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PII: S0044-8486(17)31985-3
DOI: doi:10.1016/j.aquaculture.2018.05.047
Reference: AQUA 633276

To appear in: *aquaculture*

Received date: 9 October 2017
Revised date: 3 March 2018
Accepted date: 28 May 2018

Please cite this article as: S. Albrektsen, T.K. Østbye, M. Pedersen, E. Ytteborg, B. Ruyter, T. Ytrestøyl , Dietary impacts of acid extracted soluble fish bone compounds on astaxanthin utilization and muscle quality in Atlantic salmon (Salmo salar). Aqua (2017), doi:10.1016/j.aquaculture.2018.05.047

Dietary impacts of acid extracted soluble fish bone compounds on astaxanthin utilization and muscle quality in Atlantic salmon (*Salmo salar*)

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Abstract

The aim of this study was to evaluate how a fish bone hydrolysate (FBH) from blue whiting (*Micromesistius poutassou*) fish bones may improve muscle astaxanthin (Ax) deposition in Atlantic salmon (*Salmo salar*). Fish (1.7 kg) were reared in 12 net-pens and fed with one of four practical formulated diets for 78 days. All diets (D) were balanced to meet dietary phosphorus (P) requirement (8 g kg^{-1} P): the control diet (D1) and diet D4 were added $CaPO_4$ as P source, diets D2 and D3 were added P from a spray dried FBH ingredient at 2.1 and 4.2 %, respectively, and D2 was further balanced with $CaPO_4$. Diet D4 was added K_2SO_4 to study potential impacts of sulfate in H_2SO_4 used for mineral hydrolysis. An *in vitro* cell culture trial with hepatocytes (600 g salmon) was performed to study the effects of FBH on Ax uptake and transport. The soluble fish bone compounds significantly increased specific muscle Ax retention (mg kg^{-1} weight gain) by 35 % ($P < 0.05$) in fish fed D3, possibly explained by improved Ax digestibility (ns) followed by increased circulating Ax and a tendency towards increased tissue Ax deposition (ns). Reduced metabolic turnover of Ax was indicated, with about 10 % more of absorbed Ax retained in muscle of fish fed D3. The *in vitro* trial showed no direct impact of FBH on hepatic Ax uptake. However, the FBH ingredient altered the muscle connective tissue by stimulating the production of proteoglycans and their sulfated glycosaminoglycans (GAGs) significantly. This study suggests that hydrolysed compounds from fish bones may improve Ax utilization in salmon, and that they may have an impact on the functional properties of the muscle.

Keywords: Atlantic salmon; astaxanthin utilization; soluble fish bone compounds; fish nutrition

1 Introduction

The market value of Atlantic salmon (*Salmo salar*) products is closely related to the pink flesh pigmentation (Steine et al., 2005; Alfnes et al., 2006). Normal or above normal fillet redness is therefore essential for consumer acceptance. However, it is occasionally difficult to acquire satisfying fillet pigmentation, which is set by the industry to 6-7 mg carotenoid kg^{-1}. In salmonids, digestive and absorptive processes, and metabolic turnover of Ax, influence utilization and flesh deposition of carotenoids (Torrissen et al., 1989; Bjerkeng et al., 1992; Bjerkeng 2008; Ytrestøyl et al., 2004, 2005, 2006). Usually, less than 10 % of ingested Ax is efficiently retained in the flesh of Atlantic salmon (Torrissen et al., 1989; Wathne et al., 1998). A low Ax deposition rate in the flesh may be a result of low intestinal Ax uptake or high metabolic turnover of digested Ax. The carotenoid retention vary considerably during the seawater production phase (Torrissen et al., 1995; Mørkøre and Rørvik 2001; Nordgården et al., 2003; Ytrestøyl et al., 2008) and there are indications of a higher Ax turnover during periods with increased oxidative stress (Nordgården et al., 2003). In addition, Ax may also be used as a powerful antioxidant in biological reaction systems (Camera et al., 2008; Goswami et al., 2010) and as a precursor of vitamin A in salmonid fishes (Schiedt et al., 1985; Guillou et al., 1989).

In Atlantic salmon, phosphorus (P) in fish bone hydrolysate (FBH) produced by acid hydrolysis of blue whiting (*Micromesistius poutassou*) and Atlantic herring (*Clupea harengus*) fish bones, was highly available and covered the P requirement for growth, mineralization and skeletal development (Albrektsen et al., 2013, 2017; Ytteborg et al., 2016). The fish bone ingredients also improved nutrient digestibility (Albrektsen et al., 2013) and significantly increased plasma, liver and whole body Ax concentration in A. salmon postsmolt (un-published data). The lipid digestion in salmon was correlated to dietary P contents

(Albrektsen et al., 2017), possibly explaining the increased growth. As dietary Ax is lipid-soluble and absorbed and transported by the same mechanisms as dietary lipids, the improved pigmentation may be explained by improved lipid digestion. The fish bone ingredients contain a range of macro and micro-minerals, bone proteins/collagens, amino acids, non-protein N compounds, glycoproteins and other soluble compounds, and it is possible that some of these compounds may be involved in the regulation of Ax uptake, transport and metabolism. The amino acid profile of the fish bone ingredients from blue whiting confirms presence of bone collagen proteins with characteristic high levels of Hydroxyproline (Hyp), Proline (Pro) and Glycine (Gly) (Toppe et al., 2007). Some of these non-essential amino acids has improved growth (Aksnes et al., 2008) and/or muscle quality (Albrektsen et al., 2010; Larsson et al., 2014; Østbye et al., 2017a) in Atlantic salmon. A changed composition of the connective tissue compounds in skeletal muscle may be of importance for the textural properties and quality of the flesh (Moreno et al., 2012; Tingbo et al., 2005; Torgersen et al., 2013).

In vitro studies have shown positive impacts of fish bone ingredients on hepatocytes by down-regulation of genes involved in stress and inflammation, and up-regulation of genes involved in the energy metabolism, suggesting potential health effects (Østbye et al., 2017b). In this study we wanted to investigate if the soluble compounds in the fish bone ingredients could affect factors regulating energy metabolism, since this process is also closely connected to the formation of reactive oxygen species and inflammatory stress responses. We have previously shown that increased oxidative stress in salmon can induce apoptosis, reduce ß-oxidation, damage the mitochondria membrane and change the muscle structure (Kjær et al., 2008; Todorcevic et al., 2009; Østbye et al., 2011).

The main aim of the present study was to evaluate possible impacts of fish bone ingredients on pigment utilization in large, close to slaughter size salmon. This was evaluated by measuring dietary impacts on growth, feed intake, feed conversion, Ax digestibility, Ax retention, and specific Ax contents in muscle, liver and plasma in salmon. Dietary impacts of the fish bone ingredients on connective tissue composition in skeletal muscle, a parameter known to influence fish fillet quality, was analysed by FT-IR spectroscopy. An *in vitro* study with hepatocytes from salmon was performed to study effects of the fish bone ingredients on genetic markers for uptake and transport of Ax at the cellular level, to reveal the mechanism of improved Ax utilization. Genetic markers of health and inflammation was included both *in vivo* and *in vitro*.

2 Materials and methods

2.1 Fish bone hydrolysate (FBH) production

A fish bone meal was separated from a commercial produced blue whiting fish meal (Karmsund Fishmeal, AS, Avaldsnes, Norway) by sieving (4.6 mm). The sifted fish bone meal contained 41.5 % ash and was mixed with water (1:5 w/w) in 100 L plastic barrels, added 95 % H_2SO_4 (5 % w/w) and hydrolysed (18h) at room temperature (18-20°C) to solubilize the minerals from hydroxyapatite. The soluble fraction was manually separated from the solids by sieving over a fine mesh cloth (70 µm). The solid phase was washed twice with cold water to collect all soluble compounds. The liquid phase was adjusted to pH = 2.1 with NH_3 –solution (25 % w/w) by titration, and to a final pH = 2.8 with KOH (33 % w/w), and then stored overnight to precipitate solid material. Floating lipids were manually removed, and the clear liquid was pumped into a 100 L steel kettle, and sterilized by heating to 90 °C for 5 min. The solid material contained some liquid that was collected by ultra-centrifugation (10.000 rpm, 10 min) prior to sterilization. The liquid was concentrated by evaporation (33 °Brix) and spray dried (Niro Atomizer, Denmark) into a powder (20 – 60 µm). About 20 kg FBH was produced. The P content in FBH was 9.6 % total P and 9.5 % soluble P, which make it suitable as alternative P source (Albrektsen et al., 2017). The chemical composition is presented in Results (3.1).

2.2 Experimental diets

Four experimental diets were produced at Nofima's Aquafeed Technology Centre, Bergen, Norway. The FBH ingredient was added as an alternative P source at two dietary levels, equivalent to 2 and 4 g P kg^{-1} diet. All experimental diets were balanced to meet the requirement of available P (8 g kg^{-1}), NRC (2011), as the main aim was to study potential

pigment enhancing effects of the ingredient that we expect is not explained by dietary P. The basal diet (D1) contained 4 g kg^{-1} soluble P, and was added 4 g kg^{-1} P from either $CaPO_4$ (D1, D4) or from FBH (D2, D3). In D2, FBH provided 2 g kg^{-1} P and was added another 2 g kg^{-1} P from $CaPO_4$. Diet D4 was supplemented with K_2SO_4 to evaluate possible impacts of potassium (K) in KOH used to adjust pH and SO_4 in sulfuric acid used for acid hydrolysis. The feed formulation reflected composition of a commercial diet for Atlantic salmon > 1.5 kg. The basal diet (D1) contained 15 % fish meal (Karmsund fishmeal, AS, Norway), and the main plant ingredients was pea protein concentrate (Agrimarin Nutrition, Stavanger, Norway), SPC (Imcopa, Hertogenbosch, The Netherland) and wheat gluten (Amygluten 180, Syral Belgium N.V., Aalst, Belgium), Table 1. Betafin was added as choline source and soybean based lecithin (Agrosom, Mölln, Germany) as phospholipid source due to low fish meal inclusion. Yttrium oxide was added to the feed as an inert marker at a level of 0.2 g kg^{-1}. The experimental diets were all supplemented with synthetically produced free Ax in Carophyll Pink (CP 10 % (80 % All-E, 5 % 9-Z, 15 % 13-Z), DSM Nutritional Products, France SAS) at 55 mg kg^{-1}. The experimental diets were balanced to meet the requirements of essential amino acids (Lys, Thr), NRC (2011). All diets were extruded in a Wenger TX-52 co-rotating twin-screw extruder with die size 4.5 mm in diameter. The diets were stored frozen at -20 °C during the trial, in order to avoid loss of Ax.

