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Effects of phosphorus and vitamin C deficiency, vitamin A toxicity, and lipid peroxidation on skeletal abnormalities in Atlantic halibut (*Hippoglossus hippoglossus*)

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Summary

Dietary nutrients play an important role in skeletal tissue metabolism of fish. Deficiency and toxicity of certain nutrients have been linked to bone deformities in larval and juvenile fish. The pathogenesis of skeletal disorders in larval and juvenile fish from the same genetic stock, cultured under similar environment conditions is often difficult to distinguish when marginal deficiencies of multiple nutrients are involved. A study was conducted to characterize the skeletal deformities linked to the deficiency of phosphorus and ascorbic acid, vitamin A toxicity and lipid peroxidation in juvenile halibut. Five experimental diets containing a low level of phosphorus (0.5% dry matter basis), no vitamin C supplement, high level of vitamin A (80,000 IU kg^{-1}) and oxidized marine fish oil (peroxide value, 7.53 meq kg^{-1}) and a control diet based on cod fillet and vitamin free casein were fed to juvenile Atlantic halibut for 14 weeks in an attempt to characterize the skeletal deformities. Phosphorus, ascorbic acid, retinol, and α-tocopherol concentrations of liver and kidney were measured at 0 and 14 weeks. Reduced vertebral ash and phosphorus content were observed in fish fed the low phosphorus diet. Skeletal abnormalities included abnormal hemal and neural spines in the hemal region and scoliosis in the cephalic and hemal regions of the vertebral column. Hepatic and kidney ascorbic acid concentrations were significantly lower in the fed group no ascorbic acid supplement. Skeletal abnormalities were scoliosis and lordosis primarily in the hemal region of the vertebral column. High levels of vitamin A in the diet caused increased hepatic retinol content and scoliosis spanning the cephalic/prehemal and anterior hemal regions of the vertebral column. Fish fed the oxidized oil diet showed increased thiobarbituric acid (TBA) value in the liver and muscle tissue with no significant

decrease in hepatic vitamin E concentration. The most frequent skeletal deformity observed was scoliosis, spanning the cephalic/prehemal regions as well as the anterior hemal region of the vertebral column. The pattern and type of abnormalities observed in fish fed these experimental diets were similar to those observed in a commercial halibut hatchery.

Keywords

Fish bone, skeletal abnormalities, scoliosis, halibut, vitamin C, vitamin A, phosphorus, oxidized oil

Introduction

In intensive Atlantic halibut culture, the prevalence of several malformations including incomplete eye migration, malpigmentation, jaw and fin deformities affects growth, survival, overall performance and market value of the final product, which may have a large economic impact on marine fish aquaculture. Several factors are known to induce skeletal abnormalities during larval and juvenile stages in marine fish including nutrient deficiencies and toxicities, water quality, stress, infectious diseases, pollutants, mechanical lesions and high temperature during egg incubation. The effect of dietary factors related to nutrient deficiencies or toxicities was recently reviewed (Lall and McCrea-Lewis, 2007).

Calcium and phosphorus are the most abundant minerals in fish and their functions are closely related, particularly in the development and maintenance of the skeletal system. They are complexed in a matrix, hydroxyapatite: the main inorganic matter in teleost bone and scales. Although aquatic organisms have the ability to absorb

Ca and P from water, the concentration of this element is low in both freshwater and seawater to meet the nutritional requirements of these minerals of most fish. The calcium requirement of fish is met in large part by absorption through gills and skin in freshwater and by drinking seawater and the deficiency of this mineral has not been detected in marine fish. The amount of P in feeds must be carefully balanced to prevent deficiency signs (reduced growth rate, decreased feed efficiency, skeletal deformities, low phosphorus and ash content of vertebrae and the whole body) as well as to minimize the urinary and fecal excretions to reduce P discharge in natural waters (reviewed by Lall, 2002). The availability of P from various feed ingredients varies significantly including fish meals that contain high amounts of P. Rapidly growing salmonids fed diets based on fish meal of low bioavailability gradually develop soft bones and skeletal deformities (Lall, 2001).

Vitamin C or ascorbic acid (AA) is a water soluble vitamin that acts as a reducing agent as well as an antioxidant in teleosts (reviewed by Halver, 2002). Most fish including halibut are unable to synthesize ascorbic acid therefore it must be supplied within the diet (NRC, 1993; Mæland and Waagbø, 1998). AA is also a cofactor in hydroxylating amino acids for collagen synthesis, which is required for wound repair, formation of connective tissues and bone matrix. The AA requirement for optimal biological and physiological functions in juvenile fish is 25mg ascorbic acid kg^{-1} diet (NRC, 1993). Common signs of ascorbic acid deficiency include: reduced bone collagen, hemorrhaging, increased feed conversion, lower weight gain, reduced ascorbate tissue storage, and increased mortality. Symptoms such as lordosis, scoliosis and broken back

71 can be consequences of poor collagen formation common to ascorbic acid deficient fish
72 (reviewed by Halver, 2002).

73 Vitamin A (VA) is a fat soluble vitamin that regulates cellular differentiation and
74 proliferation, reproduction, vision, embryonic development, and resistance to infections
75 (reviewed by Halver, 2002). However, consumption of large doses of vitamin A causes
76 hypervitaminosis A toxicity signs including: decreased growth, increased mortalities,
77 pale-yellow liver and a decrease in haematocrit and hemoglobin levels (Hilton, 1983;
78 Poston et al., 1965). Excess retinoid consumption increases bone resorption by
79 increasing the number of osteoclasts causing inhibition of bone formation and increased
80 skeletal turnover (Hough et al., 1988). A toxicity of vitamin A induces skeletal
81 deformities including vertebral curvatures, vertebral compression, vertebral fusion, and
82 jaw deformities (Dedi et al., 1995; Takeuchi et al., 1995).

83 Polyunsaturated fatty acids (PUFA; specifically n-3 PUFAs) are essential for
84 optimal fish growth, skeletal development, health, and feed utilization. PUFAs are more
85 susceptible to oxidation than monounsaturated and saturated fatty acids (Porter *et al.*,
86 1981). The oxidative breakdown product of PUFAs is malonaldehyde (MDA), which is
87 measured by TBA (thiobarbituric acid) reactive substances concentrations (TBARS; de
88 Zwart *et al.*, 1999). Primary effects of feeding fish oxidized lipids include: elevated MDA
89 levels in tissues, liver degeneration, anemia, and spleen abnormalities (Hamre *et al.*,
90 2001; reviewed by Sargent et al., 2002). Increased bone resorption and cartilage
91 mineralization is stimulated by peroxidation of fatty acids resulting in a net bone loss
92 (Garette et al., 1990). Dietary intake of oxidative products causes a depletion of
93 antioxidants such as vitamins E and C, which aid in preventing lipid peroxidation

(reviewed by Sargent et al., 2002) and protecting bone cells, such as osteoblasts, from being damaged by free radicals (Arjmandi et al., 2002).

In this study, the role of biotic determinants, specifically nutrient toxicity of vitamin A and oxidized dietary lipid as well as deficiencies of phosphorus and vitamin C, were examined for their effect on the development of skeletal abnormalities in juvenile Atlantic halibut.

