Cottonseed Oilcake as a Protein Source in Feeds for Juvenile Tilapia (Oreochromis niloticus): Antinutritional Effects and Potential Detoxification by Iron Supplementation

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Abstract

Crude cottonseed oilcake, a protein-rich by-product of cottonseed oil production, was used to replace fishmeal in diets for juvenile tilapia (Oreochromis niloticus). Crude cottonseed oilcake was included in the diets at levels of 0% (control), 15%, 30%, and 45%, as an alternative protein source. The impact of the main antinutritional component of cottonseed oilcake, gossypol, on growth and its chronic adverse effects on blood hematocrit and tissue histology were assessed and compared with the documented effects of cottonseed meal. The detoxifying effect of iron (Fe) on gossypol was evaluated in the diets containing 30% and 45% cottonseed oilcake, which were supplemented with ferrous sulfate heptahydrate. As the rate of oilcake rose, the specific growth rate and protein efficiency ratio slightly, but significantly, dropped. Ferrous supplementation compensated for this effect in the 30% diet, but not in the 45% diet. However, iron supplementation led to mild inflammation in the intestine (infiltration of neutrophilic granulocytes), fatty degeneration of the liver, and a lower hepatosomatic index. Nevertheless, due to the beneficial effect on growth, iron supplementation is critical. No histopathological effects of the dietary cottonseed oilcake were observed in the gills, gonads, or heart. In conclusion, the effects of cottonseed oilcake supplementation were moderate when compared to the effects of cottonseed meal and a replacement level of 15-30% is recommended to obtain non-detectable effects.

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Introduction

Diets are generally the largest expenditure in intensive aquaculture operations (40-70% of the production costs), and protein is the most expensive dietary component (Shamshak and Anderson, 2009). In view of depleting wild stocks, fishmeal, the conventional protein source in fish feeds, needs to be substituted to allow for sustainable aquaculture development (FAO, 2009). Thus, the replacement of fishmeal with less costly plant feedstuff must be emphasized. However, the presence of antinutritional factors and other active biological compounds limit the use of substitutes, and toxic effects have been reported in fish (Francis et al., 2001; Lee et al., 2006).

Turkey is one of the biggest cotton producers in the world, producing 1,440,000 tons of cottonseed in 2008 (FAO database). Crude cottonseed oilcake is an inexpensive and readily available by-product of oil extraction from cottonseed. Annual production of oilcake is projected to grow an average of 2.3% annually (Ramachandran et al., 2007). Above all, the increased production of organic cotton over the last decade provides pesticide-free quality oilcake, imperative for the production of human food products (OTA, 2009). Cottonseed by-products rank third in the world among produced vegetable protein concentrates (Chhorn, 1996) and are available at much lower costs than animal proteins. In addition, the use of by-products is a dictate of environmental sustainability, particularly with regard to the declining water resources in the eastern Mediterranean region.

Traditionally, undecorticated cottonseed is crushed and screw-pressed, providing a protein-rich crude oilcake that is characterized by high fiber and protein contents. In recent years, additional extraction procedures (fat, protein) deliver a dry cottonseed meal of potentially better quality (Lynn and Wedegaertner, 1986). Due to their high protein content of up to 40% (Ramachandran et al., 2007), cottonseed products - oilcake and meal - could be used as an inexpensive protein source in fish feeds. However, gossypol, a secondary metabolite stored in the pigment glands of cottonseed, is well-known for numerous antinutritional effects that limit its use as an alternative protein source (Rinchard et al., 2003; Yue and Zhou, 2008).

While non-ruminant animals such as swine and poultry are particularly sensitive, fish can tolerate relatively high levels of free gossypol (Roehm et al., 1967; Cheng and Hardy, 2002). Studies on fish have focused on cottonseed meal supplementation, reporting adverse effects on blood parameters (hematocrit and hemoglobin), growth, and development (Blom et al., 2001; Rinchard et al., 2003). The addition of iron salts to the diet reduces gossypol toxicity (Wenegaertner, 1981; Yildirim et al., 2003). Still, reports are controversial and the level of dietary iron necessary to prevent toxic effects has not been conclusively determined.

