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Impact of Different Dietary Phospholipid Levels on Cholesterol and Canthaxanthin Lipoprotein−Serum Transport and Muscle Deposition in Rainbow Trout

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The study was designed to assess the effect of a progressive increase of dietary phospholipid (PL) levels in the transport of cholesterol and canthaxanthin by serum lipoproteins and their deposition in trout muscle. Three groups of 30 immature rainbow trouts, in triplicate, with a mean body weight of 195 g were fed three experimental diets containing 0, 4, and 8% extra PL contents for 6 weeks. The two major lipoprotein classes in rainbow trout were HDL and LDL. Both lipoproteins were the main transporters of serum canthaxanthin, whereas cholesterol was transported principally by LDL. Serum cholesterol contents remained constant, whereas serum canthaxanthin was increased when the PL amount augmented. In muscle, PL seemed not to have an effect on cholesterol and canthaxanthin deposition. Therefore, as an extra-PL contribution in the diet did not increase relative percentages of cholesterol and/or canthaxanthin in trout muscle, the results support the hypothesis that dietary extra-PL addition is not necessary to increase cholesterol and canthaxanthin and thus fish flesh pigmentation. However, a saturation effect of diet PL contents was found on muscle canthaxanthin deposition.

KEYWORDS: Lipoproteins; phospholipids; cholesterol; canthaxanthin; muscle; rainbow trout

INTRODUCTION

The pink to red color of salmonid (*Salmo* spp., *Oncorhynchus* spp.) flesh is one of the distinguishing features of these fishes and makes a major contribution to their elite image. It is therefore of great economic importance that salmonid flesh is pigmented to meet consumer preferences (*1*). Salmonid muscle pigmentation is due to deposition of dietary carotenoids that fish cannot synthesize de novo (*2*). In the wild, carotenoids are obtained mainly from feeding on crustaceans. In intensive fish farms the use of nature identical synthetic canthaxanthin (*β*,*β*-carotene-4,4′-dione) is a source of carotenoid in the diet of farmed salmonids (*3*). Canthaxanthin is permitted in animal feed to color food of animal origin. Its utilization was revised at the European Union level for salmon, trout, laying hens, and other poultry (*4*).

In rainbow trout carotenoids are thought to be absorbed from the diet by passive diffusion into the intestinal mucosal cells, and their blood transport is associated with lipoproteins (*5*−*8*). In addition, carotenoids were found to be associated with serum albumin in the plasma of Atlantic salmon (*Salmo salar* L.) (*9*). Therefore, these results have never been confirmed in immature rainbow trout. However, large variations in the apparent digestibility of canthaxanthin have been reported, ranging from 4.8 to 71% (*5*, *10*−*12*). These variations were mainly attributed to carotenoid destruction in the intestinal tract. Canthaxanthin retention, defined as the proportion of the ingested pigment retained in the flesh of the fish, ranges from 2 to 7% (*11*−*15*), which is low when compared with the retention of other lipids, such as fatty acids (*16*).

Canthaxanthin with two polar groups at the end of the molecule is a typical amphiphilic compound analogous to phospholipids (PL) and, thereby, forms a stable monolayer. PL combine within them by interactions between the hydrophilic phosphate chain and the hydrophobic fatty acid chains. The amphipathic structure of PL, having both hydrophilic (sn-3 phosphate/headgroup) and hydrophobic (sn-1 and -2 fatty acids) regions, means they have the same central role in the structure of cell membrane bilayers in fish as they do in mammals (*17*, *18*).

PL metabolism is relatively poorly studied in fish. However, there is evidence that all of these pathways occur in fish and that the PL-derived mediators play similar roles in fish as they do in mammals: a) PL are digested by intestinal phospholipase A2, secreted by the pancreas, resulting in the formation of 1-acyl lyso-phospholipids and free fatty acids that are absorbed by the intestinal mucosal cells (*19*, *20*). b) The mechanisms of

