Use of *Chlorella vulgaris* in Rainbow Trout, *Oncorhynchus mykiss*, Diets to Enhance Muscle Pigmentation

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ABSTRACT. The microalga, *Chlorella vulgaris*, which contains 0.2% carotenoids (ash-free dry-weight basis) (consisting of canthaxanthin and astaxanthin) was used as an ingredient in rainbow trout, *Oncorhyncus mykiss*, diets. A feeding trial was conducted to compare the quantitative effect of dietary algal biomass (ALG) incorporation on rainbow trout muscle pigmentation, with that obtained by feeding diets supplemented with a synthetic mixture of canthaxanthin and astaxanthin (MIX) equivalent to the quantities of these carotenoids found in the dry alga. After nine weeks of feeding, muscle of trout fed the algal diet contained slightly less total carotenoids (11.9 mg/kg dry muscle) than trout fed the diet containing synthetic carotenoids (13.3 mg/kg dry muscle). Comparison of total feed intake and weight gain were not significantly different ($P > 0.05$) among treatments. After the feeding trial, fish maintained on a diet containing no carotenoid supplements for eight more weeks (seventeen in total) had carotenoid concentrations of 9.1 mg/kg for fish fed the algal diet and 7.1 mg/kg for fish fed the diet containing

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synthetic pigments. Dry microalgal biomass was found to be a slightly less efficient muscle coloring ingredient for farmed trout than the commercially available pigments, yet proved adequate to achieve commercially acceptable color grades. *[Article copies available for a fee from The Haworth Document Delivery Service: 1-800-342-9678. E-mail address: getinfo@haworth.com]*

INTRODUCTION

The microalga, *Chlorella vulgaris*, possesses the ability to accumulate intracellular carotenoids when appropriately stimulated (Gouveia et al. In Press) and has been shown to induce pigmentation when added to rainbow trout, *Oncorhynchus mykiss*, diets (Gouveia et al. 1996). Carotenoids in *C. vulgaris* include lutein (3,3′-dihydroxy-α-carotene) but mainly canthaxanthin (β,β-carotene-4,4′-dione) and astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) (Gouveia et al. In Press). The latter are the major pigments found in the flesh of wild salmonids (Latscha 1990), which are unable to synthesize carotenoids *de novo* or to synthesize them from other compounds and thus depend on dietary intake (Choubert 1985).

A variety of natural compounds have been compared to synthetic carotenoids as alternative sources of pigment for cultured salmonids. These include paprika, dried flowers, crayfish leftovers (Kayama et al.1973), shrimp waste (Saito and Regier 1971), shrimp meal (Choubert and Luquet 1983), red crab (Kuo et al. 1976), and krill (Ellis 1979). The yeast *Phaffia rhodozyma* is a potential source of pigment for fish, but astaxanthin content in wild strains is low (Johnson and An 1991), and usage as a dietary pigment needs treatments which decrease pigment stability (Sommer et al. 1991). The alga *Haematococcus pluvialis* causes slight pigmentation (Sommer et al. 1991; 1992); however, it must be treated to ensure pigment stability and bioavailability (Choubert and Heinrich 1993). The cyanobacterium *Spirulina* sp. causes only a partial pigment deposition (Choubert 1979); final flesh color is different from the color of fish found in the wild (Sommer et al. 1992).

Pigment deposition in muscle is a relatively fast (2-3 day) process compared to the loss of pigment after pigment ingestion is discontinued (Choubert 1985). Carotenoids are unstable compounds which deteriorate rapidly on exposure to air or heat undergoing fading, darkening and hue changes (Bauernfeind 1972). Dietary carotenoids must therefore be simultaneously bioavailable and protected against deterioration during processing and feed storage. Synthetic products are protected by encapsulation, while natural products are relatively stable before processing (Choubert and Heinrich 1993).