Here, in contrast to other studies, we studied the feasibility of fishmeal replacement by cottonseed oilcake (and not cottonseed meal) at a moderate level (<50%) and iron supplementation (in diets containing 30% or 45% cottonseed oilcake) with regard to gossypol detoxification. Focusing on chronic sublethal effects, we carried out a comprehensive histopathology of the major organs reported for adverse gossypol impact in order to determine a safe substitution level on the basis of the non-detectable effect concept recently raised by animal welfare concerns.

Materials and Methods

Experimental diets. Cottonseed oilcake was obtained from a commercial feed company (Altin Yem Co. Izmir, Turkey), ground, and used to substitute fishmeal at 0% (control), 15%, 30%, or 45% (Table 1). To assess the detoxifying effect of iron on gossypol, the 30% and 45% diets were supplemented with ferrous sulfate heptahydrate - Fe₂(SO₄)₃ - at 900 and 1350 mg Fe/kg feed, respectively, on a dry matter basis. The gossypol content of the cottonseed oilcake was determined according to Kim and Calhaun (1995) with modifications according to Blom et al. (2001). The free and total gossypol contents of the oilcake were 0.08 mg/kg and 0.47 mg/kg, respectively. The six experimental diets were isonitrogenous and isoenergetic. The dry ingredients were mixed with water and
lipids in a laboratory pelleting machine (Alexanderwerk AG, Remscheid, Germany) with a 1.0 mm diameter die, dried at room temperature for three days, and stored at 4°C.

**Experimental setup.** The 8-week feeding trial was performed at the Department of Animal Breeding and Aquaculture in the Tropics and Subtropics at Humboldt University, Berlin. Eighteen 240-l tanks with two recirculation systems were randomly stocked with eight tilapia (62.95-65.66 g), each, providing triplicates for each diet. Each recirculation system comprised nine tanks, a sedimentation chamber, and a trickling filter. The mean water temperature, pH, dissolved oxygen, and conductivity were determined daily (WTW Multi 340 I, Weilheim, Germany). There were insignificant differences between the two systems (A/B: 24°C/24°C, pH 7.98/8.14, 7.17/7.25 ppm O2, 802/792 µsm/cm). The fish were fed three times a day by hand to apparent satiation, which amounted to an average of 2.0% of the total biomass, adjusted according to biweekly weighings.

For body composition analysis, three fish were pooled, homogenized, and analyzed as a pooled sample. Three pooled samples were taken before the trial and three from each treatment (one per replicate) at the end of the trial. The fish were ground whole and stored at -20°C until analyzed. At the end of the experiment, blood from five randomly sampled fish per tank was drawn from the caudal vein with heparinized syringes. Plasma was immediately separated by centrifugation (12000 g × min) for quantification of the hematocrit of an individual fish (determined as the mean of 3-5 readings). From each dietary treatment, 14 fishes (seven of each sex) were dissected to determine hepatosomatic index (HSI) and gonadosomatic index (GSI) and selected tissues (intestine, gonad, Gill, heart, liver) were sampled for histopathology. For each treatment, mortality, specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER), and condition factor (CF) were calculated as the average of the triplicates.

**Dietary and whole fish nutrient analysis.** The experimental diets and whole body samples were analyzed for dry matter (DM), ash, crude protein, crude fat, and crude fiber according to Schulz et al. (2007): dry matter was determined after drying at 105°C for 24 h, ash after combustion at 550°C for 4 h, crude protein (N × 6.25) by the Kjeldahl distillation method (Kjeltac System, Tecator, Sweden), crude fat after extraction with petroleum ether by the Soxhlet method (Soxtec System HT, Tecator, Sweden), and crude fiber in a Fibertec TM 1020 hot extractor and Fibertec System 1021 cold extractor (Tecator TM Technology, Sweden).

**Histology.** After dissection, tissue samples were fixed in Bouin’s solution for 12 h, transferred to an automated tissue processor (Bavimed) for dehydration, embedded in
paraffin (DDM-P064 embedding centre, MEDIM), sectioned into 5-μm slices (motorized rotary microtome RM 2065 SuperCut, Leica), and stained with hematoxylin-eosin. To determine iron accumulation in livers of fish fed the iron-supplemented diets, liver sections were stained with Perls’ Prussian blue (Perls, 1867) and analyzed under a Leica DMRBE microscope equipped with a digital camera.

