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Investigations on the Effects of Growth Rate and Dietary Vitamin C on Skeletal Muscle Collagen and Hydroxylysyl Pyridinoline Cross-Link Concentration in Farmed Atlantic Salmon (*Salmo salar*)

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We have investigated the interactions between dietary vitamin C levels (at 33, 79, 135, and 424 mg kg^{-1} of wet mass feed) and growth rate on the collagen and cross-link contents of fast muscle in farmed juvenile Atlantic salmon (*Salmo salar* L.). The growth rate was measured over an 11 week period using the thermal growth coefficient (TGC). Alkaline-soluble (0.1 M NaOH) (a-s) hydroxyproline (HYP) and alkaline-insoluble (i-s) HYP were determined as a measure of collagen content and hydroxylysyl pyridinoline (PYD) as a measure of mature collagen cross-link concentration. There was a ~5-fold increase in muscle vitamin C concentration at similar feed conversion ratios (~0.82) as dietary vitamin C levels increased from 39 to 424 mg kg^{-1} of wet mass feed. However, even the lowest dietary vitamin C was sufficient for normal skeletal development and growth. The lowest dietary vitamin C level tested resulted in a ~27% decrease in the a-sHYP concentration relative to the other diets, whereas there was no significant effect of vitamin C on the i-sHYP and PYD concentrations. ANOVA revealed no significant interaction between vitamin C and growth rate, whereas the covariate TGC was significant for i-sHYP and PYD but not for a-sHYP. Pyridinoline cross-link and i-s HYP concentrations were 11.1 and 7.7% lower, respectively, in high (TGC > 3.9) mass than low (TGC < 3.9) growth rate fish. These small differences in collagen cross-linking were associated with a 15.6% decrease in fillet firmness measured with an instrumental texture analyzer. It was concluded that for healthy juvenile salmon reared under controlled growth conditions, the dietary vitamin C inclusion of 79 mg kg^{-1} of wet mass feed was sufficient to produce the required synthesis of soluble muscle collagen. Furthermore, post-translational modifications of the collagen leading to cross-linking showed a small decrease with increasing growth rate but was independent of vitamin C concentration in the diet at these levels.

KEYWORDS: Vitamin C; collagen; hydroxyproline; cross-links; fish muscle; growth; farmed Atlantic salmon; texture; flesh quality

1. INTRODUCTION

Collagen is one of the two major components of the extracellular matrix, and it has a significant influence on the texture of raw fish (*1*, *2*). All collagen molecules comprise three α-polypeptide chains wound together to form a left-hand triple helix and contain at least one domain composed of repeating Gly−X−Y sequences per chain, which are arranged in a tight triple helix. Many of the X and Y positions are occupied by two uncommon residues: proline (X) and hydroxyproline (Y), which fit perfectly inside the helix (*3*−*5*). Hydroxyproline (HYP), which is critical for the stability of the triple helix, is created by modifying normal proline after the collagen chain is built (*6*). The assay of HYP forms the basis for the determination of collagen content (*7*).

The hydroxylation of proline catalyzed by prolyl-4-hydroxylase requires vitamin C, a reducing agent, which is necessary to maintain the enzyme prolyl hydroxylase in an active form, most likely by keeping its iron atom in a reduced state (*8*). Human vitamin C deficiency slows the production of hydroxy

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proline and stops the construction of new collagen, causing scurvy (*9*) and skeletal deformities such as scoliosis or lordosis (*10*). Most animals, all higher plant species, and probably all algal classes can synthesize vitamin C from glucose or other sugars. Fish, however, are not able to synthesize vitamin C as they lack the enzyme L-gulano-γ-lactone oxidase, the final step in the synthesis of ascorbic acid from glucose (*11*). Vitamin C is an essential nutrient for salmon, rainbow trout, carp, and other fish and must be supplied in the diet (*12*−*15*). In muscle cells, vitamin C has many roles in a wide variety of biochemical pathways affecting growth reproduction, immune function, and the response to stress (*16*). It is a common cofactor of many hydroxylating enzymes and a strong reducing agent providing protection against oxidative stress (*17*) and is required for ϵ-*N*-trimethyl lysine hydroxylase activity for carnitine biosynthesis, by maintaining the prosthetic metal Fe^{2+} in the reduced form (*18*).

