Appendix - reprint
Dietary vitamin A requirement of juvenile greasy grouper (Epinephelus tauvina)

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Received 7 June 2002; received in revised form 6 December 2002; accepted 9 December 2002

Abstract

A 10-week growth study was conducted to determine the dietary vitamin A requirement of juvenile greasy grouper. Eight semipurified diets containing 210, 476, 914, 1774, 3764, 7063, 14,342 and 29,193 IU vitamin A (as retinyl acetate)/kg diet were fed twice daily to triplicate groups (20 fish/group) of fish (initial weight 5.84 ± 1.5 g/fish). Fish fed the basal diet (210 IU vitamin A/kg diet) developed haemorrhages in the skin overlaying the base of the fins and erosion on the caudal peduncle. None of these deficiency signs were observed in fish fed the vitamin A supplemented diets. Significantly higher weight gain, protein efficiency ratio and survival and lower feed conversion ratio were observed in fish fed the 3764 IU/kg diet than fish fed the other diets. Whole body crude fat concentrations significantly decreased in fish fed increasing levels of dietary vitamin A. Addition of vitamin A to the basal diet did not significantly affect whole body protein and ash concentrations of the fish. However, dietary vitamin A supplementation did not significantly affect whole body moisture concentration. Serum vitamin A concentration was affected by dietary vitamin A level. Increasing serum vitamin A concentration was observed as dietary vitamin A level increased from 210 to 3764 IU/kg and tended to plateau in fish fed higher vitamin A levels. Broken-line regression analysis showed that juvenile greasy grouper require a minimum of 3101 IU vitamin A/kg diet for maximal growth. The associated serum vitamin A concentration was 179 μg/ml.

Keywords: Epinephelus tauvina; Greasy grouper; Nutrition; Retinal; Vitamin A

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0044-8486/03/$ - see front matter © 2003 Published by Elsevier Science B.V.

doi:10.1016/S0044-8486(02)00665-8
1. Introduction

Vitamin A occurs in three forms: the alcohol (retinol), aldehyde (retinal) and acid (retinoic acid) in animal tissues. The main physiological functions of vitamin A are differentiation of epithelial tissues, resistance to infections, proper growth, reproduction and vision (Olson, 1984). Fish like other animals lack the capacity for de nova synthesis of vitamin A, thus they require a dietary source for proper growth. Few studies have been conducted on the quantitative dietary vitamin A requirement in fish. For example, the vitamin A requirement of rainbow trout and salmon is 1000–3500 IU/kg (Kitamura et al., 1967; Halver, 1989), common carp 4000–20,000 IU/kg (Aoe et al., 1968; Subenda and Djajadiredja, 1985), channel catfish and sea bream is 2000–2500 IU/kg (Halver, 1989) and guppy is 2000–4000 IU/kg (Shim and Tan, 1990).

The epinepheline serranid groupers are commercially important, highly priced food fish in several countries. The greasy grouper Epinephelus tauvina is of the most economically important finfish species of India. However, information on the dietary vitamin A requirement of greasy grouper is lacking. The purpose of the present study was to estimate the dietary vitamin A requirement of juvenile greasy grouper.

2. Materials and methods

2.1. Diet formulation and preparation

The basal diet was formulated to provide 34% of crude protein (Table 1). Casein and gelatin served as intact protein sources and provided a total of 9.8% of crude protein. An amino acid mixture supplied the remaining 23.8% of crude protein. Supplementation of the dietary essential amino acids for greasy grouper was based on the highest known requirements for fish (National Research Council (NRC), 1993). Eight semipurified test diets were formulated to contain vitamin A concentrations of 0, 500, 1000, 2000, 4000, 8000, 16,000 and 32,000 IU/kg diet (0, 150, 300, 600, 1200, 2400, 4800 and 9600 retinol equivalent [RE]/kg) as retinyl acetate. The analysed dietary vitamin A concentrations of the eight diets were estimated by HPLC (Driskell et al., 1982) to be 210 (unsupplemented basal diet), 476 (500), 914 (1000), 1774 (2000), 3764 (4000), 7063 (8000), 14342 (16,000) and 29,193 (32,000) IU/kg diet. Vitamin A was added to the basal diet at the expense of cellulose. Dry ingredients were mixer in a twin shell mixed before the oils were added. Diets were then transformed to a Hobart mixer where vitamin A was added in an oil carrier. The oils and additional water were then added to the dry ingredients and mixed. Diets were adjusted to pH 7.0 ± 0.1 with saturated sodium hydroxide (Wilson et al., 1977). The resulting dough was extruded through a pelletizer with a die into small strands and pelleted. Initially, diet particle size was ~ 0.25 mm; after 3 weeks of feeding, this was increased to 0.5 mm; after 6 weeks to 1.66 mm diameter. The diets were dried at room temperature for 48 h, then packed in plastic bags, sealed and stored at ~ 20 °C until needed. Approximately 1 week’s allowance of each diet was removed from the freezer, broken into proper size and held in a refrigerator at 4 °C until fed.
Table 1
Composition of the basal diet fed to greasy grouper

