A mixture of cottonseed meal, soybean meal and animal byproduct mixture as a fish meal substitute: growth and tissue gossypol enantiomer in juvenile rainbow trout (*Oncorhynchus mykiss*)

By K.-J. Lee¹, K. Dabrowski¹, J. H. Blom¹, S. C. Bai² and P. C. Stromberg³

Summary

Diets incorporating three different sources of extracted cottonseed meal (CM), soybean meal and an animal protein mixture were evaluated for juvenile rainbow trout. Fish averaging 0.96 g were divided into groups of 30; 3 groups per treatment, and each group was fed one of four diets for a 16-week period. Fish meal (FM) was replaced on a 25% protein basis by each of three different sources of CM from California (CA), Tennessee (TN), and Arkansas (AR), U.S.A. In the three CM-containing diets another 25% soybean meal protein and 50% animal protein mixture were also incorporated to completely replace FM protein. The results of growth rate and feed utilization showed that FM could be entirely replaced by a mixture of plant proteins (CM and soybean meal) and animal by-product proteins. Hematocrit levels were significantly lower in the group fed CM-containing diets than in the control. The findings suggest that CM can be used as a good protein source by the incorporation of at least 15% in diets (25% of fish meal protein replacement), and that the nutritive values of CM in juvenile trout can be different due to their different origin. Significantly higher concentrations of total gossypol were found in faeces of CM-TN (5.8 ± 0.4 μmol/g) and CM-AR (5.6 ± 0.6) groups than in that of CM-CA (3.7 ± 0.4) group. It was documented that gossypol enantiomers, present in an equal proportion in diets, selectively accumulated in liver and bile, whereas equal proportions of (+) and (–) enantiomers were found in whole-body and faeces. Depending on CM source, fish can absorb approximately 35–50% of dietary gossypol, and the majority of the absorbed gossypol seemed to be excreted.

Introduction

Feedstuffs of animal origin are generally considered to be of higher quality than those of plant origin, primarily because of their higher protein content and superior complement of indispensable amino acids (Robinson and Li 1998). In recent years there have been efforts to increase the amount of ingredients of plant origin and studies have reported some success in replacement of fish meal (FM) in diets for large rainbow trout, *Oncorhynchus mykiss*, using soybean meals and protein concentrates (Kaushik et al. 1995; Moyano et al. 1992), soybean and corn gluten meal mixtures (Gomes et al. 1995), and a combination of several alternative protein sources (Yamamoto et al. 1995). Soybean meal was the most frequently studied dietary ingredient as a FM replacement in diets for many fish. Animal by-products, such as poultry by-product meal, meat and bone meal, feather meal, and blood meal, have
also been incorporated in practical fish feeds (Murai 1992), and have individually been used as an animal protein source for FM replacement (Davies et al., 1990; Fowler 1991; Higgs et al., 1979; Luzier et al., 1995). However, when compared to FM-based control diets, diets free of fish meal resulted in general inferior growth in salmonids, (Mambrini et al., 1999).

By-products of cottonseed are used in diets for both terrestrial animals (Colin-Negrete et al. 1996) and fish (Hendricks et al., 1980) because of its high protein content. Cottonseed meal (CM) has been examined in diets of fish such as channel catfish, Ictalurus punctatus (Dorsa et al., 1982; Robinson and Brent 1989; Robinson and Li 1994; Robinson and Tiersch 1995), rainbow trout (Hendricks et al., 1980; Herman 1970; Roehm et al., 1967), and tilapia, Oreochromis niloticus (El-Sayed 1990; Robinson et al., 1984). Despite its high nutritional value, cottonseed contains gossypol, a polyphenolic compound, which is toxic to fish (Herman 1970; Rinchard et al., 2000) and terrestrial animals (Colin-Negrete et al., 1996; Makinde et al., 1997). In most studies, gossypol concentrations in fish tissue were either not analysed or were analysed by a colorimetric method with anisidine (Chamkasem 1988; Fisher et al., 1987), which can overestimate gossypol. Data on utilization of CM in fish diets in combination with animal by-product and the resulting gossypol accumulation and/or excretion are not available. There are wide variations in the ratio of gossypol enantiomers present in different species of cotton plants (Gossypium species) and even within different tissues in the same plant (Cass et al., 1991; Jaroszewski et al., 1992). However, only few authors reported the selective accumulation of enantiomers in tissues of animals ingesting gossypol (Kim et al., 1996), and only one report exists on catfish (Robinson and Tiersch 1995). We, therefore, evaluated three different sources of solvent extracted CM containing an equal proportion of gossypol isomers, as a FM substitute, incorporated in combination with soybean meal and an animal protein mixture in diets for juvenile rainbow trout. Diets were evaluated by fish growth rate, feed utilization, gossypol 'absorption' and concentrations in tissues, and the digestibility and concentrations of protein and minerals (phosphorus and iron) in fish body and faeces. For the first time concentrations of separate gossypol isomers in tissues and faeces of fish were analysed using specific high performance liquid chromatography (HPLC) methods employing simultaneously both UV and electrochemical detectors.