This study was conducted to evaluate the efficacy of carotenoid compounds naturally present in *C. vulgaris* biomass, compared to synthetic preparations providing equivalent quantities of commercial astaxanthin and canthaxanthin, on muscle pigmentation of rainbow trout. A further aim of this work was to evaluate pigment loss after its ingestion stopped and to determine if the rate of depletion depended upon the dietary pigment source.

MATERIALS AND METHODS

Diets

The microalga *C. vulgaris* was cultivated and isolated, yielding biomass with a total amount of carotenoid pigments of 0.2% by weight (ash free dry basis) (Gouveia et al. In Press). The total amount of potential red hue inducing pigments (canthaxanthin and astaxanthin) was approximately 80% of this (50% canthaxanthin and 30% astaxanthin). Commercially available products Carophyll pink 8% and Carophyll red 10%, respectively (Hoffmann-La Roche, Lisbon, Portugal[1]) provide synthetic astaxanthin and canthaxanthin.

A basal diet (Table 1) was formulated according to the nutrient requirements established for rainbow trout (Cho and Cowey 1991). The basal diet was either without carotenoids (control diet), supplemented with 4% of *C.vulgaris* biomass (ALG) to obtain 80 mg of total pigments/kg feed (which represented 64 mg red pigments/kg feed composed of 40 mg canthaxanthin and 24 mg astaxanthin), or supplemented with a mixture of synthetic canthaxanthin and astaxanthin (40 mg canthaxanthin and 24 mg astaxanthin), referred to as MIX.

The appropriate carotenoid supplements were mixed with the basal diet in a horizontal helix ribbon mixer (CPM) for 15 minutes and processed using a pelleting machine without steam (CPM-3000), using a 4.5-mm die. Diet was prepared at the beginning of the feeding trial, kept in black plastic bags under refrigeration (4°C), and analyzed periodically every month for carotenoid content.

Fish and Experimental Conditions

The experiment on fish pigmentation took place at the Centro Aquícola de Vila do Conde (Portugal). Six groups, each consisting of thirty rainbow

1. Use of trade or manufacturer's name does not imply endorsement.

TABLE 1. Chemical composition of the basal diet.

Ingredient (g/kg diet)	
Fish meal	350
Corn gluten	50
Soybean meal	150
Soybean oil	35
Cod liver oil	15
FSPC[1]	70
Wheat	150
Wheat middlings	140
Vitamins[2]	5
Minerals[3]	10
Lignin sulfate	20
Choline chloride	5
Dry matter	91.7
Protein (%)[4]	51.5
Fat (%)[4]	12.8
Ash (%)[4]	11.0
Energy (kJ/g)[4]	21.4
Red pigments concentration (mg/kg dry diet)	64

[1] Fish soluble protein concentrate.

[2] (IU or mg/kg diet): vitamin E, 20; vitamin K_3, 5; vitamin B_1,5; vitamin B_2,5; vitamin B_3,10; vitamin B_5,100; vitamin B_6,5; vitamin B_9,2; vitamin B_{12},0.05; ascorbic acid,200; p-aminobenzoic acid, 50; inositol, 500; choline chloride, 500; vitamin A, 10,000; vitamin D_3, 2000.

[3] (mg/kg diet): Co, 0.4; Cu, 5.0; Fe, 40; F, 1.0; I, 0.6; Mg, 100; Mn, 10.

[4] Dry matter basis.

trout with mean weights of 246.2 ± 7.2 g and not previously fed any pigmenting ingredient, were organized into three treatment groups (two tanks contained the control groups, to which equivalent weight of a diet without pigment supplement was fed throughout; two tanks were fed ALG, and two tanks were fed MIX), therefore, the fish in each two tanks consisting of one group were fed one of the different diets over 9 weeks.

After this period, fish were maintained on a diet containing no carotenoid supplements for more 8 weeks (17 weeks in total). Each tank was 1.8-m^3, and a flow rate of 300-400 L/minute was maintained. The water temperature ranged between 14-15°C. Fish were hand-fed twice a day at a total rate of 2% body weight.