2.3 Experimental fish trial

The experiment was carried out in 12 experimental net-pens at Nofima's aquaculture research station at Averøy, Norway (63° 03′N, 7° 35′E). At the start of the experiment, the salmon (1.7 kg ± 0.01 kg) were randomly distributed to twelve 5 m x 5 m x 5 m (125 m^3) cages, each holding 55 fish. The fish were fed one of four experimental diets, in triplicate net-pens for a period of 78 days. In the 4 week acclimatization prior to the trial, the fish were stocked in one

7 m x 7 m x 5 m (245 m^3) cage and fed a pigment-free diet (similar to D1) with ~2.4 mg Ax kg^{-1}. The fish was fed commercial Skretting feeds during transfer and prior to the acclimatization at Averøy. The salmon were from the Salmobreed strain and was delivered as 0^+ smolts from Nofima's aquaculture research station, Sunndalsøra, Norway.

The fish were fed three 30 min meals daily by feeding automates (Betten Maskinstasjon AS, Vagland, Norway). Unconsumed feed were collected in a plastic tarpaulin connected under the net-pen, pumped back to the surface through a plastic pipe using pressurised air, collected in a fine meshed sieve and the pellet weights recorded. Recovery tests were done with each diet in empty net-pens after finishing the trial, to adjust for nutrient loss in the wet feed-waste (Helland et al., 1996). Pumping of waste feed from the bottom cone in each cage started 4 min after termination of the meal and collection lasted for 4 min. Based on the feed intake from the previous 2–3 days, and unconsumed feed collected, the feeding rations were adjusted to about 10 % in excess of fish appetite. The fish were exposed to ambient temperature and light regimes during the trial. The water temperature was measured daily and decreased throughout the trial. Average temperatures measured at 2 week intervals were 9.1, 8.1, 7.4, 5.8, 4.7 and 4.6 °C, respectively in the trial period from October to January, and average temperature in the total feeding period was 6.8 ± 1.7 °C. The ambient temperature decreased below 5 °C in the last 4 weeks of the trial and this had a negative influence on feed intake in fish.

At the start of the experiment, the salmon were dip-netted from the 7 x 7 cages, anaesthetized in Finquel (Western Chemical Inc., Scottsdale, USA, 0.35 gL^{-1}) and the individual weights (nearest 10 g) and fork lengths (nearest 0.5 cm) were measured. Fish with weights in the range of 1.5 to 1.9 kg were collected and used as experimental fish. Three samples of 10 fish

were collected for analyses of flesh pigmentation and lipid content at start of the trial. Two samples of 10 fish were sampled prior to the Ax depletion period.

At the end of the trial, continuously fed fish from each cage was anaesthetised in Finquel (Western Chemical Inc., Scottsdale, USA, 0.35 g L^{-1}) in an outdoor tank supplied with running seawater. Pooled samples of approximately 30 - 40 g of faeces (w/w) were collected by manual stripping (20-30 fish), and the samples immediately frozen at -20°C for analyses of protein, lipid and Ax digestibility. Ten fish from each cage were transported to a nearby processing room and blood withdrawn from *Vena caudalis* (5 ml heparinized syringes, 5000 IE heparin). Individual blood samples were kept refrigerated on ice (4 ° C) and centrifuged at 4000 rpm/6 min. The plasma samples were collected and stored in dry ice (-80°C) before transport and storage at -30 °C for analyses of plasma Ax at the Nofima laboratory, Sunndalsøra. Following blood sampling, the fish was killed with a blow to the head, gutted, the sex registered (mature fish was not included) and fish measured for weight, fork length, gutted weight and liver weight, and for calculation of condition factor (C-factor), hepatosomatic index (HSI) and dress out percentage (% DOP). Both sides of the fillet was collected, the left half side was used for visual (Salmofan), colorimetric (Photobox) and chemical (Ax) measurements, and for calculation of muscle Ax retention (ret) according to the following formulas (BW_2 = final body weight, BW_1 = initial body weight; Ax_2 = final Ax concentration, Ax_1 = initial Ax concentration): 1) mg Ax ret = 0.61 x $[(BW_2 \cdot Ax_2) – (BW_1 \cdot Ax_1)]$; 2) % of Ax consumed = mg Ax ret x 100/mg Ax consumed; 3) mg Ax kg^{-1} weight gain = mg Ax ret/(BW_2-BW_1); 4) % of dietary Ax absorbed = % Ax consumed x 100 / ADC_{Ax}. Muscle Ax retention was calculated as follows: Whole body Ax x 0.61 according to Wathne et al. (1998). The right half side of fillet and liver (n = 10) were immediately frozen at -30°C. Pooled samples of liver and muscle were analysed for lipid, dry matter, Ax, vitamin E and

lipid peroxidation (TBARS). Small liver and muscle samples collected for gene expression analysis, TBARS and antioxidant enzyme essays (superoxide dismutase (SOD), gluthation peroxidase (GPX) and catalase) were snap frozen in liquid nitrogen (N) and stored at -80 °C, n=5 per cage. Small samples of muscle were also snap frozen in liquid N for FT-IR spectroscopy, n=4 per cage. Pooled samples of vertebrae (n=5 per cage) were collected according to the procedure described in Albrektsen et al. (2017) for analyses of bone ash and mineral contents.

Growth and feed conversion ratio were determined according to the following formulas (BW2 = final body weight, BW1 = initial body weight): Specific growth rate, SGR = (ln BW2 - ln BW1) / feeding days. Thermal growth coefficient, TGC = ($BW2^{1/3}$ - $BW1^{1/3}$) * 1000 /∑ (temp.(°C) * feeding days) according to Cho (1992). Feed conversion ratio (FCR) = g dry feed eaten / g live weight gain.

During the last 3 weeks of the trial, mortality increased in all cages apparently without any signs of wounds or infections. The fish were diagnosed with cardiomyopathy syndrome (CMS), an infection induced by a virus, *piscine myocarditis*, that apparently have no impacts on feed intake or growth, however it cause severe damage to the heart and produce high mortality (Haugland et al., 2011). Potential impacts of *piscine m.* on pigmentation, antioxidant enzymes and TBARS in CMS infected fish is difficult to predict. The heart from each individual sampled fish (n=10) was therefore collected for gene expression analyses of *piscine m.* virus (PMCV). Individuals with relative gene expression level of PMCV > 20 were discarded, while individuals with relative PMCV > 10 were not used for the molecular analyses and TBARS.

2.4 Chemical analyses

All chemical analyses were carried out in duplicate by accredited laboratories at Nofima. In feed ingredients, diets, faeces and fish tissues, crude protein (N x 6.25) was determined by the Kjeldahl method (ISO 5983-1997), moisture gravimetrically after drying for 4.5 hours at 103 ± 1 °C (ISO 6496-1999), and ash after combustion for 16 hour at 550°C ± 20 °C (ISO 5984-2002). Lipid contents in feed and faeces were determined by respectively acid hydrolysis («EU-lipid»), (Commission Directive 98/64/EC, Part B) and by Folch extraction with acid hydrolysis (SSF-report: A-102, 1978). The lipid contents in liver and muscle were determined by Folch. Total and free Ax in the experimental feeds during storage were analyzed according to a HPLC (high performance liquid chromatography) method developed by Hoffman La. Roche (1994) following ethanol and dichloride methane extraction of Ax from the experimental diets. Carophyll Pink in the experimental diets was enzymatically treated in hot water prior to the extraction procedure. The pooled homogenized muscle, liver and faeces samples were thawed, and carotenoids were extracted from samples of accurately weighed minced salmon tissue using a 1:1:3 mixture of distilled water, methanol (containing 500 mg l^{-1} BHT), and chloroform according to Bjerkeng et al. (1997). Blood plasma samples (1.0 ml) was added methanol (1.0 ml; containing 500 ppm butylated hydroxytoluene, BHT), mixed (Whirlmixer, Fisons, England), added chloroform (3 ml) before remixed, centrifuged (ca. 1700×*g*, 10 min) and analyzed according to Wathne et al. (1998). External standard methods were used to quantify individual carotenoids and the geometrical *E/Z* isomer composition of Ax in the various samples. Two different isocratic HPLC systems were used to determine carotenoid concentrations in muscle, liver, blood and faeces samples, respectively (Bjerkeng et al., 1997). Yttrium was determined in feed and faeces by inductively coupled plasma atomic emission spectroscopy (ISO 11885-1996). Muscle and liver vitamin E was determined

by HPLC and fluorescence detection after saponification and extraction according to Lie et al. (1994). Total P in ingredients and experimental diets were determined spectrophotometrically (430nm) after ashing and acid digestion in 6 M HCL (ISO 6491-1998). Soluble P was also determined spectrophotometrically according to the same method, however, following incubation of duplicate samples (0.8 g) in 80 mL, 1 N NaOH for 16 h according to the method described in Ruban et al. (2001) and Hua et al. (2005), and later modified and validated by Hovde (2013).

2.5 Visual colour analysis

The visual colour of the fillets were measured using the Roche *Salmo*Fan^{TM} score (Hoffman La-Roche, Basel, Switzerland) and by digital image analysis using a standardized photo box, a digital camera and prediction equations for Ax (mg kg^{-1}), *Salmo*fan score and fat (%) (Folkestad et al., 2008). The left fillet side was placed in a Salmon Colour Box with standardized light conditions and Salmo*Fan* readings were performed immediately after slaughter in 3 positions (upper dorsal, NQC and tail) in the upper region of the fillet by two individual judges. The fillets were then photographed by a digital camera (Dolphin F145C, Allied Vision Technologies, Stadtroda, Germany) in a light proof aluminium box (800 mm × 830 mm × 955 mm) with standardized illumination provided by four fluorescent bulbs (OSRAM Lumilux 55 W, OSRAM, Augsburg, Germany) placed along each of the walls in the upper part of the box, as described (Folkestad et al., 2008). A calibration card, QPcard 101, (QPcard AB, Gothenburg, Sweden) was used for calibration of lightness and white balance. Any variation in illuminance (lux) of the photographed area was corrected.

