxide as an index substance in the study of digestibility of fish feed. Bull. Jpn. Soc. Sci. Fish. 32, 502–506.
Gatlin III, D.M., Barrows, F.T., Brown, P., Dabrowski, L., Gaylord, T.G., Hardy, R.W., Herman, E., Hu, G., Krogdahl, A., Nelson, R., Overturf, K., Rust, M., Sealy, W., Skonberg, D., Souza, E.J., Stone, D., Wilson, R., Wurtele, E., 2007. Expanding the utilization of sustainable plant products in aquafeeds: a review. Aquac. Res. 38, 551–579.
Gaylord, T.G., Gatlin, D.M., 1996. Determination of digestibility coefficients of various feedstuffs for red drum (*Sciaenops ocellatus*). Aquaculture 139, 303–314.
Henry, M.H., Pesti, G.H., Brown, T.P., 2001. Pathology and histopathology of gossypol toxicity in broiler chicks. Avian Dis. 45, 598–604.
Ibeas, C., Cejas, J.R., Fores, R., Badía, P., Gómez, T., Hernández, A.L., 1997. Influence of eicosapentaenoic to docosahexaenoic acid ratio (EPA/DHA) of dietary lipids on growth and fatty acid composition of gilthead seabream (*Sparus aurata*) juveniles. Aquaculture 150, 91–102.
Karalazos, V., Treasurer, J., Cutts, C.J., Alderson, R., Galloway, T.F., Albrektsen, S., Arnason, J., MacDonald, N., Pike, I., Bell, J.G., 2007. Effects of fish meal replacement with full-fat soy meal on growth and tissue fatty acid composition in Atlantic cod (*Gadus morhua*). J. Agric. Food Chem. 55, 5788–5795.
Koven, W., Barr, Y., Lutzky, S., Ben-Atia, I., Weiss, R., Harel, M., Behrens, P., Tandler, A., 2001. The effect of dietary arachidonic acid (20:4n-6) on growth, survival and resistance to handling stress in gilthead seabream (*Sparus aurata*) larvae. Aquaculture 193, 107–122.
Kramer, C.Y., 1956. Extension of multiple range tests to group means with unequal numbers of replications. Biometrics 12, 307–310.
Lee, K.J., Rinchard, J., Dabrowski, K., Babiak, I., Ottobre, J.S., Christensen, J.E., 2006. Long-term effects of dietary cottonseed meal on growth and reproductive performance of rainbow trout: three-year study. Animal Feed Sci. Tech. 126, 93–106.
Li, M.H., Robinson, E.H., 2006. Use of cottonseed meal in aquatic animal diets: a review. N. Am. J. Aquac. 68 (1), 14–22.
Li, M.H., Hartnell, E.H., Kronenberg, J.M., Healy, C.E., Oberle, D.F., Hoberg, J.R., 2008. Evaluation of cottonseed meal derived from genetically modified cotton as feed ingredients for channel catfish, *Ictalurus punctatus*. Aquac. Nutr. 14, 490–498.
Lim, S.J., Lee, K.J., 2009. Partial replacement of fish meal and soybean meal with iron and phytase supplementation for parrot fish *Oplegnathus fasciatus*. Aquaculture 290, 283–289.
Lin, H., Wedegaertner, T.C., Mao, X., Jing, X., Espinosa, A., R., 2015. A method to refine crude cottonseed oil using non-toxic polyamine-based cationic polymers. Chin. J. Chem. Eng. 23 (2), 379–383.
Lusas, E.W., Jividen, G.M., 1987. Glandless cottonseed: a review of the first 25 years of processing and utilization research. J. Am. Oil Chem. Soc. 64, 839–854.
Mbahinzireki, G.B., Dabrowski, K., Lee, K.J., El-Saidy, D., Wisner, E.R., 2000. Growth, feed utilization and body composition of tilapia (*Oreochromis* sp.) fed with cottonseed meal-based diets in a recirculating system. Aquac. Nutr. 7, 189–200.
McMichael, S.C., 1959. Hopi cotton, a source of cottonseed free of gossypol pigments. Agron. J. 51, 630.
Palle, S.R., Campbell, L.M., Pandeya, D., Puckhaber, L., Tollack, L.K., Marcell, S., Sundaram, S., Stipanovic, R.D., Hinze, L., Wedegaertner, T.C., Rathore, K.S., 2013. RNAi-mediated ultra-low gossypol cottonseed trait: performance of transgenic lines under field conditions. Plant Biotechnol. J. 11, 296–304.
Pham, M.A., Lee, K.J., Lim, S.J., Park, K.H., 2007. Evaluation of cottonseed and soybean meal as partial replacement for fishmeal in diets for juvenile Japanese flounder *Paralichthys olivaceus*. Fish. Sci. 73, 760–769.
Rathore, K.S., Sundaram, S., Sunilkumar, G., Campbell, L.M., Puckhaber, L., Marcell, S., Palle, S.R., Stipanovic, R.D., Wedegaertner, T.C., 2012. Ultra-low gossypol cottonseed: generational stability of the seed-specific, RNAi-mediated phenotype and resumption of terpenoid profile following seed germination. Plant Biotechnol. J. 10, 174–183.
