

DEPOSITION OF ASTAXANTHIN ISOMERS IN CHINOOK SALMON (*ONCORHYNCHUS TSHAWYTSCHA*) FED DIFFERENT SOURCES OF PIGMENT

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ABSTRACT

Whyte, J. N. C., D. Travers, and K. L. Sherry. 1998. Deposition of astaxanthin isomers in chinook salmon (*Oncorhynchus tshawytscha*) fed different sources of pigment. Can. Tech. Rep. Fish. Aquat. Sci. No. 2206: vi + 27 p.

Adult chinook salmon were fed diets with synthetic racemic astaxanthin at 25, 50 and 90 $\mu\text{g}\cdot\text{g}^{-1}$, with mixed canthaxanthin and astaxanthin (1:9) at 90 $\mu\text{g}\cdot\text{g}^{-1}$, with added *Haematococcus pluvialis* and with added *Euphausia pacifica* both equivalent to 90 $\mu\text{g}\cdot\text{g}^{-1}$ astaxanthin, and with no pigment added as a control during a 150 d pigmentation trial. Carotenoid deposition in the flesh increased with increased dietary concentration of racemic astaxanthin during the first 60 d. A mean carotenoid plateau of 10 $\mu\text{g}\cdot\text{g}^{-1}$ in the flesh was attained within 60 d of feeding the chinook 90 $\mu\text{g}\cdot\text{g}^{-1}$ dietary astaxanthin and within 150 d with the mixed dietary carotenoids. This plateau level was not attained by feeding either the 25 or 50 $\mu\text{g}\cdot\text{g}^{-1}$ dietary astaxanthin or by the 90 $\mu\text{g}\cdot\text{g}^{-1}$ astaxanthin from natural sources. With increased dietary concentration and exposure to racemic astaxanthin the ratio of (3R,3'R), (3R,3'S) and (3S,3'S) isomers in chinook flesh changed to reflect that of the added synthetic pigment. Chinook salmon receiving natural astaxanthin from the microalga and the krill demonstrated linear uptake of the corresponding (3S,3'S) and (3R,3'R) isomers.

RÉSUMÉ

Whyte, J. N. C., D. Travers, and K. L. Sherry. 1998. Deposition of astaxanthin isomers in chinook salmon (*Oncorhynchus tshawytscha*) fed different sources of pigment. Can. Tech. Rep. Fish. Aquat. Sci. No. 2206: vi + 27 p.

Au cours d'un test de pigmentation de 150 jours, on a nourri des quinnats adultes avec des régimes alimentaires comportant 25, 50 et 90 $\mu\text{g}\cdot\text{g}^{-1}$ d'astaxanthine racémique synthétique, 90 $\mu\text{g}\cdot\text{g}^{-1}$ d'un mélange 1:9 de canthaxanthine et d'astaxanthine, des quantités ajoutées de *Haematococcus pluvialis* et d'*Euphasia pacifica* dont chacune était équivalente à 90 $\mu\text{g}\cdot\text{g}^{-1}$ d'astaxanthine, et sans aucun pigment ajouté pour les témoins. Le dépôt de caroténoïdes dans les tissus musculaires augmentait proportionnellement à la concentration alimentaire d'astaxanthine racémique au cours des 60 premiers jours. Chez les quinnats, on a atteint un plateau moyen de 10 $\mu\text{g}\cdot\text{g}^{-1}$ pour les caroténoïdes dans les tissus en 60 jours de régime alimentaire à 90 $\mu\text{g}\cdot\text{g}^{-1}$ d'astaxanthine et en 150 jours avec le régime mixte de caroténoïdes. Ce plateau moyen n'a pas été atteint avec les régimes alimentaires à 25 ou 50 $\mu\text{g}\cdot\text{g}^{-1}$ d'astaxanthine ni avec le régime à 90 $\mu\text{g}\cdot\text{g}^{-1}$ d'astaxanthine provenant de sources naturelles. À mesure que les concentrations alimentaires et que l'exposition à l'astaxanthine racémique augmentaient, le rapport des isomères (3R, 3'R), (3R, 3'S) et (3S, 3'S) dans les tissus des quinnats changeait de façon à refléter celui du pigment synthétique ajouté. Chez les quinnats recevant de l'astaxanthine racémique naturelle provenant d'algues microscopiques et de krill, on a pu mettre en évidence l'absorption linéaire des isomères correspondants (3S, 3'S) et (3R, 3'R).

1.0 INTRODUCTION

Astaxanthin in the flesh of salmonid species is not biosynthesized *de novo* and must be obtained from dietary sources (Schiedt et al. 1981, Torrissen et al. 1989). Carotenoid sources for wild salmon are principally food chain crustaceans that contain astaxanthin either in the free form, or as mono- and di-esters of long chain fatty acids (Manzer 1968; Foss et al. 1987a). Digestive hydrolysis of these esters provides free astaxanthin that is absorbed and deposited in the muscle of salmonids (Torrissen et al. 1989). Astaxanthin, 3,3'-dihydroxy- β,β -carotene-4,4'-dione, occurs in nature as the 3*R*,3'*R* and 3*S*,3'*S* enantiomers and as the *meso* 3*R*,3'*S* form (Figure 1) (Foss et al. 1987a). Canthaxanthin (Figure 1) is also found from trace to minor amounts in skin and flesh of wild salmonids (Kitahara 1983, Matsuno et al. 1984).

Flesh pigmentation of farmed salmonids is attained by supplementing feed with synthetic canthaxanthin Carophyll Red® (Foss et al. 1984, Storebakken et al. 1986), synthetic racemic astaxanthin Carophyll Pink® (Foss et al. 1987b) and to a lesser extent with natural sources of astaxanthin such as crustaceans (Saito and Regier 1971, Arai et al. 1987, Mori et al. 1990), algae (Sommer et al. 1991, Sommer et al. 1992, Benemann 1992) and yeast (Johnson et al. 1980, Johnson and An 1991). Synthetic racemic astaxanthin consists of the (3*R*,3'*R*), (3*R*,3'*S*), and (3*S*,3'*S*) isomers in the ratio of 1:2:1 (Vecchi and Müller 1979). Studies on feeding synthetic astaxanthin and individual optical isomers of astaxanthin to rainbow trout (Foss et al. 1984, Bjerkeng et al. 1992) and Atlantic salmon (Storebakken et al. 1985) demonstrated that no epimerization at the 3,3' carbon occurred, and that the ratio of isomers in the flesh reflected the configuration of the astaxanthin in the diet. No epimerization occurred when diesters of the optical isomers were fed separately to rainbow trout (Katsuyama et al. 1987), although feeding racemic astaxanthin diesters favoured deposition of the (3*R*,3'*R*) rather than the (3*S*,3'*S*) isomer, presumably from stereospecificity of the intestinal esterases (Foss et al. 1987b).

Few studies on dietary pigmentation of species of Pacific salmon have been reported. Flesh of coho salmon, *O. kisutch*, pigmented to 4.5 $\mu\text{g}\cdot\text{g}^{-1}$ when diets supplemented with oil extracts of red crab contained carotenoid equivalent to 60 - 90 $\mu\text{g}\cdot\text{g}^{-1}$ (Spinelli and Mahnken 1978). Juvenile coho salmon cultured in net-cages with diets containing zooplankton reached carotenoid concentrations of 6 - 10 $\mu\text{g}\cdot\text{g}^{-1}$ flesh (Mori et al. 1990). Pan sized coho salmon cultured in freshwater incorporated synthetic astaxanthin into the flesh at a rate dependent on dietary concentrations and fish size (Smith et al. 1992). The proportion of astaxanthin isomers in the flesh of juvenile coho salmon was similar to that of Antarctic krill added as a pigment supplement to the diet (Arai et al. 1987). Variation in flesh colouration in strains of the chinook salmon, *O. tshawytscha*, fed the same pigmented diet, suggested genetic control over presence of carotenoid receptors in the muscle and thus expression of pigmentation (Withler 1986, McCallum et al. 1987, Iwamoto et al. 1990, Ando et al. 1994).