Analytical Methods

At three-week intervals, each group was weighed in bulk, and the daily feed was adjusted accordingly. Five fish were sampled, anaesthetized (2-hydroxyethyl phenyl ether 1:2,500), sacrificed, their dorsal muscles collected, and stored (−18°C) until chemical and carotenoid analysis was performed. Immediately before freezing, the dorsal muscle color of fish samples was subjected to a visual examination by three persons and scored according to the Salmonids Roche Color Card under natural light intensity.

Analyses of the diets and carcasses were carried out following these procedures: dry matter was determined by drying in an oven at 104°C for 24 hours; protein was determined (N × 6.25) by the Kjeldahl method after acid digestion; fat was determined by petroleum ether (40°-60°) extraction in a Soxhlet apparatus; ash was determined by incineration at 550°C for 12 hours; and gross energy was determined in an adiabatic bomb calorimeter (Parr) (AOAC 1990). Muscle carotenoid determinations were conducted on replicate 5-g samples from 5 fish per tank by extraction with acetone and spectrophotometric quantification (with a Hitachi-2000 spectophotometer) according to Choubert and Storebakken (1989) and were expressed on a dry basis using extinction coefficients (E1% 1 cm) of 2,100 for astaxanthin; 2,200 for canthaxanthin; and 2,150 for total algal pigments at their absorption maximum in dichloromethane (Choubert pers. comm.). Diet containing algae was analyzed as mentioned above for the muscle samples, and feed containing synthetic canthaxanthin and astaxanthin was analyzed according to Osadca et al. (1972) and Manz (1983), respectively.

Total apparent carotenoid retention in the fish muscle was calculated as the ratio of carotenoids accumulated in the dorsal fish muscle relative to carotenoid intake and was expressed as a percentage (Choubert and Luquet 1982): Carotenoid retention = $(P)(C)_{final} - (P)(C)_{initial}$/pigment consumption, where P represents the weight of muscle and C is the carotenoid concentration in muscle; muscle tissue weight was taken to represent a constant 53.4 ± 1.7% of total fish weight (Storebakken and Choubert 1991).

One and two months after the end of carotenoid accumulation, all groups were fed a commercial diet without added pigment, and four fish

were sampled from each group. Weight of muscle and pigment concentration were evaluated as described above.

Statistical Analysis

Data on muscle color and total pigments of dorsal muscle were analyzed by analysis of variance. Differences between means were compared using Tukey's difference test ($P = 0.05$) (Zar 1984).

RESULTS

Carotenoid contents of the diets were determined after processing and found not to have any measurable change due to storage. Fish grew normally in all experimental groups, and the results for weight, ranged from 246.2 ± 7.2 g at the beginning of the experiment to 355.4 ± 7.5 g (at nine weeks) and 407.0 ± 5.8 g by the end of week seventeen. Specific growth rate (SGR) ranged from 0.59 for the group fed ALG to 0.66 for fish fed MIX. Visual color scores revealed that a significant and apparently equal increase in muscle color was observed in each treatment until week 6, except in the control group, which maintained the same color (not pigmented). After this time, no significant visual color changes were detected.

The carotenoid concentrations in flesh are presented in Table 2 for raw muscle, and no significant differences were observed from diet to diet ($P > 0.05$) until week nine. Muscle carotenoid concentration from week 10 to week 17 was investigated using the fish which had not been sacrificed during the feeding experiment. The two remaining groups were fed exactly the same unsupplemented diet during this period and showed similar weight increase, as reported.

After this period, muscle carotenoid concentrations were found to be significantly ($P < 0.05$), higher (9.1 mg total pigments/kg dry muscle) for the group fed algal diet (ALG) than those for the group fed astaxanthin plus canthaxanthin (MIX), determined as 7.1 mg/kg.

During the whole experiment, weight measurement and carotenoid analyses of fish from the control were performed. Because carotenoid levels were not detectable and no significant ($P > 0.05$) weight differences were found, details about this data are not presented.

