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CITIZEN PETITION

May 16, 2003

The undersigned submits this Citizen Petition to the Food and Drug Administration under Section 403 of the Federal Food, Drug, and Cosmetic Act and 21 CFR 10.30 to amend or revoke the requirement for salmonid fish that have been fed feeds containing the carotenoid astaxanthin to have the label " color added' or" artificial color" declared on the label and/or placard in accordance with 21 CFR 101.22(k)(2) and 101.100(a)(2), and that the Food and Drug Administration also amend or revoke the requirement for salmonid fish fed the carotenoid canthaxanthin also require the label and/or placard declaring" artificial color' or" color added' in accordance with 21 CFR 101.22(b), (c) and (k)(2) and 101.100(a)(2).

Statement of Grounds

(1) The " color added' or" artificial color" label is at best confusing and misleads the consumer when a farm animal is in fact fed carotenoids that are part of a normal diet for that farm animal. Formulated feeds for farm animals, including salmonids, should contain all of the essential nutrients found in a natural diet including protein, fat, vitamins and carotenoids.

(2) The labels" color added' or" artificial color' implies to virtually all consumers that a coloring agent not normal to a particular product has been added in processing. Few if any would conclude from such a label that carotenoids, natural or man made, have been added to the animals feed as part of a complete and balanced diet. It is the normal deposition of these carotenoids, natural or man made, that are responsible for the color of the animals flesh or ova. Under the FDA rules (21CFR73), if the carotenoids astaxanthin and canthaxanthin from natural or man made sources have been included in the animals feed, the farm product must have the" color added' or" artificial color' labels or placards. In effect this makes the" color added' or" artificial Color' label a method of identifying farmed fish.

When wild and farmed salmonid fish fillets are displayed in a grocery store, the only real difference is that one is" wild' and the other" farmed'. The" color added' or" artificial color' label misleads the consumer into concluding that one has had a coloring agent not natural to the product added in processing when in

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fact the color of both fillets are the same, the result of the natural deposition of carotenoids contained in their diet. A more appropriate label for the farmed fish would be just that, " farmed' . Most consumers would assume that all farm animals are fed formulated diets, pellets of some sort, containing various ingredients to produce a complete and balanced diet including man made vitamins and so on. Few if any would conclude that a " color added' label means if s a farm product fed prepared feed that includes carotenoids. The carotenoids found in wild salmonids are identical to those found in farmed that have had appropriate levels of carotenoids included in their diets. The only difference being that the optical isomer ratios may differ. The optical isomer ratios differ depending on the source of the carotenoids, but regardless of the carotenoid source, all isomers exist in natural or man made sources of the carotenoids.

Both wild and farmed fish products have been known to have artificial color added in processing. Under these conditions a " color added' or " artificial color" label accurately projects to the consumer what they are looking at. When the same label is applied to farmed fish that have consumed carotenoids as part of a balanced diet, the label becomes deceptive, confusing and misleading.

Food labels should be brief, concise, informative and project accurately what a food is or isn' t. It should not confuse or mislead. Labeling farmed fish " color added' or " artificial color' solely because it is a farm product fed a complete formulated diet that includes carotenoids, is misleading. A more accurate and informative label is required.

(3) The labeling requirement regarding the addition of carotenoids to animal feed and the resultant farm products should be consistent between USDA and FDA. Carotenoids may be fed to chickens to provide a natural color to chicken flesh (21CFR73-35). Under USDA rules, the " color added' or " artificial color' label is not required. There is no difference between feeding carotinoids to terrestrial farm animals or aquatic farm animals, the deposition of carotenoids in the animal' s tissue and ova is the same, and the presence of carotenoids in the foods of all wild aquatic and terrestrial animals is normal and natural. Conversely, the omission of carotenoids from the diets of chickens and salmonids results in an unnatural color and appearance. To avoid confusion and misleading consumers, the labeling requirements for terrestrial and aquatic animals regarding carotenoids being included in their feed should be the same, regardless of the fact that these food items are regulated by different agencies.

Carotenoids are frequently destroyed in the processing of feed stuffs used in animal feeds. In many cases they may not be present in the native feed stuff or other components of the formulated animal feed, or only present at low levels. This results in the need to provide the appropriate carotenoids in the feed from other sources.

(4) Carotenoids are a source of pro-vitamin A and are important anti-oxidants in nature. As such they have been suggested to be essential for the good growth and survival for salmonids. The attached scientific literature documents that growth rates and survival of salmon fry and parr is significantly diminished if these essential carotenoids are omitted from their diet. The inclusion of astaxanthin and canthaxanthin is now the norm for virtually all state and federal enhancement and restoration salmon hatcheries as well as commercial salmon aquaculture facilities.

(5) Selling farmed fish and shellfish, including but not limited to salmonids that contain a natural color from the inclusion of astaxanthin and canthaxanthin in their diets should be labeled to inform the consumer that these are not wild fish, but are a farm product. Fish and shellfish are the only items in a supermarket or restaurant where wild and farmed products compete on a large scale side-by-side.

The health benefits of fish and omega-3 fatty acids is well documented. The risk associated with consumption of some species that are high in mercury is a serious health risk and is also well documented. Salmon, farmed and wild, are both rich sources in omega-3 fatty acid and are exceptionally low in mercury, none detectable according to a recent Harvard Health Letter article on farmed and wild salmon. For this reason consumers should be encouraged to eat more salmon, farmed and/or wild. Farmed Atlantic salmon are the richest source of omega-3 fatty acids as listed in the National Fisheries Institute data sheet, and along with being exceptionally low in mercury, they are affordably priced and available fresh every day. It is this competitive advantage over a seasonal wild product that has to a great extent fueled the disinformation campaign against farmed salmon.

It is important that consumers not be dissuaded from considering farmed salmon because of a misleading label, but at the same time they should be informed that it is not a wild salmon. Consumers make decisions on which food items they will purchase for many reasons, and there is a need for FDA to be increasingly more precise and consistent in its labeling requirements.

(6) An appropriate label informing the consumer the fish or shellfish product is an aquaculture product vs. a wild product is important and appropriate.

There are essentially three (3) categories related to accurate fish labeling:

(a) Wild– fish that are the product of wild reproduction and spend their entire lives in the wild eating natural foods found in their wild environment.

(b) Ranched– this includes hatchery selection and egg fertilization (vs. natural selection) incubation and hatching of eggs in a protected hatchery

environment, and feeding formulated feeds in a hatchery environment for the entire fresh water portion of the salmonids lifecycle. The young salmonids are released at or near the end of this period to migrate out to sea and grow into adults over a period of one or more years. As adults returning to fresh water to repeat the cycle adults are captured by a commercial fishery. State, Federal and private salmon hatcheries in the Northwest and Alaska combined with commercial fishing are a good examples of ocean ranching. Technically this is a form of aquaculture although these fish are commonly sold as wild.

Ocean ranching also includes capture of wild fish and holding them and feeding them for a period of time prior to harvest. Blue fin Tuna is a good example of this type of ocean ranching.

(c) Farmed, Cultured or Aquaculture. Finfish that are the product of animal husbandry in a controlled environment in which the entire life of the animal , including selective breeding and fed formulated diets, is under human control are typically sold as farmed, cultured or aquaculture.

With shellfish, farmed, cultured or aquaculture typically refers to a product of wild reproduction and natural food sources, but are raised on man made substrate specific for that purpose. Some shellfish are the product of hatchery reproduction and special live feeds for a short period.

(6) The labels associated with these categories are self explanatory and quickly and easily convey to a consumer important information about the fish or shellfish. To label a farmed salmon or other fish " farmed' ," cultured' or" aquaculture' conveys an image of a farm animal fed a prepared diet. This immediately and accurately differentiates it from a wild fish or fish product. If additional information is needed regarding the differences between wild, ranched, farmed, aquaculture or cultured, what the ingredients are in fish food, antibiotic use and history and other information that may be of importance or significance to consumers, that information can be provided from other sources, the fish and shellfish supplier or the aquaculture company.

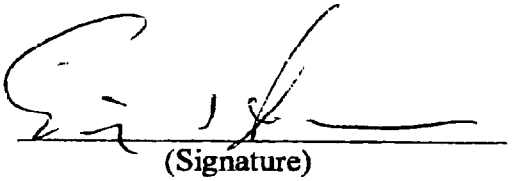
(7) The" color added or" artificial color' label should be consistent in relating that a color agent has been added to the food product as a result of or as part of processing. FDA labeling related to the inclusion of carotenoids in animal feed should be consistent with USDA labeling requirements and different from labels associated with the addition of food coloring agents added during or as a result of processing.

(8) Some environmental groups opposed to finfish aquaculture in general and salmon aquaculture in particular, and associations and companies which sell wild fish directly in competition with aquaculture fish aggressively work to discredit the aquaculture industry and use this labeling requirement to further their cause. It is of great importance to the consumer that labeling requirements are not used to malign or

otherwise mislead consumers about competing industries and products. FDA labeling rules are required to inform consumers with timely, accurate and correct information about a product, not to give one producer a competitive advantage over another or to further the political agenda of a particular special interest group through misleading labels. The consumers right to know must come first through accurate and consistent labeling that accurately projects what a farm product is or isn't.

(9) Environmental Impact. Claim for categorical exclusion under sec. 25.30(k)

The undersigned certifies that, to the best of my knowledge and belief, this petition includes all information and views on which the petition relies, and that it includes representative data and information known to the petitioner which are unfavorable to the petition.



(Signature)

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_____	Erick C Swanson
(Signature)	Name of Petitioner

Aquaculture Nutrition 1995 1, 189–198

Growth and survival of Atlantic salmon, *Salmo salar* L., fed different dietary levels of astaxanthin. First-feeding fry

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Abstract

Atlantic salmon fry hatched from pigment-free eggs and from eggs containing the pigment astaxanthin were fed eleven casein/gelatine-based purified diets with varying levels of astaxanthin, ranging from 0 to 317 mg kg^{-1}, to determine the optimum dietary astaxanthin level for satisfactory growth and survival during the start-feeding period. The fish were fed the experimental diets for a period of 11 weeks.

No difference in performance was found between the two types of fry originating from the pigment-free eggs and those containing pigment. However, the dietary astaxanthin concentration was found to have a significant effect on both the growth and the survival of fry. Fish fed diets with astaxanthin concentrations below 5.3 mg kg^{-1} were found to have marginal growth. In addition, mortality was high in the groups fed diets with astaxanthin concentrations below 1.0 mg kg^{-1}. The specific growth rate (SGR) was also affected by the dietary treatment. The lipid content was higher and the moisture content was lower in the fish fed the diets containing astaxanthin concentrations above 5.3 mg kg^{-1}. The vitamin A and astaxanthin concentrations in whole-body samples of the fry were significantly affected by the dietary level of astaxanthin. A plateau level in whole-body vitamin A concentration was observed at dietary levels of approximately 80 mg astaxanthin kg^{-1} and higher, while no maximum astaxanthin concentration in whole-body samples was observed within the dietary levels used.

The results suggest the need for a minimum dietary astaxanthin concentration of 5.1 mg kg^{-1} to achieve maximum growth and survival during the start-feeding period. The results indicate a low bioavailability of vitamin A palmitate and acetate and the results also suggest a provitamin A function for astaxanthin during the same period.

KEY WORDS Atlantic salmon fry, astaxanthin, growth, survival, vitamin A

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Introduction

The natural pigment in the flesh of wild Atlantic salmon, *Salmo salar* L., is the carotenoid astaxanthin (β,β-carotene-3, 3'-dihydroxy-4,4'-dione). Astaxanthin, like other carotenoids, is synthesized by plants, algae and bacteria. Salmon, however, are not able to synthesize astaxanthin *de novo*. To date, astaxanthin has not been considered to be among the essential nutrients. The functions of carotenoids in fish have generally been associated with camouflage and behaviour related to courtship (Fujii 1969). However, Mikulin & Soin (1975) pointed out that carotenoids, including astaxanthin, are found in animals without eyes or fully developed central nervous systems. This strongly suggests that the behavioural aspects of carotenoids are merely additional functions of these compounds and that carotenoids most likely have other important functions in the biochemical and physiological mechanisms of the organism.

Several hypotheses have been proposed for the possible functions, actions or associations of astaxanthin and other carotenoids in fish (Tacon 1981; Craik 1985, Hilton 1988, Torrissen 1990). Astaxanthin has been shown to have a positive effect on growth and survival in different fish species (Torrissen 1984; Boonyaratpalin & Unprasert 1989, Christiansen *et al.* 1994). Other studies have also shown that astaxanthin has a provitamin A effect in rainbow trout, *Oncorhynchus mykiss* (Walbum) (Schiedt *et al.* 1985, Al-Khalifa & Simpson 1988, Guillou *et al.* 1989) and Atlantic salmon (Christiansen *et al.* 1994). Furthermore, astaxanthin has been reported to increase the survival rate of crustaceans (Chien & Jeng 1992; Négre-Sadargues *et al.* 1993), although the positive effect of astaxanthin on growth was not reported in the crustacean studies.

Optimization of the dietary levels of essential nutrients is a necessity for good growth and survival of fish. In a recent study

where the interactive effects of dietary astaxanthin and vitamin A were investigated during the first feeding period of Atlantic salmon fry, astaxanthin was found to be important for both growth and survival (Christiansen *et al.* 1994). In this study, offspring from pigment-free eggs, without detectable levels of astaxanthin, were used.

It is possible that the adequate storage of astaxanthin or other carotenoids in salmon eggs is important for good performance of the fry during the first-feeding period. The present study was undertaken to determine the dietary astaxanthin level required for normal growth, development and survival during the first-feeding period of Atlantic salmon fry. We also compared the performance of offspring from both pigment-free eggs and normally pigmented eggs to determine the fry's need for an astaxanthin store in the yolk sac during the first-feeding period.

Materials and methods

Experimental design

Atlantic salmon swim-up fry originating from two different groups of broodstock fish were used in the experiment. One group had been reared on a commercial extruded diet without astaxanthin supplementation, and the other group had been reared on a commercial extruded diet supplemented with 100 mg astaxanthin kg^{-1}. The first broodstock group produced pigment-free eggs and the second group produced normally pigmented eggs. A total of 13 000 fry (0.23 g) originating from the pigment-free eggs and 5000 fry (0.23 g) originating from the normally pigmented eggs were randomly distributed into 18 fibreglass tanks (1 × 1 × 0.4 m), with 1000 fry in each tank.

Each tank was supplied with approximately 10 L min^{-1} of fresh water supplemented with sterilized sea water to a conductivity of approximately 1600 μS cm^{-1}. The supplemental sea water was provided to improve the water quality. The average pH of the mixture was 6.0 ± 0.1 (standard deviation) during the experimental period. The temperature of the water supply was elevated to an average of 12.7 ± 0.4°C using a heat exchanger and a heat pump. Both the water temperature and the pH were monitored daily. The fish were subjected to a 24-h light regime using fluorescent daylight tubes throughout the experimental period of 11 weeks.

Preparation of the diets and feeding procedure

Eleven purified test diets containing different levels of astaxanthin were formulated using vitamin-free casein and gelatine as the protein sources. The diets were formulated and prepared as described by Shearer *et al.* (1993). Sardine oil supplemented with 200 mg kg^{-1} ethoxyquin (antioxidant) was used as the lipid source. The ingredients and proximate composition of the basal diet are given in Table 1. Astaxanthin (CAROPHYLL Pink®, F.Hoffmann-La Roche, Basle, Switzerland) was added to the diets in concentrations ranging from 0 to 317 mg astaxanthin kg^{-1} dry diet (Table 2). The vitamin A source used was a mixture of palmitate and acetate (1:1) and was added to the diet at a level of 4128 μg (12 000 IU) kg^{-1}. Dry ingredients were mixed, and an emulsion of gelatine dissolved in 400 mL warm water (including the 100 mL containing the trace minerals), sardine oil, astaxanthin (dissolved in hot water, 50°C), choline chloride, L-lysine and

Table 1 Ingredients and proximate composition of the basal diet (g kg^{-1} of dry matter). Standard deviations are shown in parentheses

Ingredient	g kg^{-1}
Casein, vitamin-free	465
Gelatine	100
Sardine oil (200 ppm ethoxyquin)	180
Dextrin	120
Carboxymethylcellulose	10
α-cellulose	20
L-arginine	10
L-histidine	2
L-lysine HCl	12.5
L-methionine	4
L-phenylalanine	5
L-threonine	10
Vitamin mix[1]	10
Ascorbic acid (phosphate esters)	1
Choline chloride (70%)	10
KCl	15
NaCl	3
$NaHCO_3$	2.5
$CaHPO_4 H_2O$	15
MgO	5
Trace mineral solution[2]	
Analysed proximate composition (g kg^{-1} of dry diet)	
Dry matter	724 (13)
Ash	40 (3)
Crude protein	603 (42)
Lipid	207 (8)
Carbohydrate	150

[1]Vitamin mixture supplied the following kg^{-1} of dry diet: vitamin A (acetate and palmitate 1.1, 500 000 IU g^{-1}) 24 mg (12 000 IU), vitamin D_3 (500 000 IU g^{-1}) 4 mg (2000 IU), α-tocopherol acetate (50%) 100 mg (50 mg), vitamin K_3 (51%) 12 mg (6.1 mg), thiamine-HCl 15 mg, riboflavin 30 mg, pyridoxine hydrochloride 15 mg, calcium D-pantothenate 45 mg, nicotinic acid 150 mg, biotin (2%) 40 mg (0.8 mg); folic acid 4 mg; cyanocobolamin (vitamin B_{12}) (1%) 3 mg (30 μg), inositol 300 mg.

[2]Trace minerals were dissolved in distilled water with 0.5% HCl and supplied as 100 mL kg^{-1} dry diet containing: Fe (as $FeSO_4 \cdot 7H_2O$) 115 mg, I (as KI) 1.9 mg; Mn (as $MnSO_4 \cdot H_2O$) 32.5 mg, Se (as Na_2SeO_3) 4.2 mg, Co (as $CoCl_2 \cdot 6H_2O$) 4.0, Cu (as $CuSO_4 \cdot 5H_2O$) 11.8 mg, Zn (as $ZnSO_4 \cdot 7H_2O$) 88.0 mg.

Table 2 Analysed astaxanthin concentrations (mg kg^{-1} dry diet) in the test diets Standard deviations are shown in parentheses

Diet	Analysed astaxanthin (mg kg^{-1} dry diet)
1	0.0
2	0.2 (0.1)
3	0.4 (0.1)
4	0.7 (0.1)
5	1.0 (0.3)
6	5.3 (0.4)
7	13 7 (1.2)
8	36.0 (0 6)
9	81.4 (5 1)
10	190 1 (17.5)
11	317.3 (1.4)

$NaHCO_3$ were added. The moist mixture was blended well and frozen at –20°C prior to granulation. The diets were minced with a rotating knife and crushed in a granulating machine (Frewitt, Switzerland) using a 1.5 mm mesh. The feeds were passed through a sieve (Sweco, Belgium) equipped with screens of 1 and 2 mm mesh giving three fractions of particles which were then fed to the fish according to fish size. The diets were stored at –20°C prior to use.

Fish in the groups originating from pigment-free eggs received all of the eleven dietary treatments. The zero diet (diet 1) was fed in triplicate while the other ten diets were fed to one group each. Of the fish originating from the normally pigmented eggs, two groups were fed the zero diet, while three groups were fed either diet 3, 5 or 7. The test diets were randomly assigned and the fish were fed the diets from the time of first feeding Feeding was continuous, using automatic feeders (Aquaprodukter, Sunndalsøyra, Norway) on a 24-h cycle. The amount of feed offered was calculated using standard tables to determined the percentage daily weight gain (Austreng *et al.* 1987) and a feed conversion of 0.9 was used.

Growth parameters

Each group was weighed at 14-day intervals except the first and last intervals which were 28 and 7 days, respectively. At each weighing a sample of 100 fry was counted, pooled and weighed to determine the mean weight of the fry in each group At the end of the experiment random samples of 100 fish from each tank were weighed individually. The growth rates (g) and specific growth rates (SGR) for the experimental period were calculated as:

growth rate (g) = $(\ln W_f - \ln W_i) / t$ (Bagenal & Tesch 1978),

$SGR = (e^g - 1) * 100$ (Houde & Schekter 1981),

where W_i and W_f are the average fish weights at the beginning and the end of the experiment and t is the duration of the experiment in days.

Sampling and analyses of feed and fish

The levels of proximate constituents and of astaxanthin in the diets were determined in triplicate using 100-g samples of homogenized feed. Percentage dry matter was determined after heating the samples to 105°C for 24 h. Ash content was determined after combustion at 550°C for 12 h. Lipid content was determined gravimetrically by extraction of dry matter with chloroform/methanol (3.1) (Lie *et al.* 1988). The protein content (N × 6.25) of the dry matter was determined using a Micro-Kjeldahl technique (Crooke & Simpson 1971). Carbohydrate content was determined by difference.