Flesh pigmentation influences the market value of salmonids and is an important economic factor in fish farming. This trial was designed, not as a nutritional study, but to determine the deposition of astaxanthin and its isomeric forms in chinook salmon when fed diets supplemented with carotenoids of synthetic or natural origin. Natural sources used in this study were the crustacean *Euphausia pacifica* and the alga *Haematococcus pluvialis*, which contain predominantly the (3*R*,3'*R*) and (3*S*,3'*S*) isomer of astaxanthin, respectively (Renstrøm et al. 1981, Matsuno et al. 1984). The meso (3*R*,3'*S*) form constitutes about 50% of the isomers in farmed salmon exposed to racemic astaxanthin and has been used to differentiate wild and farmed Atlantic salmon (Lura and Sægrov 1991). A measure of the time to reduce this level to 5% of total isomers, a level indistinguishable from that of a wild chinook salmon, was also investigated by feeding the salmon the other isomeric forms present in the algal and krill diets.

2.0 MATERIALS AND METHODS

2.1 Nature of Diets

Pigmented diets were prepared as batch lots by White Crest Mills, using the same basic meal (48% protein, 18% lipid, 14% carbohydrate, 12% ash, and 6% moisture) for all formulations. Carophyll Pink was added to an astaxanthin equivalent of 90 (As90), 50 (As50), and 25 (As25) $\mu\text{g}\cdot\text{g}^{-1}$. Carophyll Pink and Red, added at 9:1 (Asca), provided a combined carotenoid level of 90 $\mu\text{g}\cdot\text{g}^{-1}$. *Haematococcus pluvialis* (Algaxan Red™, kindly provided by Microbio Resources, Inc., San Diego, California) (Alga) and local frozen at sea commercial *Euphausia pacifica*, obtained by the feed manufacturer (Euph) were added to provide an astaxanthin equivalent of 90 $\mu\text{g}\cdot\text{g}^{-1}$. Krill used in the Euph diet contained 0.001 % astaxanthin as 70% diesters, 15 % monoesters and 15 % as the free form, with isomers (3*R*,3'*R*), (3*R*,3'*S*), and (3*S*,3'*S*) in the ratio of 7:1:2. The algal product used in the Alga diet contained 0.8 % astaxanthin as 15 % diesters, 45 % monoesters and 40 % as the free form exclusively in the (3*S*,3'*S*) form. Non-pigmented diet (Nopg) was used as a control. Analysis of the prepared diets contained pigments within 7 % of requested levels. All diets were processed into 8.0 mm pellets and stored at -20°C prior to use. Diets for each pen were picked at random and fish were fed by hand at 1% of body weight per d.

2.2 Feeding Trial and Environmental Conditions

Feeding trials were conducted from April to September, 1990, at the experimental sea-farm adjacent to the Pacific Biological Station, Nanaimo, B.C. Salmon, a Big Qualicum strain of 1.5 y old red-fleshed females, were reared previously on commercial diets containing 50 $\mu\text{g}\cdot\text{g}^{-1}$ synthetic canthaxanthin (Carophyll Red®),

10%, Hoffmann-La Roche Ltd., Basle, Switzerland) then later with racemic astaxanthin (Carophyll Pink®, 5%). Experimental salmon were fed a non-pigmented diet for 6 months prior to the trial. The fish (1490 ± 126 g) injected with oxytetracycline (Liquamycin /LA, Rogar/STB Inc., $50 \mu\text{g}\cdot\text{g}^{-1}$) as a prophylactic treatment against bacterial disease on the experimental farm, were segregated into 7 net-cages (55 in each $4 \times 4 \times 6$ m deep). Salmon (10) selected at random from each pen after 0, 60, 115 and 150 d were gutted, weighed, vacuum wrapped and held at -20°C for analyses. Gutted weight was used to eliminate interference from variable residual food in the intestines. Growth rate (GR), the percentage of growth per day, was calculated as:

$$\text{GR} = (e^g - 1) * 100, \text{ with } g = (\ln W_2 - \ln W_1) / D$$

where W_2 was the final gutted weight, W_1 was the initial gutted weight and D was the number of days in the sampling period. After thawing, flesh on either side of the dorsal ridge to the lateral line was excised for extraction of carotenoids. Mean temperatures and salinities in the pens from d 0 - 60, d 60 - 115 and d 115 - 150 were 10.9 ± 1.6 , 15.6 ± 2.5 and $17.6 \pm 1.6^\circ\text{C}$, and 28.6 ± 0.4 , 25.1 ± 1.6 and 28.0 ± 1.1 ppt, respectively.

2.3 Analysis of Carotenoid in Flesh

Extractions and analyses were conducted in subdued light. Fish flesh (10 - 25 g) was extracted twice with acetone (50 mL) in a Waring blender. The slurry was filtered (GF/A) on a Buchner funnel, and the combined filtrates extracted with pet ether ($40-60^\circ\text{C}$, 50 mL) in a separating funnel. The aqueous phase was re-extracted and the combined ether extracts washed twice with distilled water (20 mL) and dried with anhydrous sodium sulphate (25 g). The ether solution was filtered through a sintered glass funnel and rotary evaporated at 35°C , with nitrogen used to break the vacuum. The residue was dissolved completely in hexane:ethanol (95:5) and absorption recorded at 470 nm. Pigment concentration was determined from linear regression values from standardized graphs of absorbance versus weight of authentic crystalline astaxanthin kindly provided by Hoffmann-La Roche Ltd. Carotenoid esters in the alga and euphausiid were hydrolyzed anaerobically by the procedure of Renstrøm et al. (1981) before determination of astaxanthin content. Pigment concentration in the flesh is expressed as μg total carotenoid per g of flesh. Rate of pigment deposition in the flesh, as μg total carotenoid deposited per g weight gain in flesh per day was calculated from the equation:

$$(F_2 W_2 C_2 - F_1 W_1 C_1) [D * (F_2 W_2 - F_1 W_1)]^{-1}$$

where W_1 , W_2 , and D are as mentioned previously, F_1 and F_2 are the respective flesh percentages of the gutted fish, and C_1 and C_2 are the corresponding carotenoid

concentrations ($\mu\text{g}\cdot\text{g}^{-1}$) in each flesh. Approximate percentage retention of carotenoid in flesh for the trial was calculated from the equation (Torrissen et al. 1995):

$$(F_2W_2C_2 - F_1W_1C_1) \cdot 100 [\text{FCR} \cdot C_D \cdot (F_2W_2 - F_1W_1)]^{-1}$$

where W_2 and W_1 were the final and initial weights of fish for the trial, C_D was the concentration of astaxanthin in the diet, and FCR was the feed conversion ratio for chinook salmon at 1.39 (Kreiberg 1991). Given the errors in assessment of flesh content and the short period between sampling, F_1 and F_2 were considered equal and cancel out in both equations. This overcame the need to know feed intake per fish, which is virtually impossible to measure in an open ocean pen culture.