2.6 Digestibility

After the feeding period of 79 days, fish from each tank were anaesthetized in Finquel (Western Chemical Inc., Scottsdale, USA, 0.35 g L^{-1}), manually stripped for feces (20 - 30 g wet material) and immediately frozen at -30°C. Apparent digestibility coefficients (ADC) of lipid, protein and Ax were determined. Yttrium, lipid, Kjeldahl-N and Ax were analysed in the four experimental diets and in pooled samples of faeces from each replicate net-pen (n=3 per dietary treatment). Samples of frozen faeces were lyophilized (final plate temperature 24 °C) and homogenized prior to chemical analyses. Apparent digestibility coefficient (ADC) of nutrients were calculated according to the formula:

$$ADC = 100 - 100\ x\ \frac{Y_d\ x\ CX_f}{CX_d\ x\ Y_f}$$

Where d is diet, f is faeces, Y is yttrium concentration and CX nutrient concentration.

2.7 Real time qPCR

Total RNA was isolated using RNeasy® Mini Kit and RNase-Free Dnase set (Qiagen, Hilden, Germany) according to the manufacturer's protocol. cDNA was made from 500 ng RNA in a 20 µL reaction volume by using AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies, CA, USA). The cDNA synthesis was run under following conditions: 25 °C for 5 minutes, 42 °C for 45 minutes, and 95 °C for 5 minutes. The reaction mix for qPCR consisted of 4 µl diluted (1:10) cDNA, 1 µl forward and reverse primer (final concentration of 0.5 µM), Table 2, and 5 µl SYBR Green-I Master (Roche Applied Science, Germany). The qPCR reaction was run on a LightCycler®480 (Roche Diagnostics Gmbh, Germany) under the following conditions: 95 °C for 5 minutes, 45 cycles at 95 °C for 15 seconds and 60 °C for 1 minutes. A melting curve analysis was performed to confirm amplification of only one PCR

product. The gene expression level was calculated according to the ΔΔCt method (Pfaffl, 2004), using *nduf* (*in vitro* trial) or *eif3* (*in vivo* trial) as reference genes and by calculation of gene expression in the various treatments relative to the control treatment. The treatment responses was tested for significant differences at a significance level of $P < 0.05$.

2.8 Measurements of antioxidant enzymes

SOD enzyme activity was measured using Superoxide Dismutase Assay Kit (Cayman Chemicals, Ann Arbor, MI, USA) according to the manufacturer's protocol. The enzyme reaction was measured at 450 nm in a Spectrostar Nano plate reader (BMG LABTECH GmbH, Ortenberg). Enzyme activity of GPX was measured using a commercial kit (Cayman Chemicals, Ann Arbor, MI, USA), following the manufacturers' protocol. The reaction was read at 340 nm in a Spectrostar Nano plate reader from BMG LABTECH GmbH (Ortenberg). Catalase enzyme activity was measured according to a method described in Baudhuin et al. (1964). Hydrogen peroxide produced in the peroxisomes, is decomposed by catalase into oxygen and water. The reaction is stopped by addition of a saturated solution (0.45 %) of titanium oxysulfate in 2 M sulfuric acid. Titanium oxysulfate reacts with hydrogen peroxide and produces a yellow solution of peroxy titanium sulfate. This was measured spectrophotometrically at 405 nm in a Spectrostar Nano microplate reader from BMG labtech GmbH (Ortenberg).

2.9 TBARS

Thiobarbituric acid reactive Substances (TBARS) was determined by using a commercial colorimetric assay kit (Cayman Chemical, Ann Arbor, MI, USA) following the manufacturers' protocol. Tissue samples for TBARS analysis were prepared by homogenizing in phosphate-buffered saline (PBS). Hepatocytes were washed once in 1 % albumin in PBS,

and then twice in PBS. Finally, the cells were harvested in 1 mL PBS and centrifuged for 2 minutes at 107 x g and 4 °C. Pelleted cells were resuspended in 250 µl PBS and sonicated three times for 5 seconds on ice. The absorbance at 532 nm was measured in a Spectrostar Nano microplate reader from BMG LabTech GmbH (Ortenberg, Germany).

2.10 Fourier transform infrared spectroscopy (FT-IR)

Muscle tissue cryo sections from fish fed different diets was analysed by FT-IR. An optical IR spotlight 400 microscope (Perkin Elmer) coupled to a Spectrum 400 FT-IR spectrometer (Perkin Elmer, UK) was used to measure the tissue sections. Spectra were collected from different connective tissue regions in the frequency range 4000 to 750 cm^{-1} using a mercury cadmium telluride (MCT) detector, and with spectral resolution of 8 cm^{-1}, 64 scans per pixel and spectral interval of 4 cm^{-1}. A background spectrum of the ZnSe substrate was recorded before each sample measurement in order to account for variation in water vapour and CO_2 level. Second derivative of the spectra were taken applying the Savitzky-Golay algorithm before further preprocessing by extended multiplicative signal corrections (EMSC) in The Unscrambler version 9.2 (Camo Process AS, Oslo, Norway) to remove multiplicative and wavenumber-independent and -dependent baselines. To analyze the main variation in FT-IR absorbance bands of connective tissue between fish fed different diets, data analysis of connective tissue parameters was performed according to Camacho et al. (2001) and principal component analysis (PCA) without further standardization of variables.

2.11 *In vitro* hepatocyte study

To understand the mechanisms of FBH stimulated uptake and transport of Ax at the cellular level, a separate *in vitro* study with hepatocytes was conducted. Atlantic salmon (ca. 600 g) reared at NIVAs Research Station (Drøbak, Norway) were kept in seawater at 10°C and fed a

commercial diet prior to isolation of hepatocytes. The hepatocytes were isolated according to the method of Dannevig and Berg (1985). After isolation, the cells were filtrated through a 100 µM nylon filter. The cells were washed three times in L-15 medium (Sigma-Aldrich, St. Louis, USA) and centrifuged for 2 minutes at 100g between each wash. Finally, the cells were added Glutamax growth media containing 10 % fetal calf serum, 1 % bicarbonate, 5mM Hepes and 1 % PenStrep (Sigma-Aldrich, St. Louis, USA). Approximately 4 x 10^7 cells were seeded in 9.6 cm^2 wells coated with laminin (Merck Millipore, Darmstadt, Germany). Cells were cultured at 13°C overnight and then added the experimental growth media. The experimental growth media consisted of 2 % foetal calf serum, 1 % bicarbonate, 5mM Hepes and 1 % PenStrep, and either FBH, Ax or a combination of FBH + Ax. The concentration of FBH was adjusted to provide 600 µg/mL P to the cells. Ax (Hoffman LaRoche) was complexed with bovine serum albumin (BSA, Sigma Aldrich) and added to the cell culture medium at a concentration of 1.5 mg L^{-1}. Cells were cultured in experimental growth media for 48 hours before harvesting of the cells to the different analysis; including genetic markers for uptake and transport of Ax (Harrison et al., 2012), for stress and inflammation, and for activity of enzymes involved in the antioxidant defense of fish (Østbye et al., 2011; Todorcevic et al., 2009).

2.12 Stability of total astaxanthin during feed production and storage

Stability of free Ax during incremental heating of the mixed feed ingredients prior to extrusion (maximum 88°C, 3 min), extrusion (approximately 120°C, 1 min) of the feed blend, drying of the extruded pellets in a hot air drought (maximum 70°C, 45 min) and during coating with heated oils (45°C, < 2 min) were calculated from the analysed free Ax values, only corrected for variable moisture during feed production. The contents of free Ax in the extruded, dried feeds were corrected for variable lipid levels during coating.

Batches of experimental diets were also stored in cabinets equipped with heating elements to keep the temperature under controlled conditions at 15°C and 25°C during storage. Small samples (100 g) of diets were collected at start and after 4, 8 and 12 weeks of storage (n=2 per feed). All samples were homogenized and analysed within one day for accurate measurements of Ax.

2.13 Statistical analyses

Biological and analytical data were subjected to regression analyses and one-way analysis of variance (ANOVA) using Statistica software (Ver 7.1, StatSoft, Tulsa, OK, USA) and SAS 9.4 software (SAS Institute Inc., Cary, NC, 2002-2012). Significant differences in body weight, growth parameters and feed intake were tested by using Tukey HSD Test with probability $P < 0.05$. Significant differences between diets were otherwise tested by using Duncan's Multiple range Test with the probability of a P value < 0.05 or $P < 0.01$ considered as significant (Zar, 1999). Pearson correlation and Spearman's rank correlation analysis was used to examine possible relationships between feed variables and the dietary responses.

3 Results

3.1 Composition of FBH ingredient and experimental feeds

Total protein content in the hydrolysed fish bone ingredient was 10.8 % (corrected for total volatile nitrogen (TVN=2.7 %)), total sum amino acids 6.2 $g100g^{-1}$ protein, lipid 1.1 %, ash 61.5 %, salt 1.7 % and dry matter 97.9 %. The collagen protein characteristic amino acids Glutamine (Glut), Glycin (Gly), Arginin (Arg), Alanin (Ala) and Prolin (Pro) accounted for 60 % of total protein (Fig. 1). The FBH mineral contents reflected the mineral contents in blue whiting FBH in Albrektsen et al. (2017) and contained 27.1 % potassium (K). The calcium (Ca) level in FBH was low (0.4 %), as most of Ca was precipitated as $CaSO_4$ during processing.

The ingredient composition and the chemical contents of the diets are shown in Table 1. The diets were iso-caloric and the energy content average 26.2 ± 0.2 MJ kg^{-1} on dry matter (DM) basis. The diets contained average 392 ± 2 g kg^{-1} protein and 351 ± 6 g kg^{-1} lipid, respectively. Besides from slightly higher Arg and Gly levels (13-14 %) in diet D3 due to inclusion of FBH, the non-protein N compounds creatinine, taurine and anserine were about 20 – 50 % higher as compared to the control diet (D1), however, accounted for only 0.25 % of diet D3.

Dietary Ax was average 59.2 ± 1.9 mg kg^{-1} (DM) for all diets following pellet extrusion, while the dietary level of soluble P was average 8.7 ± 0.4 g kg^{-1} (DM), Table 1. The low basal level of free Ax in all diets (2.4 ± 0.2 mg kg^{-1}) originated from Ax in the fish oil, and was also present in the "pigment free" diet (formulated as D1) used for depletion of muscle Ax during the 4 week acclimatization period prior to the trial.