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Table 1. Experimental Diet Composition (Amounts Expressed in Percentage)

ingredient	0% PL	4% PL	8% PL
fish meal	58.0	58.0	58.0
gelatinized corn starch	13.5	13.5	13.5
raw corn starch	24.0	20.0	16.0
soybean lecithin[a]	0.0	4.0	8.0
vitamin mix[b]	1.0	1.0	1.0
mineral mix[b]	1.0	1.0	1.0
sodium alginate	1.0	1.0	1.0
fish oil	1.5	1.5	1.5
canthaxanthin[c]	0.008	0.008	0.008
composition (%)			
proteins	40.6	40.6	40.6
lipids	7.3	11.3	15.3
phospholipids	2.5	6.3	10.5
cholesterol	0.3	0.3	0.3
carbohydrates	37.5	33.5	29.5

[a] DAFA LPR, Nickerson S.A., Marne-la-Vallée, France. [b] Choubert et al. (*55*). [c] Rhône Poulenc Animal Nutrition, Commentry, France.

absorption of the products of PL digestion, 1-acyl lysophospholipids and free fatty acids, will be associated with all of the other products of fat digestion in mixed micelles with bile salts, which diffuse to the intestinal mucosa where uptake into the enterocytes occurs, probably mainly by passive diffusion (*20*). c) PL are transported in the blood of fish as lipoproteins as they are in mammals (*17*).

Cholesterol is also an amphipathic compound integral in membranes. The hydrophobic moiety of cholesterol includes the steroid nucleus and an alkyl side chain attached at C_{17}. A hydroxyl group attached at C_3 of the steroid nucleus represents the hydrophilic moiety. Membranes are stabilized by hydrophobic interactions between fatty acyl chains of PL and the steroid nucleus of sterols on the interior of the bilayer, whereas the polar headgroup hydrogen bonds with water (*21*). Amphipathic properties of PL, sterols, and bile salts are important in the intestinal lumen for the formation of mixed micelles and liposomes during emulsification and digestion of lipids and lipid-soluble compounds such as canthaxanthin (*22*).

There is the hypothesis that large doses of dietary PL inhibit the intestinal absorption of cholesterol in both humans and rats and stimulate the capacity of the organism to eliminate cholesterol (*23, 24*). The purpose of this experiment was to investigate whether extra-dietary PL addition (4 and 8%) may modify cholesterol and canthaxanthin blood concentration and therefore flesh pigmentation in rainbow trout, after an administration period of 6 weeks (usual pigmentation delay for trout of 200 g).

MATERIALS AND METHODS

Fishes and Diets. Rainbow trout (*Oncorhynchus mykiss*, Walbaum) with a mean body weight of 195 g were settled in nine tanks (1 m diameter, 30 fish/tank), set in parallel. Tanks were gravity fed with spring water (temperature = 17 ± 1 °C; O_2 = 8−9 mg L^{-1}; pH = 7.4; Cl^- = 22.5 mg L^{-1}; Ca^{2+} = 75 mg L^{-1}) at a rate of five volume changes per hour.

Compositions of the experimental diets are given in **Table 1**. PL (soybean lecithin, DAFA LPR, Nikerson S.A., Marne-la-Vallée, France) were added to the diet as an extra-addition of 0 (control), 4, and 8%. Cholesterol amount was constant for each diet (0.3%). The diets were supplemented with 80 mg of canthaxanthin (Rhône Poulenc Animal Nutrition, Commentry, France) per kilogram of diet, which is the maximum amount permitted to be supplied to fish in the European Union. Diets were pelleted using a steamless pelleting machine (M-Labor, Simon Heesen B.V., Boxtel, The Netherlands) through a 4.5 mm dye. Fishes were hand fed ad libitum two meals per day during 6 weeks.

Table 2. Fatty Acid Composition of Dietary Fish Oil (Percentage by Weight of Total Fatty Acids)[a]

fatty acid	%
12:0	0.2
14:0	15.4
14:1	0.2
15:0	0.7
16:0	19.7
16:1	9.4
16:2n-7	0.4
16:2n-4	1.0
16:4n-1	0.3
17:0	0.2
17:1	0.3
18:0	1.3
18:1	16.1
18:2n-6	1.8
18:3	0.3
18:3n-3	1.1
18:4	3.1
20:0	0.1
20:1	8.1
20:2	0.1
20:4	0.2
20:5	4.7
22:1	7.4
22:5	0.2
22:6n-3	3.5
SFA[b]	37.6
MUFA[c]	41.5
PUFAn-6[d]	4.1
PUFAn-3	13.2
SFA/PUFA	2.2
PUFAn-3/n-6	3.2

[a] Mean of duplicate samples. [b] Saturated fatty acids. [c] Monounsaturated fatty acids. [d] Polyunsaturated fatty acids.