2.4 Analysis of Carotenoid Isomers

Carotenoid isomers were determined by HPLC of camphanoyl derivatives (Vecchi and Müller 1979). Salmon flesh (3-10 g) was extracted with acetone (30 mL) then pet ether (30 mL). After washing, drying and evaporation in a tared flask, the weight of extract was determined. Derivatization of astaxanthin with (-) camphanoyl chloride (Fluka Chemical Corp.) was performed without further fractionation. Pyridine used as solvent was refluxed over KOH, distilled and stored over a molecular sieve. Complete acylation was provided using reactants at levels equivalent to 100 mg residue in 1.0 mL dry pyridine with 100 mg (-) camphanoyl chloride. Reactants stirred for 1 h at 0°C were checked for complete derivatization by T.L.C. on silica gel (hexane : ethyl acetate, 1:1). Derivatized astaxanthin was separated from the excess camphanoyl chloride and pyridine by extraction with hexane : ethyl acetate (1:1, 10 mL). The solvent extract was washed several times with saturated sodium bicarbonate solution, dried over anhydrous sodium sulphate, and rotary evaporated. Derivatives were dissolved in the mobile phase solvent (hexane : isopropyl acetate : acetone, 76:17:7) filtered through 0.5 μm Millipore filters and applied to the HPLC column. Canthaxanthin, astaxanthin, astaxanthin as esters and dicamphanoyl derivatives were identified by co-chromatography with authentic reference compounds using a Spectra Physics SP 8100 chromatograph, SP 8400 UV/VIS detector, SP 4100 integrator. Carotenoids were resolved on bonded phase Spherisorb 5 μ CN columns (250 x 4.6 mm) with a Spherisorb 5 μ CN guard column (50 x 4.6 mm). Isocratic separation for 40 min at a flow rate of 1.0 mL min⁻¹ provided peaks detectable at λ_{max} 470 nm. The *cis* and *trans* isomers (generally 7:93) of the diastereomeric derivatives were resolved but were summated for each optical isomer as the *trans* configuration exists *in vivo* (Henmi et al. 1990b) and the *cis* is likely an artifact of work-up (Bjerkeng 1992). Data were treated statistically, using Tukey or Newman-Keuls multiple comparisons of variables (Zar 1984). A level of significance ($P < 0.05$) was used for all data.

3.0 RESULTS

3.1 Growth

Growth from the initial mean 1490 g averaged $0.47\% \cdot d^{-1}$ from all diets during the first sampling period, d 0 - 60. During the second sampling period, d 60 - 115, growth in fish fed all diets was $0.12\% \cdot d^{-1}$, and during the third sampling period, d 115 - 150, was $0.22\% \cdot d^{-1}$. Final weights of fish fed all diets were not significantly different at a mean 2250 g. With no significant difference in growth of fish between pens, no pen effects were indicated.

3.2 Flesh Content of Carotenoids from Synthetic Astaxanthin

Flesh of salmon fed the Nopg diet for 6 mo prior to the trial had $4.86\ \mu g \cdot g^{-1}$ total carotenoid, of which 15.1 % was canthaxanthin and the remainder astaxanthin (Figure 2). Carotenoid concentrations in fish fed As90, As50 and As25 for the first, second and third sampling periods averaged 9.88, 8.87 and 7.73; 9.98, 9.17 and 8.53; and 10.27, 9.56 and $9.18\ \mu g \cdot g^{-1}$, respectively (Figure 2a). Carotenoid levels in flesh from the As90 diet were significantly different from the As50 only during the first two sampling periods, but from the As25 diet were significantly different throughout the trial. Flesh concentration in fish fed the Asca diet during the first and second period had 7.34 and $8.32\ \mu g \cdot g^{-1}$, which were not significantly different from those obtained using the As25 diet (Figure 2a). However, the final concentration after 150 d, $10.13\ \mu g \cdot g^{-1}$, was not significantly different to that obtained from the As90 diet.

Percentage canthaxanthin in total flesh carotenoids declined rapidly during the first sampling period to mirror the uptake of carotenoid from the different diets (Figure 2b). Canthaxanthin was $2.8 \pm 0.3\%$ of total carotenoids after feeding for 60 d with As90. Contents in salmon fed As50 and As25 during the second and third periods declined from 6.1 - 4.3 % and 11.1 - 5.3 %, respectively (Figure 2b). When fed the Asca diet canthaxanthin concentration remained at $9.6 \pm 0.6\%$ from d 60 - 150, similar to the diet concentration.

3.3 Flesh Content of Carotenoids from Natural Sources

Carotenoid concentrations from the Alga and Euph diets were significantly different from that obtained by feeding the Nopg and As90 diets (Figure 3). For the three sampling periods carotenoid concentrations in fish fed Alga and Euph diets were 6.60, 8.13, 8.73, and 7.15, 7.64 and $7.99\ \mu g \cdot g^{-1}$, respectively, with only the levels at d 150 significantly different (Figure 3a). Levels of carotenoid in fish fed Alga and As25 for 150 d, were not significantly different but both were significantly higher than the levels provided by the Euph diet (Figures 2a and 3a). Levels of carotenoids in fish fed

Nopg were constant at $4.86 \mu\text{g}\cdot\text{g}^{-1}$ for the first 60 d, declined to $4.05 \mu\text{g}\cdot\text{g}^{-1}$ by d 150, but were significantly lower than those from all other diets (Figure 3a). Percentage canthaxanthin declined from 15.1 to 12.4 of the total carotenoids in fish fed the Nopg diet for 150 d. Canthaxanthin in the flesh of fish fed Alga was always higher than that fed the Euph diet (Figure 3b) because of the presence of canthaxanthin as 3% of the total carotenoid in the alga.

Deposition rates of carotenoids in fish fed As90, As50, As25, Asca, Alga and Euph during the first sampling period were 0.51, 0.31, 0.25, 0.25, 0.20 and $0.28 \mu\text{g}\cdot\text{g}^{-1}\cdot\text{d}^{-1}$, for the second period were 0.18, 0.49, 0.46, 0.45, 0.43 and $0.25 \mu\text{g}\cdot\text{g}^{-1}\cdot\text{d}^{-1}$, and for the third period were 0.36, 0.41, 0.56, 0.74, 0.54 and $0.34 \mu\text{g}\cdot\text{g}^{-1}\cdot\text{d}^{-1}$, respectively. Percentages of carotenoid retained in flesh of fish fed As25, As50, As90, Asca, Alga, and Euph diets for the entire trial were 50, 26, 18, 16, 13, and 12 %, respectively.