3.2 Stability of Ax during feed extrusion and storage

During extrusion, no diet dependent differences in stability of total Ax were found. Dietary total free Ax was average 55.5 ± 1.3 mg/kg following extrusion, and the processing loss was very low, < 5 % and showed no diet dependent differences. Therefore, these data are not presented. During storage of the feeds, dietary total free Ax recovered after 12 weeks at 15 °C was similar, i.e. 87, 88, 82 and 89 % for D1, D2, D3 and D4, respectively, Fig. 1A. At 25 °C, dietary Ax recovered after 12 weeks of storage was 80, 80, 62 and 84 % for D1, D2, D3 and D4, respectively, indicating lower Ax stability in D3, Fig. 1B. The stability of dietary all-E and Z-isomers closely resembled free Ax stability in all diets, and are therefore not presented.

3.3 Growth and feed utilization

During the feeding trial the fish showed a high growth rate relative to the low water temperature, with an average specific growth rate (SGR) of 0.52 ± 0.03 % and thermal growth coefficient (TGC) of 3.19 ± 0.22 for all diets, Table 3. The fish increased the body weights from 1.7 to 2.5 kg during the feeding trial. The final weights and growth measurements was not significantly different between experimental diets, $P > 0.05$, Table 3. However, fish fed D3 and D4 both improved SGR by about 4% and showed lower in between net-pen variation as compared to D1 and D2. In fish fed the K_2SO_4 supplemented diet (D4), the feed intake (%/BW/d) was significantly increased by 17 %, $P < 0.05$. The increased feed intake was not reflected in increased growth, thereby increasing FCR by about 12 % ($P < 0.05$). No obvious explanation for the feed intake stimulation in fish fed D4 has been found.

3.4 Mortality

Total mortality in the experiment ranged from 9 to 14 % (Table 3) and started to appear after about 8 weeks of feeding in all dietary groups. The fish was diagnosed with cardiomyopathic

syndrome (CMS), a common virus infection in Norway (Haugland et al., 2011). The mortality showed no diet dependent differences ($P > 0.05$), but the mortality was lowest in fish fed D3.

3.5 Nutrient digestibility, Ax retention and tissue Ax composition

At the end of the trial, the feed intake was low in the fish reared at temperatures below 5 °C, and in some fish, the faeces content was low or absent. The digestibility measurements varied between tanks, and in D4, one sample regarded as an outlier was omitted from the data set. The apparent digestibility of protein, lipid and Ax was not significantly different between dietary groups ($P > 0.05$), Table 4. However, apparent Ax digestibility was increased about 18 % in fish fed D3 (FBH, 4 %), Fig. 2, as compared to the control diet (D1), and a tendency towards lower levels of both Ax trans and Ax cis isomers in faeces was observed ($P > 0.05$). Plasma, liver and muscle Ax in fish fed D3 were non-significantly increased by 22, 30 and 7 %, respectively, compared to fish fed D1 ($P > 0.05$), Fig. 2. Muscle Ax retention was significantly increased ($P < 0.05$) by 40, 35 and 35 %, respectively, when measured as mg Ax retained ($P < 0.05$), % of dietary Ax consumed ($P < 0.05$) and mg Ax kg^{-1} weight gain ($P < 0.05$), Table 4. Muscle Ax retention in fish fed D3 was numerically higher than in fish fed D4, while significant different only when measured as % of Ax consumed ($P < 0.05$). Muscle Ax calculated as % of dietary Ax digested (≈ utilization of Ax absorbed), was weakly increased in fish fed D3 versus D1 ($P > 0.05$).

Plasma and liver Ax correlated ($R = 0.7$, $P < 0.05$), while no such relation was found between plasma and muscle Ax ($P > 0.05$). Plasma and muscle trans Ax accounted for average 92.8 ± 0.8% and 92.0 ± 0.3%, respectively, for all diets. In liver, the relative amount of trans Ax was average 59.3 ± 3.7 % for all diets. The metabolite of Ax, Idoxanthine (Idox), ranged from 3 –

6 % of total carotenoids and was consistently lowest in fish fed FBH (D2 and D3). As no significant differences was found ($P > 0.05$), these data are not presented.

3.6 Visual (Salmofan) and colorimetric (Photobox) flesh pigmentation

Muscle pigmentation evaluated by Salmofan in the upper dorsal, NQC and tail region correlated significantly to muscle Ax concentration ($r > 0.70$, $P < 0.05$). Visual colour measured by Salmofan was average 28.2 ± 0.4 for all groups, and showed no diet dependent differences ($P > 0.05$). Pigmentation measured colorimetric (Photobox) was average 26.2 ± 0.7, and reflected muscle Ax ($R = 0.54$) with 10 % higher muscle Ax in fish fed D3 as compared to D1 (ns).

3.7 Oxidation, inflammation and antioxidant enzymes in salmon

There were tendencies towards higher levels of TBARS in liver and muscle of salmon fed D3 compared to the other dietary treatments, but these changes were not significant ($P > 0.05$), Table 5 and Table 6. However, salmon fed D3 (FBH 4.2 %) showed reduced activity of GPX in liver as compared to salmon fed the control diet (D1) and diet D2 (FBH 2.1 %), Table 6. There was no significant difference in SOD activity between salmon fed Diet 1-3, whereas fish fed D4 supplemented with K_2SO_4 showed lower SOD activity than fish fed D2 (FBH 2.1 %), Table 6. There were no differences in catalase activity between the diets ($P > 0.05$), Table 6.

Gene expression analysis of liver showed small differences in markers for antioxidant defence mechanisms (*gpx3* and *nfe2*) and inflammation (*nfkb*), Table 6. However, salmon fed D3 (FBH 4.2 %) had significantly lower gene expression of *gpx3* compared to salmon fed D2 (FBH 2.1 %). Muscle related genes were only slightly affected by the dietary treatments,

Table 5. Gene expression for the muscle structural protein myosin was significantly down regulated in salmon fed D3 compared to D1 and D2 ($P < 0.05$). No significant differences were detected for the muscle regulatory genes (*myog* and *myod1b*).

3.8 *In vitro* hepatocyte study

The Ax uptake in the hepatic cells measured *in vitro* did not show any significant impact by addition of FBH ($P > 0.05$), but tended to be slightly decreased, Table 7. Salmon hepatocytes cultured in FBH and FBH + Ax showed reduced activity of GPX compared to the control ($P < 0.05$). SOD activity was significantly increased in hepatocytes added FBH + Ax as compared to the control ($P < 0.05$), and SOD was also markedly increased by FBH alone, Table 7. Catalase activity was significantly reduced only by FBH alone ($P < 0.05$). Addition of Ax alone did not change GPX, SOD or catalase activity relative to the control. There was no effect of FBH on gene expression of an inflammatory marker (*nfkb*), but FBH and FBH + Ax significantly increased expression of some markers of the antioxidant defence mechanism (*gpx3* and *nfe2*), and reduced mitochondrial and peroxisomal β-oxidation (*cpt1* and *acox*), Fig. 2A, B. FBH and FBH + Ax also reduced the gene expression level of the Ax receptors (*cd36* and *scarb1*) and Ax transport proteins (*abca1)* significantly, Fig. 2C, 2D.

3.9 Connective tissue of skeletal muscle analysed by FT-IR

Skeletal muscle of fish fed the experimental diets D1 (control), D3 (FBH 4.2 %) and D4 (K_2SO_4) were analysed for connective tissue components by FT-IR. A significant difference in the structural density of the connective tissue, calculated as GAG/amid 1 ratio was detected in fish fed D3 as compared to the control diet (D1), with a significant higher ratio of GAG/amid 1 in fish fed D3, Table 8. This change indicate a higher amount of sulfated GAGs

relative to collagen. The collagen crosslinks ratio (maturation) revealed no significant diet dependent differences ($P > 0.05$).

The main variation in FT-IR absorbance bands of single connective tissue components between fish fed different diets were analysed by principal component analysis (PCA). A tendency of 3 separate groups was observed by comparing the carbohydrate of proteoglycans/glycoproteins, collagen (amid III) and sulfation of proteoglycans/ glycoproteins region in a combined PCA plot (Fig. 3). Along the X-axis (PC-1), explaining 95 % of the variation, diet D3 appeared to group differently from D1 and D4, which may reflect the significant impact on GAG/Amid 1.

3.10 Bone ash and mineral contents

Vertebra ash, P and Ca showed no diet dependent differences ($P > 0.05$). Mean values for all diets were respectively; Ash: 16.6 ± 0.6 %; Ca: 54121 ± 3199 mg kg^{-1} and P: 29509 ±1299 mg kg^{-1}, and the Ca:P ratio was also equal between diets (Ca:P = 1.83 ± 0.05), $P > 0.05$.

4 Discussion

The present study suggest that fish bone ingredients produced from blue whiting may improve the Ax utilization by increasing the intestinal Ax uptake and muscle Ax retention in Atlantic salmon. More extensive improvements in Ax utilization has previously been observed in 0^+-smolt fed acid hydrolysed fish bone compounds, with respectively 55, 29 and 37 % higher plasma, liver and whole body Ax levels (unpublished data). A high Ax deposition rate in the flesh may be a result of increased uptake from the intestine, decreased metabolic turnover of the digested Ax or increased Ax uptake in the tissues. All of these potential altered mechanisms were studied and the results used to understand the physiological impacts of the fish bone ingredients on Ax utilization. The results also showed that the hydrolysed fish bone compounds altered the connective tissue compounds in the muscle of Atlantic salmon. These results are also discussed, as they may be of relevance for flesh quality and texture in salmon.