Materials and Methods

Experimental Conditions

The experiment was conducted with Atlantic halibut (*Hippoglossus hippoglossus*) obtained from the Scotian Halibut Ltd. Hatchery (Clark's Harbor, NS, Canada) and transferred to the National Research Council's research station at Sandy Cove, Halifax, NS, Canada. Fish were maintained on commercial diet while acclimating to experimental conditions over a 21 day period. The juvenile Atlantic halibut (4.61 ± 0.09g) were randomly distributed into 15, 350 L tanks. Each of the tanks were supplied with UV-treated, filtered (60μm) seawater at 4.0 L / min and a renewal rate of 1 turnover per hour. Average water temperature was 11.80±0.06 °C, dissolved oxygen concentration 11.15 ± 0.92 mg of dissolved oxygen /ml water and 24 hours of dim light throughout the 14-week experiment. The experimental diets were hand-fed to satiation three times daily (0830, 1230, and 1630) from week 1 to 7 then twice daily from week 8 to 14 (0830 and 1630). Fish from each tank were counted and batch weighed at the beginning of the experiment, every three weeks and at the end of the fourteen weeks.

Diet Formulation and Preparation

Five, freeze-dried cod muscle and casein-based diets were formulated (control, low phosphorus, low vitamin C, high vitamin A, and oxidized oil; Table 1). Diets were prepared by mixing the dry ingredients in a Hobart mixer (Hobart, Troy, OH). Monocalcium phosphate was added to the diets except the low phosphorus diet which was supplemented with celufil (cellulose). Ascorbic acid was added at 200 mg kg^{-1} diet to all diets except the low vitamin C diet, in which celufil was added instead of ascorbic acid. Additional vitamin A, in the form of retinyl acetate, was incorporated into the high vitamin A diet to increase the vitamin A concentration to 80,000 IU VA kg^{-1}. Finally, 12% of 16.5% anchovy oil was replaced with oxidized anchovy, herring, and mackerel oil (POV = 62.6 meq kg^{-1}) according to the methods of Koshio et al. (1994) by bubbling with air, agitation with stirring and heating of the oil at 50°C for approximately 24h to produce an oxidized oil diet with a peroxide value of 7.53 meq kg^{-1}. The diets were cold formed through a Hobart mixer with a meat grinder attachment to obtain 1, 2, and 4 mm pellet size. Pellets were freeze dried for 48 hours and stored in a -20°C freezer. Moisture and ash was determined according to AOAC (1995), crude protein was measured with a Leco nitrogen determinator (model FP-528, Leco Corporation, St. Joseph, MI) and energy content determined with an adiabatic bomb calorimeter (Model 1261, Parr Instruments, Moline, IL).

Analytical Methods

In order to determine phosphorus concentrations in the skeletons, flesh was removed by partially heating the halibut carcasses in a microwave. The bone lipid was extracted in chloroform:methanol (2:1, v/v). Moisture and ash determinations of skeletons were in accordance to AOAC (1995). Phosphorus content of the resulting

skeletal and diet ash was determined using a spectrophotometric method (Taussky and Shorr, 1953).

Ascorbic acid content of the experimental diets, liver and kidney was determined by a modified dinitrophenylhydrazine (DNPH) spectrophotometric method (Dabrowski and Hinterleitner,1989). Lipids were extracted from tissues and experimental diets using the method of Bligh and Dyer (1959). Fatty acid compositions of the diets were estimated from the fatty acid methyl ester (FAME) derivatives of the transesterified lipids. The FAME's were prepared using 7% boron trifluoride in methanol and heating to 100°C for 1-h (Kirsch et al., 1982). The FAME were separated by a gas chromatograph equipped with a flame-ionization detector (Hewlett Packard 6890 GC system, Wilmington, DE) on an Omegawax 320 capillary column (30m x 0.32mm x 0.25 μm; Supelco, Bellefonte, PA). FAME's were identified by comparison of retention times with those of known standards (Supelco 37, Menhaden Oil; Supelco, Bellefonte, PA).

Lipid extracted from both diets and liver tissues were analyzed for vitamin A and E content. The lipid extraction was performed under yellow light and butylated hydroxytoluene, an antioxidant, was mixed at 0.01% with methanol and chloroform to minimize oxidation. The vitamin concentrations were determined using a reverse phase high performance liquid chromatography (HPLC). Samples (1 μl) were injected on a Phenomenex Synergi 4 μ Hydro-RP 80 A column using an isocratic elution of acetonitrile and methanol (75:25) at a flow rate of 0.5ml/min at 25°C. A fluorescence detector (FLD) was used at 285 and 335 nm, excitation and emission respectively, to quantify α-tocopherol acetate in the diet and the excitation and emission of 294 and 330nm, respectively, for quantification of α-tocopherol in the liver. For determination of

retinol in liver and retinol acetate in the experimental diets a diode-array detector (DAD) was used at a wavelength of 330nm. Malonaldehyde (MDA) concentrations in the liver and muscle tissues were measured using the TBA method (Lemon, 1975; Williamson *et al.*, 2003). The concentration of MDA in the tissues was determined comparing standards made up with 1,1,3-tetraethocypropane (TEP).

Classification of fish abnormalities

At the beginning of the experiment, 20 juvenile halibut were randomly selected from the tanks and preserved in neutral buffered 10% formalin (Fisher Scientific, Fairlawn, NJ) for 24 hours at room temperature. A whole mount bone staining was applied to each fish to examine the initial baseline number and type of abnormalities present. The application of this technique for halibut has been described in an earlier report (Lewis et al., 2004). After 14 weeks, a total of sixteen fish per diet were sampled for examination of abnormalities by x-ray and removal of flesh to expose the bone.

In order to classify abnormalities, the vertebral column was divided into four regions: cephalic, prehemal, hemal, and caudal region as described in Lewis et al. (2004). Table 5 contains the alphanumeric dichotomic key used to classify and quantify observed abnormalities in the juvenile Atlantic halibut fed the five experimental diets. A letter indicates the type of abnormalities observed and a number represents the region in which the abnormalities were present. In total there were 17 types of skeletal abnormalities considered.

Statistical analysis

All statistical analyses were executed with SYSTAT 10 (SPSS Inc., 2000). The growth, feed efficiency, hepatosomatic index, haematocrit, liver lipid levels, fatty acid profiles, and micronutrient concentrations in various tissues were compared from fish fed the low phosphorus, low vitamin C, high vitamin A, and oxidized oil diets to the control using t-tests because of the nature of the experimental diets ($P<0.05$). Kruskal-Wallis tests were used to determine significance in the number of meristic characters between fish fed the five diets ($P<0.05$).

Results

Growth, feed utilization and hepatosomatic Index

The lowest weight gain (27g $fish^{-1}$), average percent weight gain (493%), and specific growth rate (SGR; 1.8 %) were found for fish fed the low vitamin C diet (Table. 6). The feed conversion ratio (FCR), percent survival and hepatosomatic index (HSI) of fed various experimental diets were not significantly different ($P>0.05$). Diets containing no phosphorus or ascorbic acid supplements resulted in significantly lower haematocrit values (29.9% and 26.2%, respectively) as compared with fish fed control diet (34.3%).