Materials and methods

Diets

Four experimental diets were formulated to be isonitrogenous and isocaloric in terms of crude protein (47%) and gross energy (17.5 MJ/kg) (Table 1). The energy value of each diet was estimated on the basis of mammalian physiological fuel values, i.e. 16.7 KJ/g protein or carbohydrate and 37.7 KJ/g lipid (Lee and Putnam 1973). Dietary FM protein was substituted with 50 or 100% animal protein mixture (APM) and the diets marked as APM50 and APM100, respectively. The APM consisted of equal amounts of meat and bone meal (50% protein, 8.5% lipid), blood meal (92% protein, 0.3% lipid), poultry by-product meal (58% protein, 14% lipid) and feather meal (85% protein, 2.5% lipid). For CM-containing diets, FM was replaced on a 25% protein basis by one of three different sources of CM from California (CA), Tennessee (TN), and Arkansas (AR), USA. These diets are referred to as CM-CA, CM-TN, and CM-AR, respectively. In the CM-containing diets the remaining protein consisted of 25% soybean meal (SM) and 50% APM. The three different sources of CM were solvent extracted meals and the total gossypol concentrations were 1.07, 1.65, and 1.53%, for the CM from California, Tennessee, and Arkansas, respectively. The proportions of (+)- and (-)-isomers of the CM were 53 : 47, 54 : 46, and 53 : 47, respectively. The analysed concentrations of dietary total gossypol were 0.11, 0.16, and 0.16% for the CM-CA, CM-TN, and CM-AR, respectively. Experimental diets were
cold-pelleted into 2.0 mm diameter size, freeze-dried to approximately 5% moisture, crushed into desirable particle size (0.4–2.0 mm), and stored at 20°C until use.

Fish, facility and feeding trial

The feeding trial was performed at the Piketon Research and Extension Center aquaculture facility with juvenile rainbow trout (London, Ohio, registered strain) averaging 0.96 ± 0.07 g initial weight. Prior to the feeding trial fish were fed a commercial diet (Bioproducts, Inc., Warrington, OR) for two weeks to allow for adjustment to the experimental conditions. Fish were randomly distributed into groups of 30; 3 groups per treatment. Each