Absorptive processes in the gut constitute a major limitation for the effective absorption of carotenoids in salmonid fishes. The ADC_{Ax} vary considerably, but typically range from 20 to 60 % (Bjerkeng et al., 1997; Bjerkeng and Berge 2000; Ytrestøyl et al., 2005, 2008). In the present study, the ADC_{Ax} was average 24 % in fish fed D1, D2 and D4, and 29 % in fish fed D3, all below the Ax digestibility of 40 % reported in Atlantic salmon reared at the same location and time of the year (Ytrestøyl at al., 2006, 2008). Ax digestibility is positively correlated to water temperature, and negatively to feed intake and diet concentration (Torrissen et al., 1995; Ytrestøyl et al., 2005, 2006; Rørvik et al., 2010). Despite low water temperature and consequent low ADC_{Ax} values in the present trial, the moderate increase in ADC_{Ax} in fish fed D3 was followed by moderate increased plasma and tissue Ax levels, and significant increased muscle Ax retention. All diets were stored frozen until used, hence no loss of dietary Ax due to reduced storage stability that may otherwise explain the tendency

towards improved Ax digestibility, occurred (Bjerkeng et al., 1997; Østerlie et al., 1999). The results suggests improved Ax utilization in large salmon, although the impact of FBH is less pronounced as compared to the previous observations in smolt. The FBH ingredient contains acid hydrolyzed amino acids characteristic of collagen, one of the most important protein in the connective tissues. In human nutrition, hydrolyzed collagen proteins appear to have a great potential as nutraceutical ingredients, among others by preserving of high quality of skin and bone (Bello and Oesser, 2006). Potential impacts of the acid hydrolysed fish bone compounds on the gut integrity and digestive functions in fish has to be further studied to understand the mechanism behind the tendency towards increased Ax digestibility as indicated in the present study.

The Ax digestibility was measured once, whereas the retention efficiency in the flesh was measured during a period of two months and is therefore a more reliable measure of Ax bioavailability. The muscle retention of Ax is normally less than 10 % of the ingested amount in Atlantic salmon, but may vary from 2 to 15 % depending on fish size, diet concentration, growth rate and water temperature (Torrissen et al., 1989; Wathne et al., 1998; Bjerkeng et al., 1999; Bjerkeng and Berge 2000). In the present study, the retention of Ax ranged from 4-6 % in the muscle, which is higher than the 3% observed at this location in large salmon (< 3 kg) in October and December (Ytrestøyl et al., 2008). The daily feed intake and presumably also growth was high in the initial 6-7 weeks of feeding, which may explain that the total growth and flesh Ax retention was fairly high despite the low temperature in the last weeks of feeding. In the present study, 21.7 % of the digested Ax was retained in the flesh of fish fed D3 (FBH 4.2 %) which is more than twice as high compared to that reported by Ytrestøyl et al. (2008); who found that 9.5 % of digested Ax was retained in the flesh at a dietary Ax concentration of 50 mg kg^{-1}. Considering the amount of absorbed Ax calculated, about 10 %

more of the absorbed Ax was deposited in the muscle in fish fed D3 compared to the control diet (D1).

The plasma concentration of carotenoids is considered to be a good indicator of bioavailability of carotenoids in salmonid fishes (Kiessling et al., 1995, 2003; Storebakken and Goswami 1996; Ytrestøyl and Bjerkeng 2007). In the present study, plasma total Ax concentration was increased from 1.4 mg kg^{-1} in the control diet (D1) to 1.7 mg kg^{-1} in fish fed D3 (FBH 4.2 %), and a similar increase in liver total Ax from 1.3 to 1.7 $mgkg^{-1}$ was also observed. At the same time, muscle Ax increased moderately from 4.6 to 5 mg kg^{-1}. Despite non-significant increases in tissue Ax concentrations, muscle Ax retention was significantly increased by 35 - 40 % in fish fed D3. The present results suggests increased Ax utilization in response to dietary FBH despite the fact that the results are statistically weaker than previously observed in 0^{+}-smolt.

About 60 to 80% of the absorbed Ax is metabolically transformed into other compounds. Idox is the first reductive product of Ax, and accumulates in plasma and tissues of Atlantic salmon (Schiedt et al., 1988, 1989) and Arctic charr (Aas et al., 1997; Bjerkeng et al., 2000). It is detected in the blood of Atlantic salmon only 6 hours after force-feeding a meal containing 14C-Ax (Aas et al., 1999), and is an indication of the metabolism at the time of sampling. In this study, slightly less Ax seem to be metabolized into Idox in the tissues of fish fed FBH. The small reduction in formation of Idox is not likely to explain 35 - 40 % increase in Ax retention as observed in fish fed D3. However, a major fraction of the ingested Ax may be metabolized into other colorless compounds, and the amount of Idox deposited in muscle and other organs therefore represents only a fraction of the total metabolized Ax. Potential effects

of FBH on Ax metabolism cannot be ruled out on the basis of the results from the present study.

A selective accumulation of all-E-Ax takes place in plasma and muscle whereas the Ax Z-isomers accumulates in intestine and liver of rainbow trout (Bjerkeng et al., 1997; Østerlie et al., 1999) and Atlantic salmon (Bjerkeng and Berge, 2000). This was also the case in the present study, cis-isomers constituted 38-42 % of the Ax in liver and less than 10 % in muscle and plasma, irrespective of the diets. The FBH ingredient apparently did not alter the Ax isomers in any of the measured tissues.

Several receptors and transport proteins such as scarb1, sd36, abca1 and rbp1/2 are involved in carotenoid/retinol uptake and transport (reviewed by Harrison et al., 2012). Mechanisms for Ax uptake and transport are still not known, but these receptors and transporters may be potential genetic markers. In the *in vitro* study, no positive effect of FBH alone or FBH + Ax on genes anticipated to be involved in tissue Ax uptake and transport (*cd36, scarb1, rbp1/2* and *abca1*) could be detected. Instead there was a significant down-regulation of gene expression for the receptors *cd36* and *scarb1*, as well as for the transport protein *abca1* with FBH alone, and for *scarb1* and *abca1* in FBH + Ax. Gene expression in Ax alone was not significantly different from the Control. This may indicate that Ax do not regulate expression of these genes or that these proteins are not essential for Ax uptake and transport. Although not significant, a tendency towards reduced uptake of Ax in the hepatic cells incubated with FBH was detected.

Antioxidants systems, like the enzymes catalase, GPX and SOD, are essential in all organisms in order to prevent reactivate oxygen species from interfering with biological structures (review by Chaudiere and Ferrari-Iliou, 1999). In this study, the enzyme activity of the

intracellular antioxidant GPX was significantly reduced in hepatocytes cultivated in a medium supplemented with FBH alone and with FBH + Ax. These results mirrored what was found in the *in vivo* trial. Salmon fed D3 (FBH 4.2 %) showed reduced liver GPX activity and increased TBARS as compared to salmon fed the control diet (D1) and D2 (FBH 2.1 %), indicating oxidative stress despite no negative effect on growth. The results may suggest a dose dependent response to increased dietary FBH ingredient, confirming previous results on activity of this antioxidant enzyme *in vitro* due to the presence of high sulfate level (Østbye et al., 2017b). The tendency towards reduced hepatic uptake of Ax in the cell culture trial may be explained by reduced intracellular antioxidant capacity, i.e. lower GPX activity, which may lead to increased usage of Ax as antioxidant and consequently lower hepatic Ax uptake.

Despite the indications of oxidative stress in liver, the hepatic Ax concentration in salmon fed D3 (FBH 4.2 %) was elevated by 22 % as compared to D1. This suggests that the modulatory role of the FBH ingredient may be explained by improved digestibility of Ax, leading to higher circulating Ax and increased tissue Ax deposition. In smolt, plasma and liver Ax were significantly increased by 55 and 29 %, respectively, in response to FBH (un-published data). Different fish sizes and in particular the low temperature at the time of sampling in the present study, may explain such differences.

The *in vitro* study showed that FBH alone and FBH + Ax reduced gene expression of *cpt1* and *acox*, involved in the mitochondrial and peroxisomal β-oxidation. This may indicate that the fish bone ingredients reduce fatty acid oxidation, although this has to be further confirmed. The oxidative stress induced by the FBH ingredient may also explain the result. Among other, it has been reported that increased oxidative stress may damage the mitochondrial membranes and reduce the fatty acid ß-oxidation capacity (Kjær et al., 2008;

Østbye et al., 2011). In the storage trial, dietary Ax in D3 appeared to be less stable at high temperature (25 °C), as compared to the other diets. This may be explained by increased Ax oxidation or destabilization of Carophyll Pink. In salmon fed FBH ingredients, no negative impacts on growth has been observed in juvenile fish from 15 g size (Ytteborg et al., 2016) or in 0^+-smolt (Albrektsen et al., 2017). The method for extraction of the nutrients in the fish bones has however been changed, mainly by reducing the concentration of sulfuric acid, inevitably also reducing the need to adjust pH, as previously discussed (Ytteborg et al., 2016; Albrektsen et al., 2017). By using significantly less chemicals, the minerals can still be efficiently extracted and it has been shown that the FBH ingredient no longer affects hepatic GPX activity *in vitro* in a negative way (unpublished data).

In addition to improving the muscle Ax retention, the D3 (FBH 4.2 %) diet affected connective tissue compounds in the muscle with regards to proteoglycans and their glycosaminoglycans (GAGs). The GAGs interact with a number of different factors, such as growth factors, cytokines, enzymes and lipids, and the biological roles of proteoglycans are diverse and fine-tuned by the sulfate pattern of the GAGs (Campo et al., 2004; Kreuger et al., 2006; Lindahl, U. and Li, J.P., 2009; Rønning et al., 2013). The expression of different GAGs varies and impact the different stages in the myogenesis (Melo et al., 1996; Brandan et al., 2008). In this study, mRNA expression of the muscle structural protein myosin was down-regulated in fish fed D3 (FBH 4.2 %), as compared to the control diet (D1). Myosin is one of the main proteins in mature myofibers, important for muscle contraction. Down-regulation of myosin may indicate a change in the regulation of this signaling pathway of myogenesis, however, more experimental work is required to confirm this. The potential change in the signaling pathway of myosin may be associated with the changed amount of GAGs, its sulfation or the expression of different proteoglycans, as shown by FT-IR analyses in fish fed

D3 (FBH 4.2 %). Addition of K_2SO_4 in D4 did not have any impact on connective tissue composition, suggesting that dietary sulfate is not responsible for this physiological response. The effect of the FBH ingredient on the connective tissue compounds in the skeletal muscle may result from soluble compounds other than the minerals. Among other, FBH contains high levels of amino acids (Hyp, Pro, Gly, Ala, Glut) characteristic of bone collagens (Toppe et al., 2007), of which some has been reported to improve growth or to affect muscle quality in fish (Aksnes et al, 2008; Albrektsen et al., 2010; Larsson et al., 2014; Østby et al., 2017). Some of the non-essential amino acids may become conditionally essential or physiologically advantageous in farmed fish exposed to extensive handling and stress (Li et al., 2009). However, the observed change in the connective tissue may also be explained by other soluble compounds released from the bones, such as non-protein N compounds, GAGs, specific microelements or combinations thereof. A changed composition of proteoglycans in skeletal muscle may be important for the textural properties of the flesh (Tingbo et al., 2005; Torgersen et al., 2013). GAGs are highly water binding due to its linear long unbranched polysaccharide chain structure with distinct sulfation pattern (Yanagishita, M., 1993). The increased GAG/amid ratio in our study may suggest higher water binding capacity of the fillet, and possibly improved fillet quality (Moreno et al., 2012). Flesh texture and color are two of the most important quality criteria in salmon. Confirmation of the physiological impacts of the acid extracted fish bone compounds on flesh quality and texture would be needed to claim such physiological properties of the fish bone ingredient.