Tissue lipid and ascorbic acid concentration

In fish fed diets containing no AA supplement, significantly lower liver lipid level (7.0 %) was observed as compared to fish maintained on the control diet (11.2%). The liver lipid content of fish was also not affected in the remaining three dietary groups (low phosphorus, high vitamin A, and oxidized oils) and it ranged between 11.2-12.2%. Liver lipid results showed a small inter-individual variation (Table 7). The fatty acid

composition of liver lipid is summarized in Table 8. Feeding of diet containing oxidized oil to juvenile halibut caused a significant reduction in their liver docosahexaenoic acid (DHA) content, the ratio of eicosapentaenoic acid (EPA) and DHA (DHA:EPA) and total ω-6 fatty acid content including arachidonic acid (20:4 ω–6). Significantly lower levels of total n-6 and monounsaturated fatty acids were observed in liver of fish fed low AA diet as well as higher levels of total n-3 fatty acids and DHA:EPA ratio.

Total bone ash and phosphorus content of the stripped skeleton is summarized in Table 7. The lowest bone ash and phosphorus was observed in fish sampled from the low phosphorus diet. Bone ash from fish receiving low P diet had 9.2 % P (DM basis), which was significantly lower than fish from the control diet (11.5 % P; P=0.000). From the other four diets, percent P and ash in the vertebrae ranged between 11.2 to 12.3% and 47.8 to 51.2%, respectively but the difference among dietary treatments was not significant.

The oxidative breakdown product of lipid was measured as malonaldehyde levels in muscle and liver tissues. Average liver and muscle MDA concentrations were analyzed was higher in liver than the muscle for all dietary treatments. MDA concentration was significantly higher in both the tissues from fish fed the oxidized oil diet (liver, 44.3 nmol g^{-1}; muscle, 0.80 nmol g^{-1}) than fish from the control diet (liver, 25.0 nmol g^{-1}; muscle, 0.6 nmol g^{-1}; $P<0.05$). There were no significant differences in MDA content of either liver or muscle of fish fed low phosphorus, low vitamin C, high vitamin A and control diet.

Average AA content of liver and head kidney of fish fed various experimental diets are summarized Table 7. In general, the hepatic AA concentration was higher than

231 kidney AA concentration, regardless of the diet fed. Total AA concentrations in both the
232 tissues (liver, 23 μg g^{-1} and kidney, 11 μg g^{-1}) were significantly lower in fish fed the low
233 vitamin C diet than the fish fed the control diet (liver, 76 μg g^{-1} and kidney, 66 μg g^{-1};
234 $P<0.05$). There were no significant differences in AA concentration in all the three
235 tissues of fish fed low phosphorus, high vitamin A, oxidized oil and control diet.

236 The high concentrations of dietary vitamin A caused a significant increase in
237 accumulation of retinol in halibut liver (Table 7). The concentration reached 11.4 μg
238 retinol g^{-1} liver fish fed the high level of vitamin A (80,000 IU kg^{-1}). Whereas the liver
239 retinol concentration in fish fed in remaining diets including control diet ranged from 4.1
240 to 5.6 μg retinol $^{-1}$g liver and there was no significant difference among the diets. The
241 liver α-tocopherol concentrations of fish fed various experimental diets were not
242 significantly ($P>0.05$) affected by changes in dietary AA, P and vitamin A levels and
243 feeding of diet containing oxidized lipid (Table 7). In halibut fed various experimental
244 diets for 14 weeks, the concentration ranged from 2.8 to 3.5 μg α-tochopherol/g liver with
245 the lowest values found in fish fed the oxidized oil diet.

246 ***Vertebral Characters and Skeletal Abnormalities***

247 The skeleton of halibut was divided into four distinct regions: cephalic, pre-
248 hemal, hemal and caudal region. The number of vertebrae within each region was
249 counted using x-rays and bone that was stripped from flesh (Table 9). The average
250 vertebrae number from each region was similar with small ranges and standard errors,
251 regardless of the diet fed. The number of cephalic vertebrae ranged from 3 to 4
252 vertebrae, 11 to 13 vertebrae in the pre-hemal region, 29-33 vertebrae in the hemal
253 region, 3 to 4 vertebrae in the caudal region regardless of diet interaction. Using the

Kruskall-Wallis statistic test for non-parametric data, no significant differences ($P>0.05$) in vertebral characters were observed.

Initial fish were examined for skeletal abnormalities using a bone staining technique. Common abnormalities observed include those of the neural spines (abnormality types G and H) at a low frequency of occurrence. No serious types of abnormalities, such as scoliosis and lordosis, were observed in fish at the initial stage of the experiment. At 14 weeks, 16 fish per diet were examined for skeletal abnormalities. Fish fed the control diet possessed only one abnormality, bifurcated neural spines (abnormality type G), which was observed in the cephalic and pre-hemal regions of the vertebral column (Table 10). Vertebral columns from this group were unaffected by scoliosis or lordosis.

Fish fed the low phosphorus diet had twisted neural (F) and hemal spines (L) in the pre-hemal as well as the hemal region of the vertebral column with a frequency of occurrence of 43.7% and 41.2% respectively (Table 10). Throughout the cephalic to hemal region, the frequency of scoliosis (B) was 14.0 %. Although the abnormalities occurred throughout the vertebral column, the hemal region was primarily affected by the majority of abnormalities. Only one fish possessing lordosis was observed in this group.

Scoliosis (B) was prominent within the hemal region in fish fed the low vitamin C diet at a percent frequency of 59.5% (n=16). Bifurcated (G) and supernumerary (H) spines were common in the cephalic and hemal regions, respectively, although at a lower percent frequency of occurrence (11.9% and 9.5%, respectively; Table 10). Other abnormalities present at a low percent frequency include vertebral body fusion and

compressed vertebrae (Table 10). In general, abnormalities were evenly distributed throughout the vertebral column for the low vitamin C dietary treatment.

The high vitamin A diet resulted in fish possessing scoliosis (B) within the cephalic and pre-hemal regions and the anterior hemal region of the vertebral column (FA = 92.9%). The other types of abnormalities present in fish fed the high vitamin A diet were fused vertebrae (C) and compressed vertebrae (D) at a lower percent frequency. All abnormalities observed were vertebral element abnormalities (Table 10). Skeletal abnormalities were primarily present in the cephalic/pre-hemal as well as anterior hemal region.

Fish fed the oxidized oil diet possessed both lordosis (A) and scoliosis (B). In total, 6.3% of the fish examined for abnormalities had lordosis, which was commonly observed in the anterior hemal region of the vertebral column. Conversely, scoliosis was present in the cephalic and pre-hemal regions of the vertebral column as well as in the anterior hemal region. Other types of abnormalities observed in fish fed oxidized lipid included bifurcated (G) and supernumerary (H) neural spines and fused vertebrae, which were at lower frequency of occurrence than scoliosis and lordosis. In total, 97% of the abnormalities observed were vertebral in origin while the remainder was neural elements (Table 10).