<table>
<thead>
<tr>
<th>Experimental diets</th>
<th>Control</th>
<th>APM50</th>
<th>APM100</th>
<th>CM-CA</th>
<th>CM-TN</th>
<th>CM-AR</th>
</tr>
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<tbody>
<tr>
<td>Ingredients¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fish meal, menhaden</td>
<td>20.00</td>
<td>10.00</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Fish meal, herring</td>
<td>20.00</td>
<td>10.00</td>
<td>–</td>
<td>–</td>
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<td>–</td>
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<tr>
<td>Animal Protein Mixture²</td>
<td>0.00</td>
<td>19.84</td>
<td>39.67</td>
<td>19.84</td>
<td>19.84</td>
<td>19.84</td>
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<tr>
<td>Cottonseed meal-CA³</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>15.66</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Cottonseed meal-TN³</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>14.71</td>
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<td>Cottonseed meal-AR³</td>
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<td>–</td>
<td>–</td>
<td>15.97</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>14.72</td>
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<td>Krill meal (hydrolysate)</td>
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<td>Wheat middling</td>
<td>28.00</td>
<td>27.00</td>
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<td>11.80</td>
<td>13.00</td>
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<td>Corn gluten meal</td>
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<td>11.80</td>
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<td>15.30</td>
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<td>Yeast (brewer)</td>
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<td>Vitamin mixture⁴</td>
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<td>0.50</td>
<td>0.50</td>
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<td>0.50</td>
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<tr>
<td>Mineral mixture⁵</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
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<td>0.50</td>
</tr>
<tr>
<td>Vitamin C⁶</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Menhaden fish oil</td>
<td>8.00</td>
<td>8.90</td>
<td>9.80</td>
<td>10.50</td>
<td>10.50</td>
<td>10.50</td>
</tr>
<tr>
<td>Cellulose</td>
<td>0.25</td>
<td>0.31</td>
<td>0.38</td>
<td>0.03</td>
<td>0.08</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Proximate analyses

- Crude protein: 46.8, 47.2, 48.3, 48.2, 48.3, 47.4
- Crude Lipid: 14.4, 14.4, 14.3, 14.0, 14.0, 14.0
- Gross energy (MJ/kg): 17.5, 17.5, 17.5, 17.5, 17.5, 17.5

¹The ingredients were purchased from: fish meal (herring), Ampro Fisheries Co. Reedville, Virginia, USA; fish meal (menhaden), Baker Co., Stamford, Connecticut, USA; soybean meal, Archer Daniels Midland Co., Fostoria, Ohio, USA; Krill meal, Specialty Marine Products Ltd, Vancouver, Canada; wheat middling, ADM Co., Loudonville, Ohio, USA; corn gluten meal, Baker Trading Co., Dayton, Ohio, USA; Yeast (brewer), Alltech, Chicago, Illinois, USA; mineral mixture (Bernhart Tomarelli salt mixture), choline chloride, and cellulose, ICN Pharmaceuticals Inc., Costa Mesa, California, USA; fish oil (menhaden), Cereal By-products Co., Chicago, Illinois, USA

²Animal protein mixture was composed of equal amounts of blood meal (American Protein Co., Ames, IA), meat & bone meal (Inland Products Co., Clyde, OH), feather meal (American Protein Co., Ames, IA), and poultry by-product meal (Holmes By-products Co., Millersburg, OH). Its crude protein and lipid levels were 68.25% and 6.5%, respectively

³CA, California source; TN, Tennessee source; AR, Arkansas source

⁴Roche Performance Premix composition per g of the vitamin mixture: vitamin A, 2646 IU; vitamin D₃, 221 IU; vitamin E, 66.1 IU; vitamin B₁₂, 13 µg; riboflavin, 13.2 mg; niacin, 61.7 mg; d-pantothenic acid, 22.1 mg; menadione, 1.32 mg; folic acid, 1.76 mg; pyridoxine, 4.42 mg; thiamin, 7.95 mg; d-biotin, 0.31 mg. Hoffman-La Roche, Inc., Nutley, NJ

⁵Five mg Se in the form of sodium selenite per kg Bernhart Tomarelli salt mixture (ICN Pharmaceuticals Inc., Costa Mesa, CA)

⁶Phospitan C (Mg-L-ascorbyl-2-Phosphate), Showa Denko K. K. Tokyo, Japan

⁷Calculated based on compositions of the ingredients used (NRC 1993)
experimental diet was fed to triplicate groups of fish with the feeding rates ranging from 4% of fish weight at the beginning to 2% at the end of the feeding trial (NRC 1993). All procedures and handling of animals were conducted in compliance with the guidelines of the Institutional Laboratory Animal Care and Use Committee, The Ohio State University. The fish were fed three times per day, 7 days a week, for 16 weeks. The feeding trial was conducted in 50 l flow-through circular fibreglass tanks, supplied with well water at a flow rate of 1.8–2.0 l/min. Supplemental aeration was also provided to maintain dissolved oxygen levels near saturation. Water temperature increased gradually from 8 to 15 °C during the experiment and a diurnal light:dark cycle was regulated at 12 h:12 h. Total fish weight in each tank was determined every 4 weeks to check their growth and to adjust the feeding rate. Feeding was stopped 24 h prior to weighing.

