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Dietary carotenoid pigment supplementation influences hepatic lipid and mucopolysaccharide levels in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

We assessed the effects of dietary carotenoid pigment supplementation on liver histochemistry in the rainbow trout. One hundred and eight rainbow trout (mean mass 266 ± 10 g) were assigned to each of three replicate tanks for each of three dietary treatments; astaxanthin, canthaxanthin, or control at a target dietary inclusion of 100 mg/kg, by top-coating a pigment-free commercially extruded basal diet (Trouw Aquaculture, U.K.). Fish were fed for 3 weeks at a ration of 1.2% body mass/day, in a recirculating freshwater system maintained at 16 °C. Frozen liver sections were stained for total lipids, unsaturated lipids, glycogen, mucopolysaccharides, glycogen phosphorylase and aspartate aminotransferase. Relative amounts were measured quantitatively by image analysis. Carotenoid treatment significantly ($P < 0.05$) altered the total lipid profile and hepatic mucopolysaccharide contents of livers of rainbow trout. Results are discussed in relation to the catabolic potential of the liver in carotenoid pigment metabolism.
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Keywords: Astaxanthin; Canthaxanthin; Glycogen phosphorylase; Liver; Mucopolysaccharides; Rainbow trout

1. Introduction

The characteristic pink colour of salmon flesh is caused by the deposition of ingested carotenoids (mainly astaxanthin) in the muscle. However, fish are unable to synthesize pigmenting carotenoids de novo, and must therefore obtain these from dietary sources.

Beyond their functions as flesh pigmenters, carotenoids have also been shown to have various other functions in the animal kingdom, including provitamin A (Al-Khalifa and Simpson, 1988; Guillou et al., 1989; Schiedt et al., 1985), antioxidants (Christiansen et al., 1995); O_2 quenchers/free radical scavengers (Miki, 1991), skin coloration during sexual maturation, and as signal substances in reproduction (Torrissen, 1990).

The liver, being the central organ of intermediary metabolism (including the biotransformation of carotenoids), should reflect the effects of carotenoids on fish metabolism (Segner et al., 1989). Histological techniques are considered to be an important determinant for the evaluation of the effects of food additives (Johnson and Bergman, 1984). Indeed, livers of *Oreochromis niloticus* and *Colisa labiosa* were found to have higher levels of glycogen when the fish were fed diets with increasing levels of astaxanthin, and this finding suggested that dietary astaxanthin supplementation improved the liver structure and had a positive nutritional function in the intermediary metabolism of the preceding fish species (Segner et al., 1989).

The liver adapts to fluctuating environmental conditions by continuously regulating hepatocellular structures and functions such as: metabolism of nutrients; storage of energy (e.g. glycogen and lipid); synthesis and secretion of proteins (e.g. albumin, vitellogenin, lipoproteins); maintenance of plasma glucose levels; metabolism of xenobiotics; and bile formation (Bruslé et al., 1996; Segner, 1998). Somatic factors including body and liver weight, as well as metabolic factors such as metabolite and enzyme activity, are currently used to determine the capacity for metabolic adaptation to dietary supply in fish (Méton et al., 1999). The liver-somatic index (LSI), and hepatic glycogen content and various liver enzyme activities of intermediary metabolism have been shown to correspond well

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with the nutritional status and growth rates of fish (Brauge et al., 1994; Pelletier et al., 1994). Glycogen phosphorylase (an enzyme involved in glycogenolysis; EC 2.4.1.1) is proposed to play a role in carotenoid pigment metabolism as an enzyme responsible for the provision of the required substrate (UDP-glucuronid acid) for glucuronidation (Bánhegyi et al., 1988). Aspartate aminotransferase (glutamine-oxaloacetic transaminase (GOT); EC 2.6.1.1), an indicator of liver function in rainbow trout, has previously been shown to be influenced by dietary carotenoid pigment supplementation (Nakano et al., 1995).

No direct observations of the effects of dietary carotenoids on hepatic histology and function have been performed in salmonid fish, despite these fishes having intrinsically different physiology relating to carotenoids (i.e. high pigment retention in flesh and gonads). Therefore the objective of this study was to determine the effects of dietary astaxanthin and canthaxanthin supplementation on liver histochemistry in rainbow trout.

2. Materials and methods

2.1. Fish and diets

One hundred and eight rainbow trout (*Oncorhynchus mykiss* Walbaum, initial mean mass 266±10 g), obtained from Hatchlands Trout Farm, Devon, U.K., were allocated to three replicate tanks for each of three individual treatments (astaxanthin, canthaxanthin, and control; $n=3$ (12 fish per tank)) and maintained for 2 weeks prior to the start of the experiment. Rainbow trout were held in 400 l semi-square fiber glass tanks supplied with 10 l/min recirculating fresh water (15±0.2 °C) at the University of Plymouth. Ten percent of the total system volume was replaced per week. Fish were maintained on a 12 h light : 12 h dark photoperiod, and fed a ration of 1.2% body mass/day for 21 days prior to tissue sampling. Fish were starved for 24 h prior to sampling.