DISCUSSION

Under certain culture conditions (such as nitrogen depletion, high salinity and light intensity) the alga *C. vulgaris* becomes progressively yellow,

TABLE 2. Mean weight (g), muscle color (Salmonids Roche Color Card), total carotenoid concentration (mg/kg dry matter), and carotenoid retention (%) in muscle of rainbow trout fed two experimental diets: ALG = diet containing the alga; MIX = diet containing the synthetic mixture. Means followed by different letters, are significantly different ($P < 0.05$).

Time (weeks)	Weight (g)		Muscle color		Carotenoid concentration		Carotenoid retention	
	ALG	MIX	ALG	MIX	ALG	MIX	ALG	MIX
0	250.0	236.2	–	–	nd	nd	–	–
3	271.2	292.5	12.8a	13.2a	6.0±1.4a	7.9±2.2a	1.4	1.9
6	305.6	324.8	14.3b	14.2b	9.0±2.8b	12.8±3.7b	2.3	3.5
9	362.5	358.8	14.6b	14.5b	11.9±2.7b	13.3±3.4b	3.6	4.0
13	380.3	392.6	–	–	11.1±1.8	12.0±1.4	–	–
17	401.2	412.8	–	–	9.1±0.6	7.1±0.9	–	–

nd = not detectable

yellow-orange, and orange, due to the formation of secondary carotenoids. Lutein, which is initially the main pigment (with chlorophyll *a* and β-carotene) decreases, while canthaxanthin and astaxanthin progressively increase, eventually amounting to 80% of total carotenoids (Gouveia et al. In Press). Dry algal biomass is a naturally encapsulated process-stable ingredient that may be used to supply carotenoids.

The supplement of 4% (w/w) of alga to the basal diet apparently produces no significant alterations in growth rates and voluntary feed intake. The results obtained from the visual score test show similarity between diets, with increase in grades until the sixth week. After that, visual score is almost constant (Gouveia et al. 1996). However these results are only qualitative, and the measurement of color was performed under environmental light conditions which were not constant (Skrede et al. 1990).

Chemical analysis was used to determine that trout fed the algal diet contained 11.9 mg/kg of carotenoids in muscle (dry weight), and trout fed the diet supplemented with the carotenoid mixture contained 13.3 mg/kg carotenoids. These results were determined not to differ and may be considered commercially acceptable (Foss et al.1984). The amount of carotenoids in the muscle was found to be higher than that reported by Sommer et al. (1991, 1992) (0.63-1.22 mg/kg dry muscle after 66 days) and by

Choubert and Heinrich using the microalga *Haematococcus* sp.(1993) (6.2 mg/kg after 4 weeks), and higher than that found for *Spirulina* sp., whose efficiency was limited to the pigmentation of trout skin (Choubert 1979). Carotenoid retention coefficients at the end of the 9-week feeding trial are also similar at comparable periods of time and are within the range previously reported (Hardy et al. 1990; Choubert and Storebakken 1989).

The relatively high carotenoid content found in muscle after the further additional 8 weeks without carotenoid supplements in feed, during which mean fish weight increase was 11.5%, confirms earlier observations that pigment depletion is a very slow phenomenon (Sommer et al. 1992). Thus, correcting for weight increase, it is possible to calculate that muscle carotenoids will have decreased by 15% in fish fed ALG and 35% in fish fed MIX, showing a much slower carotenoid loss from fish fed the algal diet. This difference may be due to the fact that in the former, some astaxanthin ester accumulated in skin and viscera (and later suffered translocation to muscle, partially compensating depletion). Unfortunately, the experimental data which were collected is insufficient to demonstrate this point, which will need further experimentation.

Microalgal biomass from carotenogenetic *C. vulgaris,* appears suitable for fish pigmentation and feeding a diet containing the algae (at 4% of total feed totaling 9 to 14 g algal biomass per fish, depending upon feeding period and mean fish weight) for 6-9 week appears to be a suitable method for enhancing pigmentation in rainbow trout.

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