Sample collection and analysis

Analyses of crude protein, moisture and ash were performed by standard procedures (AOAC 1995). At the end of the feeding trial all fish were weighed and counted to calculate percent body weight gain (PWG; body wt gain × 100/initial body wt), feed conversion (FC; body wt gain/dry feed consumed), protein efficiency ratio (PER; body wt gain/protein intake), specific growth rate (SGR; \[ln \text{final body wt} – ln \text{initial body wt}\] × 100/ days), and survival. Hematocrit was determined by the microhematocrit method (Brown 1980) on three fish randomly selected per group (total 9 fish per treatment). For histological examination, livers from three randomly selected fish in each dietary replicate were used. Tissues were preserved in 10% neutral buffered formalin, dehydrated through a graded series of alcohol, and embedded in paraffin. Sections were cut at 3–4 μm, mounted on glass slides and stained routinely with hematoxylin and eosin followed by clearing through xylene and cover slipped over Permount medium.

For gossypol analysis, two fish were randomly selected from each dietary group (total 6 fish per treatment) and killed to collect the liver and bile samples. Three fish per each dietary group (total 9 fish per treatment) were killed for the whole-body analysis of gossypol. For gossypol analysis, faeces were collected after 16 weeks of feeding. Mineral compositions of diets and whole-body was determined by the inductively coupled plasma (ICP) emission spectrophotometric method with the use of ARI-3560 Spectrometer (Applied Research Laboratories, Valencie, CA) according to Watson and Isaac (1990).

Faeces collection and apparent digestibility test

The indirect method described by Cho and Kaushik (1990) was used to calculate the apparent ‘digestibility’ coefficient (ADC), with chromic oxide (0.5 g per 100 g feed on dry matter basis) as the inert indicator. The apparent ‘digestibility’ coefficient of protein and gossypol ‘absorption’ was calculated using the following formula:

\[ \text{ADC}_{\text{nutrient}} = [1 – (\text{NF}/\text{ND} \times \text{CrD}/\text{CrF})] \times 100 \]

where: NF = % nutrient in faeces, ND = % nutrient in diet, CrD = % chromic oxide in diet, and CrF = % chromic oxide in faeces. Faeces were collected with a modified faecal collection system (Yamamoto et al., 1998). From the 12th week of feeding trial, fish were fed with the 0.5% chromic oxide-containing diets to facilitate the apparent ‘digestibility’ test until the end of the feeding trial. After 16 weeks of feeding, all fish of each treatment (three groups) were transferred to three 50 L collection tanks having a steep conical bottom connected to a faeces collecting chamber. Water flowed to the top and out at the bottom of the conical tanks at a rate of less than 0.4 l/min. Fish were prevented from stirring the faeces by a circular net placed at the base of the tank. To collect faeces, all the fish were fed their respective diets containing 0.5% chromic oxide to satiation each morning at 08:00, and again at 11:00. The faeces were collected every 40 min for 7 h, and immediately frozen at –20 °C.
The fish were fed again their respective diets at 20:00 on the same day, collection tanks cleaned, and the faeces were collected the next day at 08:00. The collected faeces were immediately frozen at −20 °C and then stored at −80 °C until analysis.