Conclusions

In this study, a tendency towards improved Ax concentrations in plasma, liver and muscle of Atlantic salmon fed FBH (4.2 % of the diet) was indicated, and was followed by significantly

increased Ax retention (35 - 40 %) in salmon muscle. The results suggests that the increased Ax utilization in the fish was explained by slightly improved Ax digestibility (ns), possibly combined with lower metabolic loss of Ax. The fish bone ingredient also altered the structural connective tissue compounds in muscle by stimulating the production of proteoglycans and their sulfated glycosaminoglycans (GAGs) significantly. Confirmation on how the fish bone compounds may affect connective tissues quality in the body is required to understand potential physiological implications. Refining of the process method used to hydrolyse the fish bones is in progress, aiming to maintain high processing yield of phosphorus (> 85 %) with minimal use of chemicals, and with no negative impacts on feed stability or any oxidation parameters in fish. This will contribute to make the fish bone ingredients attractive as sustainable feed additives.

5 Acknowledgements

The authors wish to thank Sissel Nergaard at Nofima's aquaculture research station at Averøy for very skillful, cautious and ethical handling of the fish, Rolf Erik Myrmel and Hilde Njøsen for excellent and accurate work with the pellet extruder, and Inger Øien Kristiansen with highly precise and accurate work with the cell culture trials. The project was carried out with financial support from The Norwegian Research Council (NRC project 217532). The financing from NRC is greatly appreciated.

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Figure abbreviations:

Figure 1 Amino acid (g/100 g protein) composition of spray-dried fish bone hydrolysate (FBH) produced from blue whiting fish meal

Figure 2 Apparent Ax digestibility (ADC Ax) and tissue Ax concentrations in fish fed D2 (2.1 % FBH), D3 (4.2 % FBH) and D4 (K_2SO_4) shown as the difference relative to fish fed D1 (control=100), $P > 0.05$.

Figure 3 PCA plot of a region (1400-1185 cm^{-1}) of connective tissue representing carbohydrate groups (from proteoglycans), amid III from collagen and sulphation of proteoglycans in fish fed D1 (control), D3 (4.2 % FBH) and D4 (K_2SO_4).

Table 1. Diet ingredients and composition

Diets	D1 Control	D2 FBH 2.1 %	D3 FBH 4.2 %	D4 K_2SO_4
Ingredients, gkg^{-1}				
Fish meal[a]	150	150	150	150
FBH Blue whiting[b]		21	42	
SPC	190	190	190	190
Wheat gluten	67	65	63	71
Pea protein concentrate	100	100	100	100
Lysine-HCl	8	8	8	8
Thr	4	4	4	4
Fish oil[c]	66	66	66	66
Canola oil	236	236	236	237
Soya lecithin	5	5	5	5
Wheat	119	112	105	98
Betafin	5	5	5	5
Vitamin mix[d]	20	20	20	20
Mineral mix[e]	5.2	5.2	5.2	5.2
$CaPO_4$ - MCP (T29/10)	2.4	1.2	0	2.4
K_2SO_4 (18 %)				1.6
Carophyll Pink, 10%	0.55	0.55	0.55	0.55
Y_2O_3	0.2	0.2	0.2	0.2
Diet composition				
Protein, g kg^{-1}	366	369	366	371
Lipid, g kg^{-1}	334	329	319	336

Water, g kg^{-1}	64	64	70	49
Ash, g kg^{-1}	62	68	73	79
Total P, %	11.9	11.5	10.8	12.1
Soluble P, %	8.6	8.1	7.6	8.4
Total energy[1], MJ kg^{-1}	24.8	24.6	24.2	24.8
Dietary carotenoids, mg kg^{-1}				
Total free Ax	56	55	57	54
All-E Trans Ax	43	42	43	41
9Z Cis Ax	2.3	2.2	2.4	2.4
13Z Cis Ax	10	11	12	11

[a] LT fish meal, SILFAS, N-5892, Bergen, Norway. Protein: 708 gkg^{-1}, Lipid: 110 gkg^{-1}, Ash: 108 gkg^{-1}, Water: 87 gkg^{-1}.

[b] FBH Blue whiting: Protein: 89 gkg^{-1}, Lipid: 16 gkg^{-1}, Ash: 677 gkg^{-1}, Water: 38 gkg^{-1}.

[c] Norsalmoil, Norsildmel AL, N-5141 Fyllingsdalen, Norway

[d] Vitamins added per kg feed: vitamin D_3, 3000 I.E., 160 mg; vitamin E, 136 mg; thiamin, 20 mg; riboflavin, 30 mg; pyrodoxine-HCl, 25 mg; vitamin C, 200 mg; calcium pantothenate, 60 mg; biotin. 1 mg; folic acid, 10 mg; niacin, 200 mg; vitamin B_{12}, 0,05 mg; menadion bisulphite, 20 mg

[e] Minerals added per kg feed: magnesium 500 mg; potassium 600 mg; zinc 120 mg; iron 60 mg; manganese 30 mg; copper 6 mg.

Table 2. Primers for gene expression analysis. *eukaryotic translation initiation factor 3 (eif3), nadh:ubiquinone oxidoreductase (nduf), glutathione peroxidase 3 (gpx3), catalase (cat), nuclear factor kappa b (nfkb), nuclear factor, erythroid 2 (nfe2), myogenic differentiation 1b (myod1b), myogenin (myog), myosin (myl), acyl-coa oxidase (acox), carnitine palmitoyltransferase 1 (cpt1), scavenger receptor class b member 1 (scarb1), retinol binding protein 1 (rbp1), retinol binding protein 2 (rbp2), atp binding cassette subfamily a member 1 (abca1).*

Gene	Genbank accession no.	Forward (5'-3')	Reverse (5'-3')
Eif3	DW542195	CAGGATGTTGTTGCTGGATGGG	ACCCAACTGGGCAGGTCAAGA
nduf	DW532752	CAACATAGGGATTGGAGAGCTGTACG	TTCAGAGCCTCATCTTGCCTGCT
gpx3	CA345853	CCTTCCAGTACCTGGAGTTGAATGC	CTCATGATTGTCTCCTGGCTCCTGT
cat	est04a09	CCAGATGTGGGCCGCTACAA	TCTGGCGCTCCTCCTCATTC
nfkb	CA341859	CAGCGTCCTACCAGGCTAAAGAGAT	GCTGTTCGATCCATCCGCACTAT
nfe2	BT059007	CCGGACTCCTCGCCTTCGGA	GTGGATAGTTGGCTTGTCCCTTCGT
myod1b	AJ557150	CCGCAACACGAAGCAACTATTACAGC	GGAACCCTCCTGGCCTGATAACAC
myog	DQ294029	ATTGAGAGGCTGCAGGCACTTG	GTGCGGTAGTGTAAGCCCTGTGTT
myl	NM_001123716	CCATCAACTTCACCGTCTTCCTCAC	CAGCCCACAGGTTCTTCATCTCC
acox	DQ364432	CCTTCATTGTACCTCTCCGCA	CATTTCAACCTCATCAAAGCCAA
cpt1	AM230810	GTACCAGCCCCGATGCCTTCAT	TCTCTGTGCGACCCTCTCGGAA
scarb1	DQ266043	AACTCAGTGAAGAGGCCAAACTTG	TGCGGCGGTGATGATG
cd36	AY606034	GGATGAACTCCCTGCATGTGA	TGAGGCCAAAGTACTCGTCGA
rbp1	NM_001141480	CATGGTACAGTGCAGGGTGA	TGGACACAGAACAGCCACAT
rbp2	BT056990	GGCAATAAATAAGCCATGGAGGTG	TGCTCTTTTGCACCCCAGTA
abca1	BI468033	CACCAGTGGTCTTCCTGGAC	GTACACAGGGCCTCACACTC

Table 3. Daily specific growth rate (SGR, %), daily feed intake per fish and feed conversion rate (FCR) in Atlantic salmon fed experimental diets D1 (Control), D2 (FBH 2.1 %), D3 (FBH 4.2 %) and D4 (K_2SO_4) for a feeding period of 11 weeks. Mean values and STD (x) is given for each respective diet, n=3.

Diets	D1 Control	D2 FBH 2.1 %	D3 FBH 4.2 %	D4 K_2SO_4	ANOVA $P^1 < 0.05$
Initial weight, g	1707 (7)	1693 (11)	1694 (13)	1698 (5)	ns
Final weight, g	2543 (93)	2489 (108)	2565 (55)	2560 (26)	ns
Weight gain, g	836 (89)	795 (99)	871 (44)	861 (29)	ns
SGR, %	0.51 (0.04)	0.49 (0.05)	0.53 (0.02)	0.53 (0.02)	ns
TGC	3.16 (0.29)	3.04 (0.32)	3.29 (0.13)	3.26 (0.10)	ns
FCR	1.01 (0.02)	1.04 (0.04)	1.01 (0.03)	1.13 (0.13)	ns
Feed intake, %/BW/d [2]	0.57 (0.06)[a]	0.56 (0.05)[a]	0.56 (0.01)[a]	0.66 (0.03)[b]	0.05
Feed intake, g/fish/d[3]	12.0 (1.5)	11.8 (1.5)	11.9 (0.3)	14.0 (0.6)	ns
Mortality, no. fish/diet	21	23	15	22	ns
Mortality, %	12.7 (4.8)	13.9 (1.0)	9.1 (3.6)	13.3 (7.3)	ns

[1] Significant differences within rows are shown with different superscript letters, $P < 0.05$, ns = not significant.