Discussion

The effects of certain nutrient deficiencies and toxicities known to cause skeletal abnormalities in other fish, produced different patterns of abnormalities in the vertebral column of juvenile Atlantic halibut. The four experimental diets, low phosphorus, low

299 vitamin C, high vitamin A and oxidized oil diets, elicited responses in both tissue and
300 bone mineral concentrations and produced specific patterns of skeletal abnormalities
301 which have not been reported before in halibut. Even though the low phosphorus diet
302 maintained a relatively high amount of phosphorus (0.5 %) originating from the cod
303 fillet, deficiency symptoms were observed in fish fed this diet for 14 weeks. Reduced
304 weight gain and increased feed conversion ratios are initial indicators of phosphorus
305 deficiency as identified in a variety of teleost species, including European white fish
306 (*Coregonus lavaretus* L.) and common carp (*Cyprinu carpio*) (Takeuchi and Nakazoe,
307 1981; Borlongan and Satoh, 2001; Vielma et al., 2002). The above mentioned effects on
308 performance of other fish species were not observed in halibut despite a significant
309 decrease in vertebral P and total ash content (9.2% and 31.7%, respectively). A
310 significant decrease in vertebral P and total ash content were also observed in
311 hypophosphatemic whitefish (Vielma et al., 2002).

312 In the liver of fish fed the low phosphorus diet, an increase in oleic acid was
313 observed, which has been also found in common carp suggesting that phosphorus
314 deficiency has an inhibitory effect on β-oxidation of fatty acids or increased fatty acid
315 synthesis (Takeuchi and Nakazoe, 1981). Information on the effect of phosphorus on
316 lipid metabolism in teleost fish is lacking and must be further examined regarding how
317 hypophosphatemia affects fatty acid metabolism.

318 For optimal bone mineralization and growth, phosphorus is an important mineral
319 (reviewed by Lall, 2002). Phosphorus deficiency has been shown to increase the number
320 of bone cells responsible for bone resorption, osteoclasts, in haddock (*Melanogrammus*
321 *aeglefinus* L.), which results in increased matrix resorption and degradation, ultimately

affecting bone growth and formation of bone (Roy et al., 2002). The Atlantic halibut juveniles fed the low phosphorus diet often showed hemal and neural spine abnormalities, specifically twisted spines, primarily in the hemal region of vertebral similar to haddock (Roy and Lall, 2003). These bones of the vertebral column were soft, likely causing muscular action within the hemal region of the vertebrae to distort the thinnest parts of the skeleton, which includes the tips of the hemal and neural spines. Although common abnormalities observed in other species of hypophosphatemic fish were not observed including deformities of the frontal bone and compressed vertebrae (Ogino and Takeda, 1976; Roy and Lall, 2003), scoliosis was frequent in phosphorus deficient halibut throughout the cephalic, prehemal and hemal regions of the vertebral column with no distinct pattern of occurrence. The nutritional status of phosphorus in fish is best represented by bone ash and phosphorus concentrations as they are sensitive indicators of dietary phosphorus intake (Vielma et al., 2002; Borlongan and Satoh, 2001). Since Atlantic halibut lack the enzyme L-gulonolactone oxidase for ascorbic acid synthesis, AA must be supplied in the diet (Mæland and Waagbø, 1998). Fish fed the low vitamin C diet had lower weight gain as compared to the control group. This was also reported in a variety of scorbutic teleost species including olive flounder (*Paralichthys olivaceus*; Wang et al., 2002) and red drum (*Sciaenops ocellatus*, Aguirre and Gatlin III, 1999). Other symptoms observed in this study include lower percent survival and reduced hematocrit levels, which are supported by Aguirre and Gatlin III (1999) and Adham *et al.* (2000). It appears that the number of red blood cells decreases with prolonged exposure to vitamin C deficient diet, which ultimately progresses into an anemic condition (Adham et al., 2000).

345 Both hepatic and anterior kidney AA concentrations were used to assess the
346 vitamin C status of Atlantic halibut because among fish species whether liver or kidney is
347 the most sensitive indicator has not been established (Lim and Lovell, 1978). Liver
348 ascorbic acid (AA) content was directly related to dietary AA levels as observed in
349 channel catfish (*Ictalurus punctatus*; Lim and Lovell, 1978). Liver AA was significantly
350 lower in halibut fed the low vitamin C diet (22.8μg AA g^{-1} liver), below the suggested
351 vitamin C deficiency concentration of 30 ug AA g^{-1} of tissue (Lim and Lovell, 1978).
352 AA concentration was higher in the liver as compared to the kidney, although the
353 reduction of this vitamin was comparable regardless of tissue for fish fed the low vitamin
354 C diet as also observed by Mæland and Waagbø (1998). AA deficiency in this study,
355 increased PUFA and total n-3 fatty acids as well as decreased total monounsaturated and
356 n-6 fatty acids. These results are inexplicable as vitamin C deficiency results in increased
357 lipid peroxidation that is associated with increased concentrations of monounsaturates
358 and saturates due to the degradation of PUFAs (Chien and Hwang, 2001).
359 Spinal abnormalities can occur in response to vitamin C deficiency since
360 development of the bone matrix is impaired as vitamin C is a cofactor in the synthesis of
361 collagen (NRC, 1993). Both scoliosis and lordosis were present primarily in the hemal
362 region of the vertebral column in scorbutic juvenile Atlantic halibut, however the
363 prevalence of lordosis was lower. Similar to these observations, scorbutic olive flounder
364 possessed scoliosis in the hemal regions of the vertebral column (Wang et al., 2002).
365 Atlantic halibut deficient in AA showed that abnormalities were evenly distributed along
366 the vertebral column with no specific pattern of occurrence. This finding is consistent
367 with observations made by Dabrowski et al. (1990) in scorbutic rainbow trout. However,

Madsen and Dalsgaard (1999) observed the highest incidence of skeletal deformities in the posterior to mid-hemal regions of the vertebral column. Prolonged exposure to low vitamin C diets may decrease vitamin C stores affecting skeletal development in a variety of teleost species. It is essential to maintain optimal dietary levels of this vitamin to prevent pathogenesis of common scorbutic symptoms.

In the past decade, several studies have focused on the effect of vitamin A toxicity on pigmentation and vertebral morphogenesis in larval Japanese flounder but not in other teleosts. In post-embryonic Japanese flounder, excess dietary retinoic acid resulted in decreased length and weight gain (Haga et al., 2002), which was not observed in halibut. In response to increased levels of VA in the diet, liver retinol level was twice as high as compared to the control diet. This increase was expected due to mega dose of vitamin A level in the diet, however the magnitude of increase in liver retinol accumulation did not reflect the eight times increase in the dietary level of this vitamin.

Vitamin A is known to have an important function in regulating normal cellular differentiation and proliferation during the skeletal and cartilage development (reviewed by Halver, 2002). In early stages of development, retinoid compounds alter gene expression, primarily *homoebox* (*Hox*) genes that are involved in neural crest positioning and differentiation, which play a role in cartilage and bone development (Marshall et al., 1996). This may explain the absence of craniofacial abnormalities commonly associated with hypervitaminosis A in larval development as this study was executed with juvenile fish.