Analysis of gossypol

Gossypol in diets, liver, bile, whole-body, and faeces were determined by HPLC according to the method described by Kim and Calhoun (1995), with some modifications. Wet liver tissue was used for analysis of (+)- and (−)-enantiomers of gossypol because preliminary assays revealed that the gossypol concentration in freeze-dried liver samples were lower than that from wet samples. The preliminary assay was conducted in triplicate to compare the two processing methods. The wet liver and freeze dried diets, whole-body, and faeces were weighed, and 5–10 volumes of complexing reagent added to obtain the 2-amino-1-propanol derivatives of (+)- and (−)-enantiomers of gossypol. The complexing reagent was composed of 2 ml 2-amino-1-propanol (Sigma Chemical, St. Louis, MO), 10 ml glacial acetic acid (Sigma Chemical) and 88 ml N, N-dimethylformamide (Sigma Chemical). The samples were homogenized in complexing reagent on ice for 10–40 s, heated at 95 °C for 30 min, cooled on ice, and then centrifuged at 1500 × g for 5 min. For determination of gossypol from bile, the homogenization step was omitted. After centrifugation, an aliquot of the supernatant was diluted with mobile phase to obtain a desirable concentration, centrifuged again at 1500 × g for 5 min, and filtered through a syringe filter (0.45 μm, Whatman Inc., Clifton, NJ) before injection to HPLC.

The HPLC system consisted of a Beckman 506 A solvent delivery system equipped with a 20 μl injection loop connected to a 4.6-mm × 150 mm Shodex C-18 column (Showa-Denko, Shoko Co. Ltd, Tokyo, Japan) packed with an octadecyl-bonded porous silica gel (5 μm), and both a UV detector (Programmable detector module 166, Beckman Instruments Inc., San Ramon, CA) set at 254 nm and an electrochemical detector (Model LC-4C; BAS, West Lafayette, IN) set applied potential at 0.75 V. The mobile phase was made of 80 ml acetonitrile and 2 mM KH₂PO₄ (final concentration) dissolved in 100 ml distilled water (HPLC grade) adjusted to pH 3.0 with H₃PO₄. Standards of (+)- and (−)-enantiomers of gossypol were provided by Dr Quezia B. Cass, Departamento de Química, Universidade Federal de São Carlos, São Carlos, Brazil (Cass et al., 1999). The retention time for (+)- and (−)-enantiomers of gossypol were 2.1 and 3.4 min, respectively, with a flow rate of 1.8 ml/min. Recovery rates were higher than 92% for both gossypol enantiomers and the detection level was 1 ng/20 μl of injection volume with a signal-to-noise ratio of 3.

Statistical analysis

Each experimental diet was fed to three groups of fish by a completely randomized design. Differences among dietary treatments were tested by one-way ANOVA, and means were compared using Tukey’s multiple comparison test by the SPSS statistical package (Version 9.0, SPSS Inc., Chicago, IL). The percentage data of weight gain, specific growth rate, and hematocrit were arcsine transformed before the ANOVA analysis. Differences were considered significant at $p < 0.05$.

Results

No significant differences in fish body weight were found among all groups until week 8, but fish began to show differences in growth rates from the 12th week, followed by significant differences at the 16th week (Fig. 1). Final body weight gains and feed conversion by fish during the 16-week feeding trial are shown in Table 2. Body weight gain, feed conversion and specific growth rate of fish fed CM-TN and CM-AR diets were not significantly different compared to those of fish fed the FM-based control diet. The
protein efficiency ratio of fish fed the CM-AR diet was also not significantly different compared to that of fish fed the control diet. However, fish fed APM50, APM100, and CM-CA diets exhibited significantly lower growth performances than the fish fed the control diet. A significant decrease in hematocrit was found in fish fed diets containing CM and/or APM compared to fish fed the control diet. No differences were observed in whole-body protein and ash concentrations among all the groups (results not presented). No mortality was observed and differences in the palatability of the diets were not noticed during the 16 weeks of the feeding trial.