Statistical analysis. Data are presented as means±standard deviations (SD) of triplicate groups or individuals evenly sampled from each replicate. Data were analyzed for normal distribution by Kolmogorov-Smirnov and equal variance by Kruskal-Wallis one-way analysis of variance (p<0.05) using SigmaStat 2.0 software (Jandel Scientific, San Rafael, CA). Multiple comparison was carried out by parametric Dunnett’s Test or nonparametric Dunn’s Test.

Results

Growth. No acute toxicity was observed and survival was comparable in all treatments and tanks (Table 2). Final body weight was comparable in all groups, but there were significant differences in SGR, FCR, PER, and CF. In general, growth parameters were low; SGR was highest in fish fed the control diet and significantly lower in fish fed diets with high oilcake supplementation levels. Iron supplementation compensated for this impact in the 30% diet but not in the 45% diet. Generally, FCR negatively correlated with SGR. PER decreased as the oilcake replacement increased. Again, iron compensated for this impact in the 30% diet but not in the 45% diet. Whole body compositions were not significantly influenced by treatment, except for the extremely low lipid content in the 30% group.

Table 2. Growth and feed efficiency (means±SD) of Nile tilapia juveniles fed diets containing different levels of cottonseed oilcake as a replacement of fishmeal, with or without iron (Fe) supplementation.

<table>
<thead>
<tr>
<th>Diet</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>30+Fe</th>
<th>45</th>
<th>45+Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial wt (g)</td>
<td>64.33±2.63</td>
<td>65.66±2.99</td>
<td>65.04±3.21</td>
<td>63.79±2.82</td>
<td>62.95±1.13</td>
<td>64.21±4.58</td>
</tr>
<tr>
<td>Wt gain (g)</td>
<td>37.82±0.84 ab</td>
<td>33.80±1.63 ab</td>
<td>30.63±2.38 a</td>
<td>34.13±3.51 a b</td>
<td>30.22±4.86 a b</td>
<td>29.73±2.77 a b</td>
</tr>
<tr>
<td>SGR (%/d)</td>
<td>0.83±0.04 a</td>
<td>0.74±0.04 ab</td>
<td>0.69±0.03 a</td>
<td>0.76±0.06 a b</td>
<td>0.70±0.10 a b</td>
<td>0.68±0.01 a b</td>
</tr>
<tr>
<td>FCR</td>
<td>1.48±0.06 a</td>
<td>1.61±0.09 ab</td>
<td>1.73±0.07 ab</td>
<td>1.55±0.15 ab</td>
<td>1.73±0.28 ab</td>
<td>1.79±0.04 b</td>
</tr>
<tr>
<td>PER</td>
<td>2.33±0.17 ab</td>
<td>2.10±0.10 ab</td>
<td>1.88±0.15 b</td>
<td>2.25±0.23 a</td>
<td>1.84±0.30 ab</td>
<td>1.77±0.05 a b</td>
</tr>
<tr>
<td>CF</td>
<td>1.77±0.10</td>
<td>1.78±0.02</td>
<td>1.84±0.09</td>
<td>1.73±0.03</td>
<td>1.73±0.04</td>
<td>1.74±0.03</td>
</tr>
<tr>
<td>Survival (%)</td>
<td>95.83±7.22</td>
<td>100±0.00</td>
<td>100±0.00</td>
<td>100±0.00</td>
<td>100±0.00</td>
<td>100±0.00</td>
</tr>
</tbody>
</table>

Whole body composition

<table>
<thead>
<tr>
<th>Diet</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>30+Fe</th>
<th>45</th>
<th>45+Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry matter</td>
<td>295±5</td>
<td>312±24</td>
<td>262±30</td>
<td>309±38</td>
<td>316±16</td>
<td>304±7</td>
</tr>
<tr>
<td>Crude protein</td>
<td>138±4</td>
<td>160±6</td>
<td>133±11</td>
<td>156±17</td>
<td>169±15</td>
<td>160±7</td>
</tr>
<tr>
<td>Crude lipid</td>
<td>107±2 ab</td>
<td>112±17 a</td>
<td>70±7 a</td>
<td>113±27 a</td>
<td>102±17 a</td>
<td>105±8 ab</td>
</tr>
<tr>
<td>Crude ash</td>
<td>65±1</td>
<td>62±3</td>
<td>44±6</td>
<td>40±25</td>
<td>59±19</td>
<td>52±19</td>
</tr>
</tbody>
</table>

Means in row with different superscripts significantly differ (p<0.05).