[2] Feed intake (% of mean body weight (BW), per day) = % feed intake / days / ((BW2 + BW1)/2 * 100) / fish no.

[3] Feed intake per fish per day = g feed intake / days / no. of fish.

Table 4. Digestibility of protein, lipid and astaxanthin (Ax), Ax carotenoids in faeces and total Ax utilization measured by muscle Ax retention in Atlantic salmon fed experimental diets D1 (Control), D2 (FBH 2.1 %), D3 (FBH 4.2 %) and D4 (K_2SO_4) for a feeding period of 11 weeks. Mean values and STD (x) is given for each respective diet, n=3.

Diets	D1 Control	D2 FBH 2.1 %	D3 FBH 4.2 %	D4 K_2SO_4
Digestibility, %				
Protein	86.3 (1.0)	86.2 (0.3)	86.6 (0.8)	87.9 (1.2)
Lipid	96.6 (0.7)	96.4 (0.5)	96.6 (0.4)	96.6 (0.6)
Total Ax	24.4 (1.4)	23.9 (6.1)	28.7 (0.5)	24.4 (3.8)
Faeces carotenoids, mgkg^{-1}				
Total Ax	13.28 (1.54)	12.01 (1.44)	11.63 (0.71)	12.11 (1.60)
All-E Trans Ax	10.18 (1.27)	9.05 (1.19)	8.64 (0.79)	9.32 (1.19)
9Z Cis Ax	0.60 (0.10)	0.60 (0.08)	0.55 (0.07)	0.63 (0.11)
13Z Cis Ax	2.27 (0.21)	2.07 (0.40)	2.03 (0.13)	1.98 (0.30)
Total Ax utilization				
Muscle Ax ret, mg	113.6 (6.5)^{a}	93.6 (34.7)^{a}	159.3 (19.1)^{b}	128.6 (29.5)^{ab}
Muscle Ax ret, % of Ax eaten	4.62 (0.18)^{a}	3.87 (1.16)^{a}	6.24 (0.69)^{b}	4.44 (0.72)^{a}
Muscle Ax ret, mg/kg weight gain	2.56 (0.14)^{a}	2.20 (0.59)^{a}	3.46 (0.28)^{b}	2.77 (0.60)^{ab}
Muscle Ax ret, % of Ax absorbed	19.0 (1.4)	16.5 (4.1)	21.7 (2.1)	18.2 (6.8)

[1] Significant differences is shown by different superscript letters (Duncan's Post Hoc test, $P < 0.05$), ns = not significant.

Table 5. Chemical composition and gene expression in muscle of Atlantic salmon fed experimental diets D1 (Control), D2 (FBH 2.1 %), D3 (FBH 4.2 %) and D4 (K_2SO_4) for a feeding period of 11 weeks. Mean values and STD (x) is given for each respective diet, n=3.

Diets	D1 Control	D2 FBH 2.1 %	D3 FBH 4.2 %	D4 K_2SO_4	ANOVA $P^1 < 0.05$
Chemical composition, %					
Lipid	11.0 (1.0)	11.3 (0.6)	10.5 (0.7)	10.8 (0.4)	ns
Dry matter	31.7 (0.6)	31.9 (0.6)	31.6 80.5)	31.7 (0.3)	ns
Tocopherol, $mgkg^{-1}$	21.0 (4.0)	19.5 (0.7)	18.7 (1.1)	19.2 (1.4)	ns
Gene expression relative to control					
myog	0.00 (0.89)	-0.23 (0.94)	-0.37 (1.30)	0.05 (0.89)	ns
myod1b	0.00 (0.62)	-0.01 (0.56)	-0.11 (0.43)	-0.13 (0.51)	ns
myl	0.00 (0.51)^{a}	-0.01 (0.39)^{a}	-0.29 (0.47)^{b}	-0.15 (0.39)^{ab}	0.05
Lipid peroxidation, µg per g muscle					
TBARS	0.42 (0.28)	0.46 (0.35)	0.58 (0.26)	0.41 (0.19)	ns

[1] Significant differences is shown by different superscript letters ($P < 0.05$), ns = not significant.

Table 6. Chemical composition, gene expression and antioxidant defence enzymes (SOD, GPX and Catalase) in liver of Atlantic salmon fed experimental diets D1 (Control), D2 (FBH 2.1 %), D3 (FBH 4.2 %) and D4 (K_2SO_4) for a feeding period of 11 weeks. Mean values and STD (x) is given for each respective diet, n=3.

Diets	D1 Control	D2 FBH 2.1 %	D3 FBH 4.2 %	D4 K_2SO_4	ANOVA $P^1 < 0.05$
Chemical composition, %					
Lipid	9.6 (0.6)[a]	8.3 (0.9)[ab]	7.0 (0.6)[b]	7.9 (1.4)[ab]	0.05
Dry matter	27.6 (0.9)	26.5 (0.6)	25.6 (0.6)	26.4 (1.3)	ns
Tocopherol, $mgkg^{-1}$	513 (134)	409 (10)	457 (51)	526 (118)	ns
Gene expression (relative to control)					
gpx3	0.00 (0.73)[ab]	0.12 (0.63)[a]	-0.33 (0.81)[b]	0.00 (0.77)[ab]	0.05
nfkb	0.00 (0.50)	0.00 (0.45)	-0.01 (0.46)	0.18 (0.58)	ns
nfe2	0.00 (0.43)	0.09 (0.43)	0.08 (0.48)	0.11 (0.56)	ns
Antioxidant defence enzymes					
SOD (% inhibition rate/mg protein)	0.40 (0.08)[ab]	0.44 (0.12)[a]	0.40 (0.09)[ab]	0.38 (0.08)[b]	0.05
GPX (nmol/min/mg protein)	2.61 (0.81)[a]	2.62 (0.72)[a]	2.10 (0.58)[b]	2.41 (0.71)[ab]	0.05
Catalase (U/g protein)	87.6 (21.8)	84.1 (22.2)	88.0 (19.9)	84.5 (17.2)	ns
Lipid peroxidation, μg per g liver					
TBARS	6.07 (1.60)	5.66 (1.53)	6.96 (1.57)	6.08 (1.84)	ns

[1] Significant differences is shown by different superscript letters ($P < 0.05$), ns = not significant

Table 7. Astaxanthin (Ax) uptake, antioxidant defence enzymes (SOD, GPX, Catalase) and gene expression measured *in vitro* in hepatocyte cell cultures.

	Control	FBH Blue whiting	Ax	FBH Blue whiting + Ax	ANOVA $P^1 < 0.05$
Ax uptake µg Ax/µg protein % of added Ax	 nd nd	 nd nd	 $5.2*10^{-3}$ ($0.5*10^{-3}$) 53.9 (4.7)	 $4.5*10^{-3}$($0.6*10^{-3}$) 46.3 (6.0)	 ns ns
Antioxidant defence enzymes					
SOD (% inhibition rate/mg protein)	2.94 (0.67)^{bc}	5.54 (1.90)^{ab}	2.28 (0.94)^{c}	7.07 (2.77)^{a}	0.05
GPX (nmol/min/mg protein)	40.6 (5.1)^{a}	24.9 (5.6)^{c}	39.3 (15.6)^{ab}	25.7 (3.8)^{bc}	0.05
Catalase (U/g protein)	0.79 (0.15)	0.90 (0.18)	0.70 (0.19)	0.94 (0.19)	ns

[1] Significant differences is shown by different superscript letters ($P < 0.05$), ns = not significant

Table 8. GAG/Amid1 ratio and collagen crosslink in the skeletal muscle of Atlantic salmon fed the control diet (D1), and with diets added FBH 4.2 % (D3) or K_2SO_4 (D4).

	GAG/amid1	Std error	Collagen crosslinks	Std error
D1: Control	0.34	0.06	3.12	0.62
D3: FBH 4.2 %	0.46 *	0.07	3.82	0.57
D4: K_2SO_4	0.35	0.05	4.27	0.60

*Significant difference compared to the control diet (D1), $P < 0.05$. Glycosaminoglycan (GAG)

Highlights of the manuscript

- The process for extracting of nutrients in fish bones aid to increased utilization of available marine resources and novel applications of fish bone nutrients in aquaculture
- Extracted fish bone compounds increase Ax digestibility and the circulating Ax levels (ns) and is followed by a significant increase in muscle Ax retention in Atlantic salmon
- Extracted fish bone compounds change the connective tissue structures in the muscle by stimulating the production of proteoglycans and their sulfated glycosaminoglycans (GAGs) significantly