Diets were prepared as described by Page and Davies (2002) using a commercially extruded basal diet (formulated for rainbow trout nutritional requirements; 4–6 mm pellet, Trouw Aquaculture, Cheshire, U.K.), top-coated with purified cod liver oil (Seven Seas, Hull, U.K.), which had been supplemented with either Carophyll® Pink (8% astaxanthin *w*/*w*), Carophyll® Red (10% canthaxanthin *w*/*w*), or 'placebo' powder (added to control diet to equalize the dietary composition of all groups; kindly donated by Hoffman La-Roche, Basle, Switzerland), at a target inclusion of 100 mg/kg. The carotenoid beadlets or control powder were first dissolved in warmed water (20 ml at 35 °C), followed by dispersion in cod liver oil (380 ml, heated to 35 °C, to provide a final oil percentage of 30% in the feed, and containing 90 mg/kg ethoxyquin as antioxidant) using a magnetic stirrer. The carotenoid-in-oil emulsion was gradually added in a continuous stream to pre-heated feed (4 kg batch; 60 °C for 1 h) in a Hobart A120 food processor (Hobart manufacturing Company Ltd., London, U.K.) for 30 min. Measured dietary carotenoid pigment (according to Page and Davies, 2002) contents and proximate compositions are presented in Table 1. Moisture and ash determinations were carried out according to AOAC (1990). The protein content of fish feeds was determined by the Kjeldahl method. Lipid determinations in dry feed were determined gravimetrically according to Folch et al. (1957).

Table 1
Basal diet formulation[a] and proximate analysis of experimental diets

Proximate analysis	Astaxanthin	Canthaxanthin	Control
Crude protein (%)	45.2±0.5	46.3±0.7	45.0±0.3
Crude lipid (%)	30.6±0.4	30.8±0.3	30.5±0.2
Ash (%)	9.8±0.0	9.9±0.1	9.8±0.0
Moisture (%)	5.7±0.4	6.5±0.1	6.3±0.1
Astaxanthin (mg kg^{-1})	84.3±1.0	N.D.	N.D.
Canthaxanthin (mg kg^{-1})	N.D.	90.8±1.2	N.D.

N.D. indicates not detectable.
Values are expressed as means±S.D. of triplicate determinations (as fed basis).
[a] Basal diet formulation common to all experimental diets was comprised of LT fish meal (Norse LT94, Egersund, Norway; 613 g/kg), wheat gluten (56 g/kg), wheat (216 g/kg), fish oil (100 g/kg), vitamin premix (7 g/kg) and mineral premix (8 g/kg). Vitamin and mineral premixes (proprietary formulation) were formulated to meet or exceed NRC (1993) requirements. The basal diet was supplemented with 95 g cod liver oil per kg to achieve 300 g/kg lipid level.

2.2. Tissue sampling and histochemical staining

At the termination of the feeding trial, fish were killed with 2-phenoxyethanol (Sigma, Poole, Dorset, U.K.) followed by a blow to the head, according to Home Office Animals (Scientific Procedures) Act 1986 regulations, and immediately weighed. Fish that showed no sign of growth (emaciation) or feed in the gastrointestinal tract were removed from the trial as these fish had evidently not fed (only 3 fish from various treatments omitted). Specific growth rate, feed conversion efficiency, and relative liver masses (g/100 g) were calculated (data reported in Page and Davies, 2002).

Livers were dissected out, blotted dry, and weighed. Livers were sectioned medially, and the left lobe used for histological analysis. Liver samples were immediately frozen in liquid nitrogen, stored (−80 °C), and sectioned on a Leica 2700-frigocut cryostat at 12 μm, prior to histochemical staining, and image analysis by light microscopy. Firstly a 1% Methylene Blue (*w*/*v*; for 10 s) stain was used to examine the general histology of the livers prior to further analysis. Unsaturated lipids and phospholipids were stained according to High (1984) using osmium tetroxide and sudan black, respectively. Glycogen in frozen sections was stained using the periodic acid-schiff (PAS) method of High (1984). Glycogen phosphorylase and aspartate aminotransferase enzymes were stained according to Pearse (1972). Liver sections were mounted using DPX (Gurr, Merck Ltd., U.K.), prior to image analysis.

2.3. Image analysis

PAS stained slides were examined with a Zeiss photomicroscope II and the images captured using a Hitachi 3CCD (model HC-C 20) colour camera. The analogue signal was then imported into a Quantimet 570 image analyser (Leica), and the color image was detected by thresholding for mainly the red component. Once the binary image was created, it was

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