The ratio of (+)- and (–)-enantiomers was equal in the three CM-containing diets. Gossypol concentrations in liver, bile, whole-body, and faeces are presented in Fig. 2. No differences in gossypol concentrations were found among treatments in liver, bile and whole-body of the fish. Significantly higher concentrations of total gossypol were found in faeces of CM-TN (5.8 ± 0.4 μmol/g) and CM-AR (5.6 ± 0.6) groups than in that of CM-CA (3.7 ± 0.4) group. This trend in total gossypol was also observed in both (+)- and

![Fig. 1](image_url)

Changes in mean body weight of rainbow trout fed the experimental diets for 16 weeks. Values are means ± SD of triplicate groups. Different letters (a, b) indicate significantly different values (p < 0.05)

<table>
<thead>
<tr>
<th>Diets</th>
<th>BWG (%)</th>
<th>FC</th>
<th>PER</th>
<th>SGR (%)</th>
<th>Hematocrit (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1370 ± 17.00 a</td>
<td>1.03 ± 0.01 a</td>
<td>2.28 ± 0.02 a</td>
<td>2.40 ± 0.01 a</td>
<td>44 ± 1.10 a</td>
</tr>
<tr>
<td>APM50</td>
<td>1129 ± 14.21  c</td>
<td>0.87 ± 0.00  c</td>
<td>1.86 ± 0.00  c</td>
<td>2.24 ± 0.01  c</td>
<td>40 ± 0.73  b</td>
</tr>
<tr>
<td>APM100</td>
<td>1235 ± 27.23 bc</td>
<td>0.92 ± 0.02 bc</td>
<td>1.94 ± 0.04 bc</td>
<td>2.31 ± 0.02 b</td>
<td>36 ± 1.52 c</td>
</tr>
<tr>
<td>CM-CA</td>
<td>1235 ± 39.01 bc</td>
<td>0.91 ± 0.03 bc</td>
<td>1.92 ± 0.06 bc</td>
<td>2.31 ± 0.03 b</td>
<td>39 ± 1.04 b</td>
</tr>
<tr>
<td>CM-TN</td>
<td>1330 ± 16.17 ab</td>
<td>0.96 ± 0.01 ab</td>
<td>2.03 ± 0.02 b</td>
<td>2.37 ± 0.01 ab</td>
<td>39 ± 0.94 b</td>
</tr>
<tr>
<td>CM-AR</td>
<td>1350 ± 10.93 a</td>
<td>1.00 ± 0.01 a</td>
<td>2.18 ± 0.01 a</td>
<td>2.39 ± 0.01 a</td>
<td>39 ± 1.09 bc</td>
</tr>
</tbody>
</table>

Means of triplicate groups; Values ± SD in the same column with different superscript are significantly different (p < 0.05)

Table 2. Percentage body weight gain (BWG), feed conversion (FC), protein efficiency ratio (PER), specific growth rate (SGR) and hematocrit of juvenile rainbow trout fed experimental diets for 16 weeks.
A significant correlation ($r^2 = 0.85, p < 0.001$) was found between gossypol concentrations in diet and faeces. Gossypol was not detected in tissues or faeces of the fish fed the control diet. Interestingly, the ratio of (–)-enantiomers to total gossypol differed, depending on tissues or faeces. In liver and bile, the percentage ratios of (–)-enantiomers to total gossypol were less than 30% which means that over 70% of (+)-enantiomer was selectively retained in tissue. However, an approximately equal proportion of each (+)- and (–)-enantiomer were found in the whole-body and faeces of fish. No significant histopathological changes were found in liver tissues of fish examined. The colour of livers were not different between fish fed CM-containing and control diets, however, yellowish liver due to the gossypol deposition was found in tilapia, *Oreochromis* spp. fed the same amount of CM for 16 weeks in our previous study (Mbahinzireki et al., 2001).

Phosphorus and iron concentrations in diets, whole body and faeces are shown in Fig. 3. Phosphorus in faeces of the control group was significantly higher than those of other groups, and iron concentration in faeces was significantly higher only in CM-TN group.
compared to the control, APM50 and CM-CA groups. No significant differences were observed in whole body concentrations of phosphorus and iron among treatments. The apparent ‘digestibility’ of dietary protein and ‘absorption’ of phosphorus and gossypol after 16 weeks of feeding were different among treatments (Fig. 4). Protein ‘digestibility’ of diet CM-AR (89.6 ± 1.29%) was significantly higher than those of the control (85.0 ± 1.88%), APM50 (83.4 ± 0.41%), APM100 (80.6 ± 2.10%) and CM-CA (83.5 ± 1.34%) diets. The phosphorus ‘absorption’ was higher in diets containing CM and/or APM than in the control. The ‘absorption’ rate of gossypol ranged from 35 to 50% depending on the CM source, and was significantly higher in CM-TN and CM-AR groups than in CM-CA group.