The mechanical strength of the connective tissue is provided by reducible and non-reducible (mature) cross-links between adjacent collagen and elastin molecules. For example, the strength of collagen depends on covalent cross-links between the helical or the non-helical ends of the molecule, which occur in an end-to-end fashion and connect the rigid regions of the molecule (*19*). There is only a single enzyme, lysyl oxidase, required for the initiation of cross-link formation. In addition to affecting the synthesis of HYP, vitamin C maybe indirectly involved in the cross-linking of collagen molecules by affecting the formation of hydroxylysine, which is one substrate of lysyl oxidase. It has been suggested that the hydroxylation of lysine in collagen is related to vitamin C via the action of lysyl-4-lydroxylase (*20*). The resulting hydroxylysine residues, through a series of reactions, form the stable cross-link deoxypyridinoline with two lysine residues or form pyridinoline with one lysine residue and another hydroxylysine residue (*21*). Therefore, different levels of dietary vitamin C could potentially change the ratio of different collagen cross-links, deoxypyridinoline and pyridinoline, as was observed in the bone and urine of guinea pigs (*22*).

Previously, we have shown that hydroxylysyl pyridinoline cross-link concentration is positively correlated with fillet firmness in fresh and smoked Atlantic salmon (*23*). The trend in salmon farming has been to reduce production time through improvements in growth rate involving the development of high-energy diets. However, the influences of growth rate on factors that affect raw material properties and flesh quality have been relatively little studied. According to the National Research Council (*24*) of the United States, the minimal vitamin C required by rainbow trout and Pacific salmon is 50 mg kg^{-1} of dry mass feed, which was determined with highly purified ingredients of nearly 100% bioavailability. Growth rate could potentially affect the turnover of water-soluble vitamins such as vitamin C. We therefore tested the hypothesis that the effects of dietary levels of vitamin C on the hydroxyproline and pyridinoline content of salmon flesh would vary with individual growth rate.

2. MATERIALS AND METHODS

1. Fish Husbandry, Harvest, and Carcass Processing. Atlantic salmon (*Salmo salar* L.) (Salmobreed strain) were transferred to seawater following smoltification on September 2, 2003 at Sjøtroll Havbruk Avd, Flatråker, Norway. Fish were transferred to the Fiskeriforskning feed trial center, Austevoll, Norway to investigate the effect of different levels of dietary vitamin C on skeletal muscle collagen biochemistry. Fish of about 160 g (19% coefficient of variation) were identified with a PIT-tag (Fish-Eagle Ltd., Gloucestershire, England) and divided between eight tanks (1000 L, 49 post-smolts per tank). Fish were acclimatized for 2 weeks prior to the start of the trial during which time they were fed a basal diet. The feeding trial lasted 80 days under similar environmental conditions at a mean water temperature of 10.6 °C and continuous light. Each diet (**Table 1**) (containing 33, 79, 135, or 424 mg of vitamin C kg^{-1}) was fed to two tanks for an 11 week period. To facilitate accurate calculations of feed utilization, feed waste was collected using a lift-up system, and the wasted pellets were counted and registered. The feeding succession among the tanks was varied to avoid any systematic differences among treatments. At trial termination, the fish were sacrificed at approximately 670 g. Immediately after death, 0.5 g of fast (white) muscle samples for vitamin C analysis was excised from the dorsal epaxial myotomes anterior to the dorsal fin, and a 0.5% (v/v) meta-phosphoric acid extract (1:10 m/v tissue/acid) was prepared and then frozen. The eviscerated fish were transported on ice to St. Andrews within 3 days. Samples of fast muscle from the dorsal epaxial myotomes at the level of the dorsal fin were dissected 3 days post-rigor and stored in sealed plastic bags at −80 °C until analysis of hydroxyproline and hydroxylysyl pyridinoline and vitamin C concentration.