3.4 Isomers of Astaxanthin in Fish Flesh

Synthetic astaxanthin with 25 % (3*R*,3'*R*), 50 % *meso* (3*R*,3'*S*) and 25 % (3*S*,3'*S*) influenced the composition of isomers in salmon flesh as a function of dietary concentration and time of exposure to the dietary pigment (Figure 4). Salmon fed the Nopg diet for 6 mo prior to the trial contained 28.57, 47.15 and 24.28 % of the (3*R*,3'*R*), (3*R*,3'*S*) and (3*S*,3'*S*) isomers, respectively (Figure 4a). Fish feeding on residual organisms in the pens caused an initial increase in the (3*S*,3'*S*) form to d 60, which was reversed and replaced by an increase in the (3*R*,3'*R*) form to d 150 (Figure 4a). Combined percentages of the (3*R*,3'*R*) and (3*S*,3'*S*) forms were generally higher than the *meso* form that was less prevalent in the sea-pen environment. Percentages of isomers varied only slightly from those of the Nopg diet throughout the trial in fish fed the As25 diet (Figure 4b). The diet As50 altered the (3*R*,3'*R*) and (3*S*,3'*S*) forms to 26.98 and 24.36 % of the total only after 150 d of feeding (Figure 4c). Similarly the Asca diet, with $81 \text{ mg}\cdot\text{kg}^{-1}$ astaxanthin, required 150 d to change the (3*R*,3'*R*) and (3*S*,3'*S*) forms to 26.00 and 24.75 % of the total isomers, respectively (Figure 4d). Using the As90 diet only 60 d exposure was required to alter the (3*R*,3'*R*) and (3*S*,3'*S*) forms to 25.25 and 26.12 % of the total isomers in the flesh (Figure 4e). From d 60 - 150 the diets Nopg, As25, As50, Asca and As90 caused a positive change in the (3*R*,3'*R*) form of 0.089, 0.038, 0.025, 0.002 and $0.002 \text{ \%}\cdot\text{d}^{-1}$, a negative change in the (3*S*,3'*S*) isomer of 0.133, 0.085, 0.053, 0.037 and $0.016 \text{ \%}\cdot\text{d}^{-1}$, and a positive change in the (3*R*,3'*S*) isomer of 0.044, 0.035, 0.028, 0.035, and $0.014 \text{ \%}\cdot\text{d}^{-1}$ (Figure 4).

The ratio of the (3*R*,3'*R*) and (3*S*,3'*S*) isomers relative to the *meso*-isomers as unity were compared with time of exposure to different levels of synthetic astaxanthin in the diets of the salmon (Figure 5). The (3*R*,3'*R*) isomer at 0.57 - 0.69 ratio in flesh of fish fed the non-pigmented diet shifted towards the 0.50 ratio expressed by synthetic astaxanthin as both the astaxanthin content and time exposed to this diet increased

(Figure 5a). Similarly, as the amount and time of exposure to synthetic astaxanthin increased the 0.75 - 0.42 ratio of the (3S,3'S) isomer in the flesh of non pigmented fish shifted towards the 0.50 ratio of the synthetic pigment (Figure 5b). In this trial, ratios at 0.50 for both isomers were not attained using the As25 diet, were only attained after 150 d of feeding with diets As50 and Asca, but were attained after only 60 d of feeding with diet As90 in accord with the previously mentioned deposition data. The higher than expected ratios for both isomers resulting from the mixed Asca diet ($81 \mu\text{g}\cdot\text{g}^{-1}$ astaxanthin) at d 60 and 115 are clearly visible above the 3D-planes joining the points for the all astaxanthin diets in Figure 5a and 5b. These results are in accord with the reduced deposition of carotenoid from the mixed diet observed during the 115 d of feeding (Figure 2a).

During feeding with Euph the (3R,3'R) isomer increased from 28.6 - 50.3 % of total isomers in the flesh and uptake from regression analysis was $0.144 \text{ \%}\cdot\text{d}^{-1}$, $r^2 = 0.9907$ (Figure 6a). Over the same period the (3R,3'S) form declined from 47.2 - 30.1 % at a linear rate of $0.113 \text{ \%}\cdot\text{d}^{-1}$, $r^2 = 0.9894$. Similarly, the (3S,3'S) form declined linearly from 28.6 - 19.6 % at $0.031 \text{ \%}\cdot\text{d}^{-1}$, $r^2 = 0.9942$. Feeding with the Alga diet with this diet the isomer (3R,3'R) in the flesh decreased from 28.6 - 10.0 % of total isomeric forms at $0.113 \text{ \%}\cdot\text{d}^{-1}$, $r^2 = 0.9217$ (Fig. 6 b). Similarly, the (3R,3'S) isomer declined linearly at $0.169 \text{ \%}\cdot\text{d}^{-1}$, $r^2 = 0.9975$, from 47.2 - 22.4 % and the uptake of (3S,3'S) isomer was linear at $0.282 \text{ \%}\cdot\text{d}^{-1}$, $r^2 = 0.9897$, with an increase from 24.3 - 67.7 %. The higher rate of increase in the (3S,3'S) isomer from the Alga diet, relative to that of the (3R,3'R) isomer from the Euph diet, confirmed the presence of other isomeric forms in the latter diet.

4.0 DISCUSSION

Carotenoid deposition in species of *Oncorhynchus* has focused mainly on uptake in juvenile fish (Spinelli and Mahnken 1978, Torrissen et al. 1989; Mori et al. 1990, Smith et al. 1992). Similarly, uptake of carotenoid in the Atlantic salmon, *Salmo salar*, has been conducted on juvenile fish except for the recent study by Torrissen et al. (1995). In this study, information is presented on pigmentation of adult chinook salmon grown under farm conditions and closer to harvest, when colour is an important quality criterion for consumers and processors. Aquacultured Pacific salmon at this age have been fed commercial diets containing dietary canthaxanthin and/or astaxanthin. The chinook used in this study had been fed both. A control level was obtained by feeding all salmon a non-pigmented diet for six months prior to the trial. Carotenoid concentration in the fish fed the non-pigmented diet did not decline but remained relatively constant during the trial. A constant value was achieved by incidental consumption of marine organisms present in the pens, which was

corroborated by changes in percentages of the (3*R*,3'*R*), (3*R*,3'*S*) and (3*S*,3'*S*) isomers of astaxanthin in the flesh of the control fish. Isomers of astaxanthin deposited reflected those inherent to different environmental feed organisms associated with the pens (Foss et al. 1987a). All chinook salmon exposed to pigmented diets during the first period deposited carotenoid rapidly from the baseline concentration. No significant difference in growth of fish fed the diets supplemented with different types and concentrations of carotenoids was observed, in agreement with the absence of any correlation between dietary carotenoid levels and growth of Atlantic salmon (Storebakken et al. 1985, Storebakken et al. 1987).

Apart from any genetic influence, the carotenoid concentration in this group of chinook was a function of content, nature of dietary carotenoid and the duration of feeding the pigment in accord with studies on rainbow trout, *O. mykiss* and Atlantic salmon, as reviewed by Torrissen et al. (1989). A plateau of 10 $\mu\text{g}\cdot\text{g}^{-1}$ was attained within 60 d of feeding the chinook 90 $\mu\text{g}\cdot\text{g}^{-1}$ dietary astaxanthin. An upper storage capacity at this level was suggested for this group of experimental chinook, in agreement with the average 11 ± 2 $\mu\text{g}\cdot\text{g}^{-1}$ carotenoid in the flesh of commercially farmed red-fleshed chinook examined to date (Whyte, unpublished data).

Carotenoid concentrations in farmed fish are generally higher than in wild chinook reported at 5 - 9 $\mu\text{g}\cdot\text{g}^{-1}$, a range that is intermediate between those for wild sockeye, *O. nerka*, at 26 - 39 $\mu\text{g}\cdot\text{g}^{-1}$ or coho, *O. kisutch*, at 9 - 21 $\mu\text{g}\cdot\text{g}^{-1}$ and wild chum, *O. keta*, at 5 - 8 $\mu\text{g}\cdot\text{g}^{-1}$ or pink, *O. gorbuscha*, at 4 - 7 $\mu\text{g}\cdot\text{g}^{-1}$ (Kanemitsu and Aoe 1958, Schiedt et al. 1981). Plateau levels for carotenoids have been reported in the flesh of other cultured species of salmon. Seawater cultured juvenile coho attained a constant 11 $\mu\text{g}\cdot\text{g}^{-1}$ when fed 40 $\mu\text{g}\cdot\text{g}^{-1}$ carotenoid from zooplankton (Mori et al. 1990). Rainbow trout attained a plateau level of 14 $\mu\text{g}\cdot\text{g}^{-1}$ when fed 40 $\mu\text{g}\cdot\text{g}^{-1}$ canthaxanthin (Storebakken et al. 1986). A mean upper storage capacity of 8.5 $\mu\text{g}\cdot\text{g}^{-1}$ was recently reported for Atlantic salmon after an intensive study of deposition using dietary carotenoid concentrations up to 200 $\mu\text{g}\cdot\text{g}^{-1}$ (Torrissen et al. 1995). This upper storage level was within the 3 - 11 $\mu\text{g}\cdot\text{g}^{-1}$ reported for wild Atlantic salmon (Torrissen et al. 1989).