Fish oil composition of the experimental diets is given in **Table 2**. Fatty acid methyl esters were prepared by acid-catalyzed transmethylation of total lipids using boron trifluoride methanol according to the method of Santha and Ackman (*25*). The chromatograph (model 3400, Varian Inc., Palo Alto, CA) was equipped with a DB-Wax fused silica capillary column, 30 m × 0.25 mm i.d., film thickness = 0.25 μm (J&W Scientific, Folsom, CA). Helium was used as carrier gas (1.4 mL min^{-1}), and the thermal gradient was from 100 to 180 at 8 °C min^{-1} and from 180 to 220 at 4 °C min^{-1} at a constant temperature of 220 °C during 25 min. Injector and flame ionization detector temperatures were 260 and 250 °C, respectively. Fatty acid methyl esters were identified and quantified by comparison with known standard mixtures (189-19, Sigma-Aldrich Co., Saint Quentin Fallavier, France).

Lipoprotein Isolation and Characterization. Lipoprotein isolation and characterization were performed at the end of the trial. Blood samples (3 mL of blood per fish) were withdrawn from the caudal vein with nonheparinized syringes and maintained during 24 h at 4 °C to clot. All samples were then centrifuged (model 5702, Eppendorf AG, Hamburg, Germany) at 16000*g* for 10 min. Serum samples were pooled to isolate the lipoproteins. Serum lipoproteins were separated by density-gradient ultracentrifugation according to the procedure described by Salvador et al. (*26*).

The amount of total cholesterol in each lipoprotein was determined using a cholesterol enzymatic kit (Merckotest, Merck, Darmstadt, Germany) according to the CHOD-iodide method (*14*). Triglycerides were estimated by an enzyme kit (Triglyceride UV, Sigma Diagnostics, St. Louis, MO) according to the method of Bucolo and David (*27*). Phospholipid phosphorus was analyzed according to the method of Böttcher et al. (*28*). Protein was measured following the Lowry et al. method (*29*) using bovine serum albumin (1 g L^{-1}) as standard.