Table 1. Feed Components, Vitamin C Content, Proximate Compositions in Experimental Diets, and Collagen and Cross-Link Contents

diets (g/100 g of diet)	1	2	3	4
Fishmeal	49.5	49.5	49.5	49.5
Wheat	12.5	12.5	12.5	12.5
corn gluten	8.0	8.0	8.0	8.0
wheat gluten	8.0	8.0	8.0	8.0
fish oil	19.0	19.0	19.0	19.0
Lysine	0.4	0.4	0.4	0.4
vitamin and trace element premix (no vitamin C)	1.35	1.35	1.35	1.35
vitamin C (target) (mg kg^{-1} of wet mass diet)	20	75	150	500
vitamin C (analyzed) (mg kg^{-1} of wet mass diet)	33	79	135	424
protein (%)	48.6	47.7	49.4	48.2
oil (%)	26.4	25.5	26.2	27.3
ash (%)	7.7	7.9	8.0	7.7
moisture (%)	4.8	6.2	5.5	5.0

2. Diet Formulation. Four commercial-extruded 4.5 mm diets were formulated (BioMar Ltd., Grangemouth, UK) and manufactured (BioMar A/S, Brande, Denmark) to provide 48% crude protein and 26% lipid and differed only in their content of vitamin C (STAY-C35) through the addition of individual premixes supplied by DSM Nutritional Products Ltd., Heanor, Derbyshire, UK. The other premix constituents were identical for each diet. The diets were designed to satisfy the nutritional requirements of salmonid fish (NRC). The proximate compositions of the experimental diets are shown in **Table 1**.

3. Calculation of Individual Growth Rate and Feed Utilization. Thermal growth coefficient (TGC) (*25*) was calculated according to the formula $TGC = [(M_2^{0.333} - M_1^{0.333}) (\text{degree days})^{-1} \times 1000]$, where M_1 and M_2 were the initial and final body weights for each period, respectively. Degree days is the accumulative sum of the average daily temperature readings. Feed utilization was based on the amount of feed consumed during the trial with the increase in biomass, taking into account the feed waste and biomass lost through sampling and mortalities. The feed conversion ratio (FCR) was calculated as FCR = feed eaten (kg)/biomass gain (kg). The condition factor (CF) was calculated as $CF = \text{body mass (g)}/(\text{fork length (cm)})^3 \times 100$.

4. X-ray Analysis of Potential Skeletal Deformities. Following the resolution of rigor, 25 fish from each tank were selected at random and X-rayed using a Portable Direct Digital Radiography (DR) System kindly provided by Xograph Imaging Systems Ltd., Tedbury, Gloucestershire, UK. The instrument settings were 50 Kvp, 2 MAS at a distance of 100 cm, using a Canon CXDI-31, direct digital plate. The digital X-ray images were analyzed for vertebral, spinal malformations, and neck disorders using eFilmLite software (version 1.8.3 (Build 8) CDFIX, eFilm Medical Inc, Toronto, ON).

5. Measurement of Fillet Firmness. The flesh quality attribute of texture was measured as fillet firmness. Briefly, the firmness was

evaluated by a compression test using a TA-HDi Texture Analyzer controlled with Texture Expert Exceed 2.52 software from Stable Micro Systems, Surrey, UK. To ensure that the flesh temperature was kept constant during testing, each fish was kept on ice until the flesh samples were excised and the temperature of the muscle tissue was measured for individual fish chosen at random. Because of the small size of the fish, a compression test was selected to be the most appropriate method. The test was performed in duplicate for each fish using two 2.5 cm × 2.5 cm × 1.5 cm blocks of fast muscle excised from the epaxial myotomes below the dorsal fin. The probe used was a 150 mm diameter platen, with a 100 kg load cell, and the compression was to 60% of sample thickness at 1 mm s^{-1} representing unrecoverable deformation. Each texture profile was analyzed using Texture Expert Exceed 2.52 software, and fillet firmness was determined as the maximum compression force, measured in Newtons, as the highest peak of the curve, representing the maximum resistance of the sample to compression.