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount g/kg dry mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>80.0</td>
</tr>
<tr>
<td>Gelatin</td>
<td>18.0</td>
</tr>
<tr>
<td>Dextrin</td>
<td>335.0</td>
</tr>
<tr>
<td>Fish oil</td>
<td>60.0</td>
</tr>
<tr>
<td>Corn oil</td>
<td>30.0</td>
</tr>
<tr>
<td>Carboxy methyl cellulose</td>
<td>20.0</td>
</tr>
<tr>
<td>Alpha cellulose</td>
<td>105.7</td>
</tr>
<tr>
<td>Amino acid mixture</td>
<td>238.0</td>
</tr>
<tr>
<td>Vitamin premix</td>
<td>3.3</td>
</tr>
<tr>
<td>C2MP-Na</td>
<td>20.0</td>
</tr>
<tr>
<td>Mineral premix</td>
<td>80.0</td>
</tr>
</tbody>
</table>

1 Proximate analysis of basal diet (%): moisture, 13; crude protein, 34; ether extract, 8; ash, 4; crude fiber, 7.
2 Essentially vitamin-free (89% crude protein).
3 L-Amino acid mixture supplied the diets with (g/kg dry diet): leucine, 30.7; valine, 22.8; phenylalanine, 20.3; tyrosine, 19.2; isoleucine, 18.2; threonine, 17.8; aspartic acid, 15.0; glutamic acid, 15; serine, 14.9; proline, 14.8; arginine - HCl, 14.0; lysine - HCl, 11.0; histidine, 9.4; cysteine, 5.1; methionine, 4.9; tryptophan, 4.9.
4 Vitamin premix supplied the diets with (mg/kg dry diet): choline chloride, 600; inositol, 450.0; niacin, 168; calcium pantothenate, 102; dl-alpha-tocopheryl acetate, 80; thiamine mononitrate, 45; pyridoxine HCl, 40; riboflavin, 22; menadione, 15; folic acid, 10; biotin, 0.4; cholic acid, 0.1; cyanocobalamin, 0.04.
5 Contained 25 g/100 g of ascorbic acid equivalent, as l-ascorbyl-2-monophosphate-Na.
6 Mineral premix supplied the diets with (g/kg of premix): CaCO3, 350; Na2HPO4, H2O, 200; KH2PO4, 200; NaCl, 12; MgSO4.7H2O, 10; FeSO4.7H2O, 2; MnSO4.7H2O, 2; AlCl3.6H2O, 1; CuCl2.2H2O, 1; KF, 1; Na2MoO4.2H2O, 0.5; Na2SeO3, 0.4; CoCl2.6H2O, 0.1; KI, 0.1.

2.2. Experimental fish and husbandry

All greasy groupers (E. tauvina) hatched from a single egg mass were used in this experiment. All fish were acclimated to laboratory conditions for 1 month in two plastic tanks (75 W × 95 L × 50 H cm) prior to initiation of the experiment and fed a commercial diet. The proximate composition (%) of the commercial diet was moisture, 13; crude protein, 33; ether extract, 9; ash, 3; crude fibre, 6. After acclimatization, groups of 20 randomly chosen fish ranging from 5.7 to 5.9 g/fish (5.84 ± 1.5 g) were stocked into 21 flow-through 85-l aquaria. Each aquarium, filled with 80 l of seawater, was covered with a wire-mesh lid and provided with continual aeration from an air blower. Water was pumped at 21/min per aquarium through two separate bio filters to remove impurities and reduce ammonia concentration. Fish were acclimated to the experimental system for 2 weeks prior to the experiment. During the first week, all fish were fed the commercial diet and, during the second week, all fish were fed the basal diet to reduce body stores of vitamin A. After acclimatization, all fish were fed their respective test diet, which had been randomly assigned to triplicate aquarium. Water quality was monitored daily and was within acceptable limits throughout the experiment. Temperature was 28 ± 1 °C, dissolved oxygen concentrations were >6.0 mg/l, ammonia-nitrogen concentrations were not >0.22 mg/l and nitrite-nitrogen did not exceed 0.07 mg/l at any point during the study. The diurnal light/dark cycle of the aquaculture facility remained at 12-h light/dark.
throughout the study. Fish were fed their respective diets at a rate 5% (weeks 1–6) and 4% (weeks 7–12) of their body weight per day during the 12-week experiment. The daily ration was subdivided into two equal feedings and fed at 0900 and 1700 h. Daily feed allowances were adjusted every 2 weeks based on sampling weight. Impurities were removed at 1500 h each day to maintain water quality. Unfed diet was collected 1 h after each feeding and the dry matter content was determined for both supplied and uneaten diet (dried at 105 °C). When mortality occurred, dead or moribund fish were examined to determine if the cause of death was from overt nutritional deficiency signs or from infections by visual inspections.