Rats exposed to VA toxicity showed an increase in osteoclast number resulting in increased bone resorption and skeletal turnover with decreased bone formation (Hough et

391 al., 1988). Excess retinoic acid can reduce both the activity and proliferation of
392 osteoblasts and chondrocytes (Takaki et al., 1996) which are considered as the basis of
393 many skeletal malformations in a variety of animals. Common signs of hypervitaminosis
394 A in Japanese flounder include compressed vertebrae, central fusion between the last
395 vertebrae and the urostyle, vertebrae hypertrophy, and abnormal caudal fin development
396 (Dedi et al., 1995; Takeuchi *et al.*, 1995; Haga et al., 2002). Vitamin A toxicity signs in
397 rainbow trout include lordosis and scoliosis in the mid to anterior hemal regions of the
398 vertebral column however, these major skeletal abnormalities developed at a low
399 frequency in rainbow trout (Hilton, 1983). Abnormalities common to Atlantic halibut
400 exposed to high VA levels was primarily scoliosis spanning the cephalic and pre-hemal
401 regions as well as the anterior hemal region of the vertebral column. Vertebral body
402 fusion and compressed vertebrae were also present at significantly lower frequency in
403 these fish. Early larval exposure to high VA diet increased the number of vertebrae in the
404 however, there was not an increase in the number of vertebrae observed in this diet as the
405 number of vertebrae was already established prior to beginning of the feeding trial as
406 observed in the initial examination of the skeleton.
407 Less than 50,000 IU VA kg^{-1} *Artemia* may be considered the safe level of vitamin
408 A to prevent bone abnormalities in Japanese flounder larvae (Dedi et al., 1995). In this
409 study, a slightly higher amount of VA (52,873 IU VA kg^{-1} diet) was incorporated into the
410 diet and resulted in major skeletal abnormalities even though many studies have used
411 higher amounts of VA incorporated into the diet to induce toxicity. It is recommended
412 that the overall concentration of this vitamin should be determined in manufactured feed,

413 because the concentration of this vitamin varies in fish oil and meal and certain fish liver
414 oils contain relatively high level of VA (Lall and Parazo, 1995).

415 A response to feeding a diet containing low levels of oxidized dietary lipid was
416 observed in both the liver and muscle tissue of the fish. MDA concentration in fish fed
417 the oxidized lipid diet showed a higher increase in the liver as compared to the muscle
418 tissue although Messager et al. (1992) observed an opposite effect in sea bass
419 (*Dicentrarchus labrax*). They attributed these results to liver lipid being more protected
420 against peroxidation than muscle, which was not supported by the findings of this study.

421 In fish fed oxidized fish oil, antioxidant stores are reduced from protecting bone
422 cells such as osteoblasts from incurring damage since they are contain PUFAs, which are
423 more susceptible to oxidation (Jilka et al., 1996). Interestingly, the present study, as well
424 as Hamre et al. (2001), did not observe a significant decrease in liver vitamin E
425 concentrations of fish fed the oxidized oil diet even though an oxidative response was
426 observed in hepatic and muscle tissue MDA concentration. Vitamin C also functions to
427 regenerate vitamin E from the vitamin E radical produced when reacting to lipid peroxide
428 radical (Packer et al., 1979). In an earlier study, rainbow trout fed slightly or moderately
429 oxidized oil showed little reduction in liver ascorbate concentration and hematocrit
430 values (Hung and Slinger, 1980) similar to the results of this study. Juvenile Atlantic
431 halibut showed a reduction in hepatic ascorbate concentration, suggesting that in response
432 to slight oxidative stress Atlantic halibut utilize the hepatic ascorbate stores not kidney
433 stores to either regenerate vitamin E radicals or combat oxidative products.. This may
434 explain the lack of reduction in hepatic Vitamin E because the oxidized α-tocopheroxyl

435 radicals produced in this process may be recycled back to the active reduced form
436 through reduction by antioxidants, such as ascorbate.
437
438 The oxidized oil used to partially supplement the herring oil in the test diet had
439 higher PUFAs and total n-3 fatty acids and lower concentration of saturated fatty acids.
440 Feeding an oxidized oil diet should have produced a response to oxidative products
441 within the body, thus lowering the amount of PUFAs and increasing the saturated fatty
442 acids in tissues as compared to the control diet. However, this response was not observed
443 in halibut probably due to low absorption and elimination of oxidized lipid components
444 in feces. It would be interesting to determine the effects of oxidized lipid on digestibility
445 of lipid and fatty acids.
446 Oxidized lipid has been suspected to inhibit differentiation of osteoblasts, thus
447 reducing bone formation and stimulate the formation of osteoclasts causing bone
448 resorption (Parhami et al., 1997). Scoliosis was commonly observed spanning the
449 cephalic/pre-hemal and anterior hemal region of the vertebral column. Since there has
450 been limited research on the effect of oxidized oil on fish bone lipid, it is not possible to
451 explain the cause for the effects of this dietary factor on pathogenesis of bone
452 abnormalities.
453 Studies on oxidative stress have previously focused on moderately to high
454 inclusion of oxidized dietary lipid. Although the present study was able to elicit an effect
455 of ingesting slightly oxidized oil (POV of 7.53 meq kg^{-1}) on skeletal formation, which is
456 lower than the recommended POV for oils in fish feed being less than 10 meq kg^{-1} feed

(Hilton and Slinger, 1981). Examination of toxic levels of oxidized lipid causing skeletal abnormalities should be further examined in Atlantic halibut.

In conclusion, this study designed to investigate potential causative dietary factors that might induce skeletal abnormalities in Atlantic halibut showed that fish fed diets containing oxidized oil developed patterns and types of abnormalities similar to a previous study on the early stages of spine and vertebral development in hatchery-reared larval and juvenile halibut (Lewis et al., 2004). Additional studies are needed to examine the molecular and biochemical basis of the pathogenesis of skeletal disorders caused by lipid peroxidation in juvenile fish tissues as well as the efficacy of antioxidants in preventing these abnormalities.

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646 **Table 1**
647 Formulation of the experimental basal diet fed to juvenile Atlantic halibut for 14 weeks
648 (as fed basis)
649

Ingredients	Amount (%)
Cod muscle [a]	30.0
Casein, vitamin free [b]	14.0
Corn gluten meal [c]	12.5
Corn starch, pre-gelatinized [d]	5.4
Squid meal [e]	4.0
Gelatin [b]	3.0
Krill hydrolysate [f]	3.0
CPSP-G [g]	3.0
Cellulose [b]	4.3
Vitamin premix [h,i,j]	1.5
Macro mineral mix [k]	1.6
Trace mineral premix [l]	1.0
Choline chloride [b]	0.2
Fish oil [m,n]	16.5