6. Hydroxyproline and Pyridinoline Assay. Collagen contents were assayed by determining the hydroxyproline (HYP) concentration with HPLC after complete hydrolysis by 6 M HCl. The total collagen was fractioned into two components following extraction with cold 0.1 M NaOH: alkaline-soluble collagen, which can be extracted by 0.1 M cold NaOH, and alkaline insoluble collagen, which contains most of the cross-links (*23*). The analysis of hydroxyproline and hydroxylysyl pyridinoline was based on an HPLC method as described previously (*23*). Briefly, a 2 μL aliquot of hydrolysate was dried, and the pellet was resuspended in 200 μL of 0.1 M boric buffer, pH 11.4 containing 11.2 μM homoarginine as an internal standard. The fluorenylmethoxycarbonyl (FMOC) derivatized amino acids were separated using a ternary gradient described by Bank et al. (*26*). The peak for hydroxyproline was quantified with reference to a calibration curve prepared with collagen hydrolase (*23*). For the hydroxylysyl pyridinoline (PYD) assay, duplicate samples of 1 g of fast muscle were homogenized in cold water (4 °C) for 1 min at 30 000 rpm using a Polytron PT2100 (Kinematica AG, Switzerland). Ten milliliters of ice cold 0.2 M NaOH was added immediately, and the sample was mixed on a wheel roller at 4 °C for 4 h. After centrifugation, the pellet containing the alkaline-insoluble collagen was hydrolyzed at 110 °C for 20 h, dried, and extracted using cellulose CF1 columns (Varian) based on published methods (*27*, *28*). The PYD eluted was loaded onto ProStar HPLC system (4.6 mm × 150 mm; Micropak ODS-80TM column, Varian, Surrey, UK). The column was eluted using a binary solvent system. Solvent A was 5% (v/v) CH_3CN and 0.1% (v/v) heptafluorobutyric acid (HFBA), and solvent B was 95% (v/v) CH_3CN and 0.1% (v/v) HFBA. The gradient and flow rate was as follows (v/v): 0−15 min, 100% A, 1 mL min^{-1}; 15−30 min, 85% A, 15% B, 1 mL min^{-1}; 30−31 min, 20% A, 80% B, 1.4 mL min^{-1}; 31−40 min, 20% A, 80% B, 1.4 mL min^{-1}; 40−41 min, 100% A, 1.4 mL min^{-1}; and 41−49 min, 100% A, 1.4 mL min^{-1}. Fluorescence of the eluted peaks was monitored at λ_{ex} = 295 nm and λ_{em} = 400 nm. The pyridinoline peak was quantified with respect to a calibration curve prepared with purified pyridinoline (kindly supplied by Simon Robbins, Rowett Institute, Aberdeen, Scotland).

7. Determination of Vitamin C in Fast Muscle. The fast muscle tissue samples, stabilized in metaphosphoric acid and frozen, were analyzed for total ascorbic acid at the Analytical Research Center, DSM Nutritional Products Ltd., Basel, Switzerland using a C_{18} RP-HPLC internal method (*34*). Briefly, at the time of analysis, the fast muscle samples were thawed and centrifuged at 2000*g* for 10 min at 4 °C. A total of 500 μL of the supernatant was reduced with 500 μL of 0.13 M dithiothreitol-EDTA-NaAc buffer at pH 4.8, then filtered (0.45 μm, Millipore) prior to injection of 10 μL of filtrate in a Jasco HPLC system (Jasco, Cambridge, UK). The column (4.6 mm × 150 mm; YMC−-ODS-AQ, 120 Å, S-3 μm column, YMC Europe GMBH, Schermbeck, Germany) was eluted using a binary solvent system. Solvent A was 3% (v/v) MeOH, 8 mM sodium acetate, and 0.15% (v/v) 1,5-dimethylhexylamine at pH 4.8, and solvent B was 70% (v/v) MeOH, 8 mM NaAc, and 0.15% (v/v) 1,5-dimethylhexylamine at pH 4.8. The gradient was as follows (v/v): 0−5 min, 80% A, 20% B; 5−15 min, 0% A, 100% B; 15−20 min, 0% A, 100% B; 20−21 min, 80% A, 20% B; and 21−30 min, 80% A, 20% B. The flow rate was 0.6 mL min^{-1}, the column oven was set to 28 °C, and the eluted peaks were monitored by absorbance at 254 nm. Multichrom software (VG Laboratory Systems, Cheshire, UK) was used to identify and quantify the peaks with a calibration curve prepared previously with an ascorbic acid standard (F. Hoffmann, La Roche AG, Switzerland).

8. Statistics. The data were analyzed by a General Linear Model analysis of variance (ANOVA) by the method of sequential sums of squares with the tank nested within dietary vitamin C treatment as a fixed factor, TGC as a covariate, and a vitamin C × growth rate interaction term. Post-hoc testing was by Tukey's multiple comparison tests. Plots of residuals versus fitted values, the normal probability of residuals, and histograms of residuals were examined to ensure that the data fulfilled the assumptions of ANOVA.