Carotenoids in the feed were determined in samples of approximately 0.2 g taken from a feed sample of 50 g ground into a powder. Dimethyl sulphoxide (DMSO) was added to the feed samples, which were then vortex agitated and placed in a water bath for 1 h. The samples were cooled to ambient temperature and diethylether and an ascorbic acid solution (5 g L^{-1}) were added The suspension was again vortex agitated, centrifuged and the supernatant transferred to a volumetric flask. The extraction process was repeated and diethylether was used to adjust to volume (Sedmak *et al* 1990). The astaxanthin concentrations were determined using the HPLC method described in Torrissen (1986) with external standards. The standards were prepared from crystalline all-trans astaxanthin dissolved in chloroform and acetone The astaxanthin concentrations in the external standards were calculated using a spectrophotometer set to 476 nm and an extinction coefficient (E1%1cm) of 2105 (Manz, 1983)

A total of 250 fish were sampled from both the fry originating from the pigment-free eggs and the pigmented eggs at the initiation of the experiment and 50 fish were sampled from each of the dietary groups at the termination of the experiment. The fry from the different groups were pooled and homogenized for whole-body analyses Dry matter, protein and ash were determined in triplicate from the homogenates using the same methods as described previously for the feed. Homogenized samples of about 1 g were dried and the lipid extracted by adding ethyl acetate followed by vigorous agitation for 1h The solvent was evaporated from an aliquot of the sample and the lipid content was determined gravimetrically.

The astaxanthin content of the fry in the different dietary groups was determined in triplicate from pooled homogenized samples of 10 fish. The stomach and intestine of each fish were removed prior to homogenization to avoid inflated astaxanthin levels resulting from the presence of astaxanthin in the gastrointestinal tract. Astaxanthin was extracted from the samples using acetone supplemented with 100 mg L^{-1} of the antioxidant BHT (2,[6]-di-tert-butyl-p-cresol) as described in Christiansen *et al.* (1995b) The astaxanthin concentrations were then determined as described above for the feed samples.

The initial vitamin A concentration in whole-body samples from a pooled sample of 10 fish was determined according to Lambertsen (1983). To approximately 0 5 g of the homogenate were added ethanol, EDTA, pyragalloll, ascorbic acid and KOH (200 g L^{-1}) before it was saponified at 100°C. Retinol was extracted from the suspension with hexane and the concentration was determined by HPLC (column: SiO_3, 3 μm; eluent. isopropanol in hexane, 100 mL L^{-1}). At the final sampling, the vitamin A concentrations were determined from pooled samples of 10 fish from each dietary group. The vitamin A concentration in sardine oil was determined using the same method.

Statistical analyses

The mean body weights were transformed to $\log_{10}$ and the log-transformed weight data were subjected to linear regression analysis. Analyses of covariance were used to test for differences in the slopes of the regression lines (Zar 1984) of the different dietary groups to reveal differences in growth rates. The optimum dietary level of astaxanthin was determined using broken line regression analysis (Zeitoun *et al.* 1976). The survival data were arcsin transformed prior to the statistical analyses (Zar 1984). The relationship between the vitamin A and astaxanthin concentration in the fry and the dietary astaxanthin content was described using non-linear regression analyses. One-way analysis of variance (ANOVA) was used to test for differences in the proximate composition of the fry Where significant effects of the dietary treatment on the proximate composition were found, the Student–Newman–Keuls multiple range test was used to test for differences among the groups. The statistical analyses were performed using the software packages CSS (Statistica, Complete Statistical Systems) and RS1 (BBN Software Products Corporation). Statistical significance was tested at the 0.05 probability level. Where mean values are given in the text, the standard deviation is supplied in parentheses.

Results

The proximate composition of the basal diets is shown in Table 1. The dry matter content was 725 g kg^{-1}. The content of protein, lipid and ash on a dry weight basis were 603, 207 and 40 g kg^{-1}, respectively. The astaxanthin contents of the diets ranged from 0 to 317 mg kg^{-1}. Vitamin A was not detected in the sardine oil.

There were no statistical differences in growth or mortality among the three zero groups originating from the pigment-free eggs. The groups were, therefore, pooled prior to further statistical analysis Moreover, there was no significant difference found with respect to either growth or mortality between the two zero groups originating from the pigmented eggs. These two groups were also pooled prior to further statistical analysis

The average weights of fish originating from the pigment-free eggs at each weighing are shown in Fig. 1. Weights of the fish from the normally pigmented eggs are shown in Fig 2 The coefficient of determination (r^2) of the regression lines of the log-transformed weight data varied from 0 78 to 0.996 (Table 3). All slopes were significantly different from zero. Dietary astaxanthin content was found to have a significant effect on fish growth expressed as differences in the slopes of the regression lines for log-transformed weight data versus time (Table 3) There were no differences in the slopes for the groups that were fed diets containing 0 to 0 7 mg of astaxanthin kg^{-1}, regardless of the origin of the fry. The two groups of fry fed diet 5, which contained 1 0 mg astaxanthin kg^{-1}, showed improved growth, as revealed by a significantly higher slope compared with the slope

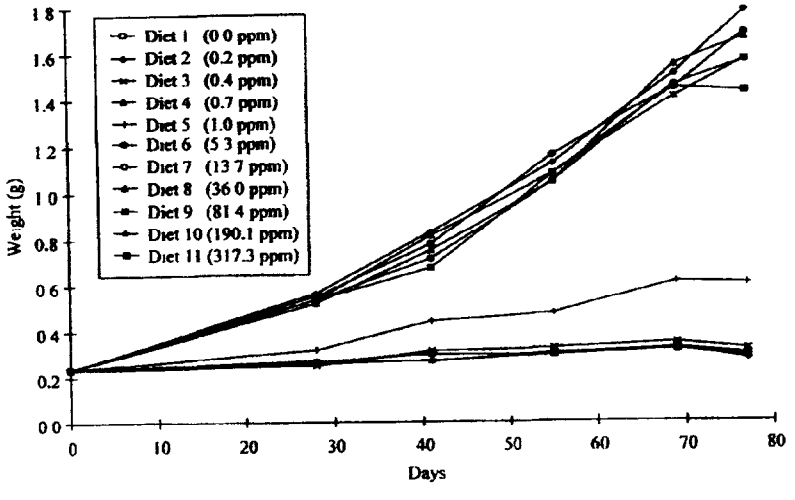

Figure 1 Mean weights of Atlantic salmon fry originating from pigment-free eggs fed diets containing different levels of astaxanthin (shown in parentheses) during the first-feeding period

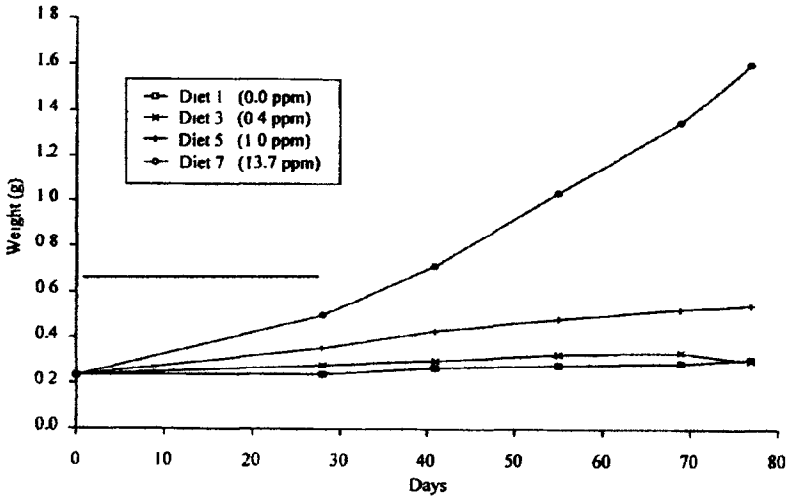

Figure 2 Mean weights of Atlantic salmon fry originating from pigmented eggs fed diets containing different levels of astaxanthin (shown in parentheses) during the first-feeding period

of the groups fed diets with astaxanthin levels below 1.0 mg astaxanthin kg^{-1}. Once again, there were no significant differences in the slopes between the groups originating from pigment-free and pigmented eggs. The best growth was observed for the groups fed diets 6 to 11, which contained from 5.3 to 317.3 mg astaxanthin kg^{-1}, and there were no significant differences in the slopes of the latter groups, regardless of the origin of the eggs Similar trends were observed for SGR, which ranged from 0.3% to 2.7% day^{-1} (Table 4). SGR values for the groups originating from pigmented eggs were in the same range as those for fry hatched from pigment-free eggs. Broken-line regression analyses, using data from the fry originating from the pigment-free eggs, indicated a maximum growth response of the fish at a dietary astaxanthin concentration of 5.1 mg kg^{-1}. This is defined as the minimum dietary level of astaxanthin for maximum growth rate in this diet.

The relationship between the mean weight of the fish at the end of the experiment and the dietary astaxanthin concentration in the feed was found to be non-linear (Fig. 3). Broken-line regression analyses, using data from the fry originating from the pigment-free eggs, indicated that the optimum dietary astaxanthin level for maximum weight gain was 5.0 mg astaxanthin kg^{-1} The correla-

Table 3 Slopes, significance levels of slopes and coefficient of determination (r^2) from the linear regression analyses of the log-transformed weight data versus time of feeding. Slopes with different letters were found to be significantly different, by analyses of covariance

Diet	Astaxanthin (mg kg^{-1})	Slope	P-value‡	r^2
		Fry from pigment-free eggs		
1	0 0	0 0017[a]	**	0.921
2	0 2	0 0014[a]	*	0 782
3	0 4	0 0024[a]	**	0.873
4	0.7	0 0018[a]	***	0 978
5	1.0	0 0059[b]	***	0.980
6	5.3	0 0112[c]	****	0.990
7	13 7	0 0110[c]	****	0.994
8	36.0	0 0114[c]	****	0.996
9	81 4	0.0109[c]	***	0.986
10	190 1	0 0115[c]	****	0 992
11	317 3	0 0107[c]	****	0 984
		Fry from pigmented eggs		
1	0 0	0.0017[a]	*	0 830
3	0 4	0 0018[a]	*	0.780
5	1.0	0 0048[b]	****	0 955
7	13 7	0 0109[c]	****	0 976

‡Regression analyses, n = 6, *, $P < 0$ 05, **, $P < 0.01$, ***, $P < 0$ 001; ****, $P < 0$ 0001

Diet	Astaxanthin (mg kg^{-1})	SGR	Survival (%)	Moisture (g kg^{-1})	Ash (g kg^{-1})	Lipid (g kg^{-1})	Protein (g kg^{-1})
			Fry from pigment-free eggs				
1	0.0	0.39	33.7	843 (4.2)^{a}	21 (0.9)^{a}	6 (0 4)^{a}	121
2	0.2	0.30	29.7	846 (0.5)^{a}	22 (0.2)^{a}	5 (0 2)^{a}	118
3	0.4	0.51	49.2	835 (0.6)^{b}	22 (1.7)^{a}	7 (0.1)^{a}	128
4	0.7	0.43	10 4	844 (4.1)^{a}	21 (0.4)^{a}	7 (1.0)^{a}	119
5	1.0	1.34	84.3	806 (1.1)^{c}	22 (1.2)^{a}	28 (1.0)^{b}	136
6	5 3	2.61	96.0	767 (0.9)^{d}	20 (0.1)^{a}	46 (3.9)^{c}	140
7	13.7	2.52	93.7	759 (0.8)^{e}	21 (0.4)^{a}	56 (1.9)^{d}	141
8	36.0	2.60	98.4	758 (5.0)^{e}	20 (0.5)^{a}	60 (1 2)^{e}	138
9	81.4	2.54	98.1	755 (3.7)^{e}	21 (0 7)^{a}	64 (0 3)^{f}	143
10	190 1	2.71	96.4	757 (4.2)^{e}	20 (1 2)^{a}	63 (0.5)^{f}	140
11	317.3	2.40	89.6	758 (4.1)^{e}	21 (0 4)^{a}	59 (0.2)^{e}	139
			Fry from pigmented eggs				
1	0 0	0.37	17.0	834 (1.5)^{b}	21 (0 5)^{a}	6 (1 0)^{a}	128
3	0.4	0.34	27.4	837 (1.3)^{b}	22 (0 7)^{a}	7 (0 2)^{a}	123
5	1 0	1.10	87.4	812 (1.9)^{f}	22 (1.0)^{a}	22 (0 2)^{g}	138
7	13.7	2.53	98.3	759 (2.9)^{e}	20 (1.0)^{a}	73 (1 6)^{h}	144

Table 4 Daily percentage increase in weight (SGR), percentage survival and whole body composition (kg^{-1} of wet weight) of Atlantic salmon fry fed diets with different levels of astaxanthin for 11 weeks Means in columns that have different letters are significantly different ($P < 0.05$; Student–Newman–Keuls multiple range test) Standard deviations are shown in parentheses

tion coefficient (r) was 0.988, and 97.7% of the variance was explained by the model

Dietary astaxanthin content was also found to have a significant effect on fish survival. Cumulative percentage survival for the different dietary groups is shown in Table 4. Mortality was low in all groups during the first 3 weeks of the experiment. Three weeks after feeding was initiated, the fish in groups fed the diets supplemented with less than 1 0 mg astaxanthin kg^{-1} began to die and the mortality in these groups remained high throughout the experiment More than 50% of the fry fed diets with less than 1.0 mg astaxanthin kg^{-1} died during the experimental period The survival of the group fed the diet containing 1.0 mg astaxanthin kg^{-1} was 84%, while all the groups fed diets with higher astaxanthin concentrations had survival percentages above 90% There were no observed differences in survival related to the origin of the fry.

At the beginning of the study, the fry from the pigment-free eggs contained, on a wet weight basis, 817 (SD1) g kg^{-1} moisture, 26 (1) g kg^{-1} fat and 14 (0.1) g kg^{-1} ash. Fry from the pigmented eggs contained 811 (0) g kg^{-1} moisture and 28 (1) g kg^{-1} lipid on a wet weight basis, and 13 (0.1) g kg^{-1} ash. The whole-body compositions of the fry at the end of the experiment are given in Table 4 according to each experimental diet One-way ANOVA showed that the percentages of moisture and lipid were significantly influenced by the dietary treatment. There were no significant effects on the ash contents In the groups

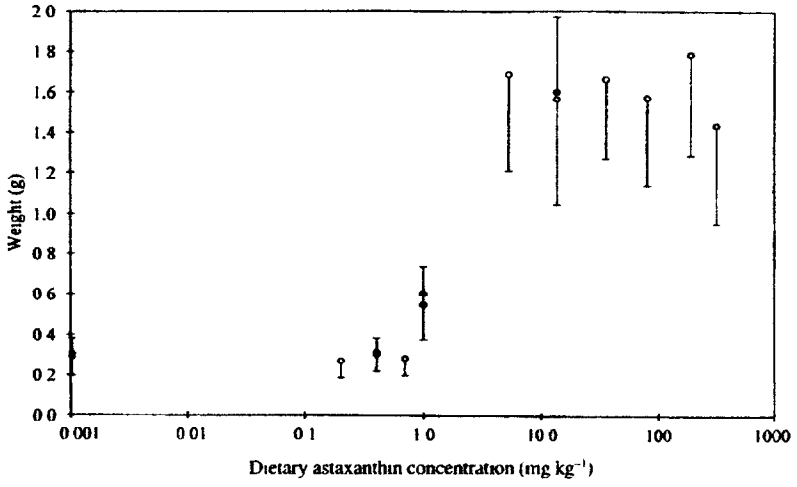

Figure 3 Mean final weights of Atlantic salmon fry originating from pigment-free eggs (○) and fry from pigmented eggs (● versus dietary astaxanthin (mg kg^{-1}) concentration after 11 weeks of feeding Horizontal lines show standard deviation ($n = 100$)

fed diets containing less than 1.0 mg astaxanthin kg^{-1} the lipid content decreased and moisture content increased from the start to the end of the experiment. The ash content increased in all groups throughout the experimental period

The initial mean vitamin A concentration in the fry originating from the pigmented eggs was 0.72 (0 12) $\mu g\ g^{-1}$ wet body weight, which is significantly higher than the initial concentration found in the fry from the pigment-free eggs, 0.54 (0.12) $\mu g\ g^{-1}$ wet body. The dietary treatment had a significant effect on the determined vitamin A concentrations analysed in whole fry at the end of the experimental period (Fig. 4). Non-linear regression analyses gave the following relationship between the vitamin A content in the whole body at the end of the experiment and the dietary astaxanthin content

$$\text{Vitamin A } (\mu g\ g^{-1}) = 0.127 + 0.237 \log_{10}(x),$$

where x is the dietary astaxanthin concentration. According to this relationship, a plateau level of whole-body vitamin A is reach at a dietary astaxanthin concentration of 80 mg kg^{-1}.

The concentrations of free astaxanthin in the whole bodies of fry originating from both pigmented and pigment-free eggs at the start of the experiment were below the detection limit of the methods used The relationship between the astaxanthin concentrations in the fry and the dietary astaxanthin concentration was non-linear (Fig 4) and was described, using data from the fry originating from the pigment-free eggs, by the following equation

$$\text{Astaxanthin (mg kg}^{-1}) = (2.0{*}10^{-5}{*}x^2) - (1.0{*}10^{-3}{*}x) - 0.02,$$

where x is the dietary astaxanthin level. The whole-body astaxanthin concentration was not observed to reach a plateau within the dietary levels used Free astaxanthin was not detected in the fry originating from pigment-free eggs when the dietary astaxanthin concentrations were less than 36 mg kg^{-1} diet. However, the concentration in the group originating from pigmented eggs which had been fed a diet with an astaxanthin concentration of 13 mg kg^{-1} was 0.2 mg kg^{-1}. Red pigmentation of the skin, particularly at the edges of the fins, was observed in the groups fed the diet with high levels of astaxanthin. The degree of skin pigmentation in the different groups was not quantified.

Discussion

A casein/gelatine based diet was used in this experiment to ensure low levels of carotenoids. The analyses of the diets did not reveal the zero diet to contain astaxanthin. Sardine oil was chosen as the lipid source because of its low carotenoid concentrations, vitamin A was not detected in the oil This is in agreement with previous analyses of sardine oil which have shown vitamin A levels to be below the detection limit of the method used (Lie unpublished) The growth of the fry in the groups fed diets with a sufficient amount of astaxanthin was comparable to that of fry from other studies where purified diets based on casein and gelatine were used (Rumsey & Ketola 1975; Christiansen *et al.* 1994).

The eggs were obtained from two groups of broodstock fish, one of which had been fed a diet with astaxanthin supplementation and one which was fed a diet without astaxanthin supplementation It was anticipated that offspring from the second group would show reinforced and accelerated effects of low

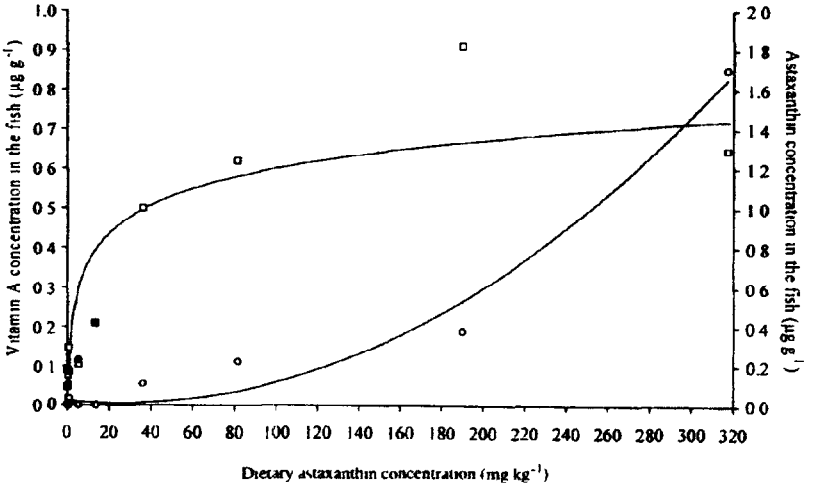

Figure 4 Vitamin A concentrations ($\mu g\ g^{-1}$ whole body) in Atlantic salmon fry originating from pigment-free eggs (□) and in those originating from pigmented eggs (■) and astaxanthin concentrations (mg kg^{-1} whole body) in fry originating from pigment-free eggs (○) and in fry originating from pigmented eggs (●) when fed diets containing different levels of astaxanthin during the start-feeding period

dietary astaxanthin levels on growth and survival due to the low level of carotenoids in the fry's yolk sacs. However, such effects were not observed and there were no differences in either growth or mortality of offspring from the two broodstock groups

Dietary astaxanthin content did have a clear effect on the survival rate of the fry, and astaxanthin was found to be essential for good survival. This finding is in agreement with Christiansen *et al.* (1994), who observed high mortality in start-feeding Atlantic salmon fed purified diets without astaxanthin supplementation. Other studies have also revealed similar effects of canthaxanthin and β-carotene on the survival of Indian carp (Goswami, pers. comm.) and of astaxanthin on the survival of kuruma prawn, *Penaeus japonicus* (Bate) (Chien & Jeng, 1992, Négre-Sadargues *et al.* 1993) and lobster, *Homarus vulgaris* (Milne-Edwards) (Uglem & Christiansen, unpubl).