**Discussion**

The results of the present study are significant because, to our knowledge, it is the first fish meal free and high dietary protein (45%) formulation for juvenile rainbow trout that resulted in comparable growth rate to the FM-based control diet. Furthermore, the fish fed
the FM-based control diet (herring and menhaden FM, 1:1) in the present study grew at a rate comparable or higher specific growth rates (2.40 ± 0.01% per day) than indicated earlier in other studies. For instance, Skonberg et al., (1997) used a similar size of rainbow trout and SGR of fish fed a control diet (herring FM) amounted to 1.42 and 2.56% on restricted or ad libitum feeding, respectively, in an 8-week-long study. Rainbow trout weighing 3 g fed a menhaden FM-based diet (ad libitum) showed a SGR of 2.77% after 13 weeks of feeding (Watanabe et al., 1993). The feeding period in our study was longer compared to other FM replacement studies that were conducted with juvenile rainbow trout (Stickney et al., 1996; Yamamoto et al., 1995).

Herman (1970) reported that in rainbow trout, growth depression did not occur until dietary free gossypol concentrations were higher than 290 mg/kg. Robinson and Tiersch (1995) found no effect of 200 mg free gossypol per kg diet in channel catfish on growth, feed consumption, feed conversion ratio, and survival. Free gossypol is defined as ‘acetone soluble gossypol’, whereas bound gossypol can be estimated by subtracting the portion of free form from total gossypol. In the present study the free gossypol concentrations of the diets CM-CA, CM-TN, and CM-AR were 60, 150, and 225 mg/kg, respectively, based on individual CM analysis. The reason for the lower growth rate in CM-CA diet group can be attributed to the inferior nutritive value of CM rather than its gossypol concentration. The nutritive values of CM can differ among different species of cottonseeds processed (Cass et al., 1991; Jaroszewski et al., 1992) and different processing methods (Cherry et al., 1978; Forster and Calhoun 1995).

Lower hematocrits were found in the group fed CM-containing diets than in the control in the present study. This result is in agreement with the results in rainbow trout broodstock (Dabrowski et al., 2000), Nile tilapia (Mbahinzireki et al., 2001), swine and rats (Skutches et al., 1973, 1974). The reason for the observed lower hematocrit in groups fed CM-containing diets in the present study could be the cumulative effect of gossypol and/or decreased availability of iron in CM causing increased erythrocyte fragility (Colin-Negrete et al., 1996; Makinde et al., 1997).