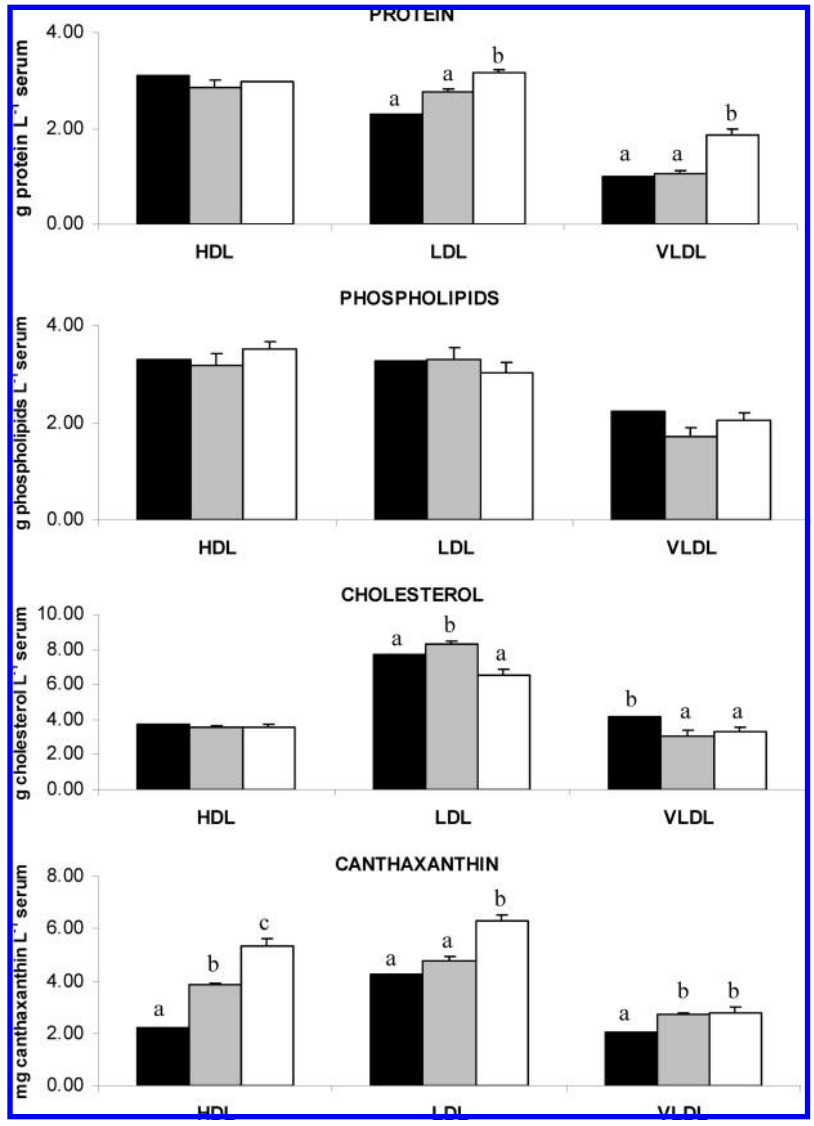

Figure 1. Protein, phospholipid, cholesterol, and canthaxanthin concentrations in the lipoprotein fraction of serum of trout fed the different experimental diets: (black bars) 0% PL; (gray bars) 4% PL; (white bars) 8% PL, after 6 weeks. Horizontal bars with different letters represent significant values ($p \leq 0.05$).

Muscle Lipid and Canthaxanthin Extraction and Determination. After blood collection, rainbow trout were sacrificed by a sharp blow to the head, and the latero-dorsal muscle was taken off. Muscle total lipids were extracted according to the method of Folch et al. (*30*).

Muscle total cholesterol was assayed according to the method of Martensson (*31*) using cholesterol (1 g L^{-1}) in chloroform as standard and acetic anhydride and sulfuric acid (10:1, v/v) as reaction mixture. Absorption was measured with a spectrophotometer (Uvikon 941 Plus, Kontron Instruments, Schlieren, Switzerland) at 620 nm after a stabilizing period of 10 min.

Canthaxanthin extraction from lipoproteins and from muscle was carried out according to the procedure described by Chávez et al. (*32*). Canthaxanthin absorbance was measured with a spectrophotometer (Uvikon 941 Plus, Kontron Instruments), and canthaxanthin concentrations were calculated by assuming a $E_{1cm}^{1\%}$ value in hexane at 474 nm of 2250 (*33*).

Statistical Analysis. To compare the three groups, statistics have been gathered according to one-way analysis of variance (ANOVA) and Tukey′s method comparison test using the SAS-GLM procedure (*34*). $p \leq 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

The effect of the progressive increase of dietary PL on cholesterol and canthaxanthin lipoprotein levels, serum transport, and muscle deposition in rainbow trout at usual pigmentation delay for a trout of 200 g (6 weeks) has been studied. PL contents were defined 4 and 8 times higher than those used in the previous experiment (*26*), resulting in final dietary PL contents of 2.5, 6.3, and 10.5%, respectively.

Lipoproteins. Lipoproteins were identified and classified according to density intervals applied to human serum (*35*, *36*). The composition of the three lipoprotein bands observed after ultracentrifugation is given in **Figure 1**. In trout serum, when PL contents increased, serum circulating protein ($p \leq 0.05$) and canthaxanthin ($p \leq 0.05$) amounts also were increased. PL and cholesterol levels were not affected when dietary PL contents increased. Serum proteins were distributed almost equally (about 40% for each fraction) between LDL and HDL ($p \leq 0.05$) and the remainder (around 20%) for VLDL. The PL transport was shared between HDL and LDL ($p \leq 0.05$), independent of the given diet, and all serum lipoprotein PL contents remained constant independent of the given diet. After 6 weeks, most of the cholesterol was transported by serum LDL ($p \leq 0.05$). The HDL cholesterol amount remained constant independent of the given diet. LDL and VLDL cholesterol amounts varied: LDL cholesterol was higher at 4% extra PL contents ($p \leq 0.05$) than