Dietary astaxanthin concentration also had a significant effect on the growth of the fry. This is in agreement with Torrissen (1984), who observed increased growth in Atlantic salmon fry fed commercial diets supplemented with 30 mg astaxanthin and 30 mg canthaxanthin kg^{-1} when compared with a control group which was fed a commercial diet without carotenoid supplementation Christiansen *et al.* (1994) also observed a positive effect of dietary astaxanthin supplementation on the growth of Atlantic salmon fry fed casein/gelatine-based diets containing 15 and 45 mg astaxanthin kg^{-1} dry matter In addition a similar positive effect of astaxanthin on the growth of parr was observed by Christiansen *et al.* (1995a), who studied the effect of astaxanthin on the antioxidant status and immune response of Atlantic salmon parr/smolt A positive effect of dietary carotenoids on growth has been found in other fish species, such as red tilapia, *Oreochromis niloticus* L. (Boonyaratpalin & Unprasert 1989) Goswami (pers. comm) found that the carotenoids β-carotene and canthaxanthin improved the growth of Indian carp Bordner *et al.* (1986) reported increased growth in lobster, *Homarus americanus* (Milne-Edwards), when carotenoids from crawfish waste were supplemented to a purified diet.

Growth rates of fry fed diets without astaxanthin or with low levels of supplemented astaxanthin were poor. Fry fed commercial diets do, however, show good growth Commercial diets for start-feeding fry have normally not been supplemented with astaxanthin The diets do, however, contain small amounts of astaxanthin and other carotenoids from the dietary ingredients, especially fish meals and oils. Previous analyses of the astaxanthin levels in some commercial start feeds have shown levels of astaxanthin ranging from 5 to 10 mg kg^{-1} (Christiansen unpubl.) Those concentrations are above the minimum dietary astaxanthin level suggested in the present study for the optimum growth and survival of Atlantic salmon fry

The proximate composition was affected by the dietary treatment. The whole-body moisture content was higher and the lipid content lower in the fry that were fed the diets low in astaxanthin. These fry also had lower specific growth rates than those fed diets with astaxanthin levels above 1 mg kg^{-1} The resulting differences in growth and proximate composition may be the result of poor feed intake due to a depressed palatability of the diets containing little or no astaxanthin. As far as we know, astaxanthin has not been shown to be a feed attractant for fish. It is also possible that the colour of the diets may have affected the feed intake of different groups, but the diets were introduced as the first feed and all groups were observed to ingest feed at the beginning of the experiment Therefore, the poor growth and nutritional status of the fish may have been the result of a deficiency for an essential nutrient.

Animals are unable to synthesize vitamin A *de novo*. The requirement must be satisfied via the diet either as vitamin A or as provitamin A Vitamin A deficiency symptoms in fish include poor growth, haemorrhaging, keratinization of epithelial tissue and visual disorders (Halver 1989). The dietary requirements of vitamin A for salmonids have been reported to vary between 2000 and 2500 IU kg^{-1} (690–860 μg vitamin A acetate kg^{-1}) of dry matter (Halver 1989) However, to our knowledge, the dietary requirement of vitamin A for Atlantic salmon has not been reported. All diets in the present study were supplemented with 4128 μg kg^{-1} (12 000 IU) of vitamin A, supplied as a mixture of vitamin A palmitate and acetate Because the sardine oil contained no detectable levels of vitamin A, the vitamin A palmitate and acetate were the only vitamin A sources in the diet The supplemented level of vitamin A in the present study should have been sufficient to satisfy the need for vitamin A.

The vitamin A levels found in whole-body analysis in the present study were comparable to those found in a study by Christiansen *et al* (1994) using a similar diet The dietary astaxanthin level was found to have a significant effect on the vitamin A content of the fry, the lowest levels of vitamin A being found in groups fed the diets with the lowest astaxanthin levels. This effect could have been caused either by the reduced intake of vitamin A resulting from poor feed intake, combined with a metabolic depletion of vitamin A in the tissue, or by a poor bioavailability of the vitamin A esters in the diet Fry fed the diets containing 5 3 and 13 7 mg astaxanthin kg^{-1} achieved satisfactory growth, showing that the low levels of vitamin A found in these fry were not the result of low feed intake The low levels of vitamin A in the groups fed the diets with low levels of astaxanthin indicate a poor bioavailability of synthetic vitamin A palmitate and/or acetate for Atlantic salmon fry, as has also been seen by Christiansen *et al* (1994) Larger Atlantic salmon (> 20 g), on the other hand, have been reported to utilize synthetic vitamin A esters (Storebakken *et al* 1993, Thompson *et al* 1994) Further studies are required

to clarify whether differences in the bioavailability of different forms of vitamin A exist for Atlantic salmon fry.

Given that all the fry were fed the same level of synthetic vitamin A, and that fry fed diets with little or no astaxanthin showed low levels of vitamin A, the results strongly suggest that astaxanthin functions as a provitamin A for Atlantic salmon fry. A provitamin A function or a vitamin A saving effect of astaxanthin during the first-feeding period of Atlantic salmon has been suggested by Christiansen *et al.* (1994). The conversion of astaxanthin to vitamin A has been reported in rainbow trout (Schiedt *et al.* 1985, Al-Khalifa & Simpson 1988; Guillou *et al.* 1989) Other studies have also shown a provitamin A function of astaxanthin in fish species such as *Gambusia holbrooki* Girard (Grangaud *et al.* 1962) and *Tilapia nilotica* (L.) (Katsuyama & Matsuno 1988)

Reduced growth is one of the vitamin A deficiency symptoms reported in fish (Kitamura *et al.* 1967, Dupree 1970, Goswami & Basumatari 1988). In the present study, with the exception of fish in groups fed diets containing 5.3 mg astaxanthin kg^{-1} diet, reduced growth and increased mortality were observed in the groups where the vitamin A levels of the fish were depleted The repressed growth and high mortality in groups fed diets with less than 5.3 mg astaxanthin kg^{-1} may be the result of a vitamin A deficiency. The good growth of the fish in the groups fed diets containing 5.3 and 13.7 mg astaxanthin kg^{-1} may be explained as a sufficient provitamin A supply in the diet which maintained the metabolic processes, including growth, despite a depletion of the vitamin A storage. The group fed the diet containing 5.3 mg astaxanthin kg^{-1} had a lower lipid content than the groups fed diets with higher astaxanthin content, possibly suggesting the initial signs of a deficiency. This observation has been confirmed in a subsequent feeding experiment where this group also showed reduced growth (Christiansen & Torrissen unpublished data).

The antioxidant properties of astaxanthin may also be important for metabolism (Terao 1989). Recent evidence suggests that astaxanthin and other carotenoids are important in the protection of membrane lipids from peroxidation (Kurashige *et al.* 1990, Miki 1991; Oshima *et al.* 1993). It has also been shown that the levels of the antioxidant vitamins A, E and C increase in Atlantic salmon fed a diet supplemented with astaxanthin when compared with salmon fed an astaxanthin-free diet (Christiansen *et al* 1995a).

The minimum dietary level of astaxanthin for satisfactory growth and survival of Atlantic salmon fry was found to be 5.1 μg astaxanthin g^{-1} of dry matter. Other carotenoids have been found to improve growth in other fish species and the role of astaxanthin may be covered by other carotenoids such as canthaxanthin, zeaxanthin and lutein which all contain two (β-ionone rings Zeaxanthin and lutein are common carotenoids in the aquatic environment. The dietary requirement was determined using a purified diet. The digestible energy content of different diets will vary depending on the proximate composition and the levels and quality of the ingredients used. This, in turn, will affect the amount of a nutrient that is necessary to satisfy the requirements of fish, and it is likely that the dietary requirement of astaxanthin will vary according to the digestible energy content of the diet. Because the requirements of the salmon for essential nutrients are related to life history stage, the astaxanthin requirement will probably change according to the size, age and maturity status of the fish.

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FOOD COMPOSITION AND ADDITIVES

Rapid Liquid Chromatographic Method to Distinguish Wild Salmon from Aquacultured Salmon Fed Synthetic Astaxanthin

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Analytical methods are needed to determine the presence of color additives in fish. We report a liquid chromatographic (LC) method developed to identify the synthetic form of the color additive astaxanthin in salmon, based on differences in the relative ratios of the configurational isomers of astaxanthin. The distributions of configurational isomers of astaxanthin in the flesh of wild Atlantic and wild Pacific salmon are similar, but significantly different from that in aquacultured salmon. Astaxanthin is extracted from the flesh of salmon, passed through a silica gel Sep-Pak cartridge, and analyzed directly by LC on a Pirkle covalent L-leucine column. No derivatization of the astaxanthin is required—an important advantage of our approach, which is a modification of our previously described method. This method can be used to distinguish between aquacultured and wild salmon. The method has general applicability and can also be used to identify astaxanthins derived from other sources such as *Phaffia* yeast and *Haematococcus pluvialis* algae.

The oxycarotenoid astaxanthin is responsible for the distinctive color of salmon flesh (1). Because salmon cannot synthesize astaxanthin de novo, their flesh color is derived entirely from astaxanthin in their diet (2). Wild salmon acquire their pink-to-red color from astaxanthin in their prey. To obtain a flesh color similar to that of wild salmon, aquacultured salmon are fed with fish feed supplemented with color additives (3). Two oxycarotenoids are believed to be widely used as color additives in fish feed to enhance the color of aquacultured salmonids: canthaxanthin, β,β-carotene-4,4′-dione (Figure 1), and astaxanthin, 3,3′-dihydroxy-β,β-carotene-4,4′-dione (Figure 2).

Only color additives are listed in the *Code of Federal Regulations* (CFR) may be used legally in the United States to enhance the color of salmon and other animals used as food (4). Astaxanthin recently was listed by the U.S. Food and Drug Administration (FDA) as a color additive in salmonid feed to pigment the flesh of salmonids in the United States (5). Because a validated analytical method was unavailable to distinguish synthetic astaxanthin in aquacultured salmon from astaxanthin in wild salmon, a method was needed to determine the presence of added synthetic astaxanthin in the fish, as required by the CFR. Canthaxanthin is listed in the CFR as a food color additive (6). A petition has been submitted for its use as a color additive to color the flesh of salmonids (7). Astaxanthin, however, and not canthaxanthin, is normally found in wild salmon (Atlantic salmon, *Salmo salar*, and Pacific salmon, *Oncorhynchus*). Canthaxanthin can be distinguished easily from astaxanthin by thin-layer chromatography (TLC; 8) and liquid chromatography (LC; 9).

All-*trans* astaxanthin is the major geometric isomer in wild salmon flesh (10) and also in the stabilized synthetic astaxanthin beadlet added to the fish feed of aquacultured salmon. All-*trans* astaxanthin has 2 chiral centers, C-3 and C-3′, and can exist as 3 configurational isomers: 2 enantiomers (3*R*,3′*R* and 3*S*,3′*S*) and a meso form (3*R*,3′*S*) (Figure 2). Synthetic all-*trans* astaxanthin consists of a racemic mixture of the 2 enantiomers and the meso form.

Studies with rainbow trout (*Oncorhynchus mykiss*; 11) and Atlantic salmon (12) have shown that when synthetic astaxanthin or the individual configurational isomers are added to fish feed, they are deposited in the flesh of the salmon with no change in the configurational isomer distribution. These results indicate the absence of selective absorption or deposition of the different configurational isomers and of epimerization at C-3 and C-3′. Therefore, the ratio of configurational isomers in salmon flesh reflects the configurational isomer distribution in the diet.

Maoka et al. (13) resolved all-*trans* astaxanthin on a covalent D-phenylglycine Pirkle-type column manufactured in Japan; however, the analysis required 70 min. Astaxanthin can also be derivatized with enantiomerically pure chiral reagents, such as camphanic acid chloride, to give diastereomers that can be separated on an achiral stationary phase (14). In our laboratory, other derivatizing reagents, such as 1-naphthoyl chloride, that enhance the affinity of the configurational analyte for the chiral stationary phase without changing the enantiomeric relationship of the configurational astaxanthin isomers to each other, were also used successfully.

Although in some cases the derivatization of synthetic astaxanthin was spontaneous, avoidance of the extra step of making and purifying an astaxanthin derivative was deemed advan-

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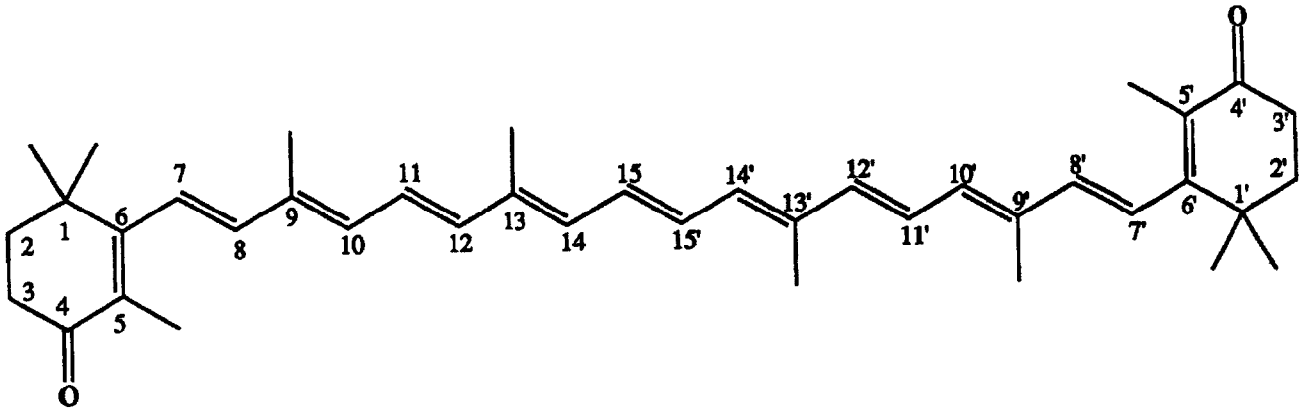

Figure 1. Canthaxanthin.

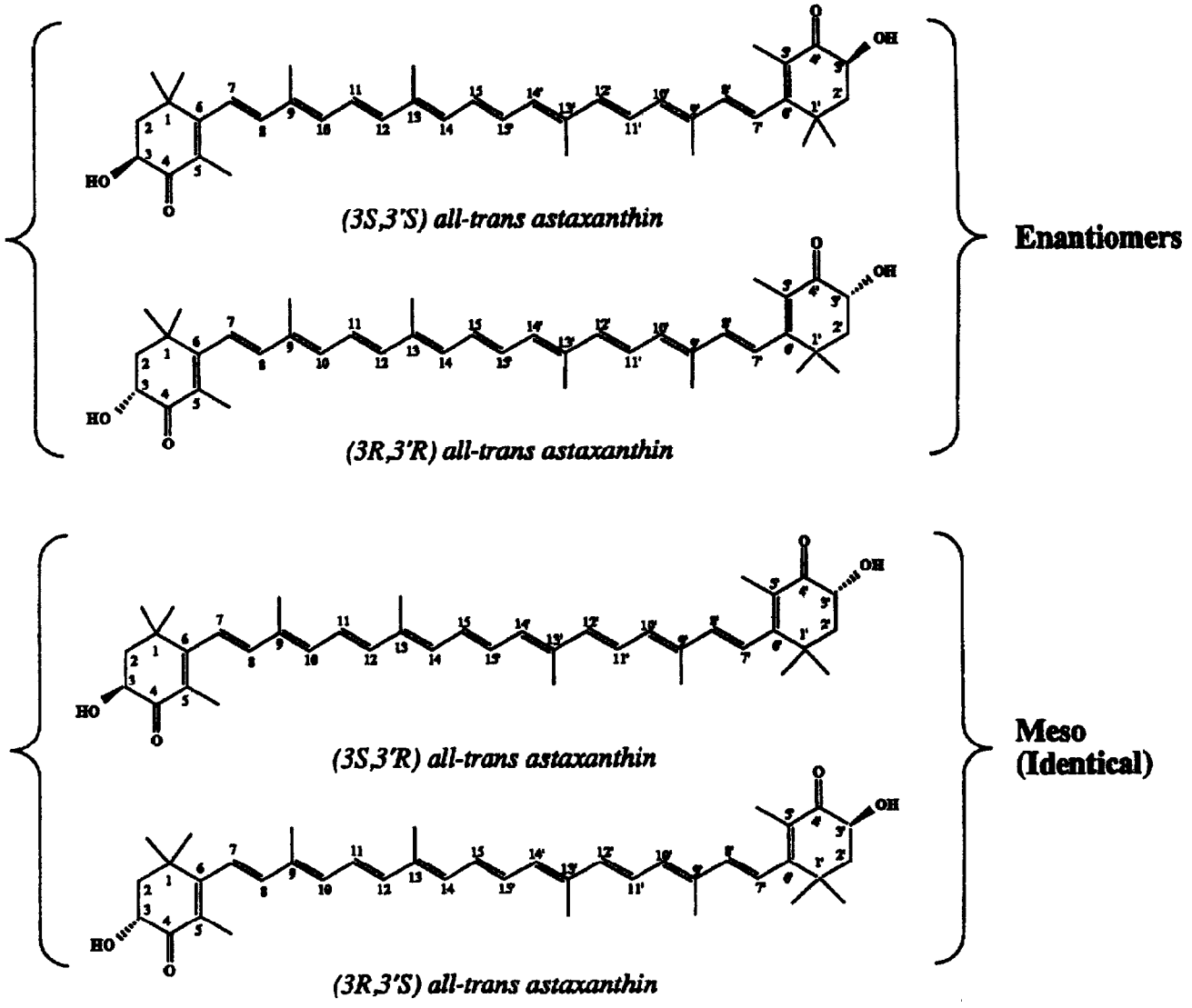

Figure 2. Configurational isomers of all-*trans* astaxanthin.

tageous. Moreover, when the astaxanthin extracted from the flesh of salmon was used, residual fish oil frequently interfered with and sometimes inhibited the derivatization reaction.

This paper describes an LC method based on direct resolution of the configurational isomers of underivatized astaxanthin (15). We previously described an LC method for efficiently separating and identifying the configurational isomers of synthetic astaxanthin in salmon (16). The present method is faster than the LC method of Lura and Saegrov (17), in which astaxanthin is derivatized before LC analysis. It entirely avoids the derivatization step in which a residual amount of colorless lipids may interfere and, therefore, must be removed before derivatization (10). Previously, we reported that mobile phases of similar polarity allowed direct resolution of the configurational isomers of all-*trans* astaxanthin on a covalent L-leucine Pirkle column in 10–15 min (15). However, when a significant amount of *cis*-astaxanthin was present, analysis time was sometimes as long as 25 min (Figure 3).

With the modified method described here, we can distinguish between synthetic astaxanthin extracted from the flesh of salmon and naturally occurring astaxanthin extracted from the flesh of wild salmon by comparing their chromatographic profiles. During the method development phase of this study, the color extracts from the flesh of salmon and the synthetic astaxanthin standard were chromatographed by using mobile phase A described in the **Experimental** section.

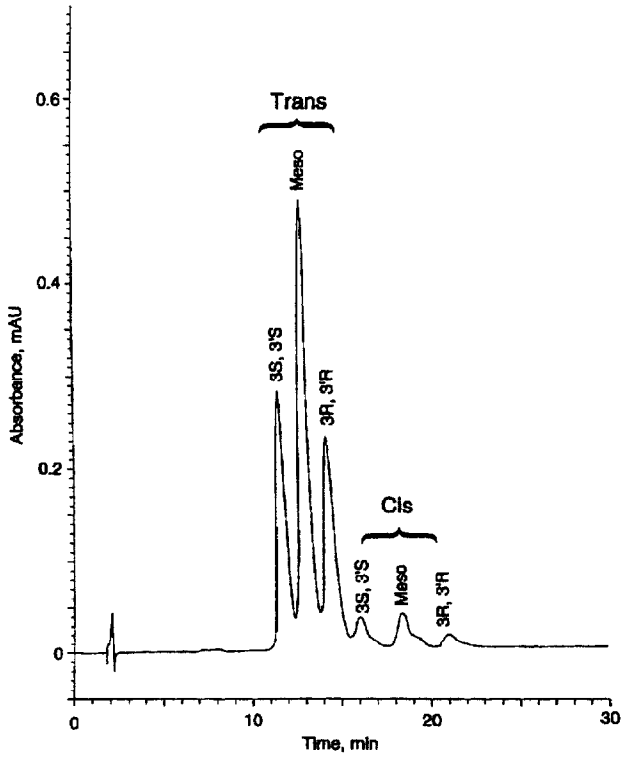

Figure 3. Chromatogram of synthetic astaxanthin. LC conditions: Pirkle covalent L-leucine column; mobile phase B; flow rate, 1.5 mL/min; monitoring wavelength, 474 nm.

To devise a regulatory scheme to identify the color additive astaxanthin in salmon flesh and thereby distinguish between aquacultured salmon and wild marine salmon, it is necessary to know the ratio of the configurational isomers of the all-*trans* astaxanthin in each species of salmon from a broad-based set of authentic wild marine Atlantic and Pacific salmon. A range of each of the configurational isomers in astaxanthin extracted from wild salmon was determined by Schiedt et al. (10): 78–85% of the (3*S*,3′*S*) enantiomer, 12–17% of the (3*R*,3′*R*) enantiomer, and 2–6% of the (3*R*,3′*S*) meso form. This pioneering work, however, was based on 4 Atlantic salmon and 1 salmon from each of 3 Pacific species. The data set was too narrow to be generalized to the larger population of wild salmon.