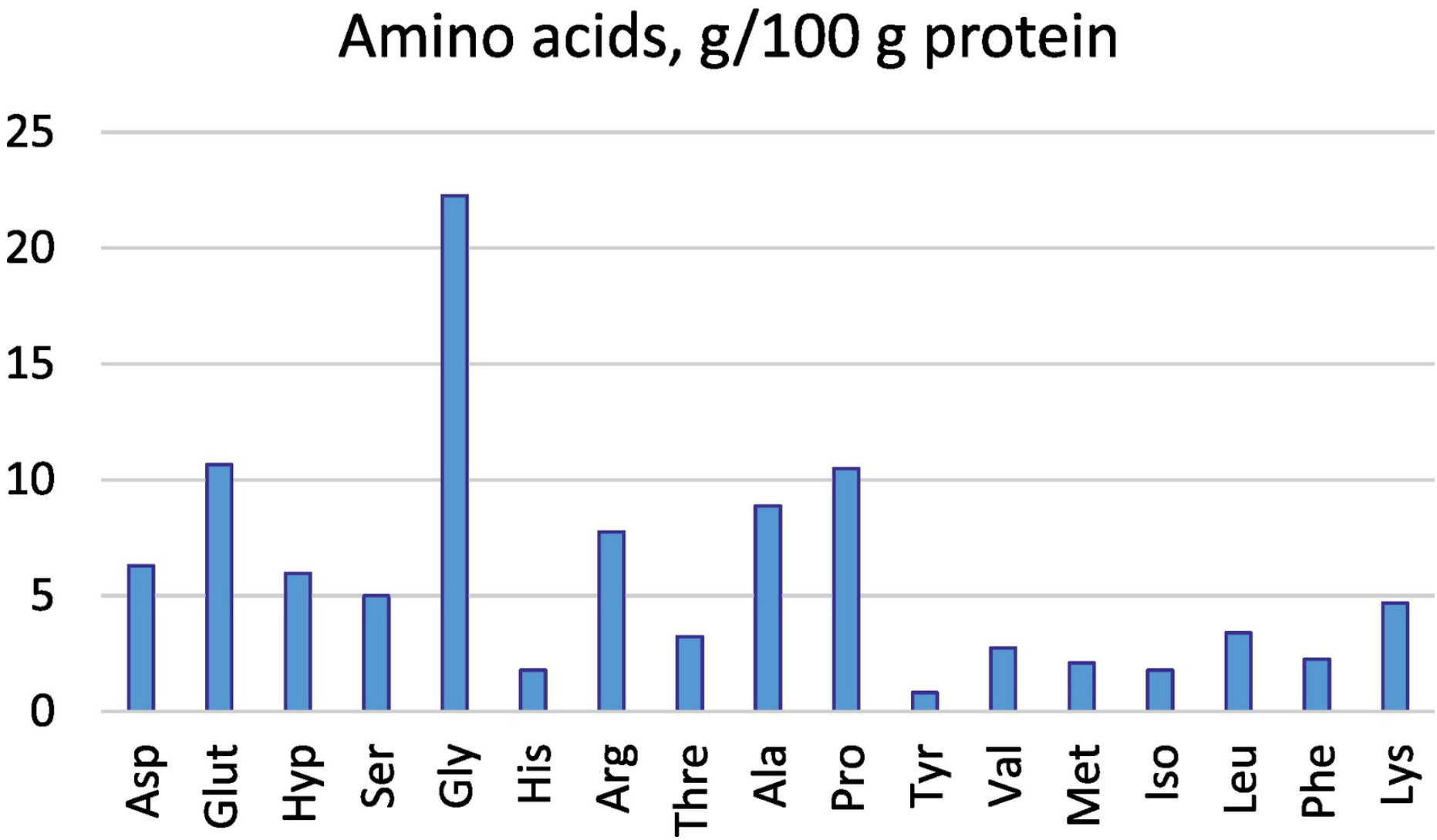
Amino acids, g/100 g protein
25
20
15
10
5
0
Asp
Glut
Hyp
Ser
Gly
His
Arg
Thre
Ala
Pro
Tyr
Val
Met
Iso
Leu
Phe
Lys

Figure 1

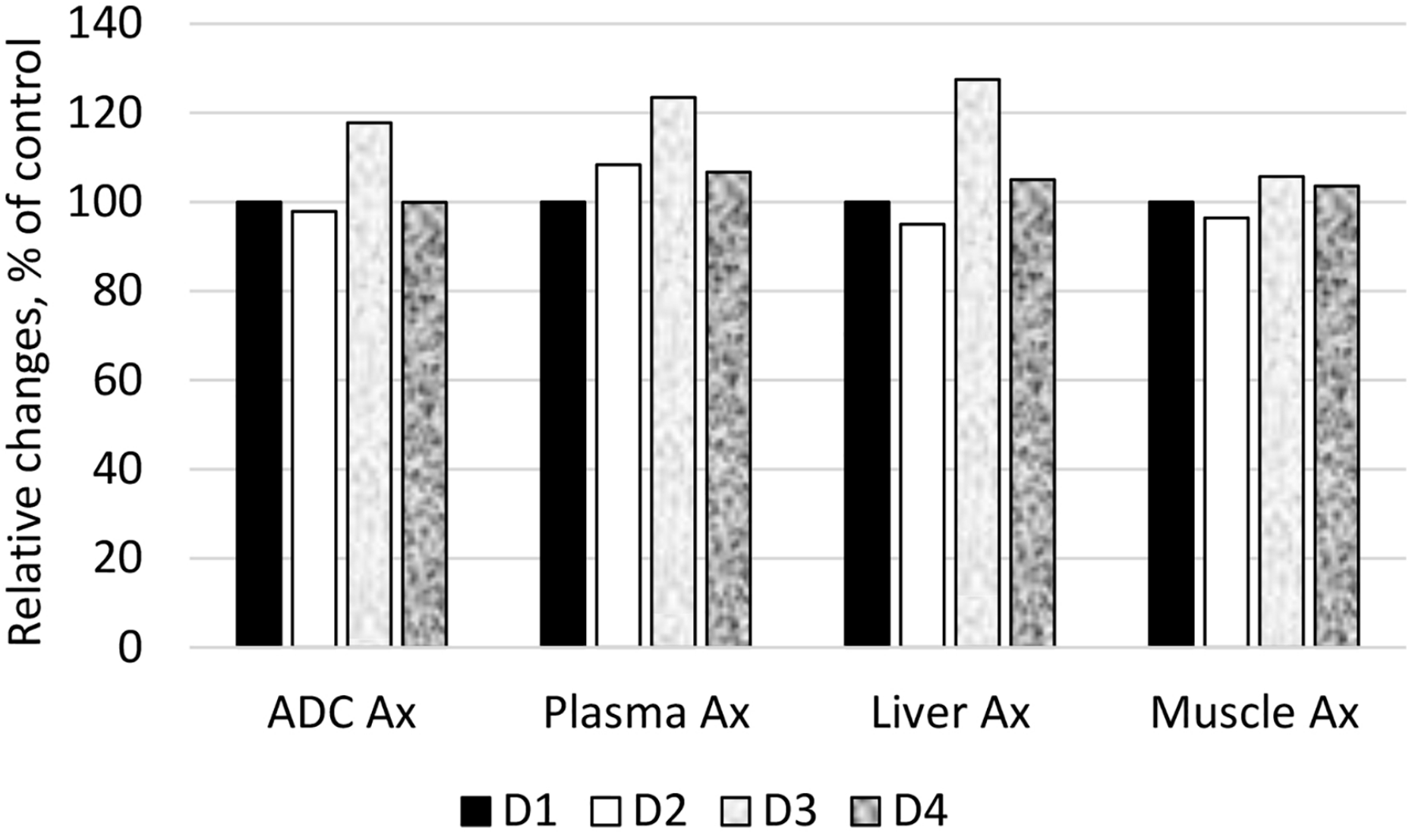
140
120
100
80
60
40
20
0
Relative changes, % of control
ADC Ax
Plasma Ax
Liver Ax
Muscle Ax
D1
D2
D3
D4

Figure 2

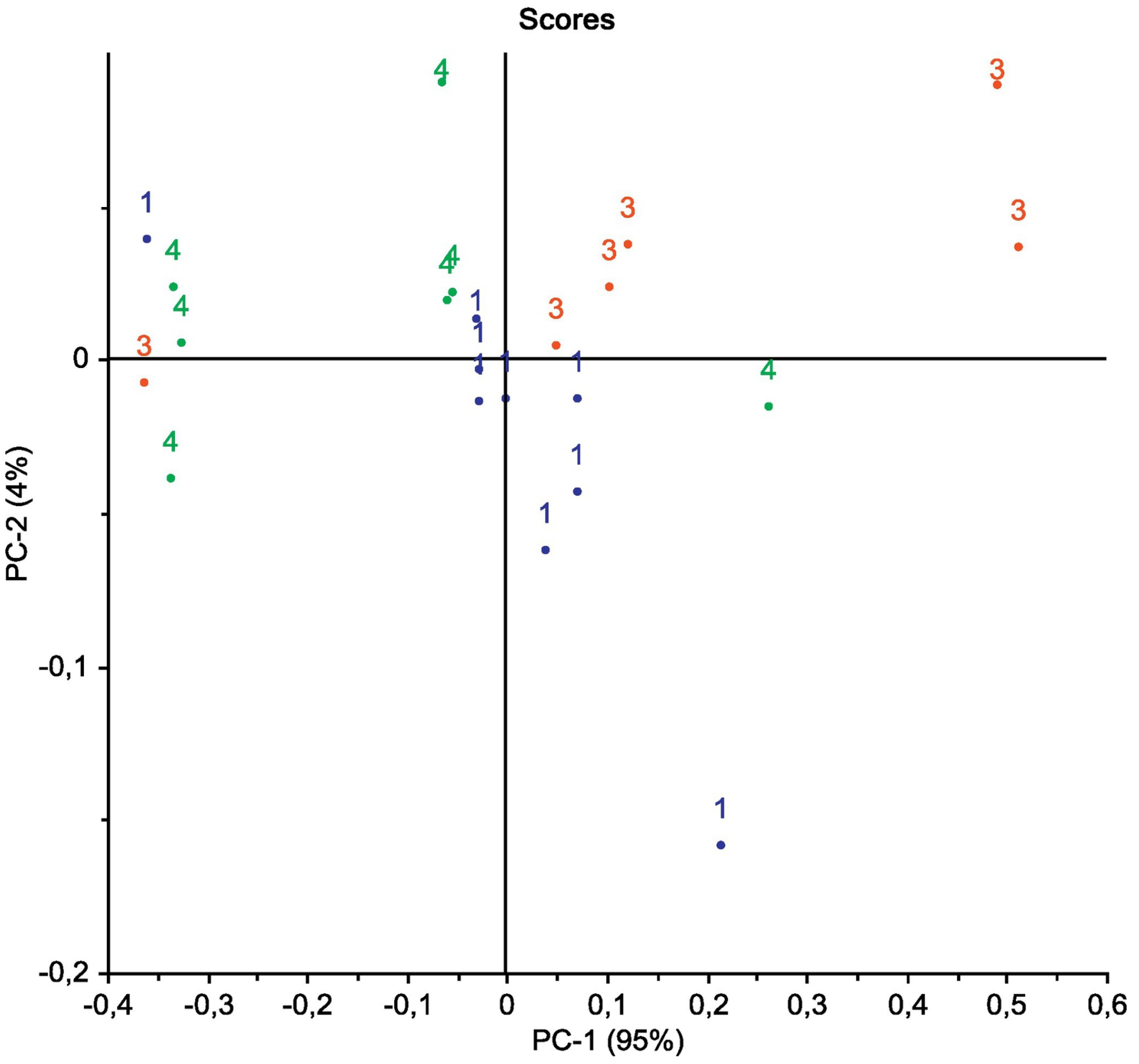

Figure 3