Table 2. Growth Parameters and Biometrics[a]

biometrics	1	2	3	4
start weight (g)	164 ± 3	161 ± 3	161 ± 3	161 ± 3
end weight (g)	679 ± 14	689 ± 3	674 ± 14	654 ± 14
growth rate (TGC)	3.90 ± 0.04	3.97 ± 0.05	3.90 ± 0.05	3.79 ± 0.05
TGC range	2.83–5.28	2.11–5.18	1.83–5.27	1.35–4.91
FCR	0.81 ± 0.01	0.82 ± 0.01	0.82 ± 0.01	0.82 ± 0.01
condition factor	1.47 ± 0.01	1.44 ± 0.01	1.44 ± 0.01	1.40 ± 0.01
fillet firmness (*N*)	15.9 ± 0.5	16.2 ± 0.52	17.1 ± 0.7	18.3 ± 0.9

[a] The values represent mean ± SE; n = 94–98 per treatment for biometric data and n = 51 for measurements of fillet firmness.

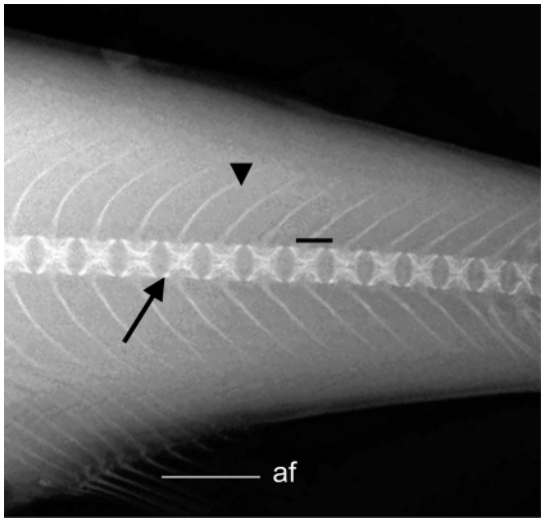

Figure 1. Digital X-ray of Atlantic salmon fed 33 mg of vitamin C kg^{-1} of wet mass feed. No deformation was found in the vertebrae (the line represents a single vertebrate). The arrowhead indicates a vertebral spine and the arrow an inter-vertebral disc. af: Anal fin.

3. RESULTS AND DISCUSSION

There was no significant difference in growth rate, feed conversion ratio (FCR), or condition factor between vitamin C treatments (**Table 2**), and none showed evidence of skeletal abnormalities (**Figure 1**). However, increasing the dietary vitamin C from 39 to 424 kg^{-1} of feed did result in an approximately 5-fold increase in muscle vitamin C from ∼4 to 20 mg kg^{-1} of wet mass (P < 0.001). The muscle vitamin C concentration in relation to the total vitamin C consumed per increase in biomass is shown in **Figure 2**, which illustrates that most of the increase in muscle vitamin C occurred on increasing the dietary vitamin C inclusion from 39 to 79 mg kg^{-1} of wet mass feed. Vitamin C has many important biological roles, and the concentration required for each of its functions is known to vary (*30*). In fish, it is involved in collagen synthesis, growth, wound healing, immune response, disease resistance, and many other functions (*14*, *31*, *32*). Lin (*30*) reported that 45.3 mg

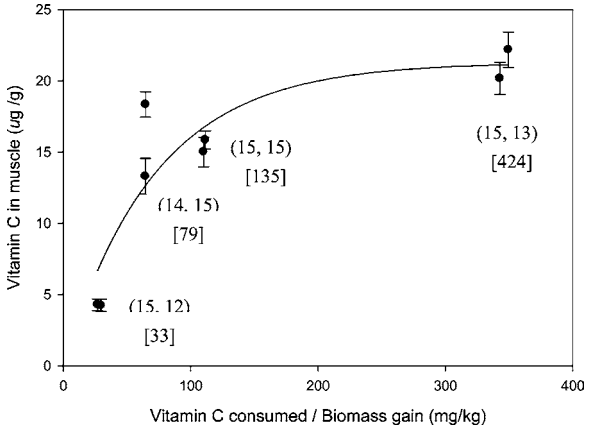

Figure 2. Relationship between muscle collagen content mg g^{-1} of wet mass muscle and amount of collagen (mg) consumed per net increase in biomass (g) over the trial for Atlantic salmon. The dietary inclusion of vitamin C (mg kg^{-1} wet mass feed) is shown in square brackets. The number of fish analyzed per diet treatment for the duplicate tanks is shown in round brackets. The reason for the large variation in muscle vitamin C concentration between tanks for the 79 mg kg^{-1} dietary inclusion level is unknown.