Rezek, T.C., Watanabe, W.O., Harel, M., Seaton, P.J., 2010. Effects of dietarydocosahexaenoic acid (22:6n-3) and arachidonic acid (20:4n-6) on the growth,-survival, stress resistance and fatty acid composition in black seabass *Centropristis striata* (Linnaeus 1758) larvae. Aquac. Res. 41, 1302–1314.
Richardson, C.M., Siccardi, A.J., Palle, S.R., Campbell, L.M., Puckhaber, R.D., Stipanovic, R.D., Wedegaertner, T.C., Rathore, K.S., Samocha, T.M., 2016. Evaluation of ultra-low gossypol cottonseed and regular glandless cottonseed meals as dietary protein and lipid sources for *Liptopenaeus vannamei* reared under zero-exchange condition. Aquac. Nutr. 22, 427–434.
Riche, M., Williams, T.N., 2010. Apparent digestible protein, energy and amino acid availability of three plant proteins in Florida pompano, *Trachinotus carolinus* L. in seawater and low-salinity water. Aquac. Nutr. 16, 223–230.
Robinson, E.H., 1991. Improvement of cottonseed meal protein with supplemental lysine in feeds for channel catfish. J. Appl. Aquac. 1 (2), 1–14.
Robinson, E.H., Rawles, S.D., 1983. Use of defatted, glandless cottonseed flour and meal in channel catfish diets. In: Proceedings of the Southeastern Association of Fish and Wildlife Agencies. 37. pp. 359–363.
Romano, G.B., Scheffler, J.A., 2008. Lowering seed gossypol content in glanded cotton (*Gossypium hirsutum* L.) lines. Plant Breed. 127 (6), 619–624.
Sargent, J.R., 1995. Origins and functions of egg lipids: nutritional implications. In: Bromage, N.R., Roberts, R.J. (Eds.), Broodstock Manageme nt and Egg and Larval Quality. Blackwell Science, Oxford, England, pp. 353–372.
Siccardi, A.J., Richardson, C.M., Dowd, M.K., Wedegaertner, T.C., 2012. Glandless Cottonseed Meal Replaces Fishmeal in Shrimp Diet Research. Global Aquaculture Alliance.
Siccardi, A.J., Richardson, C.M., Dowd, M.K., Wedegaertner, T.C., Samocha, T.M., 2016. Digestibility of glandless cottonseed protein in diets for Pacific white shrimp, *Litopenaeus vannamei*. J. World Aquacult. Soc. 47 (1), 97–106.
Sullivan, J.A., Reigh, R.C., 1995. Apparent digestibility of selected feedstuffs in diets for hybrid striped bass (*Morone saxatilis*♀ × *Morone chrysops*♂). Aquaculture 138, 313–322.
Sunilkumar, G., Campbell, L.M., Puckhaber, L., Stipanovic, R.D., Rathore, K.S., 2006. Engineering cottonseed for use in human nutrition by tissue-specific reduction of toxic gossypol. Proc. Natl. Acad. Sci. U. S. A. 103, 18054–18059.
Toko, I.M., Fiogbe, E.D., Kestemont, P., 2008. Mineral status of African catfish (*Clarias gariepinus*) fed diets containing graded levels of soybean or cottonseed meals. Aquaculture 275, 298–305.
Trushenski, J.T., Kasper, C.S., Kohler, C.C., 2006. Challenges and opportunities in finfish nutrition. N. Am. J. Aquac. 68, 122–140.
Wang, X.F., Li, X.Q., Leng, X.J., Shan, L.L., Zhao, J.X., Wang, Y.T., 2014. Effects of dietary cottonseed meal level on the growth, hematological indices, liver and gonad histology of juvenile common carp (*Cyprinus carpio*). Aquaculture 428–429, 79–87.
Watanabe, W.O., Carroll, P.M., Daniels, H.V., 2001. Sustain, natural spawning of southern flounder *Paralichthys lethostigma* under an extended photothermal regime. J. World Aquacult. Soc. 32 (2), 153–166.
Watanabe, W.O., Woolridge, C.A., Daniels, H.V., 2006. Progress toward year round spawning of southern flounder broodstock by manipulation of photoperiod and temperature. J. World Aquacult. Soc. 37, 256–272.
Wilson, R.P., Robinson, E.H., Poe, W.E., 1981. Apparent and true availability of amino acids from common feed ingredients for channel catfish. J. Nutr. 111, 923–929.
Wu, F.-C., Ting, Y.-Y., Chen, H.-Y., 2002. Docosahexaenoic acid is superior to eicosapentaenoic acid as the essential fatty acid for growth of grouper, *Epinephelus malabaricus*. J. Nutr. 132, 72–79.
Zheng, Q., Wen, X., Han, C., Li, H., Xie, X., 2012. Effect of replacing soybean meal with cottonseed meal on growth, hematology, antioxidant enzymes activity and expression for juvenile grass carp, *Ctenopharyngodon idellus*. Fish Physiol. Biochem. 38, 1059–1069.