650
651 [a] Prepared in the laboratory from boneless and skinless cod fillet collected from local
652 fishery, freeze-dried and ground to a powder
653 [b] US Biochemical, Cleveland, OH, USA
654 [c]Bunge Canada, Oakville, ON, Canada
655 [d]National Starch & Chemical Company, Bridgewater, NJ, USA
656 [e]Spray dried seafood digest, APC Natural Flavour, Denison, IA, USA
657 [f]Special Marine Products Ltd., West Vancouver, Canada
658 [g] Soluble fish protein concentrate, Sopropêche, France
659 [h] Vitamin added to supply the following (per 1.5 kg): vitamin A (retinol acetate), 8000
660 IU; vitamin D_3 (cholecalciferol), 4500 IU; vitamin E (dl-α-tocopheryl acetate), 400 IU;
661 vitamin K_3 (menadione sodium bisulfite), 40 mg; thiamin HCl, 50 mg; riboflavin, 50 mg;
662 d-calcium pantothenate, 150 mg; biotin, 1 mg; folic acid 15 mg; vitamin B_{12}, 0.15 mg;
663 niacin, 200 mg; pyridoxine HCl, 20 mg; inositol, 400 mg; butylated hydroxytoluene
664 (BHT), 15 mg; butylated hydroxyanisole (BHA), 15mg.
665 [i] For the low vitamin C diet, no ascorbic acid was added to the basal diet while the
666 remainder of the four diets was supplemented with 200 mg of ascorbic acid kg^{-1} diet
667 [j]For the high vitamin A diet, 0.084 g retinol acetate kg^{-1} diet was added to the basal diet
668 to total the vitamin A concentration to 60,000 IU VA kg^{-1} diet.
669 [k]In the low Phosphorus diet, cellulose was supplemented instead of monocalcium
670 phosphate as used in the other four diets.
671 [l]Minerals added to supply the following (per kg diet): manganous sulfate ($MnSO_4 \cdot H_2O$,
672 32.5 % Mn), 40 mg; ferrous sulfate ($FeSO_4 \cdot H_2O \cdot 7H_2O$, 20.1% Fe), 30 mg; copper
673 sulphate ($CuSO_4 \cdot 7H_2O$, 25.4% Cu), 5mg; zinc sulfate ($ZnSO_4 \cdot 7H_2O$, 22.7% Zn), 75 mg;
674 cobalt chloride ($CoCl_2 \cdot 6H_2O$, 24.8% Co), 2.5 mg; sodium selenite (Na_2SeO_3, 45.6% Se),
675 1 mg; sodium fluoride (NaF, 42.5% F), 4 mg.

676 [m]Herring oil was stabilized with 0.06% ethoxyquin; Comeau seafood, Saulnierville, NS,
677 Canada; POV 0.46 meq kg^{-1} oil
678 [n]For the oxidized oil diet 72.7% of herring oil used was replaced with oxidized anchovy,
679 herring and mackerel oil blend, Ocean Nutrition Canada, Ltd., Bedford, NS; POV 62.6
680 meq kg^{-1} oil.
681 .

682 **Table 2**
683 Proximate analysis of the five experimental diets [1]
684

	Diet				
	Control	**Low Phosphorus**	**Low Vitamin C**	**High Vitamin A**	**Oxidized oil**
Moisture (%)[2]	4.4±0.19[a]	2.6± 0.00[b]	4.3± 0.07[a]	4.1±0.05[a]	4.3±0.09[a]
Crude protein (%)[2]	57.1±0.28[a]	57.2±0.14[a]	57.3±0.26[a]	56.9±0.23[a]	56.3±0.29[a]
Lipid (%)[2]	18.1±0.07[a]	18.0±0.12[a]	18.2±0.13[a]	18.6±0.01[a]	18.2±0.07[a]
Ash (%)[2]	5.6±0.03[a]	3.2±0.00[b]	5.5±0.04[a]	5.6± 0.02[a]	5.5±0.03[a]
Energy (MJ kg^{-1})[3]	5611.5±32.20[a]	5806.8±12.90[a]	5641.9±15.40[a]	5608.2±6.34[a]	5594.9±10.58[a]

685
686 [1] Values in the same row containing different letter superscripts were significantly
687 different (P-value<0.05).
688 [2] Values are present as mean ± S.E. of three replicate samples.
689 [3] Values are presented as mean ± S.E. of two replicate samples

690 **Table 3**
691 Analyzed phosphorus, ascorbic acid, α-tocopherol acetate, and retinol acetate content of
692 the experimental diets [1,2].
693

	Diet				
	Control	**Low Phosphorus**	**Low Vitamin C**	**High Vitamin A**	**Oxidized oil**
Phosphorus (%) on DM basis	1.2 ± 0.02[a]	0.5 ± 0.04[b]	1.2 ± 0.01[a]	1.2 ± 0.02[a]	1.2 ± 0.03[a]
Total ascorbic acid (mg/kg)	217.5±2.3[a]	211.4±6.7[a]	ND[b,3]	199.7±3.6[a]	213.7±4.2[a]
α-tocopherol (IU/kg)	278±13[a]	289±6[a]	275±12[a]	288±3[a]	260±6[a]
Retinol (IU/kg)	6649±97[a]	6762±197[a]	6621±193[a]	52837±118[b]	6534±204[a]

694
695 [1] Values are present as mean ± S.E. of six replicate samples.
696 [2] Values in the same row containing different letter superscripts were significantly
697 different (P-value < 0.05)
698 [3] ND: not detected

699 **Table 4**
700 Fatty acid composition of experimental diets fed to juvenile Atlantic halibut for 14
701 weeks[1,2].
702

Fatty Acid	Diet				
	Control	Low Phosphorus	Low Vitamin C	High Vitamin A	Oxidized oils
14:0	6.3 ± 0.0[a]	6.4 ± 0.1[a]	6.2 ± 0.0[a]	6.3 ± 0.0[a]	6.51 ± 0.[a]
16:0	18.0± 0.1[a]	18.1 ± 0.2[a]	17.8 ± 0.2[a]	17.9 ± 0.1[a]	16.4 ± 0.0[b]
16:1n-7	7.3 ± 0.0[a]	7.3 ± 0.1[a]	7.2 ± 0.1[a]	7.2 ± 0.0[a]	7.5 ± 0.1[b]
18:0	4.0 ± 0.0[a]	3.9 ± 0.0[a]	3.9 ± 0.0[a]	3.9 ± 0.0[a]	3.4 ± 0.0[b]
18:1n-9	10.3 ± 0.0[a]	10.3 ± 0.0[a]	10.3 ± 0.1[a]	10.1 ± 0.0[a]	9.7 ± 0.1[a]
18:1n-7	2.8 ± 0.0[a]	2.8 ± 0.0[a]	2.8 ± 0.0[a]	2.3 ± 0.5[a]	3.0 ± 0.0[a]
18:2n-6	2.8 ± 0.0[a]	2.9 ± 0.0[a]	2.8 ± 0.0[a]	2.9 ± 0.1[a]	2.8 ± 0.0[a]
20:1n-9	1.9 ± 0.0[a]	1.9 ± 0.0[a]	1.9 ± 0.0[a]	1.9 ± 0.0[a]	1.3 ± 0.1[b]
20:4n-6	1.3 ± 0.0[a]	1.3 ± 0.0[a]	1.3 ± 0.0[a]	1.3 ± 0.0[a]	1.2 ± 0.0[b]
20:5n-3	13.0 ± 0.0[b]	13.0 ± 0.2[b]	13.1 ± 0.0[b]	13.0 ± 0.0[b]	16.4 ± 0.2[a]
22:1n-11	0.04 ± 0.0[a]	0.04 ± 0.0[a]	0.05 ± 0.0[a]	0.04 ± 0.0[a]	0.0 ± 0.0[b]
22:5n-6	0.5 ± 0.0[a]	0.5 ± 0.0[a]	0.5 ± 0.0[a]	0.5 ± 0.0[a]	0.4 ± 0.0[a]
22:6n-3	12.2 ± 0.0[a]	12.2 ± 0.2[a]	12.3 ± 0.0[a]	12.1 ± 0.1[a]	10.2 ± 0.0[b]
Unknowns	2.5 ± 0.3	1.9 ± 0.4	2.8 ± 0.3	3.5± 0.1	2.2 ± 0.5
Σ saturates	29.6 ± 0.2[a]	30.4 ± 0.3[a]	29.3 ± 0.2[a]	29.4 ± 0.1[a]	27.9 ± 0.4[b]
Σ monounsaturates	24.5 ± 0.1[a]	24.4 ± 0.4[a]	24.3 ± 0.2[a]	23.8 ± 0.4[a]	23.5 ± 0.2[a]
Σ polyunsaturates	43.3 ± 0.0[b]	43.3 ± 0.4[b]	43.6 ± 0.1[b]	43.3 ± 0.1[b]	46.4 ± 0.3[a]
Σ n-3	32.1 ± 0.0[b]	32.1 ± 0.4[b]	32.3 ± 0.0[b]	32.0 ± 0.1[b]	33.9 ± 0.2[a]
Σ n-6	5.9 ± 0.0[a]	5.9 ± 0.0[a]	5.9 ± 0.1[a]	5.9 ± 0.1[a]	5.7 ± 0.0[a]
DHA/EPA	0.9 ± 0.0[a]	0.9 ± 0.0[a]	0.9 ± 0.0[a]	0.9 ± 0.0[a]	0.6 ± 0.0[b]