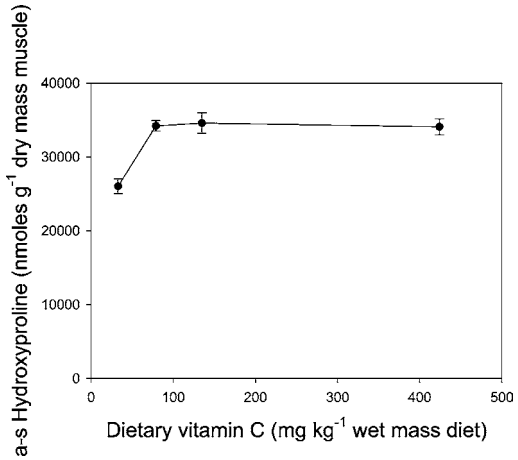

Figure 3. Relationship between dietary vitamin C concentration (mg kg^{-1} of wet mass feed) and the concentration of alkaline-soluble (a-s) hydroxyproline (HYP) in fast skeletal muscle of Atlantic salmon. The values represent mean ± SE of 28–30 fish per diet.

kg^{-1} of dietary vitamin C was required for normal growth of juvenile grouper, but 6 times that level was needed to enhance nonspecific immune responses and maintain survival of the fish infected with bacteria (*30*).

Vitamin C deficiency results in scurvy in mammals. Many of the symptoms of scurvy are related to damage to connective tissue and impaired collagen synthesis. In the present study, ANOVA revealed no significant tank effects for muscle HYP or PYD concentrations, and therefore, data from duplicate tanks were combined. The effects of dietary vitamin C on hydroxyproline concentration were different for the a-sHYP and i-sHYP fractions. We found a significant effect of dietary vitamin C on the a-sHYP concentration ($F_{3,102} = 10.72$; $P = 0.022$). Fish fed 33 mg kg^{-1} of dietary vitamin C had an approximately 27% lower a-sHYP concentration in the muscle than fish fed higher amounts (**Figure 3**) ($P < 0.05$). Increasing dietary vitamin C above the minimum level produced no further increase in the soluble HYP concentration. In contrast, there was no significant effect of vitamin C on the concentrations of either a-iHYP or a-iPYD. The alkaline-soluble collagen fraction may represent newly synthesized collagen molecules, whereas the alkaline-

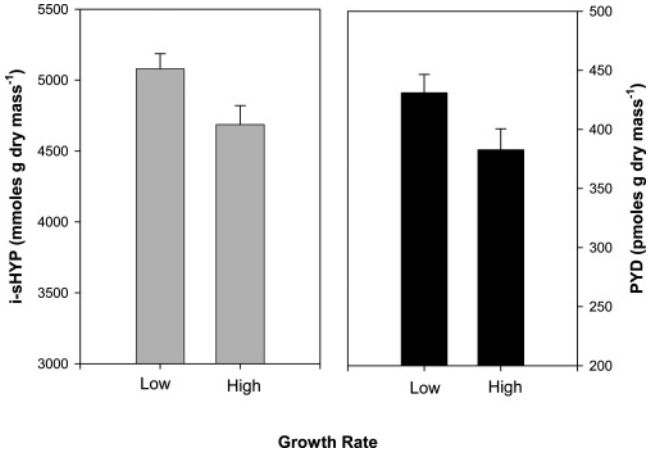

Figure 4. Alkaline-insoluble collagen and pyridinoline cross-link concentration in fast muscle for low (TGC < 3.9) (average 3.51 ± 0.04, $n = 62$) and high (TGC > 3.9) (average 4.29 ± 0.04, $n = 52$) growth rate Atlantic salmon fed. The values represent mean ± SE.