We initiated a study to determine the configurational isomer distribution that could be generalized to wild marine salmon (*Salmo salar* and *Oncorhynchus*). Such a distribution would form the reference standard with which the distribution of configurational isomers of astaxanthin in any salmon could be compared to determine whether the salmon was aquacultured or wild. Thus, a total of 80 specimens consisting of authenticated wild, male and female, Atlantic and Pacific salmon were obtained, as described in the **Experimental** section. These salmon constitute a broad-based set that can be generalized to wild marine salmon. The identification of the species of wild Pacific salmon was reconfirmed in-house by analysis of fish scale patterns (18, 19).

We report here the results of the analysis of at least 6 wild salmon from each of the 6 species.

Experimental

Apparatus

(a) *Liquid chromatograph.*—Method development was conducted and initial analysis of salmon extracts was performed by using an HP 1090 Series II/M liquid chromatograph equipped with a DR5 ternary solvent delivery system, helium sparge, autosampler, diode array detector, and workstation (Hewlett-Packard, Inc., Avondale, PA). Analysis of the astaxanthin extracts from marine-caught, authenticated wild salmon was performed by using a Waters solvent delivery system Model 510 equipped with a Waters Model 990 diode array detector, a workstation (Waters Chromatography Division, Millipore Corp., Milford, MA), and a Beckman Model 504 autosampler (Beckman Instruments, Inc., Fullerton, CA). LC conditions: Isocratic conditions and ambient temperature were used for all analyses, and solvents were filtered and sparged with He before use.

(b) *LC columns.*—For initial analyses, including analysis of derivatized astaxanthin, a Pirkle covalent D-phenylglycine column, 5 µm particle size, 25 cm × 4.6 mm (Regis Chemical Co., Morton Grove, IL) was used. For remaining analyses, including the study of the configurational isomer distribution of astaxanthin in authenticated wild salmon, the Pirkle covalent L-leucine column previously described (15) was used.

(c) *Homogenizer.*—Polytron (Brinkmann Instruments, Inc., Westbury, NY).

(d) *Centrifuge.*—Sorvall Instruments Model RC5C fitted with a GSA rotor (DuPont Co., Instruments Div., Newton, CT).

(e) *Spectrophotometer.*—Hitachi 200 (Hitachi Ltd., Tokyo, Japan).

(f) *Freezer.*—Model 8416, ultralow temperature, upright freezer (Forma Scientific, Div. of Mallinckrodt, Inc., Marietta, OH).

(g) *Microscope.*—Nikon Optiphot (Nikon, Inc., Melville, NY).

(h) *Solid-phase extraction cartridge.*— Sep-Pak, silica gel (Waters Chromatography Div., Millipore Corp.).

(i) *Rotary evaporator.*—Büchi Rotavapor R 110 (Brinkmann Instruments, Inc.).

(j) *Molecular sieve.*—Union Carbide Type 4 Å, 1/16 in., 8–12 mesh (Fluka Chemical Corp., Ronkonkoma, NY).

Reagents

(a) *Solvents.*—Hexane, tetrahydrofuran (THF), methylene chloride, and 2-propanol (all LC grade; Baxter Diagnostics, Inc., Scientific Products Div., McGaw Park, IL); triethylamine (≥99.5%; Fluka Chemical Corp.); ethanol (200 proof) and chloroform (stabilized with 1% ethanol; EM Science, Gibbstown, NJ); and pyridine (99+%; Aldrich Chemical Co., Inc., Milwaukee, WI).

(b) *Standards.*—Synthetic astaxanthin (a gift from Hoffmann-La Roche, Inc., Nutley, NJ); 4-*N*,*N*-dimethylaminopyridine (DMAP; 99+%), 3,5-dinitrobenzoyl chloride (98%), 1-naphthoyl chloride and 2-naphthoyl chloride (Aldrich Chemical Co., Inc.); (1*S*)-(−)-camphanic chloride (98%; Fluka Chemical Corp.); and L-menthoxyacetyl chloride (American Tokyo Kasei, Inc., Portland, OR).

(c) *LC mobile phase A.*—Hexane–THF–ethanol (77 + 22 + 1). The flow rate was 1.5 mL/min, and the monitoring wavelength was 470 nm.

(d) *LC mobile phase B.*—Hexane–THF–2-propanol–triethylamine (77 + 17 + 3 + 3). The flow rate was 1.5 mL/min, and the monitoring wavelength was 474 nm.

Salmon

Samples for method development were purchased from supermarkets and fish markets. The initial authenticated wild salmon used in method development were obtained through the Office of Seafood, FDA. The authenticated wild salmon used for determination of the configurational isomer distribution of astaxanthin are described below.

Determination of Configurational Isomer Distribution of All-trans Astaxanthin in Marine-Caught, Authenticated Wild Salmon

(a) *Marine-caught, authenticated wild salmon.*—A minimum of 12 authenticated wild salmon from each of the 5 species of Pacific salmon—sockeye (red), chum, pink, coho (silver), and chinook (king)—were collected in marine waters under the supervision of the Seattle District, FDA, Washington State, and shipped to Washington, DC, in dry ice. Some salmon were received whole, and others were gutted before shipping. All the Pacific salmon were measured, photographed, and weighed. The Pacific salmon were certified to be wild either through collection of the fish by FDA inspectors (Seattle District) or by purchase of the salmon directly from a boat whose itinerary at sea had been established. Speciation of the wild Pacific salmon was determined by morphological examination and by the location of the catch. Sex was also determined by morphological examination.

Twelve authenticated wild Atlantic salmon were caught off the coast of Cartwright, Newfoundland, and were filleted before being shipped to Washington, DC. The Atlantic salmon were certified to be wild through collection of the fish by scientists of the Quebec Labrador Foundation, Ipswich, MA.

The wild salmon were stored in a freezer at −77°C. Each fish was assigned a number that was used throughout the study and in data reporting. Results of the analysis of at least 6 wild salmon from each of the above-mentioned species are reported in this study.

(b) *Preparation of authenticated wild salmon flesh for extraction of astaxanthin.*—Salmon received whole were decapitated and gutted. For all salmon, whether received whole or gutted, the skin was removed from the desired sampling area (*see* **Results and Discussion**), and a portion of the flesh (≥10 g) was excised. The sample of salmon flesh was then cleaned of extraneous material (scales, fat, bones, etc.) and dried by blotting with a paper towel. A 10 g portion was accurately weighed on an analytical balance to 3 significant figures.

(c) *Extraction of astaxanthin from wild salmon flesh for chiral LC analysis.*—The 10 g test portion of wild salmon flesh was transferred to a 150 mL centrifuge tube and homogenized for 2 min with 20 mL hexane to remove a significant amount of the lipid. The homogenate was centrifuged for 5 min at 3000 rpm, and the hexane was decanted. The amount of astaxanthin extracted into hexane was determined by recording the volume and measuring the absorbance of the hexane extract at 474 nm (λ_{max} of astaxanthin in hexane). Astaxanthin was extracted from the partially delipified flesh remaining in the centrifuge tube by homogenizing the residue for 1 min with 20 mL acetone. The homogenate was centrifuged for 5 min at 3000 rpm, and the supernatant was decanted. Acetone was added to the homogenate, the homogenate was centrifuged again, and the process was repeated. The acetone extracts were combined, and the acetone was removed with a rotary evaporator. Approximately 4 mL water (extracted by acetone from the salmon flesh) remained. The wet residue was mixed with 20 mL methylene chloride, and the mixture was swirled to dissolve astaxanthin. The water layer was removed with a separatory funnel, and the organic layer was dried over ca 1 g anhydrous sodium sulfate. The amount of astaxanthin extracted into methylene chloride was determined by recording the volume and measuring the absorbance of the methylene chloride extract at 494 nm (λ_{max} of astaxanthin in methylene chloride).

The astaxanthin was purified by loading the dried methylene chloride extract onto a Waters silica gel Sep-Pak cartridge that had been pretreated with hexane. The cartridge was eluted with 20 mL methylene chloride to remove residual salmon flesh lipids in the extract. Astaxanthin was eluted from the cartridge with chloroform, which was then removed under a stream of nitrogen. The residue was reconstituted in

methylene chloride, and a portion was injected into the liquid chromatograph.

(d) *Precision of LC analysis.*—We determined the precision of the LC analysis of synthetic astaxanthin (Table 1) by using mobile phase B. Six replicate analyses were performed with the synthetic astaxanthin standard.

(e) *LC analysis.*—Each astaxanthin extract from wild salmon was analyzed in duplicate. The average of the 2 analyses is reported in all cases. Synthetic astaxanthin was used as a standard before each run. Analyses were performed with mobile phase B.

(f) *Determination of lipid content of wild salmon flesh.*—The hexane extract of the salmon flesh and the methylene chloride washes from the silica gel Sep-Pak cartridge [*see* (c) above] were placed in tared 12 × 35 mm (or ½ dram) vials. The solvent was evaporated under a gentle stream of nitrogen, and the tube was weighed. This process was repeated until a constant weight was obtained. The amount of lipid in each extract was recorded.

(g) *Identification of Pacific salmon species by microscopic examination of scales.*—AOAC Official Method **979.15** was used (17). From each salmon, a minimum of 4 scales were selected from the area beneath the dorsal fin and above the lateral line. Only well-formed scales with intact areas were used. Each scale was mounted and examined separately, and a separate worksheet was completed for each scale examined. For measurement of scale vertical dimensions, observation of circuli and wave striations, and overall scale morphology, a 2× objective lens was used with a 10× eyepiece. For inspection of reticulations, a 4× objective lens was used with a 10× eyepiece.

A minimum of one representative scale from each fish was photographed for documentation. For photomicrography of scales, a 2× objective lens was used for all except the pink salmon, for which a 4× objective lens was used.

Results and Discussion

Method Development

First attempts to resolve the configurational isomers of synthetic all-*trans* astaxanthin on a Pirkle covalent D-phenylglycine column failed to duplicate the results obtained by Maoka et al. (13) under the same LC conditions. We used a commercial column packed with chiral stationary phase from the manufacturer of the Sumipax OA-2000 column used by Maoka et al. (13).

Derivatization of astaxanthin.—When minor adjustments to the LC conditions failed to duplicate the resolution obtained by Maoka et al. (13), we experimented with various derivatizing reagents in an attempt to obtain optimum conditions for making diastereomers that could be easily resolved on a chiral or an achiral column. These derivatizing reagents included L-menthoxyacetyl chloride, 3,5-dinitrobenzoyl chloride, 1-naphthoyl chloride, 2-naphthoyl chloride, and camphanic acid chloride. Only camphanic acid chloride is described in the literature for this purpose (14). The reaction between synthetic astaxanthin and the benzoyl derivatizing agent proceeded rapidly in anhydrous pyridine with a catalytic amount of dimethylaminopyridine (*see* **Experimental** section). The derivatization reaction was also performed successfully with 3,5-dinitrobenzoyl chloride, a π acid, which enhanced interaction of the derivatized astaxanthin enantiomer with a Pirkle π-electron donor chiral stationary phase. Similarly, the reaction was performed with 1-naphthoyl chloride and 2-naphthoyl chloride, π bases, which enhanced interaction of the derivatized astaxanthin enantiomer with a Pirkle π-electron acceptor chiral stationary phase.

When derivatization was performed with astaxanthin extracted from salmon flesh, erratic results were obtained. Fortuitously, the initial derivatization of astaxanthin extracted from salmon proceeded rapidly without problems. Subsequent reactions were sometimes incomplete or did not proceed at all. Other reactions proceeded very slowly, interspersed with reactions that proceeded very quickly.

When astaxanthin extracted from salmon flesh was derivatized successfully, the derivatized astaxanthin was analyzed by LC. For example, the camphanoyl derivative of astaxanthin extracted from the flesh of salmon purchased from a fish market in Washington, DC, and labeled "Washington State" salmon was analyzed by LC under the conditions used by Maoka et al. (13) without modification. The camphanoyl derivative of synthetic astaxanthin was also analyzed under the same LC conditions. The LC profile of the extracted astaxanthin is different from that of synthetic astaxanthin, as shown by the overlay of the 2 profiles (Figure 4). The astaxanthin extracted from the "Washington State" salmon is therefore not synthetic astaxan-

Table 1. Precision of LC analysis of synthetic astaxanthin

	trans			*cis*		
Run	*S,S*, %	Meso, %	*R,R*, %	*S,S*, %	Meso, %	*R,R*, %
1	24.6	48.6	22.5	0.47	3.06	0.76
2	24.3	48.6	22.4	0.38	2.43	0.61
3	25.0	48.6	22.6	0.58	2.58	0.74
4	25.0	48.5	22.3	0.47	2.12	0.87
5	24.5	48.7	22.8	0.23	2.23	0.55
6	24.9	48.9	22.7	0.65	2.13	0.62
Av. ± SD	24.7 ± 0.3	48.6 ± 0.15	22.6 ± 0.19	0.46 ± 0.15	2.57 ± 0.66	0.86 ± 0.35

thin. The LC profile of the extracted astaxanthin resembles that of wild salmon, and the configurational isomeric ratio is within the range expected for wild salmon (*see* below).

The LC profile in Figure 4 clearly illustrates that optimization of LC conditions will reduce analysis time significantly. However, the need to ascertain that the last trace of oil was removed before derivatization led us to abandon this approach, because the amount of colorless lipid in salmon flesh was variable. This problem and the need to purify the derivative formed led us to reexamine the possibility of direct chiral LC analysis of underivatized astaxanthin. LC conditions were subsequently found that permitted chiral resolution on a Pirkle covalent L-leucine column (15). With the aging of the L-leucine column, the mobile phase (mobile phase A) was modified (to mobile phase B) to obtain the same chiral resolution of astaxanthin (*see* **Experimental** section).

Regardless of whether the astaxanthin was first derivatized or analyzed directly by chiral LC, the configurational isomer distribution of all-*trans* astaxanthin, the predominant geometric isomer, had to be established in marine-caught, authenticated wild salmon.

Determination of Configurational Isomer Distribution of All-trans Astaxanthin in Marine-Caught, Authenticated Wild Salmon

To ascertain that there was little or no variation in the configurational isomer distribution in different parts of salmon muscle, test portions were taken from 3 locations along the lateral line: near the head, at the center below the dorsal fin, and near the tail above the anal fin. At least 6 salmon were sampled from each of the Pacific salmon species for this part of the study. No appreciable variation was found, as shown in Tables 2–6. On the basis of these results, the remaining Pacific salmon were sampled at one location only, the center of the fish. The Atlantic salmon were received as filets and sampled at the center only (Table 7).

The distributions of the configurational isomers of all-*trans* astaxanthin in 38 marine-caught, authenticated wild salmon (*Oncorhynchus* and *Salmo salar*) are listed in Tables 2–7. No variation was observed between male and female salmon for any species. The ranges of the configurational isomers in Atlantic and Pacific salmon were 47.1–90.0% of the (3*S*,3′*S*) enantiomer, 7.7–45.2% of the (3*R*,3′*R*) enantiomer, and 0–8.6% of the (3R,3′S) meso form. The range of each configurational isomer is much broader than that found by Schiedt et al. (10), who used a very narrow database and did not include chinook salmon, which has a significantly wider range of each isomer than do the other species. When combined with the other 5 species, chinook salmon appreciably broadens the range, as discussed below. The result, however, supports the basic conclusion of Schiedt et al. (10): The configurational isomer distributions of astaxanthin in wild marine salmon are similiar. We have expanded it in this study to include the 6 common species of wild salmon.

The configurational isomer distributions in the 6 species of wild salmon are significantly different from that of synthetic astaxanthin, which consists of 25% of each of the enantiomers and 50% of the meso form. These results show that the distribution of the configurational isomers of astaxanthin in wild salmon flesh indeed provides a basis for distinguishing wild salmon from aquacultured salmon fed synthetic astaxanthin.

The range of each of the configurational isomers of all-*trans* astaxanthin is much wider in chinook (king) salmon than in the 5 other species (Table 6). For example, the range of the (3*S*,3′*S*) enantiomer for chinook is 47.1–80.5%, compared with 65.4–77.0% for sockeye, 77.1–89.4% for coho, 78.5–90.0% for pink,

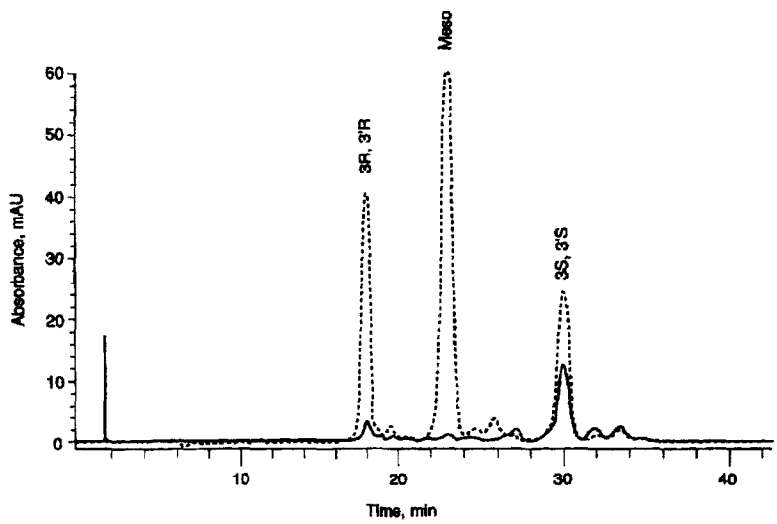

Figure 4. Overlay of LC profiles of the camphanoyl derivative of astaxanthin extracted from wild salmon and synthetic astaxanthin. Solid line = astaxanthin extracted from "Washington State" salmon purchased from the SW pier fish market, Washington, DC; - - - = synthetic astaxanthin. LC conditions: Pirkle covalent D-phenylglycine column; mobile phase, hexane–methylene chloride–ethanol (73.3 + 24.4 + 2.4), flow rate, 1.6 mL/min; monitoring wavelength, 490 nm.

Table 2. Total level (ppm) of astaxanthin and distribution of configurational isomers of all-*trans* astaxanthin in wild sockeye salmon

Salmon						
No.	Sex	Sampling location	*S,S*, %	Meso, %	*R,R*, %	Total isomers, ppm
44	F[a]	A[b]	73.8	4.8	21.4	31.1
		B[c]	74.6	4.6	20.8	31.7
		C[d]	74.6	4.6	20.9	32.4
48	F	A	76.8	3.8	19.4	30.0
		B	76.8	3.8	19.4	34.6
		C	77.0	3.9	19.1	39.2
50	F	A	72.8	4.8	22.5	45.7
		B	73.6	4.7	21.7	48.0
		C	73.5	4.7	21.8	59.7
76	F	A	71.1	4.6	24.2	33.7
		B	71.0	4.8	24.4	28.6
		C	71.3	4.6	24.2	37.7
57	M[e]	A	65.4	5.8	28.8	47.8
		B	65.6	5.8	28.6	50.9
		C	65.4	5.8	28.8	58.9
74	M	A	73.2	4.4	22.4	32.4
		B	74.5	4.6	21.2	43.3
		C	73.1	4.5	22.4	42.2
Range			65.4–77.0	3.8–5.8	19.1–28.8	30.0–58.9

[a] F = female.
[b] A = sample taken near the head, along the lateral line.
[c] B = sample taken at the center below the dorsal fin, along the lateral line.
[d] C = sample taken near the tail above the anal fin, along the lateral line.
[e] M = male.

77.4–89.8 for chum, and 79.3–82.6 for Atlantic salmon. Chinook salmon (*Oncorhynchus tshawytscha*) are different from the other salmon species because they occur along the Pacific coast of North America in 2 distinct forms known as red-fleshed and white-fleshed chinook (20). The white-fleshed chinook is the only wild Pacific or Atlantic salmon that apparently does not contain deposits of colored dietary carotenoids in the flesh of the sexually maturing adult (20). In a study of intestinal absorption of astaxanthin, investigators concluded that the poor flesh pigmentation was due to rapid metabolism of the absorbed astaxanthin to colorless derivatives rather than to failure of the salmon to absorb astaxanthin (21). Only 2 of 6 chinook salmon samples in our study had very pale flesh (No. 22 and No. 23). The amount of astaxanthin in one sample (No. 22) was too low to determine the configurational isomeric ratios (Table 6). Furthermore, there seemed to be 2 groups of configurational isomeric distributions in the red-fleshed chinook salmon (Table 6). One group resembled the rest of the wild Pacific and Atlantic salmon (samples 23, 52, and 54) with a range of 64.5–80.5% for the (3*S*,3′*S*) enantiomer compared with a range of 65.4–90.0% for the 5 other species. The other group had about equal distribution of the (3*S*,3′*S*) enantiomer (47.1–51.0%) and the (3*R*,3′*R*) enantiomer (40.6–45.2%), with the former slightly higher than the latter. Inclusion of this second group broadens the range of the entire survey. A much larger database, however, would be required to determine whether those fish with the enantiomers as the 2 major components constitute a distinct subgroup within the red-fleshed chinook salmon.