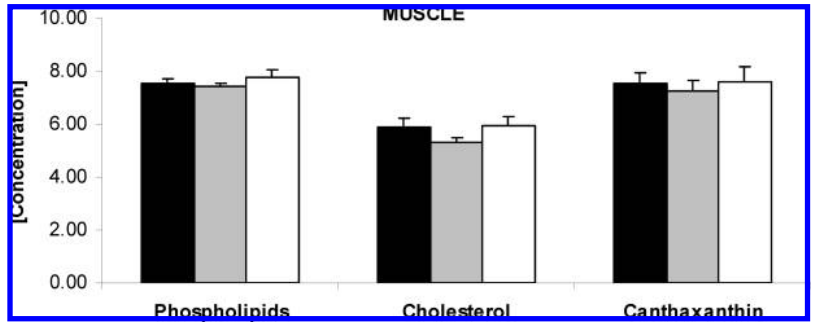

Figure 2. Phospholipid (mg of phospholipids g^{-1} of muscle), cholesterol (mg of cholesterol g^{-1} of muscle), and canthaxanthin (μg of canthaxanthin g^{-1} of muscle) concentrations in trout muscle fed the different experimental diets: (black bars) 0% PL; (gray bars) 4% PL; (white bars) 8% PL, after 6 weeks.

in the other diets, whereas VLDL cholesterol was diminished when dietary extra PL contents were augmented ($p \leq 0.05$). In humans, VLDL has the highest lipid fraction (90%) and the biggest size, followed by LDL with 78% of lipid, and, finally, HDL is the smallest in size with a lipid contents of 48% (*37*). A similar composition appeared in trout after 6 weeks of experiment: VLDL and LDL contained 89.5 and 74.3% lipids, respectively. However, HDL contained the lowest amount of lipids (66.5%). These data are in accordance with those obtained by Chávez et al. (*32*), who reported that trout serum lipoprotein lipid contents were 80.3% for VLDL, 69.8% for LDL, and 60.4% for HDL (**Figure 2**).

Studies on plasma lipoproteins during exogenous lecithin absorption in man have suggested that intraduodenal infusion of lecithin reduced dietary cholesterol absorption efficiency and that the predominant products of lecithin absorption are particles in the range of VLDL (*23*). The enhancement of lipoprotein synthesis by dietary soybean phosphatidylcholine may be related to its predominance in fish lipoproteins as compared with other PL (95% of total PL in lipoprotein) and to a possible stimulation of the apoprotein secretion as reported in mammals (*38*). Phosphatidylcholine appears to be an essential nutrient in diets fed to juvenile rainbow trout and Atlantic salmon (*39*−*41*).

After 6 weeks of experiment, plasma total cholesterol decreased. It may be designated as an increase in lipid processing such as cholesterol transport to the liver for its elimination because muscle cholesterol levels were not altered by the diet (*42*). On the other hand, both lecithin and cholesterol are natural substrates of plasma lecithin-cholesterol acyltranferase (LCAT). The reverse transport of cholesterol by HDL to the liver is intimately connected with the actions of plasma converting cholesterol and phosphatidylcholines (lecithins) of HDL into cholesteryl esters and lysophosphatidylcholines by a transesterification reaction (*43*). Therefore, PL (lecithin) induced a reduction in plasma cholesterol, possibly through increased formation of HDL particles, which were predominant in our study.

Canthaxanthin was bound to the three classes of lipoproteins present in the serum, and it is mainly transported by LDL independent of dietary PL amount ($p \leq 0.05$). This is in accordance with the idea that LDL is the lipoprotein carrying carotenoids to most tissues in humans (*7*). The highest concentration of serum canthaxanthin was observed for fish fed 8% extra dietary PL contents (**Figure 1**). The capacity of each lipoprotein for binding canthaxanthin depends on the proportion of the different components of the particle. If the surface components are considered, PL and free cholesterol, these two polar components may lead and limit the addition of canthaxanthin into the lipoprotein (*32*). There was a relationship between the amount of canthaxanthin and the amount of PL and cholesterol in each lipoprotein. A high amount of PL may enhance canthaxanthin lipoprotein binding. Their amphiphilic nature may stabilize the lipoprotein for incorporation of the carotenoid. The polar molecule canthaxanthin may be incorporated into the lipoprotein external PL monolayer, with interactions between the polar head groups, the polyenic chain of PL, and carotenoids. The incorporation of carotenoid into PL is saturable. Chávez et al. (*32*) described canthaxanthin saturation of serum lipoproteins of immature rainbow trout when one molecule of canthaxanthin is stabilized by approximately 15 molecules of PL, independent of the lipoprotein class and its relative chemical composition.