1 Specific growth rate = 100 (ln Wf - ln Wi)/duration of experiment in days
2 Feed conversion ratio = Wf/total feed supplied/(Wf - Wi)
3 Protein efficiency ratio = (Wf - Wi)/Wd supply of dietary protein

Gonadosomatic index (GSI), hepatosomatic index (HSI), hematocrit level, and nutrient composition of fish. There were no significant differences (p>0.05) among dietary treatments in terms of GSI for males or females; GSI was higher in females than in males, though not significantly. The HSI decreased from 1.9% to 0.7% in males and from 2.3% to 1% in females as the percent of oilcake supplementation increased (Fig. 1). Hematocrit levels varied 26.6-36.6% with no differences between sexes or treatments.

Histological examination. No histopathological changes were evident in the gills, gonads, or heart of fish fed oilcake supplemented diets. However, pathological changes were detected in the liver and intestine, the most prominent was a fatty degeneration within the liver (Fig. 2). There were highly vacuolated hepatocytes with severe fat
deposition predominantly in the periportal region. Further, a moderate centrolobular fat deposition was frequently observed in these samples. Iron accumulated within the centrolobular region in groups fed iron-supplemented diets. Additionally, there was mild infiltration of neutrophilic granulocytes within the lamina propria and tela submucosa of the intestine in fish fed the iron-supplemented diets (Fig. 3).

**Fig. 1.** The (a) hepatosomatic index (HSI; means±SD; n = 7; asterisks indicate statistical difference from control, p<0.05, Dunnett’s) and (b) hematocrit (means±SD; n = 7; no statistical differences, p>0.05, Dunnett’s) of tilapia juveniles (males grey, females white) fed diets with different levels of cottonseed oilcake replacement of fishmeal.

**Fig. 2.** Liver histology of juvenile tilapia fed (a) the unsupplemented control, (b) a diet with 45% fishmeal replaced by cottonseed oilcake, and (c) the 45% replacement diet enriched with 1350 mg iron per kg feed. After eight weeks of feeding, hepatocytes were unremarkable in control fish but had moderate (m) and severe (s) fatty degeneration (HE staining), and mild iron accumulation (blue color; Perls’ staining), in fish fed supplemented diets.

**Fig. 3.** Intestines of juvenile tilapia fed (a) unsupplemented control and (b) iron-supplemented diets. There was mild infiltration of neutrophilic granulocytes (nc) in the tela submucosa of juveniles fed the iron-supplemented diet. le = lamina epithelialis, lp = lamina propria.
Discussion

With regard to the political debate on sustainable aquaculture, the use of cottonseed oilcake as an alternative protein source is an economically promising application and an ecological challenge. Although previous studies in a variety of fishes have shown antinutritional effects associated with feeding cottonseed meals, results vary considerably and the potential for iron detoxification is yet unclear. In this study, crude cottonseed oilcake derived as a first and inexpensive by-product after oil extraction was used as an alternative protein source. This product is characterized by a relatively moderate gossypol concentration (0.08 mg/kg free gossypol, 0.47 mg/kg total gossypol), but is as protein-rich as cottonseed meal.

Here, 0.08% free gossypol was comparable to concentrations reported regularly for cottonseed meal (0.07-0.18%). In contrast, total gossypol was lower in the oilcake (0.47%) than the usual range (1.15-1.49%) reported for meal (Waldroup, 2002). Thus, bound gossypol is substantially lower in the oilcake. Bound gossypol is considered nontoxic to animals and toxicity is attributed exclusively to free gossypol. Still, it should be considered that bound gossypol represents mostly covalent adducts of gossypol to proteins, from which free gossypol can be (partially) liberated by acidification or microbial activity in the gut.