The results of analyses of the remaining authenticated wild salmon are not expected to appreciably affect the configurational isomeric distribution reported here. Results of all 80 samples will be reported separately.

Methodology

The LC profile of astaxanthin extracted from the flesh of salmon was examined to determine the configurational isomeric ratio and to compare the LC profile and isomeric ratios with those of synthetic astaxanthin. Aquacultured salmon fed a diet supplemented with synthetic astaxanthin would be easy to identify because the configurational isomeric ratios and the LC profile of the extracted astaxanthin would be identical to those of synthetic astaxanthin. For wild, marine-caught salmon, the ratio of configurational isomers is expected to lie within the range we have established for wild salmon. Furthermore, the LC profile of astaxanthin extracted from wild salmon would be different from the LC profile of synthetic astaxanthin. Exam-

Table 3. Total level (ppm) of astaxanthin and distribution of configurational isomers of all-*trans* astaxanthin in wild coho salmon

Salmon						
No.	Sex	Sampling location	*S,S*, %	Meso, %	*R,R*, %	Total isomers, ppm
70	F[a]	A[b]	83.2	3.2	13.6	13.0
		B[c]	81.7	2.8	15.4	12.2
		C[d]	82.8	3.0	14.2	12.7
71	F	A	77.6	3.8	18.6	13.8
		B	77.6	3.7	18.7	14.4
		C	77.1	3.7	19.2	13.0
68	M[e]	A	87.8	2.8	9.5	10.7
		B	89.4	2.1	8.6	9.9
		C	86.8	3.0	10.2	13.8
69	M	A	81.6	3.0	15.4	9.6
		B	82.9	2.8	14.2	9.8
		C	84.4	2.8	12.8	11.7
72	M	A	81.0	3.6	15.6	25.5
		B	79.9	3.2	16.9	16.7
		C	79.6	3.2	17.1	28.0
75	M	A	83.8	2.9	13.4	12.8
		B	84.4	2.8	12.7	10.7
		C	81.6	2.8	15.6	10.6
Range			77.1–89.4	2.1–3.7	8.6–19.2	9.6–28.0

[a] F = female.
[b] A = sample taken near the head, along the lateral line.
[c] B = sample taken at the center below the dorsal fin, along the lateral line.
[d] C = sample taken near the tail above the anal fin, along the lateral line.
[e] M = male.

Table 4. Total level (ppm) of astaxanthin and distribution of configurational isomers of all-*trans* astaxanthin in wild pink salmon

Salmon						
No.	Sex	Sampling location	*S,S*, %	Meso, %	*R,R*, %	Total isomers, ppm
18	F[a]	A[b]	84.8	3.4	12.5	6.5
		B[c]	83.0	3.6	13.4	6.2
		C[d]	82.5	3.4	14.2	6.5
55	F	A	83.8	3.3	13.0	5.6
		B	85.4	2.9	11.8	4.9
		C	80.4	3.2	16.4	5.3
63	F	A	84.0	4.1	11.8	5.3
		B	78.5	3.0	18.6	6.1
		C	80.8	3.2	16.0	6.9
14	M[e]	A	87.8	2.9	9.4	7.6
		B	86.4	2.5	11.0	6.2
		C	86.9	2.4	10.6	6.6
59	M	A	80.6	3.6	15.8	7.2
		B	81.3	3.8	14.9	6.9
		C	80.9	4.0	15.1	7.3
65	M	A	85.6	2.3	12.1	3.1
		B	86.6	2.6	10.8	3.4
		C	85.4	2.4	12.2	4.2
77	X[f]	A	87.1	2.0	10.8	3.9
		B	88.4	2.1	9.4	3.9
		C	87.4	2.4	10.2	3.3
78	X	A	88.6	2.2	9.2	4.5
		B	88.2	0.0	11.8	4.2
		C	90.0	1.0	9.0	5.0
Range			78.5–90.0	0–4.1	9.0–18.6	3.1–7.6

[a] F = female.
[b] A = sample taken near the head, along the lateral line.
[c] B = sample taken at the center below the dorsal fin, along the lateral line.
[d] C = sample taken near the tail above the anal fin, along the lateral line.
[e] M = male.
[f] X = sex unknown.

ples are given below for wild salmon and aquacultured salmon fed synthetic astaxanthin.

This method could be also used to determine the presence in aquacultured salmon of astaxanthin derived from other sources such as *Phaffia* yeast and *Haematococcus pluvialis* algae. Astaxanthin in *Phaffia* yeast consists of >98% of the (3*R*,3′*R*) enantiomer (22), giving it a very distinctive LC profile that is easy to recognize. Similarly, the astaxanthin extract of salmon fed *Haematococcus pluvialis* algae would consist almost entirely (99%) of the (3*S*,3′*S*) enantiomer (23). Its LC profile also would be highly distinctive and easy to characterize.

Aquacultured Salmon Fed Synthetic Astaxanthin

We extracted astaxanthin from a Norwegian salmon filet purchased from a local supermarket and analyzed it by LC on a Pirkle covalent L-leucine column eluted with mobile phase A. Synthetic astaxanthin was also analyzed under the same LC conditions. The LC profiles of the astaxanthin peaks were very similar: Each of the 3 configurational isomers eluted at practically identical retention times, and the ratios of peaks were the same in both profiles (Figure 5). Overlay of peaks eluting at 10.1, 10.8, and 11.5 min showed them to be an almost perfect match. We concluded that the astaxanthin extracted from the Norwegian flesh was synthetic astaxanthin, which must have been added to the fish feed. Consequently, the Norwegian salmon was presumed to be aquacultured and not wild. The retention time and the UV/VIS absorption spectrum of the peak at 3.87 min suggest that it is the diester(s) of astaxanthin.

Similar results were obtained for salmon purchased from a local supermarket and labeled as "imported" from Idaho, as well as for salmon purchased from the delicatessen of an up-

Table 5. Total level (ppm) of astaxanthin and distribution of configurational isomers of all-*trans* astaxanthin in wild chum salmon

Salmon						
No.	Sex	Sampling location	*S,S*, %	Meso, %	*R,R*, %	Total isomers, ppm
29	F[a]	A[b]	81.1	3.4	14.8	6.5
		B[c]	80.3	4.2	15.5	7.0
		C[d]	81.5	3.8	14.7	6.9
36	F	A	84.5	1.7	13.8	2.5
		B	84.0	1.7	14.3	1.8
		C	83.7	1.9	14.5	1.1
42	F	A	88.8	2.2	9.0	6.7
		B	89.8	2.4	7.7	6.2
		C	88.4	2.4	9.2	5.7
56	F	A	85.0	3.2	11.8	5.0
		B	85.5	3.4	11.2	4.6
		C	84.8	3.3	11.8	5.4
58	M[e]	A	81.2	3.0	15.8	7.2
		B	80.6	3.6	15.8	7.8
		C	81.2	3.8	15.0	6.8
62	M	A	79.3	3.6	17.4	7.1
		B	77.4	3.6	19.0	5.7
		C	78.2	3.7	18.1	6.6
Range			77.4–89.8	1.7–4.2	7.7–19.0	1.1–7.8

[a] F = female.
[b] A = sample taken near the head, along the lateral line.
[c] B = sample taken at the center below the dorsal fin, along the lateral line.
[d] C = sample taken near the tail above the anal fin, along the lateral line.
[e] M = male.

Table 6. Total level (ppm) of astaxanthin and distribution of configurational isomers of all-*trans* astaxanthin in wild chinook (king) salmon

Salmon						
No.	Sex	Sampling location	*S,S*, %	Meso, %	*R,R*, %	Total isomers, ppm
22	F[a]	A[b]	ND[c]	ND	ND	0.7
		B[d]	ND	ND	ND	0.8
		C[e]	ND	ND	ND	0.8
23	M[f]	A	65.6	3.6	31.1	0.9
		B	65.1	2.8	32.1	0.9
		C	64.5	2.0	33.5	1.0
17	X[g]	A	50.1	8.6	41.3	12.9
		B	51.0	8.3	40.6	13.1
		C	50.2	8.6	41.2	11.0
19	X	A	48.5	8.1	43.4	11.7
		B	48.3	7.9	43.8	10.4
		C	47.1	7.7	45.2	11.4
52	X	A	79.1	3.5	17.4	18.8
		B	80.5	3.3	16.3	19.6
		C	79.8	3.3	17.0	22.4
54	X	A	71.1	5.3	23.7	7.3
		B	70.8	5.3	23.9	8.3
		C	70.8	5.3	24.0	8.3
Range			47.1–80.5	2.0–8.6	16.3–45.2	0.7–22.4

[a] F = female.
[b] A = sample taken near the head, along the lateral line.
[c] ND = configurational isomeric ratio not determined (astaxanthin concentration too low).
[d] B = sample taken at the center below the dorsal fin, along the lateral line.
[e] C = sample taken near the tail above the anal fin, along the lateral line.
[f] M = male.
[g] X = unknown.

scale department store and advertised as being caught off the icy waters of Canada and Scotland.

Wild Salmon

We extracted astaxanthin from a wild pink salmon that had been authenticated by the Office of Seafood, FDA, but was not part of the broad-based set of specimens used for the determination of configurational isomers of all-*trans* astaxanthin in wild salmon. The extracted astaxanthin was analyzed by LC on a Pirkle covalent L-leucine column with mobile phase A. Synthetic astaxanthin was also analyzed under the same LC conditions. The LC profiles of the astaxanthin peaks were very different (Figure 6). Moreover, the LC profile of the extracted astaxanthin was similar to the LC profile of the marine-caught, authenticated wild salmon, as expected.

Concentration of Astaxanthin in Wild Salmon Flesh

We determined the amount of astaxanthin in the flesh of the 38 wild salmon studied, including 2 pale-colored chinook

Table 7. Total level (ppm) of astaxanthin and distribution of configurational isomers of all-*trans* astaxanthin in wild Atlantic salmon

Salmon					
No.	Sex	*S,S*, %	Meso, %	*R,R*, %	Total isomers, ppm
2	F[a]	79.3	4.9	15.9	5.1
6	F	82.6	3.2	14.3	7.2
1	M[b]	81.0	3.7	15.7	5.1
3	M	80.1	4.3	15.6	4.9
4	M	79.3	3.6	17.1	4.5
5	M	80.0	3.4	16.6	4.9
Range		79.3–82.6	3.2–4.9	14.3–17.1	4.9–7.2

[a] F = female.
[b] M = male.

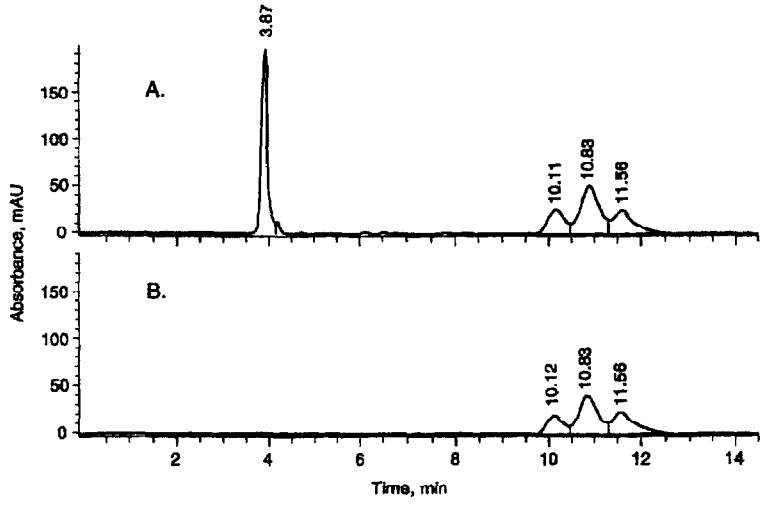

Figure 5. Comparison of LC profiles of astaxanthin extracted from Norwegian salmon and synthetic astaxanthin. (A) Astaxanthin extracted from Norwegian salmon filet, purchased from Safeway. (B) Synthetic astaxanthin. LC conditions: Pirkle covalent L-leucine column; mobile phase A; flow rate, 1.5 mL/min; monitoring wavelength, 470 nm.

salmon. The results were consistent with literature values for each of the species (10). The amounts of astaxanthin were within a defined range for each of the wild salmon species (Tables 2–7). These ranges of astaxanthin content, however, overlapped sufficiently to preclude speciation on the basis of color content alone.

Determination of Lipid Content of Wild Salmon Flesh

The amount of lipid in the flesh of wild salmon was also determined (Table 8). Results were consistent with literature values (10). The amount of lipid varied even within the same species. For example, Atlantic salmon No. 7 had twice the amount of lipid found in Atlantic salmon No. 9, whereas chinook salmon No. 21 had 6 times the amount of lipid found in chum salmon No. 35. This variation and the differences in fatty acid profiles of lipids extracted from wild and aquacultured salmon (24) may explain the difficulties encountered in attempts to derivatize astaxanthin extracted from the flesh of salmon (*see Method Development*).

Conclusions

A rapid LC method to distinguish between wild salmon and aquacultured salmon fed synthetic astaxanthin has been developed. Validation demonstrated good LC method precision. Preliminary study of the distribution of the configurational isomers

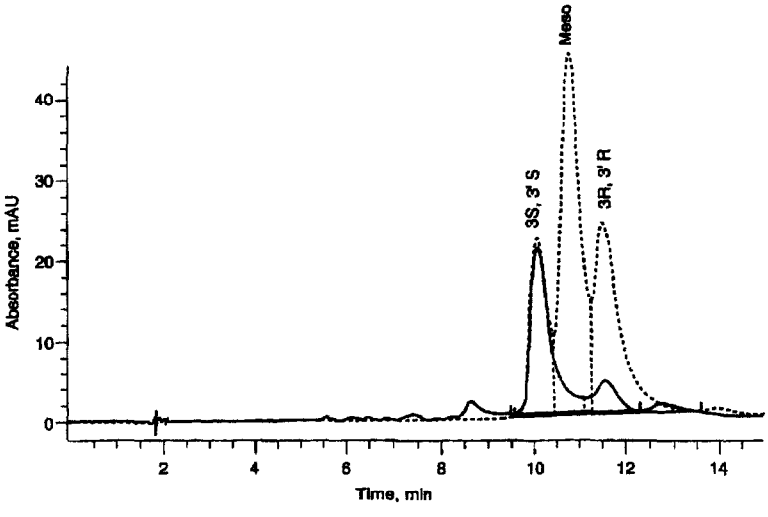

Figure 6. Overlay of LC profiles of astaxanthin extracted from wild salmon and synthetic astaxanthin. Solid line = astaxanthin extracted from wild pink salmon obtained from FDA's Office of Seafood; - - - = synthetic astaxanthin. LC conditions: Pirkle covalent L-leucine column; mobile phase A; flow rate, 1.5 mL/min; monitoring wavelength, 470 nm.

Table 8. Lipid content of authenticated wild salmon flesh

Salmon No.	Total lipid, % [a]	Lipid in hexane, % [b]	Lipid in methylene chloride, % [c]
7	4.61	74.6	25.4
8	4.39	68.4	31.6
9	2.76	50.0	50.0
11	4.66	77.0	23.0
12	4.70	70.9	29.1
13	1.86	30.2	69.8
15	2.85	46.1	53.9
16	1.64	35.8	64.2
20	3.81	62.2	37.8
21	6.68	79.4	20.6
25	3.25	72.5	27.5
26	2.03	37.1	62.9
27	5.84	80.6	19.4
28	5.13	80.7	19.4
30	1.33	16.9	83.1
31	1.23	19.6	80.4
32	1.39	61.0	39.0
33	2.52	45.7	54.3
34	1.50	34.4	65.6
35	1.09	31.3	68.7
39	1.52	20.9	79.1
41	4.67	68.5	31.5
43	4.75	61.3	38.7
45	2.72	51.8	48.2
46	2.52	52.4	47.6
47	2.78	46.5	53.5
49	2.03	32.5	67.5
51	2.51	51.1	48.9
53	3.54	54.5	45.5
61	1.96	43.0	57.0
64	2.70	50.3	49.7
66	1.43	26.2	73.8
67	2.27	38.7	61.3

[a] Total lipid, $\% = \frac{\text{g total lipid}}{\text{g sample}} \times 100.$

[b] Lipid in hexane, $\% = \frac{\text{g lipid in hexane extract}}{\text{g total lipid}} \times 100.$

[c] Lipid in methylene chloride, $\% = \frac{\text{g lipid in methylene chloride extract}}{\text{g total lipid}} \times 100.$

of astaxanthin in wild authenticated salmon confirm the basic tenet of the method: The configurational isomeric ratio falls within a defined range and can be used as a basis for determining whether the salmon is wild. This method also can be used to determine the presence of astaxanthin derived from other sources such as *Phaffia* yeast and *Haematococcus pluvialis* algae.

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Growth and survival of Atlantic salmon, *Salmo salar* L. fed different dietary levels of astaxanthin. Juveniles

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Abstract

Atlantic salmon, *Salmo salar* L., juveniles, with a mean initial weight of 1.75 g, were fed casein-based purified diets which had been supplemented with different levels of astaxanthin for a 10-week period. The astaxanthin content of the diets ranged from 0 to 190 mg kg^{-1} dry diet. The growth and survival of the juveniles were recorded throughout the experiment. The proximate composition, astaxanthin and vitamin A content were determined from whole-body samples at the start and termination of the experiment.

The dietary treatment was found to affect growth significantly ($P < 0.05$). A reduction in the mean weight of the juveniles was observed in the groups fed the diets without astaxanthin supplementation. There was no difference in growth rate between the fish in the groups fed the diets containing 36 or 190 mg astaxanthin kg^{-1} dry diet, whereas the fish in the group fed the diet containing 5.3 mg astaxanthin kg^{-1} dry diet had a lower growth rate. There was a tendency to higher survival in the groups fed the diets containing astaxanthin when compared with the groups fed the non-supplemented diets. The moisture and ash contents were significantly lower and the lipid content was higher in the groups fed the astaxanthin-supplemented diets. The astaxanthin and the vitamin A concentrations in the fish were found to be dependent upon the dietary astaxanthin dose; the highest values were found in the fish fed the diet with the highest astaxanthin content. These results strongly indicate that astaxanthin functions as a provitamin A for juvenile Atlantic salmon. The body storage of vitamin A increased in the fish fed the diets containing astaxanthin. However, the increase was low in the fish fed the diet containing 5.3 mg astaxanthin kg^{-1} dry diet.

KEY WORDS: astaxanthin, Atlantic salmon, growth, juveniles, survival

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Introduction

Evidence is accumulating to support the hypothesis that astaxanthin and other carotenoids have an important role in the metabolism of Atlantic salmon, *Salmo salar* L., and other fish species. A number of suggestions as to the functions of carotenoids in fish have been made and have been reviewed in several articles (Tacon 1981; Craik 1985; Torrissen 1990). Previous research has found evidence supporting a number of these functions. It has been shown that astaxanthin is converted to vitamin A in rainbow trout, *Oncorhynchus mykiss* (Walbaum), that are deficient in vitamin A (Schiedt *et al.* 1985; Al-Khalifa & Simpson 1988). A dose–response relationship between the astaxanthin content of the diet and the vitamin A content of the fish has been observed during the first-feeding period of Atlantic salmon (Christiansen *et al.* 1994, 1995b). Reduced growth and high mortality were observed in the fish fed the diets low in astaxanthin. In addition to the provitamin A effect observed in salmonids, a provitamin A effect of astaxanthin has been reported in other fish species (Grangaud *et al.* 1962; Katsuyama & Matsuno 1988). Furthermore, the addition of astaxanthin to the diet of Atlantic salmon parr/smolt has been shown to elevate levels of the antioxidant vitamins retinol, tocopherol and ascorbic acid in the liver when compared with fish fed a diet without astaxanthin supplementation (Christiansen *et al.* 1995a). In the same study, an increased resistance to *Aeromonas salmonicida* also was observed in the fish fed diets containing astaxanthin.

Supplementation of astaxanthin or canthaxanthin to commercial starter feeds has been shown to improve the growth of Atlantic salmon juveniles when compared with fish fed commercial diets without astaxanthin supplementation (Torrissen 1984). In a study by Christiansen *et al.* (1994), astaxanthin was found to be important for the growth and survival of Atlantic salmon fry during the first-feeding period when the fish were fed a purified casein/gelatine diet. In a later experiment, the dietary astaxanthin level necessary for satisfactory growth and survival was determined to be 5 mg kg^{-1} (Christiansen *et al.* 1995b). The supplementation of astaxanthin to the diet of Atlantic salmon parr was

also found to improve growth (Christiansen *et al.* 1995a). Other studies have shown positive effects of dietary carotenoids on the growth of tilapia, *Oreochromis niloticus* (L.) (Boonyaratpalin & Unprasert 1989) and Indian carp (U.C. Goswamional pers communication) as well as on the survival of kuruma prawn, *Penaeus japonicus* Bate (Chien & Jeng 1992; Négre-Sadargues *et al.* 1993).

In the farming of Atlantic salmon, as well as other fish species, knowledge of dietary requirements is essential for maximum growth and good health. The requirements for essential nutrients are associated with the life cycle and change with the age, size and maturational status. The objective of the present study was to clarify whether or not the need for astaxanthin in Atlantic salmon juveniles increases after the first-feeding period.