Muscle. In our study, muscle cholesterol and PL levels were not altered by dietary PL supplementation at 6 weeks of experiment. These data were in agreement with Sealey et al.'s (*44*) study concluding that dietary supplementation of lecithin (0, 2, 4, and 6%) had no substantial beneficial effect on body PL and cholesterol composition of juvenile hybrid striped bass (*Morone chrysops* × *M. saxatilis*) at 8 weeks of experiment. Carotenoid pigmentation is affected by dietary pigment source, dosage level, duration of the feeding, and dietary composition (*45*). No effects on pigmentation by proteins and carbohydrates have been reported, whereas lipids appeared to affect pigmentation appreciably (*46*). Canthaxanthin is commonly used in intensive fish farming to enhance the natural color of the flesh of rainbow trout. This lipid-soluble compound is not synthesized by the fish and must be supplied with the diet by utilizing commercial preparations of this pigment in the manufacture of pelleted feeds. Countries where canthaxanthin has been the sole pigment source for salmonids do not report incidences of canthaxanthin-related pathologies (*47*). In the European Union, canthaxanthin is currently authorized for use as a coloring agent in complete feedingstuffs (*4*).

There were no significant ($p \geq 0.05$) differences in canthaxanthin depositon among the three dietary groups. Although an increase of serum canthaxanthin amount is appraised (highest value for 8% PL at 6 weeks), this did not lead to an increase of muscle canthaxanthin deposition. In a previous work (*26*), after 12 days of experimentation, a significant increase of muscle canthaxanthin amount was obtained, using a diet with canthaxanthin alone and others with cathaxanthin plus fish oil and 1.6% of PL (2.77 ± 0.37 and 4.33 ± 0.39 μg/g of muscle, respectively). In this experiment, with a 2.5% of PL diet content, canthaxanthin muscle composition was approximately twice (7.56 ± 0.41 μg/g of muscle) the previous one, and no effect was obtained in the other ones before increasing diet PL percentages 4 and 8 times, respectively. Therefore, a saturation effect of PL on canthaxanthin muscle deposition was found, reaching a plateau when PL contents increased (7.23 ± 0.45

μg/g of muscle for the diet with 4% extra-PL contents and 7.57 $\pm$ 0.59 μg/g of muscle for the diet with 8% extra-PL contents).

The limitation of carotenoid utilization for muscle pigmentation in salmonids may be affected by (1) the rate of metabolic transformation (*48*) (2)) clearance from serum (*49*) and (3) the uptake mechanisms at the cell membrane in the muscle cells as suggested by Ytrestøyl et al. (*50*). In vitro carotenoid saturation of the muscle has been estimated at 100 mg kg^{-1} (*51*), greatly exceeding the levels found in vivo [up to 10 mg mg kg^{-1}; (*50*)]. However, Chávez et al. (*32*) obtained a 100-fold increase in canthaxanthin concentration in lipoproteins after in vitro incubation. Thus, it appears that blood transport does not constitute a limitation to carotenoid supply to various tissues and organs in vivo.

There is a relative paucity of information concerning the effect of PL on flesh pigmentation in fish. However, Abat et al. (*52*) noted a slight effect of a dietary 8% PL level to enhance canthaxanthin concentration in trout flesh during the two first weeks of treatment in terms of color. Some studies in broiler pigmentation showed that increasing dietary fatty acid composition (with increasing soybean PL in the diet) enhanced xanthophyll (xanthophylls mix, without description by the authors) absorption and deposition in the skin (*53*, *54*).

Conclusions. The data obtained from the present work showed that in nonmature rainbow trout high dietary PL (up to 10%) has an effect on cholesterol and canthaxanthin transport but not on trout muscle deposition after 6 weeks of experiment. Therefore, as an extra-PL contribution in the diet did not increase relative percentages of cholesterol and/or canthaxanthin in trout muscle, our results lead to the suggestion that dietary extra-PL addition is not necessary to increase cholesterol and canthaxanthin and then fish flesh pigmentation. In the case of muscle canthaxanthin deposition, a saturation effect of diet PL contents was found by comparison with our previous work.

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