The diversity of plateau levels and range of carotenoid concentrations cited for these salmonid species tends to suggest their variable potential to retain carotenoids. The capacity of flesh to retain carotenoid will depend fundamentally on the concentration of available carotenoid receptor sites in the flesh (Henmi et al. 1989, Henmi et al. 1990b, Henmi et al. 1991). The concentration of sites will most probably be governed by genetic traits within each species and the strains and varieties making up each species of salmon (Withler 1986, McCallum et al. 1987, Iwamoto et al. 1990, Ando et al. 1994). The ability to fill these receptor sites will depend on the circulating serum carotenoid concentrations (Kiessling et al. 1995), which in turn will depend on

the concentration and form (free, esterified or as a cellular matrix) of the dietary carotenoid supplied to healthy fish.

Deposition of racemic astaxanthin in the experimental chinook salmon was dependent on the dietary concentration of astaxanthin during the first period. Once the plateau level had been reached with the $90 \mu\text{g}\cdot\text{g}^{-1}$ diet, the majority of available receptor sites would have been bonded, and caused a decline in the replacement or deposition of carotenoid during the second and third sampling periods. Had the plateauing been caused by a reduction in growth during the second and third periods then flesh pigmentation with the less pigmented diets would have ceased totally. This was not evident in the continued deposition of astaxanthin from 25 and $50 \mu\text{g}\cdot\text{g}^{-1}$ dietary astaxanthin or in the $90 \mu\text{g}\cdot\text{g}^{-1}$ natural astaxanthin supplied. The continued deposition from these diets suggested that the serum concentrations were insufficient to fill the relatively large number of receptor sites still available for bonding, at least within the timeframe of the experiment. Percentage retention of astaxanthin in the chinook flesh increased with decreased dietary levels over the entire trial period. This agreed with the tendency towards higher retention of carotenoids in coho salmon with lower dietary carotenoid concentration (Smith et al. 1992).

In general when synthetic astaxanthin is used, the ability of fish to assimilate pigment reflects the concentration in the diet, until a plateau is reached. The inverse relationship between percentage retention and dietary level of astaxanthin may therefore be explained by the higher concentration of receptor sites in the less pigmented flesh having a greater capacity to bind or retain the relatively small number of circulating astaxanthin molecules in the serum derived from the lower dietary levels. The 26 and 18 % astaxanthin retention from 50 and $90 \mu\text{g}\cdot\text{g}^{-1}$ dietary astaxanthin fed chinook was more in accord with the 15 - 20 % retention of pigment in juvenile coho salmon (Smith et al. 1992) than the 4 - 5 % cited for Atlantic salmon by Torrissen et al. (1989). However, a higher percentage retention of carotenoid in chinook than Atlantic salmon would be expected from the naturally higher level of receptor sites in the flesh of the more pigmented Pacific species.

Canthaxanthin present in chinook salmon was displaced rapidly from 15 to 2% of total carotenoids during the first period of sampling when $90 \mu\text{g}\cdot\text{g}^{-1}$ astaxanthin was fed. Continued feeding at this level failed to reduce the level below 2%. Displacement of canthaxanthin was proportional to the level of synthetic astaxanthin in the diet. Displacement rather than dilution caused by increased growth were indicated, because the latter would have provided similar canthaxanthin levels in all fish. The β -ionone rings in astaxanthin and canthaxanthin (Figure 1) form non-covalent hydrophobic bonds with hydrophobic receptor sites in muscle actomyosin (Henmi et al. 1989, Henmi et al. 1990a). Stronger hydrogen bonding also occurs between the hydroxyl and ketone groups at C-3 and C-4 of the β -ionone ring and the protein matrix, thus astaxanthin with

two additional hydroxyl groups than canthaxanthin (Figure 1) has a higher bonding capacity to the muscle. Higher rates of deposition of astaxanthin relative to canthaxanthin would be expected with displacement of canthaxanthin in the chinook salmon as observed. However, the irreversible 2% in the chinook flesh even with sufficient astaxanthin in the diet suggests the presence of a small proportion of extremely hydrophobic receptor sites in the muscle, tending to confirm the variability in binding capacity of receptor sites in chinook.

Use of a mixed canthaxanthin and astaxanthin diet on the chinook salmon reduced carotenoid deposition by 50 % , relative to that given by astaxanthin alone during the first sampling period. This lag in deposition was overcome in the second and third sampling period to provide the plateau level by the end of the trial. Torrissen (1989) has reported a 70 % higher total carotenoid concentration in flesh of rainbow trout fed for 57 days with a dietary mix of 28 % canthaxanthin in astaxanthin compared to the level obtained using astaxanthin alone. However, as in this chinook study, the enhanced deposition was also time dependent, and resulted only from the lowest percentage of canthaxanthin (28%) to astaxanthin studied. Although astaxanthin is deposited into salmonid flesh more rapidly than canthaxanthin when administered individually (Torrissen 1989), it would appear that when small percentages of canthaxanthin are admixed with astaxanthin some lag in initial deposition occurs that is eventually overcome.

The ratios of (3*R*,3'*R*), (3*R*,3'*S*) and (3*S*,3'*S*) isomers of astaxanthin in chinook fed the non-pigmented diet varied with time and consumption of marine organisms associated with the holding pens. Studies on optical isomers of astaxanthin in rainbow trout and Atlantic salmon fed non-esterified astaxanthin had demonstrated that no epimerization at the 3,3' carbon atoms in the astaxanthin molecule occurred in the fish and no selectivity in deposition of free isomeric forms occurred, resulting in isomer ratios in flesh resembling those in the dietary astaxanthin (Foss et al. 1984, Storebakken et al. 1985). Similarly in chinook, the ratio of isomers in the flesh changed with increased dietary concentration and exposure to synthetic astaxanthin to reflect that of the synthetic pigment (1:2:1). The lag in deposition of astaxanthin from the mixed carotenoid diet that contained 81 $\mu\text{g}\cdot\text{g}^{-1}$ astaxanthin was clearly evident from ratios of the (3*R*,3'*R*) and (3*S*,3'*S*) isomers well above those obtained from the 90 $\mu\text{g}\cdot\text{g}^{-1}$ astaxanthin diet during the first two sampling periods.

Astaxanthin, as free and esterified forms, in the alga *H. pluvialis* was deposited less efficiently than synthetic astaxanthin, in agreement with the reduced deposition from palmitate esters of astaxanthin in rainbow trout (Sommer et al. 1992, Choubert and Heinrich 1993) and Atlantic salmon (Storebakken et al. 1987). As only free astaxanthin was absorbed and deposited in the chinook flesh, the reduced rate of deposition relative to synthetic astaxanthin suggested that cleavage of these esters

was the limiting step for pigmentation (Foss et al. 1987b, Storebakken et al. 1987). In chinook salmon the rate of deposition increased with time of exposure to the algal diet. This may be due to an increased efficiency in the enzymatic de-esterification process with time of feeding. Regression analysis indicated a linear increase in the (3S,3'S) isomer at 0.28 % per day, with a consequent reduction in the (3R,3'R) and (3R,3'S) forms by 0.11 and 0.17 % per day, respectively. The *meso* form is uncommon in flesh of Pacific species (Schiedt et al. 1981) and rarely constitutes more than 5 % of the total isomers in wild chinook salmon (Whyte and Sherry, unpublished data). The percentage *meso* form of astaxanthin is therefore a diagnostic marker for escaped farmed fish in the ocean. Theoretically, an escaped farmed chinook salmon, with 50% *meso* astaxanthin, would therefore take about 265 days for the *meso* form in the flesh to reach 5 % of the total isomeric forms, assuming the prey organisms contained the equivalent of $90 \mu\text{g}\cdot\text{g}^{-1}$ (3S,3'S) astaxanthin.