703
704 [1] Values presented as area percent of FAME (mean ± S.E. of three replicates)
705 [2] Values in the same row containing different letter superscripts were significantly
706 different (P-value < 0.05)

Table 5

The alphanumeric dichotomous key of the vertebral regions and considered abnormalities used to classify skeletal abnormalities in juvenile Atlantic halibut.

Region	
1. cephalic region	**3.** hemal region
2. prehemal region	**4.** caudal region
Abnormalities	
A. lordosis	**J.** absent neural spine
B. scoliosis	**K.** caudal shifting of neural arch
C. vertebral body fusion	**L.** abnormal hemal spine shape
D. compressed vertebrae	**M.** bifurcated hemal spine
E. hypertrophic vertebrae	**N.** supernumerary hemal spine
F. abnormal neural spine shape	**O.** detached hemal spine
G. bifurcated neural spine	**P.** absent hemal spine
H. supernumerary neural spine	**Q.** caudal shifting of hemal arch
I. detached neural spine	

712 **Table 6**
713 Feed efficiency, growth, survival, condition factor, hepatosomatic index and hematocrit
714 of juvenile Atlantic halibut fed different experimental diets for 14 weeks [1].
715

	Diet				
	Control	**Low Phosphorus**	**Low Vitamin C**	**High Vitamin A**	**Oxidized oil**
Weight/fish (g/fish)[2]	41.6±4.14[a]	35.0±2.64[a]	27.4±0.14[b]	41.4±3.49[a]	40.1±2.42[a]
Weight gain (%)[5]	802.9±51.82[a]	658.0±33.10[a]	493.2±1.75[b]	796.9±43.77[a]	770.6±30.29[a]
FCR[3]	0.5±0.00[a]	0.5±0.01[a]	0.5±0.01[a]	0.5±0.02[a]	0.5±0.01[a]
SGR (%)[4]	2.2±0.02[a]	2.1±0.05[a]	1.8±0.01[b]	2.3±0.04[a]	2.2±0.05[a]
Survival (%)	97.3[a]	95.0[a]	84.9[b]	96.8[a]	97.7[a]
CF (g cm $^{-3}$)[6]	1.3±0.02[a]	1.3±0.02[a]	1.3±0.01[a]	1.3±0.02[a]	1.3±0.01[a]
HSI (%)[7]	1.6±0.16[a]	1.7±0.05[a]	1.3±0.05[a]	1.4±0.04[a]	1.4±0.04[a]
Hct (%)[8]	34.3±0.54[a]	29.9±0.84[b]	26.2±0.87[c]	34.3±0.28[a]	35.0±0.20[a]

716 [1] Values in the same row containing different letter superscripts were significantly
717 different (P-value < 0.05).
718 [2] Initial weight/fish = 4.61±0.05g
719 [3] Specific growth rate (%; SGR) = 100 X [ln(final weight) – ln (initial weight)] / Duration
720 (days); n=3 tanks/diet (P=0)
721 [4] Feed conversion ratio (FCR) = dry feed intake (g) / wet weight gain (g); n=3 tanks/diet
722 (P=0.196)
723 [5] Weight gain (%) – n=3 tanks/diet (P=0)
724 [6] Condition factor (CF) = 100 X [body weight (g) / (total length (cm)) ^{3}]; CF – n=21/diet
725 (P=0.359); CF of initial fish was 1.15±0.02
726 [7] Hepatosomatic index (%; HSI) = 100 X (wet liver weight (g) / body weight (g)); initial
727 fish HSI was 2.5 ± 0.4; n=21/diet (P=0.002)
728 [8] Hematocrit (%; Hct) – n=9/diet (P=0)

729 **Table 7**
730 Liver lipid, tissue ascorbic acid concentration and TBARS values and vertebral ash and
731 phosphorus content in juvenile Atlantic halibut fed various experimental diets for 14
732 weeks [1].
733

	Diet				
	Control	**Low Phosphorus**	**Low Vitamin C**	**High Vitamin A**	**Oxidized Oil**
Liver lipid (%)[2]	11.22±0.60[a]	11.27±1.14[a]	7.04±0.84[b]	11.63±0.90[a]	12.18±1.20[a]
Liver ascorbic acid (μg g^{-1} liver)[7]	75.89±3.33[a]	72.93±3.64[a]	22.81±1.77[b]	69.75±2.42[a]	50.02±8.07[c]
Kidney ascorbic acid (μg g^{-1} kidney)[8]	66.43±4.80[a]	62.35±3.54[a]	11.67±3.02[b]	68.93±6.57[a]	71.31±6.18[a]
Liver retinol (μg g^{-1} liver)[9]	5.60±0.81[a]	5.17±0.22[a]	4.06±0.67[a]	11.37±0.73[b]	5.58±0.17[a]
Liver α-tocopherol (μg g^{-1} liver)[10]	356.1±48.7[a]	235.9±29.6[a]	247.3±31.8[a]	372.6±41.2[a]	344.9±32.1[a]
TBARS liver (nmol g^{-1} liver)[5]	24.99±0.88[a]	22.94±0.45[a]	24.07±1.39[a]	24.02±1.30[a]	44.34±3.28[b]
TBARS muscle (nmol g^{-1} muscle)[6]	0.62±0.03[a]	0.57±0.05[a]	0.57±0.05[a]	0.63±0.04[a]	0.80±0.02[b]
Vertebral ash, % (DM basis)[3]	47.78±1.58[a]	31.68±0.93[b]	49.90±1.53[a]	51.21±2.03[a]	48.64±1.73[a]
Vertebral P, % (DM basis)[4]	11.46±0.27[a]	9.22±0.20[b]	11.29±0.12[a]	11.19±0.20[a]	12.27±0.48[a]

734 [1]Values in the same row containing different letter superscripts were significantly
735 different (P-value < 0.05).
736 [2] % liver lipid, n=16/diet; Initial % liver lipid: 11.98±0.32 (n=3)
737 [3] Percent vertebral phosphorus (on DM basis), n=8/diet; Initial % vertebral P –
738 10.79±0.18 (n=3)
739 [4] Final TBARS liver concentration, n=8/diet
740 [5] Final TBARS muscle concentration, n=8/diet