2.3. Sample collection and analysis

All fish were anaesthetized with 60 mg/l tricainemethanesulfonate, weighed 24 h after the final feeding and examined externally and internally for overt nutritional deficiency signs. Five randomly chosen fish were collected from each dietary replicate and frozen at −20 °C for subsequent proximate carcass analysis. Analysis of dry matter (drying at 105 °C for 24 h), crude protein (by Kjeldhal apparatus, nitrogen × 6.25), crude fat (extraction with petroleum ether by Soxhlet apparatus) and ash (incineration at 600 °C for 24 h) were performed for both carcass and diets. Blood from five randomly chosen fish in each dietary replicate was collected from the caudal vein using 1-ml plastic sterile syringe without anticoagulant to clot (4 °C for 2 h). Serum was separated by centrifugation at 3000 rpm for 10 min and stored at −8 °C for vitamin A analysis (Driskell et al., 1982).

2.4. Statistical analysis

Data were analysed by one-way ANOVA. When the ANOVA identified differences among groups, a multiple comparisons test among means was performed with Duncan's new multiple range test. Statistical analysis was preformed using the computer software package Statistica (Statistical software Statsoft, Tulsa, OK) for windows, release 4.5. Accepted level of significance was 0.05. The broken-line regression procedure of Robbins et al. (1979) was used to determine the breakpoint that represents the minimum dietary vitamin requirement for the maximum response.

3. Results

Mortality initially began on day 49 among fish fed the basal diet and continued throughout the experiment. After 42 days of feeding on the basal diet, fish began to show vitamin A deficiency signs. By day 43, about 30–35% of fish fed the basal diet showed haemorrhages on the skin overlaying the base of the fins. By day 45, 28% of fish in this treatment had developed severe erosion on the caudal peduncle. Fish in this group had greater mortality, poorer weight gain, feed conversion ratio and protein efficiency ratio than the other groups. By the end of week 12, about 32% of fish fed the basal diet had died. None of these deficiency signs were observed in fish fed any of the vitamin A supplemented diets, which had 91% survival or more.
Dietary vitamin A supplementation significantly improved growth of juvenile greasy grouper (Table 2). Weight gain was highest for fish fed the diet containing 3764 IU vitamin A/kg, followed by fish fed the diet containing 7063 IU vitamin A/kg, intermediate for fish fed the diets supplemented with 914, 14342 and 29,193 IU vitamin A/kg, and lowest in fish fed the diet containing 476 IU vitamin A/kg. Addition of vitamin A to the basal diet significantly increased protein efficiency ratio and decreased feed conversion ratio. Feed conversion ratio was lowest \( (P<0.05) \) for fish fed the diet containing 3764 IU vitamin A/kg followed by fish fed the diet containing 7063 IU vitamin A/kg and highest \( (P<0.05) \) for fish fed the diets containing 476, 914, 1774, 14342 and 29,193 IU vitamin A/kg. The pattern of difference in protein efficiency ratio was similar to findings of feed conversion ratio.

There were significant differences between fish fed the basal diet and the diets containing vitamin A with regard to whole body crude protein, fat and ash (Table 3). However, no difference in whole body moisture between dietary treatments was observed.
Addition of vitamin A to the basal diet significantly decreased whole body fat concentration. Fish fed the basal diet significantly showed higher body fat concentration than fish fed the diets containing vitamin A. Whole body fat concentration was lower for fish fed the diets containing >7063 IU vitamin A/kg and intermediate for fish fed the diets containing <3764 IU vitamin A/kg. Addition of vitamin A to the basal diet did not significantly affect whole body protein and ash concentration of the fish. There were significant differences in serum vitamin A concentration between fish fed the control diet and fish fed the diets containing vitamin A (Fig. 1). Addition of vitamin A to the basal diet up to 3764 IU/kg significantly increased serum vitamin A concentration, thereafter no differences were observed with further supplementation. Serum vitamin A concentration was highest \((P<0.05)\) for fish fed the diet containing 3764 IU vitamin A/kg followed by fish fed the diets containing 1774, 914 and 476 IU vitamin A/kg.

The minimum dietary vitamin A requirement for maximum weight gain, feed conversion ratio and protein efficiency ratio was established by the broken-line regression procedure (Table 4). The dietary vitamin A requirement for maximum weight gain was 3101 IU/kg.