The rate of deposition of astaxanthin in chinook salmon fed *E. pacifica* was the lowest among all diets tested. A study on coho salmon pigmented by the addition of oil extracts from *E. superba*, suggested that diesters of astaxanthin that predominate mainly as the (3R,3'R) isomer were more stable and preferable to free astaxanthin as the pigment source (Arai et al. 1987). However, as with the alga, absorption of astaxanthin from krill is limited probably by the rate of hydrolysis of the esters prior to absorption of the free astaxanthin into the flesh (Foss et al. 1987b, Storebakken et al. 1987). Regression analysis indicated a linear increase in the (3R,3'R) isomer at 0.14 % per day, with a consequent reduction in the (3S,3'S) and (3R,3'S) forms by 0.03 and 0.11 % per day, respectively. From these data it would theoretically take an escaped farmed chinook salmon, with 50% *meso* astaxanthin, about 363 days for the *meso* form in the flesh to reach 5 % of the total isomeric forms, assuming the prey organisms contained the equivalent of $90 \mu\text{g}\cdot\text{g}^{-1}$ (3R,3'R) astaxanthin. As the level of astaxanthin in farmed salmon is higher than wild salmon, as mentioned previously, the level of the *meso* isomer of astaxanthin in chinook salmon would identify an escaped farmed salmon from a wild salmon for about 1 year after escapement to the ocean. This has allowed the assessment of percentage escaped farmed fish in salmon populations returning to spawning river systems (Whyte and Sherry unpublished data).

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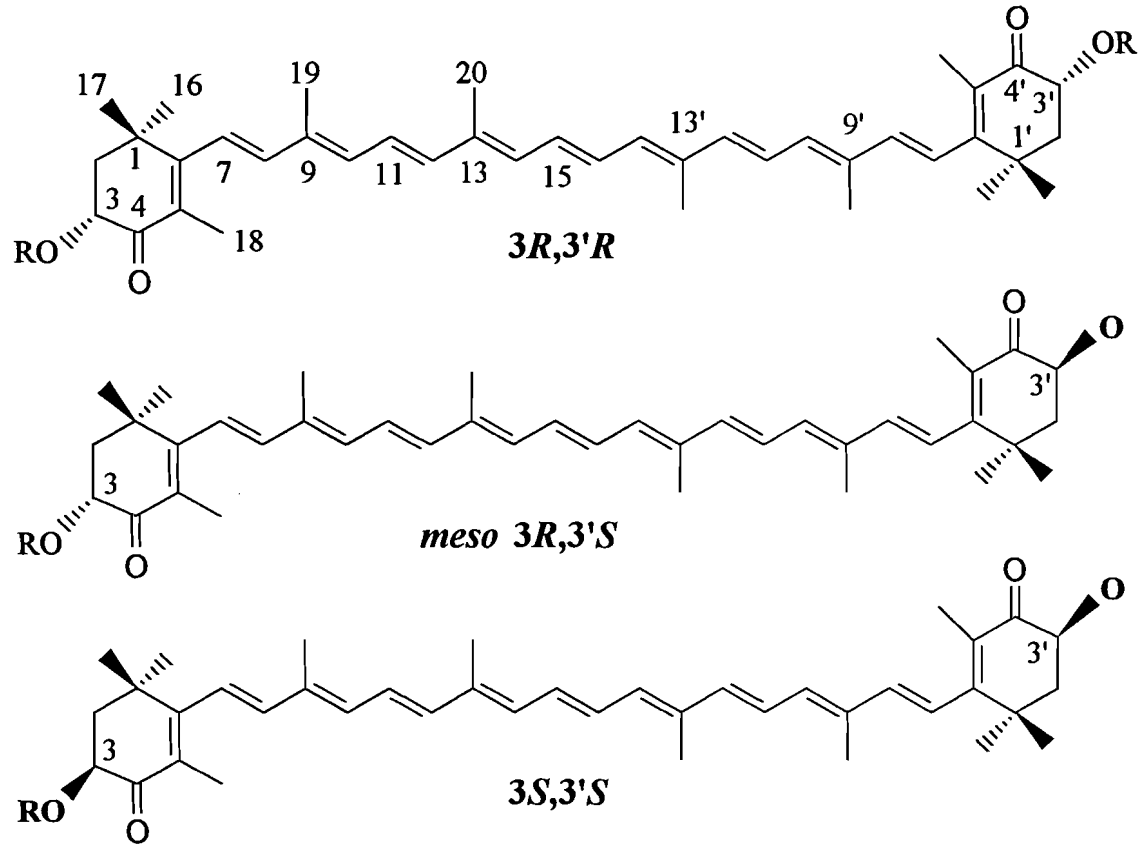
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17
ASTAXANTHIN



CANTHAXANTHIN

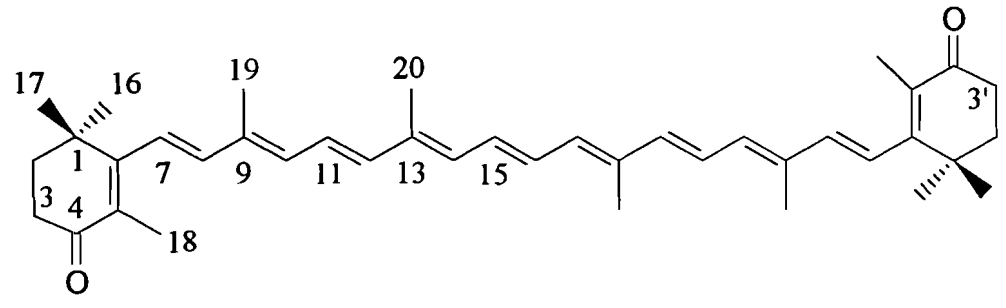


Figure 1. Structure of astaxanthin stereoisomers and canthaxanthin (R = H, or fatty acid).