Materials and methods

This experiment is an extension of a previous investigation which evaluated the requirement of astaxanthin during the first-feeding period of Atlantic salmon (Christiansen *et al.* 1995b). The fish from this previous experiment, which all originated from Matre Aquaculture Research stock, were used in the present experiment. The juveniles had a mean start weight of 1.75 g (± 0.46 g standard deviation). In the present experiment only three groups of fish were used. All the groups had been fed diets containing astaxanthin concentrations above the required level found in the earlier study. These levels were 5.3, 36.0 and 190.1 mg kg^{-1} dry diet, respectively. Each group of fish from the previous experiment was divided in two and placed into fibreglass tanks (1 × 1 × 0.4 m), 250 fish per tank, supplied with approximately 10 L min^{-1} of fresh water. The freshwater source was routinely supplemented with UV-sterilized salt water to a conductivity of 2100 µS cm^{-1} to increase the pH of the water. The mean temperature and the pH of the water were maintained at 11.5°C (± 0.7°C) and 6.0, respectively. A 24-h light regime (fluorescent daylight tubes) was used throughout the experiment and the fish were fed continuously (24 hours a day) with automatic feeders (Aqua-produkter, Sunndalsøyra, Norway). The amount of feed offered to the juveniles during the first 2 weeks was calculated according to standard tables (Austreng *et al.* 1987) using a feed conversion ratio of 1.0 during the first period until the first weight recording. In subsequent periods, the specific growth rate (SGR) of the fish was recalculated based on the weight of the fish, and the SGR of the group with the best growth rate was used to calculate the amount of feed for all groups. The feeds were stored at –20°C. Mortality

Basal diet ingredient	g kg^{-1}	Basal diet ingredient	g kg^{-1}
Casein, vitamin-free	465	L-threonine	10
Gelatine	100	Vitamin mix[1]	10
Sardine oil	180	Ascorbic acid (phosphate esters)	1
Dextrin	120	Choline chloride (70%)	10
Carboxymethylcellulose	10	KCl	15
α-cellulose	20	NaCl	3
L-arginine	10	$NaHCO_3$	2.5
L-histidine	2	$CaHPO_4.H_2O$	15
L-lysine HCl	12.5	MgO	5
L-methionine	4	Trace min. sol.[2]	
L-phenanthrene	5		

Proximate composition	(g kg^{-1} dry matter)		Diet	Analysed astaxanthin (mg kg^{-1} dry diet)	
Dry matter	724	(13)	1	5.3	(0.4)
Ash	40	(3)	1-0	0.0	(0.0)
Crude protein (N × 6.25)	603	(42)	2	36.0	(0.6)
Lipid	207	(8)	2-0	0.0	(0.0)
Carbohydrate	150		3	190.1	(17 5)
			3-0	0.0	(0.0)

Table 1 Formula of the basal diet (g kg^{-1} of dry matter), proximate composition (g kg^{-1} dry matter) of the diets and staxanthin concentration (mg kg^{-1} dry diet) in the diets. Standard deviation is given in parentheses, $n = 3$

[1]Vitamin supplement supplied the following kg^{-1} of dry diet: vitamin A (acetate and palmitate 1:1, 500 000 IU g^{-1}) 24 mg (12 000 IU); vitamin D_3 (500 000 IU g^{-1}) 4 mg (2000 IU); α-tocopherol acetate (50%) 100 mg (50 mg); vitamin K_3 (51%) 12 mg (6.1 mg), thiamine-HCl 15 mg, riboflavin 30 mg, pyridoxine hydrochloride 15 mg; calcium D-pantothenate 45 mg; nicotinic acid 150 mg; biotin (2%) 40 mg (0 8 mg); folic acid 4 mg; cyanocobolamin (vitamin B_{12}) (1%) 3 mg (30 µg); inositol 300 mg.

[2]Trace minerals were dissolved in distilled water added 0 5% HCl and supplied as 100 ml kg^{-1} dry diet containing: Fe (as $FeSO_4$ $7H_2O$) 115 mg; I (as KI) 1.9 mg, Mn (as $MnSO_4$ H_2O) 32.5 mg; Se (as Na_2SeO_3) 4 2 mg; Co (as $CoC1_2$ $6H_2O$) 4 0mg; Cu (as $CuSO_4$ $5H_2O$) 11.8 mg, Zn (as $ZnSO_4 7H_2O$) 88 0 mg

was recorded daily. The basal test diet used was based on vitamin-free casein as the protein source and was supplemented with free amino acids (Shearer *et al.* 1993) The individual diets were prepared as described in Christiansen *et al.* (1995b). Sardine oil, containing 200 mg kg^{-1} of the antioxidant ethoxyquin, was used as the lipid source. The diets were supplemented with 5.3, 36.0 and 190.1 mg astaxanthin kg^{-1} dry diet using CAROPHYLL Pink (F.Hoffmann–La Roche, Basle, Switzerland) which contains 80 g kg^{-1} astaxanthin. These dietary groups were designated as diets 1, 2 and 3, respectively. The ingredients of the diets, the proximate composition and the astaxanthin contents are given in Table 1. A zero diet without astaxanthin supplementation was also prepared. Of the two groups of fish derived from the same fish group, one was fed the zero diet and one was given a diet containing the same level of astaxanthin as they had been given in the previous experiment (5.3, 36.0 or 190.1 mg kg^{-1}) (Christiansen *et al.* 1995b). The groups fed the zero diet without astaxanthin supplementation were designated by referring to the dietary astaxanthin level fed to the other half of the original group and a zero showing that the fish were fed the zero astaxanthin diet as shown in Table 1. The addition of astaxanthin to the diets gave the diets different shades of orange, depending upon the level of astaxanthin. The zero diet was yellowish-white.

At the start of the experiment, 100 juveniles from each of the original dietary treatments were weighed individually. The total biomass for each tank was recorded at 2-week intervals and the mean weight of the fish was calculated with adjustment for mortality during that period. At the end of the experiment, both the total biomass of the tank as well as a random sample of 50 fish from each tank were weighed individually. The growth rates and specific growth rates (SGR) for the experimental period were calculated as:

growth rate (g) = $(\ln W_f - \ln W_i) / t$ (Bagenal & Tesch 1978),

SGR = $(e^s - 1) * 100$ (Houde & Schekter 1981),

where W_i and W_f are the mean fish weight at the start and the end of the experiment and t is the number of days. The body weight increase (BWI) was calculated as

$$BWI = [(W_f - W_i)/W_i] * 100.$$

The feed conversion efficiency was calculated as wet weight gain/dry feed fed. The proximate composition and astaxanthin concentration of the feed and the fish and the vitamin A concentration in fish were analysed as described in Christiansen *et al.* (1995c) The body storage of individual fish was calculated using the mean weight of the fish and the analysed vitamin A concentration. The proximate composition of the feed was analysed in triplicate from homogenized samples of 100 g feed at the start of the experiment. At the start and the end of the experiment, 10 fish were randomly sampled from each tank and killed with an overdose of (20 mg L^{-1}) metomidate hydrochloride diluted in water (Marinil, Wildlife Pharmaceuticals, Fort Collins, Colorado, USA). Fish from the same tank were pooled and homogenized prior to the determination of proximate composition and astaxanthin concentrations in triplicate.

The mean body weights were log-transformed and the $\log_{10}$ weights according to days of feeding were subjected to linear regression. Analyses of covariance (ANCOVA) were used to test for differences in the slope of the regression lines (Zar 1984) to reveal differences in growth among the different dietary groups. Differences in the mean initial and final weights were tested by analyses of variance (ANOVA). Where significant effects of dietary treatment on weight were found, Student–Newman–Keuls multiple range test was used to test for differences among the groups. Kruskal–Wallis non-parametric ANOVA was used to test for differences in the proximate composition, astaxanthin and vitamin A concentrations in whole-body samples. Differences in survival were compared using the logrank test (Peto *et al.* 1977). A probability level of 0.05 was used for all tests. The statistical analyses were performed using the software package CSS (Statistica, Complete Statistical Systems) and RS1 (BBN Software Products Corporation).

Results

There were no differences in the mean body weights of fish in the groups at the start of the experiment (Table 2). The fish in the groups fed the zero diet were observed to lose weight during the experimental period (Table 2). During the first 6 weeks of the

Table 2 Initial body weight, final body weight, percentage increase in body weight (BWI), specific growth rate (SGR), feed conversion efficiency and survival of Atlantic salmon fry fed diets with different levels of astaxanthin for 10 weeks. Initial and final body weight are given as means with SD in parentheses, $n = 100$ and 50, respectively

Diet	Astaxanthin (mg kg^{-1})	Initial body[(1)] weight (g)	Final body[(1)] weight (g)	BWI (%)	SGR (%)	Feed conversion efficiency	Survival[(2)] (%)
1	5.3	1.7 (0.5)[a]	4.4 (2.6)[a]	156.8	1.36	0.48	93.6[a]
1-0	0.0	1.7 (0.5)[a]	1.4 (0.6)[b]	−18.7	−0.27	−0.21	83.2[b]
2	36.0	1.7 (0.4)[a]	6.3 (2.9)[c]	271.1	1.90	0.83	95.2[a]
2-0	0.0	1.7 (0.4)[a]	1.7 (1.1)[b]	−2.0	−0.00	−0.06	91.6[a]
3	190.1	1.8 (0.5)[a]	5.5 (3.2)[c]	199.5	1.61	0.73	98.8[c]
3-0	0.0	1.8 (0.5)[a]	1.4 (0.8)[b]	−24.2	−0.34	−0.24	83.2[b]

Mean values within columns that have different superscript letters are significantly different ($P < 0.05$; [1]Student–Newman–Keuls multiple range test, [2]Logrank test).

experiment, there appeared to be no difference in growth among the groups fed diets containing astaxanthin. However, reduced growth was observed from week 6 to week 10 in the group of fish fed diet 1 (5.3 mg astaxanthin kg^{-1} dry diet). There were also differences in the mean weight of the fish in the dietary groups by the end of the experiment. The mean weights of the fish fed the diets supplemented with astaxanthin were significantly higher than those of the groups fed the unsupplemented diet (Table 2). The mean weight of the fish in the group fed diet 1 was significantly lower than that of fish fed diets 2 and 3 (36 and 190 mg astaxanthin kg^{-1} dry diet).

Results from the linear regression analyses and ANCOVA are shown in Table 3. The coefficient of determination (r^2) was high for the groups fed the diets containing astaxanthin showing a good relationship between log-transformed weights and days of feeding. Log-transformation of weight data is less suitable for fish with poor growth or for fish losing weight; r^2 is lower for groups fed the zero diet. The astaxanthin content of the diet was found to have a significant effect on the growth of the fish, expressed as the difference in the slope of the regression lines between fish fed the diets supplemented with astaxanthin and fish fed the diet without supplementation (Table 3). We found no differences in the slopes of the groups fed the zero diet. The slope of group fed diet 3-0, however, was not significantly different from zero. The group fed diet 1 had a significantly higher slope than the zero groups but had a lower slope than the groups fed diets 2 and 3. There was no difference in the slopes of the two last groups. The specific growth rates (SGRs) of the fish from the different dietary groups during the experimental period are shown in Table 2. The SGRs confirm the results from the analyses of covariance, with negative or zero SGRs in the groups fed the zero diets.

Survival was higher in the groups fed the astaxanthin-supplemented diets when compared with the groups fed the non-supplemented diet (Table 2). The logrank test revealed a difference in the survival rate between the fish fed diet 1 and the fish fed diet 1-0. The same relationship was found for the fish that had been fed diet 3 and diet 3-0. There was no difference in the survival rate between the fish that had been fed diets 1 and 2, while the fish fed diet 3 had a higher survival rate.

The best feed conversion efficiency was observed in the groups fed diets 2 and 3 (Table 2). A somewhat lower feed conversion efficiency was observed in the group fed diet 1, whereas negative feed conversion efficiency was observed in the groups fed the zero diet. Whole-body proximate composition at the start of the experiment and at week 10 is shown in Table 4. There were no differences in proximate composition of the fish at the start of the experiment. The dietary treatment was found to significantly affect the proximate composition of the fish. After 10 weeks of feeding, the groups fed the diets supplemented with astaxanthin had lower moisture and ash contents and higher lipid contents than the groups fed the zero diet. In general, the protein content tended to be lower in the zero groups.

Table 3 Slopes, significance levels of slope and coefficient of determination (r^2) from the linear regression analyses of the log_{10}-transformed weight data of fry fed different dietary levels of astaxanthin versus days of feeding

Diet	Dietary astaxanthin (mg kg^{-1})	Slope[1]	P-value[2]	r^2
1	5.3	0.0054[a]	0.000	0.968
1-0	0.0	−0.0017[c]	0.040	0.692
2	36.0	0.0075[b]	0.000	0.994
2-0	0.0	−0.0009[c]	0.042	0.686
3	190.1	0.0067[b]	0.000	0.988
3-0	0.0	−0.0015[c]	0.069	0.603

Means within columns that have different superscript letters are significantly different ($P < 0.05$; [1]analyses of covariance, [2]regression analyses).

Diet	Moisture (g kg^{-1})	Ash (g kg^{-1})	Lipid (g kg^{-1})	Protein (g kg^{-1})	Astaxanthin (mg kg^{-1})	Vitamin A (μg g^{-1})	Vitamin A (μg ind^{-1})
Initial sampling							
1	758 (5)[a]	21 (0.1)[a]	46 (3.9)[ab]	140	0.0 (0.01)[a]	0.10 (0.03)[ab]	0.17
2	756 (4)[a]	20 (0.2)[a]	60 (1.1)[e]	138	0.1 (0.02)[b]	0.50 (0.03)[cd]	0.85
3	756 (4)[a]	20 (0.5)[a]	63 (0.5)[a]	140	0.4 (0.08)[c]	0.90 (0.05)[e]	1.64
Final sampling							
1	736 (2)[b]	22 (0.1)[b]	91 (0.8)[c]	147	0.1 (0.01)[b]	0.07 (0.03)[a]	0.31
1-0	800 (1)[c]	27 (0.1)[c]	47 (0.2)[b]	121	0.1 (0.01)[b]	0.08 (0.01)[a]	0.11
2	730 (2)[b]	21 (0.0)[a]	103 (1.2)[d]	139	0.3 (0.04)[c]	0.74 (0.13)[e]	4.66
2-0	785 (1)[d]	27 (0.3)[c]	55 (0.2)[a]	133	0.1 (0.01)[b]	0.37 (0.20)[bcd]	0 63
3	730 (5)[b]	20 (0.3)[a]	100 (1.6)[d]	136	1.7 (0.54)[d]	0.68 (0.17)[de]	3.74
3-0	787 (1)[d]	27 (0.1)[c]	54 (0 7)[ab]	126	0.5 (0.11)[c]	0.19 (0.10)[abc]	0.28

Means in columns that have different superscript letters are significantly different ($P < 0.05$; Kruskal–Wallis ANOVA by ranks).

Table 4 Whole-body content of moisture, ash (g kg^{-1} wet tissue weight), lipid (g kg^{-1} wet tissue weight), protein (g kg^{-1} wet tissue weight), astaxanthin (mg kg^{-1} wet tissue weight), vitamin A (μg g^{-1} wet tissue weight) and vitamin A storage (μg) in individual fish of Atlantic salmon fry fed diets with different levels of astaxanthin for 10 weeks. Values are given as means with standard deviation within parentheses, $n = 3$.

The concentration of astaxanthin in whole-body samples of the fish, however, was dependent upon the dietary astaxanthin level of the feed which the fish had been fed prior to the present experiment (Table 4). Increased astaxanthin concentrations were observed in the groups fed the astaxanthin-supplemented diets (Table 4). Small changes in the astaxanthin concentration were found in the groups fed the zero diet when compared with their initial concentrations. Red pigmentation of the skin, particularly the edges of the fins, was observed in the groups fed the diets with high levels of astaxanthin. However, the degree of skin pigmentation among the different groups was not quantified.

The vitamin A concentration in whole-body samples of fish and the body storage of individual fish is shown in Table 4. The vitamin A concentrations were dependent upon the dietary astaxanthin level which the fish had been fed prior to the start of the experiment (Christiansen *et al.* 1995b). There was no difference in the vitamin A concentrations in the fish fed diet 1 and the zero diet (diet 1-0) at the end of the experiment, nor were the concentrations different from the concentrations in the same fish at the start of the experiment. However, the amount of vitamin A stored in the body increased in the fish fed diet 1, whereas the body storage decreased in the fish fed diet 1-0. Both final vitamin A concentration and body storage were higher than the initial concentration and body storage in the fish fed diet 2. In the fish fed diet 2-0, on the other hand, the vitamin A concentration was not different from the initial concentration but the body storage was lower. Similar results were found for the fish in the groups fed diets 3 and 3-0. However, there was no difference in the initial concentration and the final concentration in the groups fed diet 3. The concentration and the body storage in the group fed diet 3-0 was significantly lower at the end of the experiment.

Discussion

Lipid and protein sources in commercial fish diets are at present based mainly on fish oils and meals. They may contain substantial levels of carotenoids and vitamin A. In most cases, the use of fish meal and oil in the diets ensures an adequate level of carotenoids. There is, however, extensive research being done on the use of vegetable proteins and lipids as alternatives to fish meal and oil in salmonid diets (Thomassen & Røsjø 1989; van der Ingh *et al.* 1991; Carter *et al.* 1994). The substitution of fish meal and oil with vegetable products may reduce the natural astaxanthin/carotenoid levels in the diet, thus making it necessary to supplement the feed with carotenoids.

Previous studies have shown a requirement for astaxanthin during the first-feeding period, and have connected this requirement to a provitamin A effect of astaxanthin (Christiansen *et al.* 1994, 1995b). We chose in the present experiment to use fish that had already been fed different levels of astaxanthin during their first-feeding period. Thus the juveniles had different reserves of astaxanthin and vitamin A at the start of the experiment. If the requirement for astaxanthin increased with size, it was expected that a negative effect on growth would appear first in the group fed the diet containing an astaxanthin level close to the requirement of 5.1 mg astaxanthin kg^{-1} dry diet found in the previous experiment. By dividing the groups into two subgroups and feeding one of these a zero diet, we wanted to clarify whether or not the juveniles were able to utilize their stored astaxanthin. If the juveniles could utilize the astaxanthin reserves, the negative effects of feeding the zero diet should have been lowest in the group with the largest reserves.

The astaxanthin concentration in the juveniles fed the zero diet at the end of the experiment was the same as the initial concentration in the juveniles, indicating that previously stored astaxanthin was not metabolized during the experimental period. Astaxanthin was observed to be deposited in the skin of the juveniles. This storage may be less available than astaxanthin stored in muscle. Low levels of astaxanthin were found in the fish fed 5.3 mg astaxanthin kg^{-1} diet, which shows that this dietary level is too low to enable the juveniles to store astaxanthin. A low, yet significantly higher concentration, was observed in the fish fed the diet containing 36 mg astaxanthin kg^{-1} dry diet. The astaxanthin concentration in the juveniles fed the highest level of astaxanthin was elevated. This was also observed by Christiansen *et al.* (1995b). No attempt was made in the present experiment to determine the location of the astaxanthin storage in the juveniles. As in the preceding experiment, red pigmentation of the skin, particularly the edges of the fins, was observed in the fish fed the diets with the highest dietary level of astaxanthin.

In comparison with the growth achieved using practical diets containing fish meal and oil, the growth of Atlantic salmon fed purified diets is often lower (Rumsey & Ketola 1975; Christiansen *et al.* 1994, 1995a, b; Hamre & Lie 1995). The growth of the groups fed diets supplemented with astaxanthin was comparable to the growth observed by Christiansen *et al.* (1994, 1995b). Shearer *et al.* (1993) did not observe any differences in the growth of Atlantic salmon juveniles fed a casein/gelatine-based diet without astaxanthin supplementation when compared with other diets, including a fish-meal-based diet. The mean initial weight of the fish in Shearer *et al.* (1993) was 3.6 g, and the SGR was 2.37%. The SGR of the fish with the best growth in the present experiment was 2.0% during the last 4 weeks of the experiment when the size of the fish was comparable to that of the fish in the experiment by Shearer *et al.* (1993). Water temperatures were similar in the two experiments. In the study by Shearer *et al.* (1993), a capelin oil was used as the lipid source and the higher SGR may be explained by a better palatability of

the diet. The diet was not supplemented with astaxanthin, but there may have been astaxanthin in the capelin oil. This could explain why good growth was found in Shearer *et al.*'s experiment despite the lack of astaxanthin supplementation of the diet as compared with other studies where poor growth has been found using unsupplemented diets (Christiansen *et al.* 1994, 1995a, b).