<table>
<thead>
<tr>
<th>Response</th>
<th>Dietary vitamin A requirement (IU/kg)</th>
<th>Serum vitamin A (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight gain</td>
<td>3101</td>
<td>179</td>
</tr>
<tr>
<td>Feed conversion ratio</td>
<td>2666</td>
<td>154</td>
</tr>
<tr>
<td>Protein efficiency ratio</td>
<td>2502</td>
<td>144</td>
</tr>
</tbody>
</table>
Fig. 2. Weight gain percentage and dietary vitamin A requirement of juvenile greasy grouper fed various levels of vitamin A. Each point represents the mean of three groups of fish with 20 fish per group (n = 3). Requirement derived with the broken line method for weight gain percentage is 3101 mg vitamin A/kg diet.

diet (Fig. 2) \( Y = 250.15 + 0.0445x \), \( r = 0.98 \) and \( Y = 397.63 - 0.0029 \), \( r = -0.85 \) and for feed conversion ratio and protein efficiency ratio were 2666 \( Y = 2.0203 + 0.00015x \), \( r = 0.97 \) and \( Y = 1.5835 + 0.0000080x \), \( r = +0.75 \) and 2502 \( Y = 1.439 + 0.00016x \), \( r = 0.97 \) and \( Y = 1.8661 - 0.0000088x \), \( r = -0.75 \) IU/kg diet, respectively. Associated serum vitamin A concentration for weight gain, feed conversion ratio and protein efficiency ratio were 179, 154 and 144 \( \mu g/ml \), respectively, estimated by the broken line procedure.

4. Discussion

This study indicated that a dietary source of vitamin A is essential for juvenile greasy grouper. Nutrient requirements in fish can be estimated by broken-line regression procedure (Robbins et al., 1979). Application of this procedure resulted in minimum dietary vitamin A requirement for juvenile greasy grouper ranging from 2502 to 3101 IU/kg diet. Nutrient requirement estimates based on weight gain can be misleading, because an animal can deposit a large amount of fat without real growth (Phillips and Brockway, 1957). In this study, the weight gain of juvenile greasy grouper plateaued at a dietary level of 3764 IU vitamin A/kg and the whole body fat concentration in fish tended to decrease as the dietary vitamin A level increased. These results indicate that juvenile greasy grouper had oxidized their body fat and had showed real growth. The estimate of dietary vitamin A
requirement in this study based on weight gain must therefore be the requirement of juvenile greasy grouper and was 3101 IU/kg diet.

The dietary vitamin A requirement of greasy grouper obtained in this study is comparable to the dietary vitamin A requirements for guppy (Shim and Tan, 1990) and rainbow trout (Kitamura et al., 1967; Halver, 1989). Although, comparisons of dietary vitamin A requirements among species are difficult due to differences in the duration of the study, initial weight of fish, the initial body pool of vitamin A concentrations, dietary composition and experimental conditions. Furthermore, the disparities in methodology and assessment criteria that are used to quantify the vitamin A requirements vary with some results based on the supplementation levels that are actually analysed.

This study indicates that a dietary vitamin A deficiency caused haemorrhages in fins of greasy grouper. Vitamin A deficiency is characterized by abnormal bone formation, exophthalmia, haemorrhage in the anterior eye chamber, night blindness, poor growth and vision and retinization of epithelial tissue (Halver, 1989).

Whole body fat concentration has been used as an indicator of vitamin status in a variety of animals. Singh et al. (1969) and Ramachandran et al. (1986) observed that the higher plasma free fatty acids and liver fat in vitamin A fed rats were due to the mobilization of fatty acids from adipose tissue. A similar phenomenon was also reported in trout (Poston, 1970) and guppy (Shim and Tan, 1990). These results are in agreement with the present study. However, Thompson et al. (1995) found that differential vitamin A intake had no marked effect on the body composition of rainbow trout.

Thompson et al. (1995) suggested that dietary vitamin A supplementation reflected serum vitamin A concentrations in rainbow trout. Vitamin A concentration in the serum of fish fed the diets containing vitamin A increased as the dietary vitamin A supplementation level increased to 3764 IU/kg, and no significant change found with further increases in the dietary vitamin A level, indicating that the optimum level of dietary vitamin A was reached in terms of maximizing the serum vitamin A concentration. Thus, serum vitamin A concentration can be used as an indicator of vitamin A status in juvenile greasy grouper.

Acknowledgements

This study was financially supported by Council of Scientific and Industrial Research (CSIR), New Delhi (6/652 (10) 2000-EMR-I) to JSM and NATP (ICAR), New Delhi (28 (1) 2000-NATP/CGP-II/223) to MPM.

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