The impact on growth parameters was low and significant only in fish fed the 30% and 45% oilcake diets. At concentrations of 315 mg/kg free gossypol (30%) and 630 mg/kg (45%), growth inhibition was comparable to early findings in tilapia where tolerance for high levels (0.18%) of free gossypol was reported with no loss in growth, feed efficiency, or survival when purified gossypol acetate (0.1-0.2% diet) was used (Robinson et al., 1984). However, in the same study, growth and feed efficiency was reduced when cottonseed meal was used as a replacement, suggesting the existence of antinutritional factors other than gossypol. This is further supported by the substantially reduced growth performance observed when glandless free cottonseed was used. Feed quality and composition may have contributed to this effect. The crude, unprocessed (press) oilcake used here did not result in reduced growth in contrast to the deoiled or even solvent extracted meals commonly used. Growth was low in all dietary groups including the control, similar to findings of Mbahinzireki et al. (2001) who reported weight gains of 0.54 g/d in the control, 0.59 g/d in the 25% cottonseed meal replacement group, and 0.25 g/d in the 100% cottonseed meal replacement group.

Depending on the parameter evaluated, the toxic range for catfish is 300-1200 mg/kg free gossypol (Yildirim et al., 2003). In rainbow trout, 300 mg/kg free gossypol resulted in considerable growth depression (Rinchard et al., 2003). Compared to our results, growth inhibition in juvenile tilapia was more pronounced in most studies (Robinson et al., 1984; El-Saidy and Gaber, 2004). It might be that adult tilapia are less sensitive to gossypol or exhibit an increased detoxification mechanism. Other studies suggest that tilapia might be less susceptible than other species.

Adverse effects on blood parameters have been explained by a gossypol-mediated impact on intestinal iron absorption and reduced iron bioavailability which may lead to reduced hemoglobin formation and anemia (Skutches et al., 1974; Yildirim et al., 2003). Here, hematocrit values ranged 27-36% and were comparable to normal values reported for tilapia (Aly et al., 2008; Giron-Perez et al., 2008; Yue and Zhou, 2008). Some authors reported reduced hematocrit with increasing replacement (El-Saidy and Gaber, 2004; Yue and Zhou, 2008). Our data do not provide support of significant differences between treatments or an impact on hematopoiesis.

Iron supplementation has been suggested to compensate for gossypol toxicity by formation of an iron-gossypol complex in the intestinal tract, preventing gossypol absorption (Wenegaertner, 1981; Yildirim et al., 2003). This beneficial effect of iron supplementation has been reported in a number of studies. In the iron-supplemented 30% oilcake diet, growth parameters were indeed slightly increased compared to the unsupplemented 30% group. Still, at 45% replacement, no effect of iron was observed. The histological findings indicate potential accumulation of iron-gossypol complexes in the liver which may lead to chronic intoxication. The HSI was lowest in the iron-
supplemented 45% cottonseed group, indicating adverse effects on the liver, but further research is needed. Liver abnormalities, including liver necrosis, have been reported in rainbow trout fed a diet containing 0.0531% free gossypol (Herman, 1970). The symptoms observed here might precede such toxic effects. Mild to severe fat degeneration of the liver was observed with increasing oilcake inclusion. No other histopathological changes were observed in gills, heart, or gonads.

Gossypol can have a severe impact on reproductive parameters. Cottonseed meal significantly reduced GSI of female fish associated with depressed growth and a consequent delay in gonad maturation while males were not affected (Dabrowski et al., 2000; El-Saidy and Gaber, 2004). Estrogenic compounds, e.g., quercetin and other isoflavones, have been reported in cottonseed (Tacon, 1997), and these might compensate for some of the effects of gossypol. Histopathology of gonad tissue and GSI revealed no differences between groups or abnormalities (e.g., ovotestis).

In conclusion, moderate replacement of up to 45% cottonseed oilcake resulted in slightly reduced growth while 15-30% of the fishmeal can be replaced by cottonseed oilcake without impact. Above 30% cottonseed oilcake, the impact on growth must be considered, although no evidence of adverse chronic effects was detected in the blood parameters or histopathology of the major organs. Taking into account the variability of results reported when cottonseed meal was used as a replacement, one may speculate that the quality of cottonseed as a protein source depends on the use of pesticides, although this issue has not yet been considered.

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