The growth of the juveniles was clearly affected by the dietary treatment. The reduction in mean weight observed in the groups fed the diets without astaxanthin supplementation is in agreement with the lack of growth observed in other studies done on Atlantic salmon juveniles fed purified casein diets without astaxanthin supplementation (Christiansen *et al.* 1994, 1995b). The reduction in weight in the groups fed the zero diet could be the result of either a metabolic deficiency of the astaxanthin itself or a reduced feed intake by the juveniles. The latter could be the result of a reduced palatability of the diets without astaxanthin supplementation. Extracts of crustaceans, which contain high levels of astaxanthin, are known to increase the palatability of diets (Storebakken 1988). The improved taste has, however, been related to certain free amino acids (Adron & Mackie 1978; Ellingsen 1982). Thus far, astaxanthin has not been reported to improve the flavour of feed for fish. A different experimental design would have been necessary to investigate the effect of astaxanthin as a feed enhancer. The alteration of the feed colour from orange to yellowish for the fish fed the zero diets may also have affected the feed intake. However, fish in all groups were observed to feed at the start of the experiment, indicating that the colour of the feed was not the cause of the dramatic differences in growth observed. The fish in the groups fed the zero diets were observed to feed, but the feed intake was lower than that of the groups fed the diets containing astaxanthin. The feed intake was expressed as the feed conversion efficiency. A negative feed conversion efficiency, resulting from the weight reduction of the fish throughout the experimental period, was observed in groups that had been fed the zero diet. The highest feed conversion efficiency was 0.93 in the last 4 weeks of the experiment in the group fed 36.0 mg astaxanthin kg^{-1}, which is less than the feed conversion efficiency of 1.50 observed by Shearer *et al.* (1993). In the latter experiment, a restricted feeding regime was used. In the present study, a feeding strategy that ensured a surplus of feed was used. The lower feed conversion efficiency can be explained by a higher feed waste. It is also likely that the negative feed conversion efficiency in the groups fed the zero diet is the result of poor feed intake and high feed waste.

The minimum concentration of astaxanthin required for maximum growth of Atlantic salmon during the first-feeding period was found to be 5.1 mg astaxanthin kg^{-1} dry diet (Christiansen *et al.* 1995b). The group fed the diet containing 5.3 mg astaxanthin kg^{-1} dry diet in the present experiment showed no deficiency symptoms during the first-feeding period. However, in the present experiment, reduced growth was observed in this group following 5 weeks of feeding. This indicates that a supplementation of 5.3 mg astaxanthin kg^{-1} dry diet is insufficient to satisfy the dietary need of juveniles above 3 g when fed a casein/gelatine diet. This supports the assumption that astaxanthin has a metabolic role. Earlier studies on the pro-vitamin A function of astaxanthin in rainbow trout have shown provitamin A activity of astaxanthin and canthaxanthin in fish weighing above 200 g which were deficient in vitamin A (Schiedt *et al.* 1985; Al-Khalifa & Simpson 1988, Guillou *et al.* 1989). The provitamin A activity of astaxanthin in fish fed a sufficient amount of vitamin A is low in both rainbow trout (Schiedt *et al.* 1985; Al-Khalifa & Simpson 1988) and Atlantic salmon (Storebakken *et al.* 1993). A provitamin A function of astaxanthin has previously been suggested during the first-feeding period of Atlantic salmon (Christiansen *et al.* 1995b). In the earlier study, reduced growth and increased mortality was observed in groups of first-feeding salmon which had been fed diets low in astaxanthin. Reduced levels of vitamin A were observed in the fish from the same groups. The low levels of vitamin A indicate a poor bioavailability of vitamin A palmitate and/or acetate during the first-feeding period. The deficiency symptoms observed in the earlier study were assumed to be caused by a vitamin A deficiency which is known to result in poor growth (Halver 1989). The present study shows that there was a provitamin A effect of astaxanthin, which led to higher vitamin A concentrations and body storages in the fish in the groups that had been fed the two highest levels of astaxanthin. All of the diets contained the same amount of vitamin A, and the levels were well above the requirement of 2500 IU given by Halver (1989) for salmonids. The results from this study verify the results from the earlier studies (Christiansen *et al.* 1994, 1995b) which have indicated a poor bioavailability of vitamin A esters by first feeding Atlantic salmon. Larger salmon (0.8 kg), however, have been shown to be able to utilize vitamin A esters (Storebakken *et al.* 1993).

Low growth, high mortality and low vitamin A levels were observed in groups of first-feeding Atlantic salmon fed diets low in astaxanthin, regardless of whether the fish originated from normally pigmented eggs or eggs that contained low levels of pigment (Christiansen *et al.* 1995b). This suggests a poor bioavalability of astaxanthin from the yolk-sac for metabolic purposes during the first feeding of the fry. Other studies have shown that astaxanthin in the yolk is transferred to the chromatophores in the skin during yolk-sac absorption and is followed by esterification in the skin without any quantitatively significant loss (Steven 1949–1950; Kitahara 1984). The red fins observed in the present experiment show that dietary astaxanthin

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HARVARD MEDICAL SCHOOL

Harvard Health Letter

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The truth about mold

Most experts say there's more fear than fact to "toxic mold." But that doesn't mean that indoor mold can't cause health problems.

Mold spreads easily, but fear of it may be spreading even faster. In Texas, claims related to mold damage have created a homeowners insurance crisis as insurers have raised premiums or stopped writing policies altogether. Moldy school buildings have been torn down in California. Hearings were held in Congress this past summer, and legislation introduced to fund special mold studies.

Meanwhile, the opportunists are circling. In legal circles, they are calling it the "mold rush." In Houston last year, seven people were charged with filing fraudulent insurance claims after deliberately flooding homes and "cooking" one of them to encourage runaway mold growth.

Homegrown

Water is the essential ingredient for mold growth. Most species aren't picky eaters; as long as they can wash it down with a little water, many will feed on anything containing *cellulose*, the stringy sugar molecules that are the main component of the cell walls of all plants. Wood, paper, wallboard — they all contain cellulose. And "tighter," more energy-efficient homes and buildings are more likely to trap moisture, especially if they're not well ventilated or insulated. As a result, American homes and buildings have probably gotten moldier in recent years, although proving that is difficult. Even older buildings have new components — tighter windows, carpeting, dropped ceilings — that make them more susceptible to mold growth.

We've also become cellar-dwellers as millions have converted basements prone to dampness into family rooms, entertainment centers, and home offices. J. David Miller, a mold expert at Carleton University in Ottawa, Canada, says these transformed basements are like mushroom factories (mushrooms, like mold, are fungi): "nice and dark and wet and humid."

Poor housekeeping may also help mold grow. Air doesn't circulate as well through a cluttered room, which can lead to condensation and dampness. A moist, dirty bathroom is a mold smorgasbord. The black mold that grows on grout and caulking has an appetite for human skin cells, among other things.

Toxic chemical factories

There's no question that some molds produce chemicals that are toxic to other molds, bacteria, insects — and to people. Collectively, these chemicals are known as *mycotoxins*. (*Myco-* means anything having to do with fungi.) The *Aspergillus flavus* mold that grows on peanuts, soybeans, and cassava (a root) produces *aflatoxin* B_1, which causes liver cancer. The ergot alkaloids produced by the *Claviceps purpura* mold that grows on rye cause nerve damage, convulsions, gangrene, and even limb loss. In the 1940s, thousands of Russians got sick from eating corn and wheat contaminated with *Fusarium* and *Stachybotrys* mold after it had been stored under snow during the winter.

Of course, we've also turned mold-generated toxins to our advantage. As every schoolchild knows, Alexander Fleming discovered penicillin in a moldy Petri dish (sometimes it pays not to clean up). Other antibiotics and drugs like cyclosporin, the immune system suppressant, are mold or fungal toxins, or are derived from them.

Limited exposure

Mold grows indoors. Some molds produce toxins. Hence, "toxic mold." But that deduc-

INSIDE

The dish on fish
Eating fish is doing your health, and especially your heart, a favor. But lately some doubts have crept in. Is this another example of health advice gone awry? . 4

Simon says...
It's really your choice. Dr Harvey B Simon believes that the days of doctor knows best are long gone. An interview with the editor of the *Harvard Men's Health Watch*, who has written a new book on men's health 6

In brief
Snow shoveling and your heart, grapefruit juice and your medications, nosepicking and its consequences . . . 7

By the way, doctor
Suggestions for people with nerve pain; advice on timing your heart medications for maximum effect . 8

In future issues

Another front in the vitamin E debate: Gamma- vs alpha-tocopherol

The body mass index: Is it a fair measure of body fat?

The latest on COX-2 inhibitors

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health_letter@hms.harvard.edu

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www.health.harvard.edu

Hooked on fish? There might be some catches

Health-conscious people eat it three, even four times a week. But farm-raised fish and worries about mercury contamination are churning the waters.

The advantage of eating fish has become one of those health-advice truisms, ranking right up there with getting exercise and eating fruits and vegetables. "Studies show that fish consumption lowers your risk of..." — you can fill in the blank, although the evidence remains strongest for heart disease.

The topic has spawned plenty of research. We recently did a quick computer search of the medical literature for fish-consumption studies. Within minutes we found research papers on stroke in American women, prostate cancer in Swedish men, Alzheimer's disease in French seniors, and leptin (an appetite hormone) levels in Tanzania. Not surprisingly, all came out swimmingly for the fish eaters.

Farm vs. wild

The glowing health reports have whet the American appetite for fish, and the millions of pounds of farm-raised fish produced each year help meet that demand. In addition to farm-raised catfish, salmon, and trout, we now have tilapia, striped bass, sturgeon, and walleye on the menu and at the store. In Australia, they've started tuna "ranching" — catching the fish in large nets and herding them into pens for several months of feeding.

Dilemmas abound. Farming fish makes a healthy food less expensive for consumers. The added supply almost certainly eases overfishing of dwindling stocks of some species. But some environmental groups are critical, especially of salmon operations on the West Coast, and want consumers to boycott farm-raised salmon. They say the "floating feedlots" harm fragile marine environments. There's also an argument that raising carnivorous fish like salmon is wasteful of natural resources because it takes several pounds of wild fish like herring or anchovy to produce a pound of salmon. The industry says it has responded by cutting back on antibiotics, switching to low-phosphorous feeds that make fish waste less polluting, and experimenting with soy and other vegetable-based feeds. If you want to learn more about these environmental issues, visit our Web site at www.health.harvard.edu/health.

Nutritional issues

Coddled and cooped up, farm fish tend to be anywhere from two to five times fattier overall than wild fish, although the fat content of wild fish varies tremendously depending on the season and where the creature is in its reproductive cycle. That extra fat means more calories. But fattier (oilier) fish also tend to have more of the omega-3 fats that are the main reason fish is such a healthy food. (*See sidebar.*) A meal of an oily fish like bluefish will give you twice as many omega-3s as a like-sized serving of halibut, and four times as many as farmed catfish.

So farm-raised fish — simply because they're fattier — tend to have more omega-3s than wild fish. But actual comparisons become complicated. Both the amount and type of fat in farmed fish depend on their feed, particularly the type of oil (fat) it contains.

When we looked up the omega-3 content of farmed and wild Atlantic salmon in a nutritional database compiled by the United States Department of Agriculture (USDA), they were the same. But wild Atlantic salmon is scarce and not commercially available very often. A more realistic comparison is

What is it about fish?

When you eat carbohydrates (sugar or starch) or protein, your body shows little respect for the artistry of those molecules. It tears them apart and reassembles them to suit its own purposes. Carbohydrates and protein – they're just fodder.

But it's different with fat. Some gets roughed up during digestion and metabolism. But some gets through more or less intact, becomes part of our cell membranes, and thus has considerable say-so over how cells behave. We are the fat that we eat.

Fish is a special food because it contains two important varieties of *long-chain omega-3* fats that you won't find anywhere else in a conventional diet. *Long-chain* refers to the number of carbon atoms, *omega-3* to a position of a certain chemical bond that puts a 45-degree kink in that chain. Both attributes determine how a fat molecule is going to fit into cell membranes and what it's going to do once it gets there.

As it turns out, long-chain omega-3 fats in fish are just the sort of fat molecules that any healthy cell should gladly welcome into its membranes. One of them, *eicosapentaenoic acid,* manages to displace molecules that would otherwise give rise to active prostaglandins, leukotrienes, and other inflammatory compounds. And inflammation seems to be a root cause of many diseases. *Eicosapentaenoic acid* also seems to be the omega-3 with the most pronounced cardiac benefits.

The other main omega-3 in fish is *docosahexaenoic acid* (DHA). It's important to brain and vision development in infants and is added to infant formulas.

Sometimes there's some confusion about where the *alpha-linolenic acid* in walnuts, flaxseed oil, and soy products fits in. It's also an omega-3 fat, but has fewer carbon atoms and therefore isn't a long-chain omega-3. Being shortchanged those few carbon atoms makes a difference because alpha-linolenic acid doesn't have as many health benefits as the more carbon-blessed omega-3s in fish.

farmed Atlantic with other wild salmon species. And according to the USDA database, wild coho salmon, for example, contains half the amount of omega-3s as farmed Atlantic salmon.

Researchers at Oregon Health & Science University have made their own comparisons. So far, their tests haven't shown any difference in the omega-3 content of farmed and wild salmon, according to Dr. William E. Connor, one of the researchers. But when they tested catfish, the omega-3 content of the wild fish was much higher than the farmed.

Fish feed

Fish feeds vary tremendously with the species. There is also continual experimentation with, for example, different sorts of enzymes to make the fish metabolize feed more efficiently and thus grow faster. British scientists announced last year that they had successfully added *pheromones* to feed to make it more appetizing. Red coloring in the form of synthetic carotenoids is added to salmon feed to give the flesh that rosy color that consumers have come to expect.

For consumers, the oil content of the feed is a key issue because it influences omega-3 levels. Currently, most of the oil used for fish feed comes from small fish like herring and menhaden — and it's rich in omega-3s. But the industry is worried about dwindling supplies and rising costs and thus interested in plant-based alternatives. Researchers at the University of Stirling in Scotland have published several studies showing that replacing fish oil with plant-derived substitutes is feasible, but, not surprisingly, a high proportion of plant oil significantly reduces the omega-3 content of salmon.

Some experts we talked to said feed makers are more likely to switch from fish to vegetable (soy) sources of protein, not fat. For one thing, some species — notably salmon, trout, and steelhead — need omega-3 oil to flourish. The industry also has an interest in preserving the reputation of fish as a healthy food, which means keeping the omega-3 levels as high as possible.

As for farm vs. wild taste, we defer to the palate of Roger Berkowitz, CEO of Legal Sea Foods, a chain of seafood restaurants based in Boston. He says that wild fish, especially salmon, has a gamier, more intense flavor. It's also more expensive. Berkowitz says farm-raised flounder has foundered because of poor taste and texture.

Omega-3 and mercury content of select fish

	Omega-3 fats (grams in 3-oz. serving)*		Mercury (parts per million)**
Atlantic salmon, farmed	1.8	Tilefish	1.45
Anchovy	1.7	Swordfish	1.00
Sardines	1.4	Shark	0.96
Rainbow trout, farmed	1.0	King mackerel	0.73
Coho salmon, wild	0.9	Tuna (fresh and frozen)	0.32
Bluefish	0.8	Halibut	0.23
Striped bass	0.8	Mahi mahi	0.19
Swordfish	0.7	Tuna (canned)	0.17
Tuna, white, canned	0.7	Catfish	0.07
Halibut	0.4	Salmon	Not detectable
Catfish, channel, farmed	0.2	Tilapia	Not detectable

*Source: USDA Nutrient Database **Source: FDA

Mercury contamination

But an even bigger worry these days is that the fish we're urged to eat for health may contain some very unhealthy contaminants, particularly mercury. Most research suggests that if the mercury in fish causes harm, the danger is primarily to the developing nervous systems of children, although studies have suggested a link between mercury and the atherosclerosis that underlies heart disease. Last spring, the FDA advised pregnant women and all women of childbearing age not to eat any shark, swordfish, king mackerel, and tilefish because of their high mercury content, and to limit consumption of all fish to 12 ounces (about two servings) per week. Harvard researchers recently published a study in the *New England Journal of Medicine* showing that Americans who eat more fish have higher levels of the metal in their bodies (more specifically, in their toenails), although they don't believe the levels cause harm. No one is recommending routine mercury testing. But the contaminant does seem to pose a damned-if-you-do, damned-if-you-don't problem for people who want to eat a lot of fish for health reasons. Mercury tends to accumulate in the food chain: the higher on the chain, the greater the concentration of mercury. But species rich in omega-3 fats also tend to be the food chain's higher-ups, including swordfish, mackerel, and tuna.

The FDA is correct to take a better-safe-than-sorry approach to mercury in fish. But consider the risks and benefits. The amount of mercury you're exposed to by occasionally eating swordfish and mackerel is very small. Besides, you have other choices. Salmon, for example, is high in omega-3s and so far has tested very low for mercury. Smaller tuna are used for canning, so apart from all that mayonnaise, eating a tunafish sandwich a couple times per week isn't a major hazard.

In November 2002, the American Heart Association re-emphasized its recommendation that all adults should eat at least two servings of fish per week because of the cardiovascular benefits. The association takes the position that for adult men and older women not having children, any risk from mercury is offset by the advantages.

So you can have your fish and enjoy it, too. Eating fish remains one of the better health bets out there.

Welcome to NFI

Web Site Updates:

The Latest: Scholarship Pub. 134 Posted Online

May 15, 2003:

Scholarship Publication: The study entitled, The Implication of Omega-3 Fatty Acids in Huma (S-134), has been added to the member's portion of the site. Log on here, then go to the Techr section and click on Research Reports. From there, scroll down to paper 134.

NFI Personnel: A new photo of NFI President John Connelly has been added to the "Our Leac "Staff" pages of the "About NFI" section of the public portion of the Web site.

Industry News: NFI member Howard Johnson, president of the seafood market research com H.M. Johnson & Associates, has pondered the future of the U.S. seafood market and sees a br outlook with strong demand and increased consumption. For details, log on to the members' se the Web site by clicking here.

Scholarship Publication: The study entitled, Lipid Oxidation in Fish Muscle (S-133), has beer to the member's portion of the site. Log on here, then go to the Technical section and click on F Reports. From there, scroll down to paper 133.

NFI Community: The Convention Committee list has been updated in the members-only secti Web site. Log on here, then select "NFI Community" from the navigation bar that runs across th the Web page. From there, select "Committees," then "Convention Committee."

Scholarship Publication: The study entitled, Allergic Reactions to Seafoods: Identification of / (S-132), has been added to the member's portion of the site. Log on here, then go to the Techr section and click on Research Reports. From there, scroll down to paper 132.

NFI Events: The National Fish & Wildlife Foundation and NOAA with the support of the **Nation Fisheries Institute** will host the 28th Annual NOAA Fish Fry on June 18. For details, log on to members' section (click here) of the Web site.

Scholarship Publication: The study entitled, A Simple Procedure to Monitor Fecal Coliforms i Seafood Processing Plants (S-129), has been added to the member's portion of the site. Log o then go to the Technical section and click on Research Reports. From there, scroll down to pap

NFI Calendar: NFI's Calendar of Events page has been updated.

Scholarship Publication: The study entitled, Effect of Vacuum Packaging on Changes Associ Frozen Cod Fillets (S-128), has been added to the member's portion of the site. Log on here, th the Technical section and click on Research Reports. From there, scroll down to paper 128.

Other Updates This Week:

NFI, BWFA Dispute Overfishing Study

May 14, 2003:

NFI News: NFI and BWFA are disputing an overfishing study that is generating a substantial a major news media attention. Log on to the members' section (click here) for important details

Omega-3

Omega-3 Content of Fish and Shellfish

Finfish	grams per 3 ounce portion*
Anchovy, European, canned in oil, drained	1.7
Bass, freshwater, mixed species, cooked, dry heat	0.6
Bass, striped, cooked, dry heat	0.8
Bluefish, cooked, dry heat	0.8
Catfish, channel, farmed, cooked, dry heat	0.2
Cod, Atlantic, cooked, dry heat	0.1
Fish portions and sticks, frozen preheated	0.2
Flatfish (flounder and sole species), cooked, dry heat	0.4
Grouper, mixed species, cooked, dry heat	0.2
Haddock, cooked, dry heat	0.2
Halibut, Atlantic and Pacific, cooked, dry heat	0.4
Herring, Atlantic, pickled	1.2
Mackerel, Atlantic, cooked, dry heat	1.0
Mackerel, jack, canned, drained solids	1.0
Mackerel, Pacific and jack, mixed species cooked, dry heat	1.6
Mahi mahi, cooked, dry heat	0.1
Perch, mixed species, cooked, dry heat	0.3
Perch, ocean, Atlantic, cooked, dry heat	0.3
Pike, walleye, cooked,dry heat	0.3
Pollock, Atlantic, cooked, dry heat	0.5
Pollock, Alaska, cooked, dry heat	0.4
Rockfish, Pacific, Mixed species, cooked, dry heat	0.4
Roughy, orange, cooked, dry heat	<.01
Sablefish, cooked, dry heat	1.5
Salmon, Atlantic, farmed, cooked, dry heat	1.8
Salmon, Chinook, cooked, dry heat	1.5
Salmon, Chinook, smoked, (lox), regular	0.4
Salmon, chum, cooked, dry heat	0.7
Salmon, coho, wild, cooked, dry heat	0.9
Salmon, pink, canned, solids with bone and liquid	1.4
Salmon, sockeye, canned, drained solids with bone	1.0
Salmon, sockeye, cooked, dry heat	1.0
Sardine, Pacific, canned in tomato sauce, drained solid with bone	1.4
Sea bass, mixed species, cooked, dry heat	0.6
Smelt, rainbow, cooked, dry heat	0.8
Snapper, mixed species, cooked, dry heat	0.3
Swordfish, cooked, dry heat	0.7
Trout, rainbow, farmed, cooked, dry heat	1.0

Tuna, light, canned in water, drained solids	0.2
Tuna, white, canned in water, drained solids	0.7
Tuna, yellowfin, fresh, cooked, dry heat	0.2
Whiting, mixed species, cooked, dry heat	0.4

Mollusks

Clam, mixed species, cooked, moist heat	0.2
Mussel, blue, cooked, moist heat	0.7
Oyster, Eastern, farmed, cooked, dry heat	0.4
Oyster, Eastern, wild, cooked, dry heat	0.5
Scallop, mixed species, cooked, dry heat	0.3

Shellfish

Crab, Alaska king, cooked, moist heat	0.4
Crab, Alaska king, imitation, made from surimi	0.5
Crab, blue, cooked, moist heat	0.4
Crayfish, mixed species, farmed, cooked, moist heat	0.1
Lobster, Northern, cooked, moist heat	0.1
Shrimp, mixed species, cooked, moist heat	0.3

*Cooked without added fat or sauces
Source: USDA Nutrient Database for Standard Reference, Release 11-1

Farmed and Dangerous

Coastal Alliance for Aquac

Think Twice About Eating Farmed

What is salmon farming?