insoluble fraction is enriched with reducible and mature collagen cross-links that are known to contribute to fillet firmness (*23*). There was no significant interaction between TGC and vitamin C concentration. Currently, the NRC recommended values for minimal dietary vitamin C level in salmonids is 50 mg kg^{-1} of wet mass feed (*25*). Current practice within the salmon farm industry is to add greater than 50 mg of vitamin C kg^{-1} of feed. Our data show that 79 mg kg^{-1} of dietary vitamin C is sufficient for the synthesis of soluble collagen, whereas 33 mg of vitamin C kg^{-1} produces equal growth. Kuroyanagi and co-workers reported that in vitro vitamin C inhibited the activity of lysyl oxidase, which is required for cross-link formation (*33*). Multiple regression analysis also showed no significant effect of dietary vitamin C inclusion on a-iHYP or a-iPYD concentrations in juvenile salmon (not shown). It appears that the cross-linking of collagen was not affected by vitamin C at the levels tested. Furthermore, our original hypothesis that vitamin C requirements for collagen synthesis would vary with individual growth rate was not supported. We conclude that current levels of vitamin C added to the diet within the Industry are sufficient to maintain texture under controlled conditions, although higher concentrations may be required for proper immune function and wound healing in commercial conditions (*34*) and in larger sized fish.

ANOVA revealed a significant effect of growth rate (TGC) on both i-sHYP ($F_{1,\ 102} = 12.10$; $P = 0.001$) and i-sPYD ($F_{1,\ 102} = 7.58$; $P = 0.007$). In contrast, the covariate TGC was not significant for alkaline-soluble HYP. Similar results were obtained using multiple regression analysis ($P < 0.01$). Fish were divided into high (TGC > 3.9) and low (TGC < 3.9) growth rate groups of approximately equal size for illustrative purposes (**Figure 4**). Pyridinoline cross-link and i-sHYP concentrations were 11.1% ($P < 0.05$) and 7.7% ($P = 0.02$) lower, respectively, in high (TGC > 3.9) than low (TGC < 3.9) growth rate fish (one-way ANOVA) (**Figure 4**). The growth rate may indirectly or directly influence the synthesis of lysyl oxidase, which is required to initiate mature cross-link formation. The growth rate was only sufficient to explain 6.5% of the total variation in pyridinoline cross-link concentration. This small difference in collagen cross-linking with growth rate in juvenile salmon was, however, sufficient to produce a 15.6% reduction in fillet hardness as measured with an instrumental texture analyzer (**Figure 5**). In contrast, studies with several populations of adult salmon (4−5 kg of body mass) have shown no consistent effects of growth rate on the texture of muscle PYD concentration (I. A. Johnston and X. Li, unpublished observa-

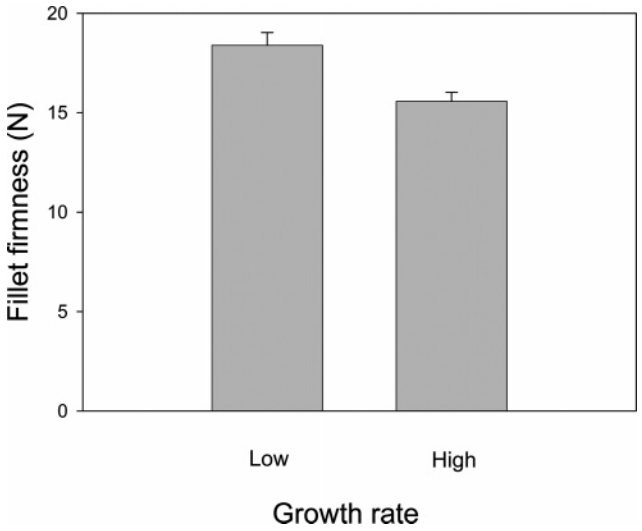

Figure 5. Values for fillet firmness (Newtons) for the high and low growth rate salmon shown in **Figure 4**. The values represent mean ± SE of 62 low growth rate and 52 high growth rate fish.

tions). In conclusion, altering vitamin C in the diet over the range tested had no effect on the formation of mature collagen cross-links or fillet texture in both low and high growth rate fish. A dietary vitamin C of 79 mg kg^{-1} resulted in the maximum concentration of soluble collagen in the muscle, whereas levels as low as 33 mg of vitamin C kg^{-1} had no detrimental effect on growth rate.

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