741 [6] Final AA liver on wet weight basis, n=8/diet; Initial liver: 83.60±1.14 (n=3)
742 [7] Final AA kidney on wet weight basis, n=8/diet
743 [8] Final retinol liver on wet weight basis, n=8/diet; Initial liver: 5.63±1.05 (n=3)
744 [9] Final α-tocopherol liver on wet weight basis, n=8/diet; Initial liver: 218.8±35.5 (n=3)
745 [10] Percent vertebral ash (on DM basis), n=8/diet

746 **Table 8**
747 Fatty acid composition of liver lipid of juvenile Atlantic halibut fed various experimental
748 diets for 14 weeks.[1,2]
749

Fatty Acid	Diet				
	Control	Low Phosphorus	Low Vitamin C	High Vitamin A	Oxidized oils
14:0	3.7±0.2^{a}	3.6±0.1^{a}	2.6±0.2^{b}	4.2±0.2^{a}	3.7±0.2^{a}
16:0	12.7±0.4^{a}	12.0±0.3^{a}	15.2±0.4^{b}	13.8±0.3^{a}	12.5±0.4^{a}
16:1n-7	7.8±0.4^{a}	7.7±0.4^{a}	4.8±0.4^{b}	8.5±0.2^{a}	7.9±0.4^{a}
18:0	3.6±0.4^{a}	4.2±0.3^{a}	4.6±0.3^{a}	3.3±0.2^{a}	4.0±0.3^{a}
18:1n-9	12.9±1.0^{a}	15.6±1.3^{b}	7.3±0.7^{c}	13.0±0.6^{a}	15.4±1.4^{d}
18:1n-7	5.3±0.4^{a}	4.8±0.3^{a}	5.1±0.3^{a}	5.4±0.4^{a}	4.0±0.8^{a}
18:2n-6	3.1±0.2^{a}	2.8±0.1^{a}	2.2±0.1^{b}	3.5±0.1^{a}	3.0±0.1^{a}
18:3n-3	0.7±0.0^{a}	0.5±0.1^{b}	0.5±0.0^{b}	0.8±0.0^{a}	0.6±0.0^{b}
20:1n-9	1.3±0.1^{a}	1.6±0.1^{a}	0.9±0.1^{b}	1.4±0.0^{a}	1.5±0.2^{a}
20:4n-6	3.1±0.2^{a}	2.7±0.1^{a}	3.5±0.2^{a}	3.1±0.2^{a}	2.4±0.2^{b}
20:5n-3	12.8±0.4^{a}	11.9±0.6^{a}	12.2±0.4^{a}	11.0±0.4^{b}	14.0±0.8^{a}
22:1n-11	0.4±0.0^{a}	0.8±0.2^{a}	0.3±0.1^{a}	0.4±0.0^{a}	0.5±0.1^{a}
22:6n-3	14.4±0.9^{b}	13.2±0.5^{b}	16.6±0.9^{a}	13.4±0.6^{b}	11.8±1.8^{c}
Unknowns	1.4±0.5	1.6±0.5	1.6±0.3	1.6±0.4	1.9±0.6
Σ saturates	22.4±0.7^{a}	22.1±1.5^{a}	25.3±1.5^{a}	23.3±0.5^{a}	21.8±0.7^{a}
Σ monounsaturates	29.9±1.2^{a}	32.4±1.9^{a}	20.4±1.2^{b}	31.1±0.7^{a}	31.1±1.8^{a}
Σ polyunsaturates	46.3±0.9^{b}	43.9±0.6^{b}	52.7±0.8^{a}	44.0±0.8^{b}	45.2±1.7^{b}
Σ n-3	34.4±0.9^{b}	31.9±0.7^{c}	42.4±0.7^{a}	31.5±0.9^{c}	34.2±1.8^{b}
Σ n-6	8.8±0.2^{a}	8.4±0.3^{a}	8.0±0.3^{a}	9.3±0.2^{a}	7.8±0.3^{a}
DHA/EPA	1.1±0.1^{b}	1.1±0.1^{b}	2.0±0.1^{a}	1.2±0.1^{b}	0.8±0.1^{c}

750
751 [1] Data presented as percent area of FAME
752 [2] Vales presented as mean ± SE (n=8) with a different superscripts in the same row
753 indicates significant differences at P-value<0.05.
754

Table 9

Meristic character[1] for juvenile Atlantic halibut fed five experimental diets for 14 weeks.

	Diet				
	Control (n=16)	**Low Phosphorus (n=16)**	**Low Vitamin C (n=16)**	**High Vitamin A (n=16)**	**Oxidized oil (n=16)**
No. cephalic vertebrae	3.86±0.10	3.79±0.11	3.86±0.10	3.79±0.11	3.79±0.11
No. prehemal vertebrae	11.86±0.10	12.00±0.00	12.14±0.14	12.14±0.10	11.79±0.19
No. hemal vertebrae	31.14±0.23	31.00±0.18	31.00±0.26	31.29±0.22	30.86±0.29
No. caudal vertebrae	3.86±0.10	3.57±0.14	3.50 ±0.14	3.57±0.14	3.79±0.11
No. total vertebrae	50.71 ±0.34	50.36±0.17	50.50±0.31	50.79±0.24	50.21±0.33

[1]Mean ± standard error

Table 10

The number of abnormalities (No.), percent frequencies of abnormalities (FA) and number of fish affected by each type of abnormality in fish fed five experimental diets for 14 weeks.[1]

	Diet													
	Control (n=10)			Low Phosphorus (n=10)			Low Vitamin C (n=10)			High Vitamin A (n=10)			Oxidized oil (n=10)	
Abnormality	No.	FA	No. of Fish	No.	FA	No. of Fish	No.	FA	No. of Fish	No.	FA	No. of Fish	No.	FA	No. of Fish
A.	0	0	0	3	0.67	1	0	0	0	0	0	0	12	8.63	1
B.	0	0	0	63	13.97	5	25	59.52	3	79	92.94	6	120	86.33	7
C.	0	0	0	0	0	0	4	9.52	2	2	2.35	1	3	2.16	1
D.	0	0	0	0	0	0	4	9.52	1	4	4.71	2	0	0	0
E.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F.	0	0	0	197	43.68	5	0	0	0	0	0	0	0	0	0
G.	3	100	2	2	0.44	2	5	11.90	4	0	0	0	3	2.16	3
H.	0	0	0	0	0	0	4	9.52	3	0	0	0	1	0.72	1
I.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
J.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
K.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L.	0	0	0	186	41.24	5	0	0	0	0	0	0	0	0	0
M.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
O.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
P.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Q.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total abnormalities	3			451			42			85			139		
Vertebral element abnormality	3	100		66	14.63		33	78.57		85	100		135	97.12	
Neural element abnormality	0	0		199	44.12		9	21.43		0	0		4	2.88	
Hemal element abnormality	0	0		186	41.24		0	0		0	0		0	0	

[1] FA was determined by dividing the number of times each abnormality was observed by the sum of the total abnormalities observed and multiplied by 100