History of salmon farming in BC

Salmon Farming and the environment

Salmon Farming and Human Health

Salmon Farming and First Nations

Salmon Farming and the Coastal Economy

Location of salmon Farms in BC

Why You Should Think Twice About Eatir Farmed Salmon

- Farmed salmon is much higher in saturated fats than wild salmon This can contr health problems
- A single serving of salmon, wild or farmed, gives you the suggested daily require omega 3 fatty acids These essential fatty acids are also found in other wild fish l sardines or anchovies Farmed salmon, however, contains more unhealthy fats Preliminary research also shows that farmed salmon has higher levels of PCBs a contaminants than wild salmon
- Farmed salmon are frequently fed antibiotics which contribute to the growth of dr resistant bacteria.

  - Click here to link to section on antibiotics
- Preliminary findings suggest that farmed salmon contain higher levels of **PCBs** a dioxins than wild salmon.

  - Click here to link to report by Michael Easton
- Farmed salmon are often given additives in their food to colour their flesh pink wi chemicals to resemble its wild counterparts - otherwise, it would remain an unap grayish-brown color
- All **Atlantic salmon** sold in restaurants and grocery stores are farmed There is r any commercial fishery for wild Atlantic salmon
- In BC, over 70% of farmed salmon are Atlantic salmon Atlantic salmon are cons exotic (non-native, or alien) species in Pacific waters since they do not naturally the Pacific Ocean
- With only a very few exceptions, farmed salmon are raised in open **net cages** in ocean These nets can tear, allowing farmed salmon to escape into the wild Ove farmed salmon have been reported by the industry to have escaped into Pacific since 1988, because many escapes over the years have gone unreported, exper the real figure is much higher
- Atlantic salmon have been found in 78 BC rivers and streams, but only a small p our rivers have been surveyed so far - meaning non-native Atlantic salmon could inhabiting many more
- Atlantic salmon compete with wild salmon for habitat, particularly steelhead, and been known to eat wild salmon fry and eggs Atlantic salmon have been found sp and juveniles surviving in the wild.

  - Click here to link to Super Unnatural Report by John Volpe
- There are risks even when native **Pacific salmon** escape into the wild Escaped

Chinook can interbreed with wild Chinook. Since farmed salmon are cultivated fr limited gene pool, this interbreeding leads to "genetic dilution", or a narrowing of genetic makeup in wild fish - which could lessen their ability to survive in the wild

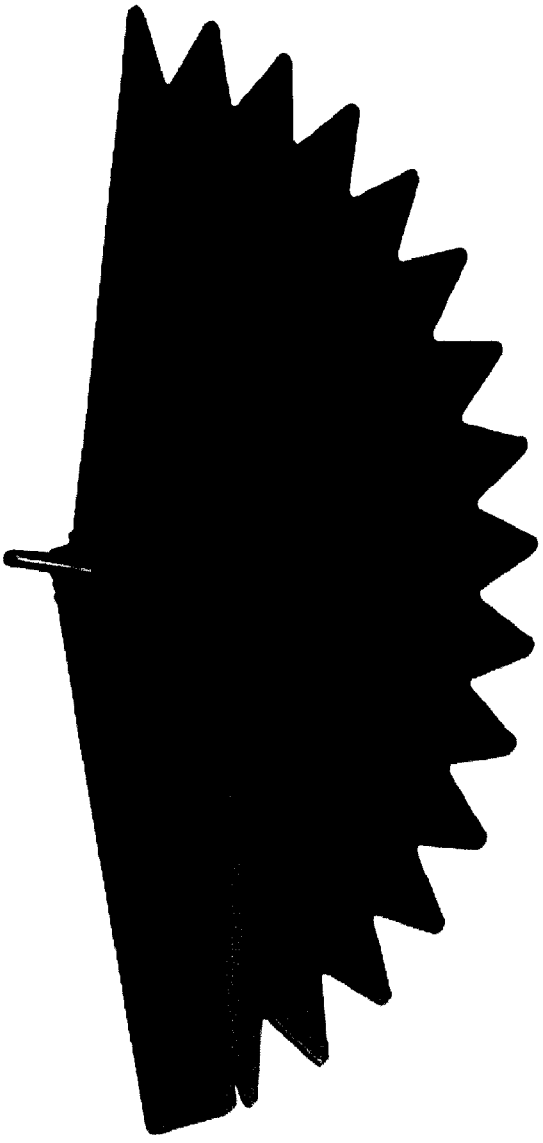
- Open netcage systems can allow for the transfer of disease and parasites from f wild salmon

  - Click here to link to Watershed Watch report

- At some farms, Acoustic Harassment Devices (**AHDs**) have been used to scare such as seals and sea lions away from salmon farms. These underwater noisem extremely loud and can lead to loss of hearing in marine mammals. Research sh they also change the behaviors in some marine mammals such as killer whales (and harbor seals, driving them out of an area

  - Click here to link to Displacement of Orcinus orca (L.) by high amplitude s BC

- Salmon farmers are granted licenses to kill predators such as sea lions and seal them eating their fish. In the spring of 2001 a mass grave containing at least 15 s killed by a farm operator was discovered in Clayoquot Sound. Since then, more dead sea lions have been found in the same area. BC salmon farmers reported k killed at least 5000 seals and sea lions in the last decade. The real figure could b higher as some kills according to fish farm employees go unreported
- The mass worldwide production of salmon in fish farms has caused a drop in wil prices. This has hurt thousands of commercial fishermen and their communities i they live and support, drawing into question the true economic value of this indus
- It takes three to five kilograms of other fish, such as herring and anchovy to mak necessary to produce one kilogram of farmed salmon resulting a loss of edible a protein worldwide
- In Canada it is illegal to make animal feed out of proteins otherwise suitable for h consumption. As a result most of the feed for BC salmon is obtained from South A This reduces the amount of food energy available to people there
- To fatten up their livestock, some salmon farmers use bright lights even at night t the salmon into thinking it is always feeding time. This attracts other fish to the fa and may disrupt their feeding and migration patterns
- In B.C. fish farms use net guards that deter predators. Some farmers coat the ne highly toxic solution to prevent naturally occurring marine organisms from growin them. This toxic solution contaminates our waters
- There are over 85 open **net cages** farms currently operating in BC waters and a farms that are dormant and could be started up at any time. This number does n all the many new farms that the industry and provincial government hope to see coast over the next few years. At the current level alone, collectively these farms discharge waste into the ocean, which is roughly equivalent in terms of pollution sewage from a city with 500,000 inhabitants. This untreated waste contaminates marine environment



The SalmoFan ® is a tool used by Salmon Farmers to decide what shade of pink they would like their Salmon to be at time of slaughter. The color selection is made and the corresponding level of colorance is then added to feed. The SalmoFan is evidence in our lawsuits.

Take Action

Do you want to join the lawsuit?

If you have purchased farm raised salmon from a st did not label the product as artificially colored, pleas an e-mail.

Please let us know:

- the store at which you purchased the salmon
- the approximate date of the purchase
- your contact information

We'll get back to you.

Send an e-mail to knoll@igc.org.

Please Donate to the Salmon Litigation Fund!!

We need donations to help cover the costs of this s could be substantial. Please give generously to the Labeling Litigation Fund!

Send your checks to:

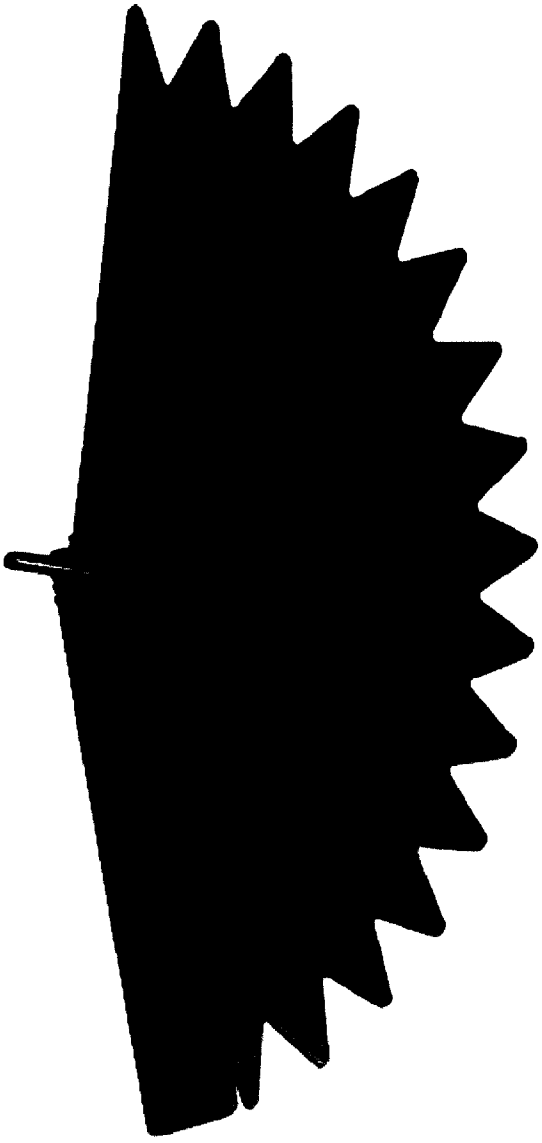
Salmon Labeling Litigation Fund
c/o Smith & Lowney PLLC
2317 E. John St.
Seattle, WA 98112

Your contribution allows this case to go the distance deceptive marketing of farm-raised salmon.

Is your retailer labeling the artificial coloring in I raised Salmon?

Please check to see if your local retailers and majo restaurant chains are labeling the artificial colors in raised / atlantic salmon and farm-raised trout. If the they should probably be targeted as well.

Send an e-mail to knoll@igc.org.



The SalmoFan ® is a tool used by Salmon Farmers to decide what shade of pink they would like their Salmon to be at time of slaughter. The color selection is made and the corresponding level of colorance is then added to feed. The SalmoFan is evidence in our lawsuits.

Nationwide class actions target majc grocery store chains for concealing artificial coloring in farm-raised salm

1. **Buyer Beware: Something Fishy about Far Salmon**
   - Read the press release
2. **See the complaints:**
   - Lori Thomas vs. Albertson's, Inc. [PDF]
   - Heather Dolin *et al.* vs. Safeway, Inc. [P
   - Christopher Krupp *et al.* vs. Kroger Co., Meyer Stores, Inc., & QFC Sub. Inc. [PD
3. **Background Information:**
   - Links and Resources
   - About Smith & Lowney
4. **Take Action:**
   - Join the lawsuit
   - Contribute to the Salmon Label Litigatio
   - Is your retaller labeling the artificial colo Farm-raised Salmon?

Wild salmon develop their trademark color naturally they feed on certain prey like krill (tiny shrimp-like crustaceans). Farmed salmon get their color from fc feed, which usually contains the chemicals astaxan canthaxanthin. (In response to concerns about adv health affects, the European Union has agreed to si reduce the level of canthaxanthin that may be fed tc raised salmon).

According to the suits' claims, lack of labeling also r the public into thinking they're buying wild salmon, a the problems associated with farm-raised salmon in

- Contamination from antibiotics and exposure t pesticides and other chemicals
- Risks to wild salmon and other aquatic specie disease and parasites which escape from fish
- Misrepresentation of health benefits - accordi US Department of Agriculture, farmed Atlantic over 200 percent higher in saturated fat than v chum salmon
- Impacts on marine ecosystems from fish farm

The lawsuits are designed to protect millions of con who purchase farm-raised salmon from the three ch call for:

- Damages for consumers, expected to exceed millions of dollars for each chain
- A court order requiring the chains to inform co that the salmon are artificially colored
- Civil penalties for violation of various consume protection statutes

Filed in the King County Superior Court in Seattle, Washington, the claims are being brought by Smith Lowney, PLLC, a law firm that practices public inter consumer and environmental law.

FOR MORE INFORMATION AND DOCUMENTATI TO: www.smithandlowney.com/salmon

##########

Is farmed salmon [illegible] for the environment

Diseases and Parasites

Living conditions are cramped inside salmon farms. These crowded conditions provide the perfect breeding ground for diseases and parasites. Naturally occurring diseases and parasites infect farmed salmon and can quickly spread throughout the farm. Since many salmon farms are located along wild salmon migration routes or near wild salmon rivers, these can also spread to wild salmon.

[illegible] Control

Every year, marine mammals, birds, and other fish are affected directly or indirectly by fish farms. Some animals such as seals and sea lions are shot, while acoustic deterrent devices may displace others, such as killer whales.

Loss of global supply of animal protein

It takes 3-4 kilograms of wild fish, such as herring and anchovy, to make the feed necessary to produce one kilogram of farmed salmon. The result is a net loss of edible animal protein worldwide. In Canada it is illegal to make fish feed out of fish otherwise suitable for human consumption, therefore, most of the feed for salmon in British Columbia is obtained from South America.

What has been done to address these problems

For many years, aboriginal groups, fishermen, and the conservation community have led efforts in British Columbia to develop a salmon farming industry that is safe for humans and the environment. However, industry has staunchly resisted change, putting human and ocean health at risk. Now, with industry planning to multiply the number of net-cage fish farms on the coast of British Columbia, Canada, groups working to promote safe farming have joined together to create the Coastal Alliance for Aquaculture Reform (CAAR). Working in partnership, we believe we can contribute to a healthy and sustainable coast.

Coastal Alliance for Aquaculture Reform

CAAR member groups are:

- BC Aboriginal Fisheries Commission (BCAFC)
- David Suzuki Foundation (DSF)
- Friends of Clayoquot Sound (FOCS)
- Georgia Strait Alliance (GSA)
- Living Oceans Society (LOS)
- Musqamagw Tsawataineuk Tribal Council (MTTC)
- Raincoast Conservation Society (RCS)
- Raincoast Research (RR)
- Society Promoting Environmental Conservation (SPEC)
- T'Buck Suzuki Environmental Foundation (TBSEF)
- Watershed Watch Salmon Society (WWSS)

what you can do

Don't eat farmed salmon until it is safe.

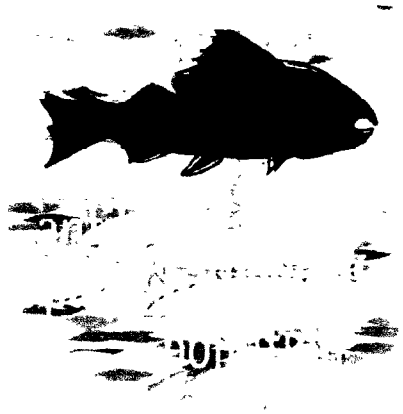
Safe for us.

Safe for the ocean.

Farmed salmon will be safe when the fish farming industry:

- uses technology that eliminates the risks of disease transfer and fish escapes
- guarantees waste is not released into the ocean
- labels their fish as "farmed" so consumers can make informed choices
- develops fish feed that doesn't deplete global fish stocks
- ensures that wildlife is not harmed as a result of fish farming
- prohibits the use of genetically modified fish
- eliminates the use of antibiotics in fish farming
- ensures contaminants in farmed fish don't exceed safe levels
- stops locating fish farms in areas opposed by aboriginal groups or other local communities

Farmed and Dangerous



Think twice about eating farmed salmon.

www.farmedanddangerous.org

Aerial view of a fish farm

Salmon aquaculture, also known as salmon "farming", is the industrial mass production of salmon. Farmed salmon are raised in net cages – floating feedlots. These are pens made of nets located directly in the ocean.

- Disease and parasites tend to multiply quickly in the net cages and because the pens are porous, can spread to wild fish outside farms.
- Antibiotics are added to the fish feed and pass along the food chain, contributing to the development of bacteria that are resistant to antibiotics.
- Fish farm waste is released directly into the ocean with no treatment, smothering marine life and passing contaminants into the ocean.

Nutrition

Increasingly, people are turning to salmon for a healthier diet. However much of the salmon available on the market comes from fish farms, not from the wild. Preliminary research shows that farmed salmon does not offer the same health benefits as wild salmon.

In a letter urging retailers to stop selling farmed salmon, Dr. Warren Bell, a medical doctor and president of the Canadian Association of Physicians for the Environment (CAPE) writes, "Not only is the fat content of farmed salmon higher than that of wild salmon but the composition of farmed salmon fat is also less healthy than that of wild salmon fat."

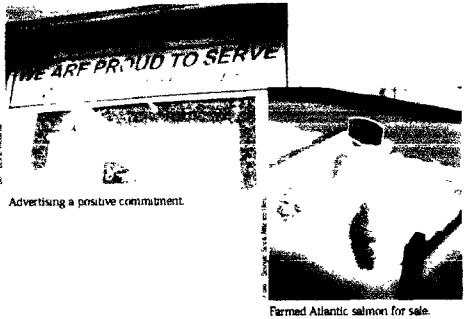
Antibiotics

Antibiotics are fed to farmed salmon to help fight disease outbreaks. When people eat farmed salmon, antibiotic residues can be passed on to them, increasing their risk of developing antibiotic-resistant bacteria and reducing the effectiveness of certain antibiotics for curing human illnesses.

Dr. Bell also writes that, "Another issue of concern to consumers is the fact that the monitoring of residues of antibiotics and other drugs in farmed salmon is inadequate."

monitoring of residues of antibiotics and other drugs in farmed salmon is inadequate

Dr. Warren Bell, Canadian Association of Physicians for the Environment



Advertising a positive commitment.

Farmed Atlantic salmon for sale.

PCBs

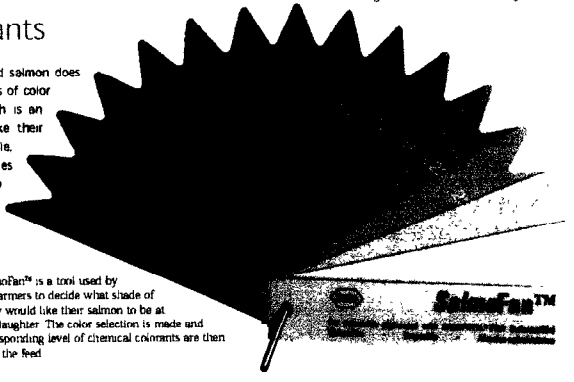
Preliminary findings suggest that farmed salmon contain higher levels of PCBs and dioxins than wild salmon. These chemicals can cause cancer, high blood pressure, strokes, immune-system problems and reproductive disorders. PCBs and dioxins can also affect normal development in children.

Farmed Atlantic salmon is 200 per cent higher in saturated fat than wild pink or chum salmon.

US Food and Drug Administration (www.fda.gov/)

Food Colorants

The food given to farmed salmon does not contain natural sources of color and as a result, their flesh is an unappetizing gray. To make their product more marketable, some fish farm companies add chemical colorants to their fish feed.



The SalmoFan™ is a tool used by salmon farmers to decide what shade of pink they would like their salmon to be at time of slaughter. The color selection is made and the corresponding level of chemical colorants are then added to the feed.

Are **you** eating farmed salmon

There are virtually no commercial Atlantic salmon fisheries in the world. So if you are eating atlantic salmon, it is farmed. Although most farms around the world raise Atlantic salmon, in British Columbia there are a few farms that raise chinook (king) and coho.

Retailers and restaurants often advertise fresh salmon, but this often means fresh from the farm, not the ocean.

To be sure, ask your restaurant or retailer if the salmon is farmed or wild.

alternatives

There is one salmon farm in BC that is addressing some of these issues by using a land-based system that eliminates escapes and reduces the risk of transfer of disease to wild salmon. This farm is working to improve its systems, and we hope to see it eliminate all harmful waste discharge and address concerns about fish feed. More pilot projects like this are needed to develop successful models for the industry.

Healthy alternatives to farmed salmon include sustainably caught wild salmon and wild halibut. Always make sure that you buy sustainably caught seafood. To learn more visit