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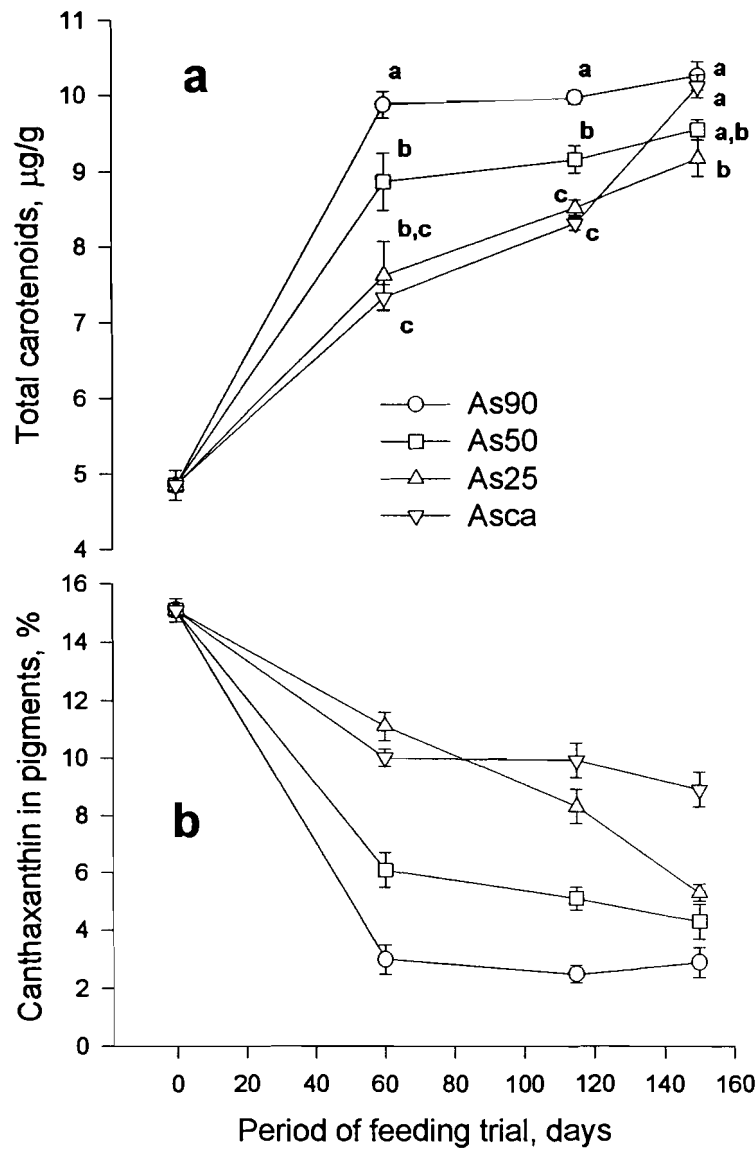


Figure 2. Mean ($\pm 1\text{SE}$) concentrations of total carotenoids (a) and percentage of canthaxanthin in total carotenoids (b) in flesh of chinook salmon fed diets with synthetic astaxanthin added; values at each sampling period not sharing a common letter are significantly different ($P < 0.05$); abbreviations for diets are 25 $\mu\text{g}\cdot\text{g}^{-1}$ (As25), 50 $\mu\text{g}\cdot\text{g}^{-1}$ (As50) and 90 $\mu\text{g}\cdot\text{g}^{-1}$ (As90) astaxanthin; and 90 $\mu\text{g}\cdot\text{g}^{-1}$ astaxanthin:canthaxanthin mixed (9:1) (Asca).

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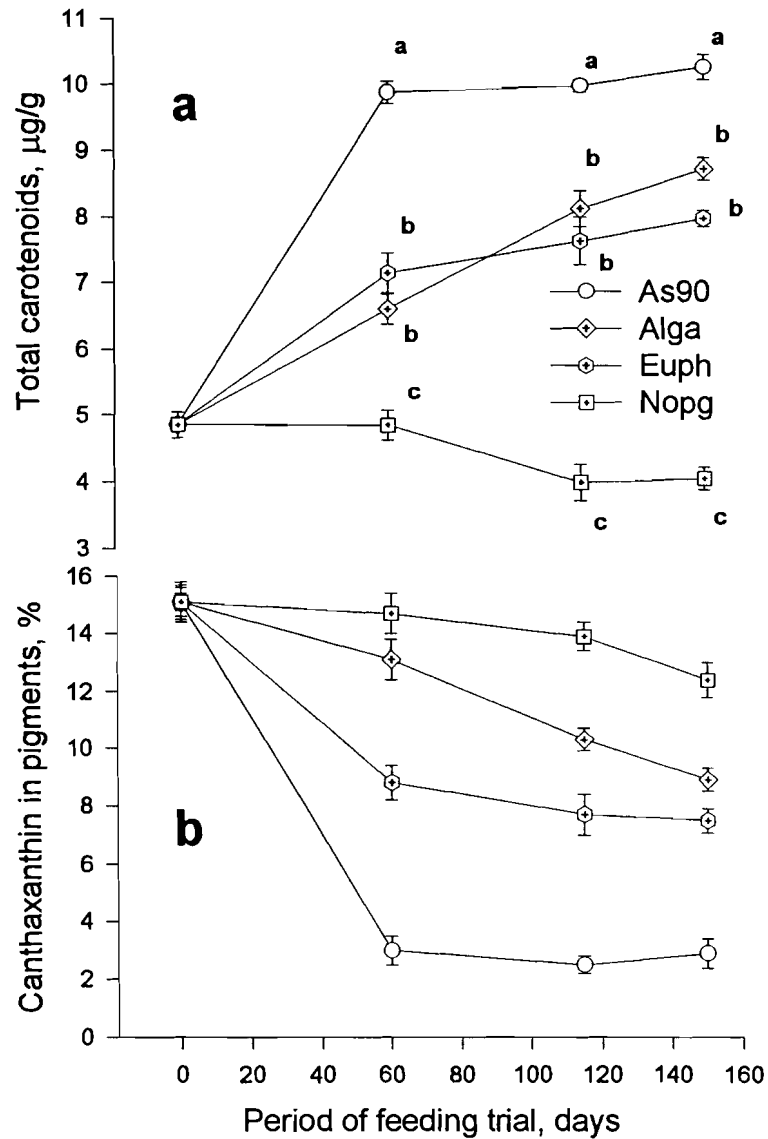


Figure 3. Mean ($\pm 1\text{SE}$) concentrations of total carotenoids (a) and percentage of canthaxanthin in total carotenoids (b) in flesh of salmon fed diets with synthetic and natural astaxanthin, and without astaxanthin added; values at each sampling period not sharing a common letter are significantly different; abbreviations are for diets with astaxanthin equivalent to $90 \mu\text{g}\cdot\text{g}^{-1}$ from a commercial alga (Alga) and commercial krill (Euph) product; and without carotenoid (Nopg).

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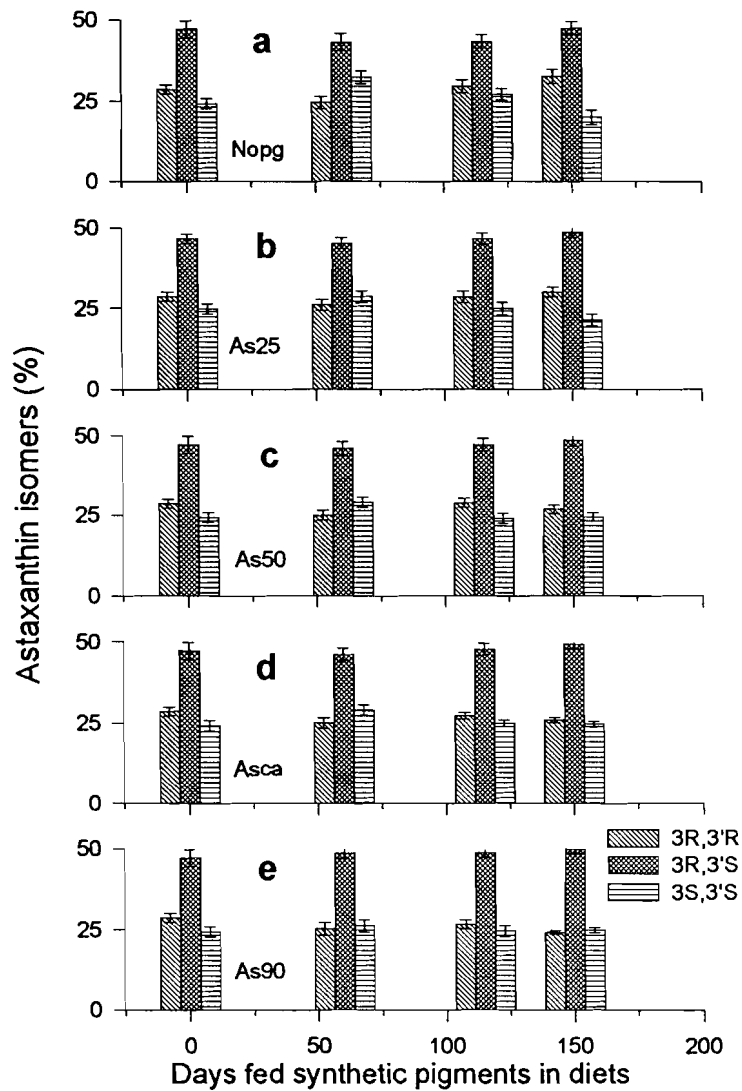