Roehm et al., (1967) reported that in rainbow trout, the liver was the main organ responsible for accumulation of gossypol and that the gossypol elimination took place
with a considerable delay in the liver. In the present study we found the percent ratio of the (−)-enantiomer to total lower in liver (26–29%) and bile (27–39%) than in the whole-body (47–52%) and faeces (49–51%). This result supported the notion that liver is the main organ for the elimination of absorbed gossypol (Roehm et al., 1967), and was consistent with the result of tissue gossypol concentrations found in rainbow trout broodstock in our laboratory. In our previous study we observed the highest gossypol concentrations in liver compared to plasma, bile, kidney, muscle, stomach and gametes (results not published). The findings reported here may suggest that liver eliminates (−)-enantiomer more actively than the (+)-enantiomer. Several studies have shown that the (−)-enantiomer has the higher biological activities, such as antifertility, antitumour and toxicity than (+)-enantiomer (Benz et al., 1990; Joseph et al., 1986; Shelley et al., 1999; Tanphaichitr et al., 1988). Smith and Clawson (1965) indicated that the primary pathway of gossypol excretion was via the biliary system. Our results of bile gossypol concentrations supported the hypothesis that the primary pathway of gossypol excretion is via biliary system. We found high concentrations of total gossypol in the faeces of rainbow trout (3.7–5.8 μmol/g dry matter). Heifers exposed to 1300 and 2000 mg of gossypol/kg excreted 0.58 and 2.3 μmol of total gossypol per g faeces, respectively (Colin-Negrete et al., 1996). Colin-Negrete et al., (1996) calculated that between 5 and 15% of gossypol was absorbed by the heifers. Abou-Donia and Lyman (1970) reported that in pigs and hens, which are sensitive to gossypol toxicity, the maximum amount of radioactivity absorbed in tissues was 32.9 and 16.8% of the orally administered dose, respectively. The approach we used to determine the ‘absorption’ rate of gossypol in trout was to compare the gossypol concentration in diets and excreted faeces by using chromic oxide as a indirect method (Cho and Kaushik 1990) and to consider the total consumption and accumulation of gossypol in the whole-body throughout the feeding trial. In the present study, we found a large proportion of dietary gossypol (35–49%) absorbed by the fish in comparison to terrestrial animals (Fig. 4). We also calculated that fish consumed on average 13.1 ± 0.26 g of diet during the 16-week period, leading to a total intake of 13.7–21.4 mg of total gossypol per fish depending on the three different sources of CM-containing diets. This would then result in gossypol intake ranging from 1.07 ± 0.05 to 1.54 ± 0.02 mg per g of fish body weight. However, fish retained only 0.8–1.6 μg gossypol per g of fish body wt indicating that the amount of gossypol that was not excreted through faeces is less than 0.2% of dietary gossypol. Therefore, it may be assumed that the majority of the absorbed gossypol is metabolized to other compounds such as gossypolone, gossypolonic acid and demethylated gossic acid as proposed by Abou-Donia and Dieckert (1975). However, we observed very low amounts of gossypolone in liver tissues (identified by internal standard) showing less than 1% of the total gossypol detected by HPLC (data not shown).

The result of the higher protein ‘digestibility’ (Fig. 4) in the fish fed CM-AR and CM-TN diets may indicate that in rainbow trout the availability of CM is comparable or a little higher than that of fish meal. Other protein sources, such as soybean, corn gluten, and poultry by-product meal had similar protein availabilities to both herring and menhaden fish meal (Riche and Brown 1999; Sugiura et al., 1998). The significantly lower phosphorus ‘absorption’ (Fig. 4) in the control in comparison to the other groups can be explained by the significantly improved utilization of fish meal phosphorus by plant ingredients (Riche and Brown 1999). This result is also in agreement with the observation by Sugiura et al., (1998) in coho salmon and rainbow trout, where phosphorus ‘absorption’ was inversely correlated with dietary levels of calcium and with phosphorus itself. The authors reported higher phosphorus availabilities in soybean and corn gluten meal, as well as poultry by-product and feather meal, than in both herring and menhaden fish meal. In the present study the concentrations of calcium and phosphorus in the control diet (1.67 and 1.63 g/100 g, respectively) was 1.5 and 2.3 times higher than those in CM-CA, CM-TN, and CM-AR diets (results not presented). The reason for the higher trend in
the faeces iron concentrations in CM-TN (significant) and CM-AR (not significant) compared to that of the control (Fig. 3) might be attributed to the characteristic of gossypol chelation by iron. Owing to the fact that gossypol readily reacts with iron, it was found that the reaction in the intestine resulted in the formation of an insoluble complex which is egested in the faeces (Muzaffaruddin and Saxena 1966; Skutches et al., 1974).

In conclusion, this study demonstrates that CM can be used as a valuable protein source by at least 15% incorporation in diets for juvenile rainbow trout. Fish meal, a traditional protein source in fish feed, can also be completely replaced by a mixture of plant protein (CM and soybean meal) and some other animal by-product proteins. Nutritive values of CM in fish can be different depending on their origin and processing. We documented that fish excrete the (–)-enantiomer faster than the (+)-enantiomer of gossypol, and that approximately 35–50% of dietary gossypol is absorbed by fish, whereas the remaining absorbed gossypol seemed to be excreted with urine and/or through gills. This needs further study.

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