Figure 4. Mean (\pm 1SE) percentages of isomers of astaxanthin in flesh of chinook salmon fed diets with and without synthetic astaxanthin added; diet abbreviations as in Figures 2 and 3.

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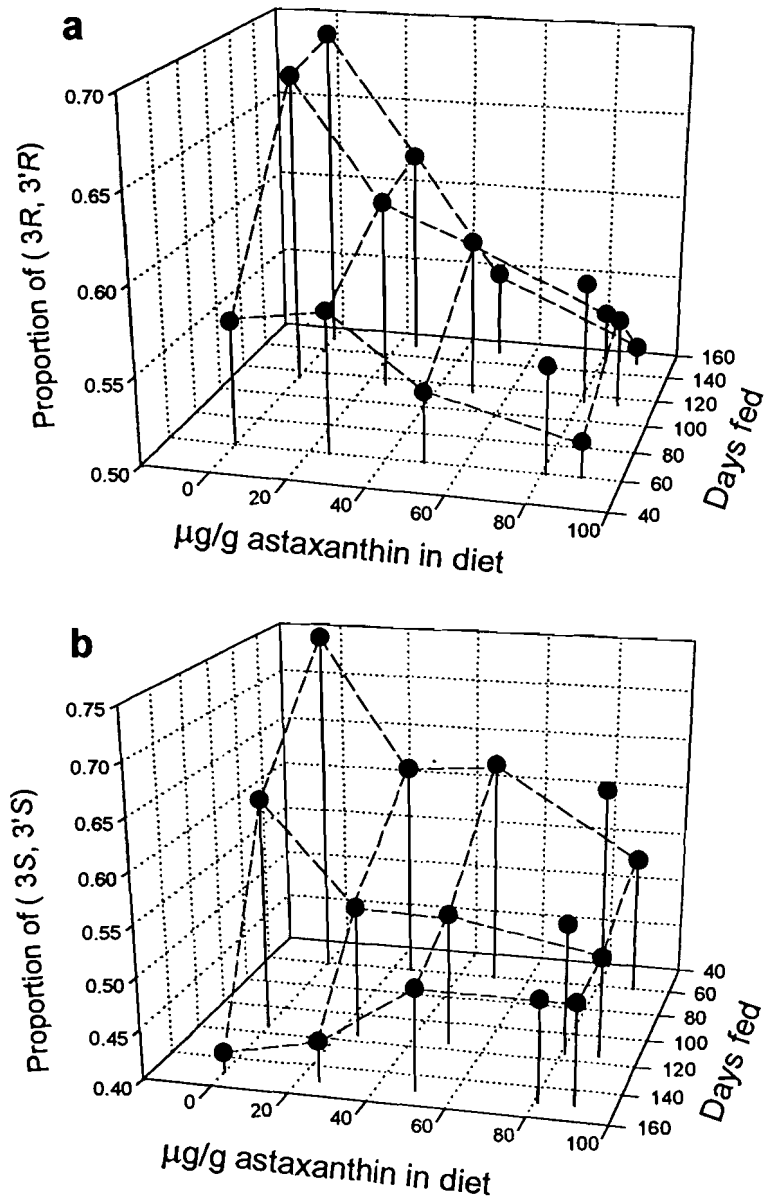


Figure 5. Change in proportion of the (3R,3'R) isomer (a) and the (3S,3'S) isomers (b) relative to the *meso* (3R,3'S) isomer of astaxanthin in flesh of chinook salmon fed diets with different levels of astaxanthin and the period of feeding.

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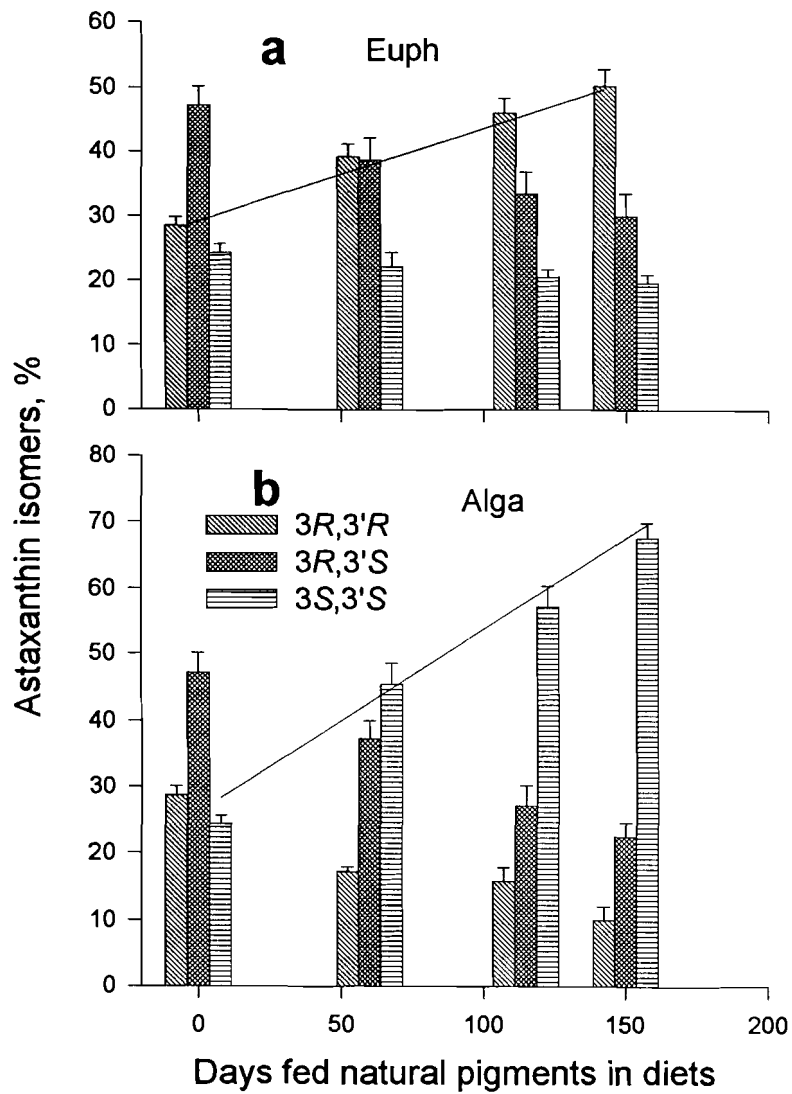


Figure 6. Mean (\pm 1SE) percentages of isomers of astaxanthin in flesh of chinook salmon fed diets with natural sources of astaxanthin; diet abbreviations as in Figure 3.