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Vertebral column adaptations in juvenile Atlantic salmon *Salmo salar*, L. as a response to dietary phosphorus

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ABSTRACT

Deficiency in dietary phosphorus (P) is considered as a nutritional risk factor for the development of vertebral column deformities in farmed Atlantic salmon *Salmo salar*, L. This mono-factorial study examines how 11-week deficiency and excess of dietary P influence the structure and microstructure of the vertebral bodies in juvenile, freshwater stages of Atlantic salmon. Animals were fed continuously with three diets containing different levels of total P (tP) and soluble P (sP), respectively: low P (LP) = 6.8 g/kg tP, 3.5 g/kg sP, regular P (RP) = 10.0 g/kg tP, 5.6 g/kg sP, and high P (HP) = 13.0 g/kg tP, 9.3 g/kg sP. Animals were analysed for plasma and bone mineral content, vertebral column deformities (x-ray), vertebral centra stiffness, bone mineralisation pattern and vertebral body microanatomy (cells and connective tissue structures). A low (background) level of deformities was observed on a gross morphological level but no increase and no specific type of vertebral column deformity was associated with either of the three groups. While feed intake was comparable among all diet groups animals fed LP showed a 50% reduction in total calcium (Ca) and P content in abdominal vertebrae and opercula. Regular P and HP animals showed similar levels of total Ca and P in abdominal vertebrae and opercula. Animals in all diet groups showed well-developed vertebral bodies. Low P animals had vertebral centra, neural and haemal arches with large areas of non-mineralised bone. Vertebral centra stiffness in LP animals was reduced accordingly. Regular P and HP animals showed comparable values for vertebral centra stiffness. Non-mineralised vertebral body end plates of LP animals developed a slight inward bending and intervertebral ligaments increased in length and thickness. The cellular and extracellular components of the intervertebral joints remained intact without structural alterations that would indicate the development of vertebral centra compression or fusion. Animals from all three diet groups showed active osteoblasts at the vertebral body growth zone. Despite the three-fold decline in plasma inorganic P in LP animals growth continued at the same rate as in RP and HP animals. It is discussed whether the use of a P-reduced diet under a continuous feeding regime can maintain growth without adverse effects for animal health and welfare. This study further discusses that a HP diet relative to an RP diet has no beneficial effect concerning bone formation, bone mineralisation, growth and prevention of vertebral centra deformities in Atlantic salmon parr.

1. Introduction

Bone is a specialised vascularised connective tissue that has several functions. It protects internal organs and is a site of muscle attachment. The principal mineral components of bone are calcium (Ca) and phosphorus (P) which precipitate as apatite ($Ca_{10}(PO_4)_6(OH)_2$) (Boskey, 2007) within an organic matrix mainly consisting of type I collagen (Young, 2003). Bone acts as a mineral storage (Lall, 2002; Lall and Lewis-McCrea, 2007; Tarlo, 1964) and has endocrine and metabolic functions (Confavreux et al., 2009; Witten and Huysseune, 2009). Teleosts can actively take up Ca from the water through the gills in addition to intestinal Ca uptake (Flik et al., 1996; Flik and Verbost, 1993; Lall and Lewis-McCrea, 2007). In contrast, gill absorption of P is minimal therefore teleosts rely on the dietary P intake (Ketola, 1975; Vielma and

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Lall, 1998a, 1998b). Apart from being essential for bone mineralisation, P is important for growth, energy metabolism, and as an essential component of DNA, proteins and cell membranes (Lall, 2002). Requirements of dietary P in the feed for Atlantic salmon changes according to the animals' life stage. Due to high maintenance energy requirement in small teleost (Cho, 1992) the required level of P in the diet for early freshwater stages of Atlantic salmon (1–5 g) is increased to estimated 9.2–11.4 g/kg of total P, 7.9–9.8 g/kg of available P (Åsgård and Shearer, 1997). Subsequently, dietary P requirement decreases to an estimated 8.3 g/kg of total P, 5.6 g/kg of available P in juvenile freshwater stages (15–40 g) (Vielma and Lall, 1998a). Phosphorus requirements increase slightly during the early seawater phase (Fjelldal et al., 2009) to values between 8.8 and 11.0 g/kg of total P, equivalent to 5.1–7.4 g/kg of soluble (≈ digestible) P levels (Albrektsen et al., 2018).

In freshwater stages of Atlantic salmon, dietary P-deficiency has been associated with vertebral column deformities such as decreased intervertebral spaces, homogenous compression of vertebral centra, hyperradiodense vertebral centra and vertebral centra fusion (Fjelldal et al., 2016; Smedley et al., 2018). In contrast, high dietary P levels are assumed to prevent vertebral column deformities (Baeverfjord et al., 1998, 2018; Fjelldal et al., 2009, 2012a). Nonetheless high levels of dietary P are also associated with decreased growth in Atlantic salmon, in rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) and in haddock *Melanogrammus aeglefinus* (L.) (Albrektsen et al., 2009; Fjelldal et al., 2016; Roy and Lall, 2003; Porn-Ngam et al., 1993; Vielma and Lall, 1998a). There are also reports that a high dietary P intake causes impaired bone mineralisation (Albrektsen et al., 2009) and increased mortality (Vielma and Lall, 1998a) in Atlantic salmon. An increased dietary P:Ca ratio is known to reduce zinc (Zn) absorption in rainbow trout (Hardy and Shearer, 1985; Porn-Ngam et al., 1993). The retention rate of P in Atlantic salmon decreases sharply when the dose of dietary P exceeds the optimum level (Albrektsen et al., 2009; Sugiura et al., 2000). As a consequence, urinary P excretion increases (Roy and Lall, 2004) which can result in unwanted P discharge from fish farms (Lazzari and Baldisserotto, 2008; Sugiura et al., 2000).

Recent studies on early seawater stages of Atlantic salmon show the decoupling of bone formation and mineralisation and suggest that dietary P-deficiency is not the single cause of the development of vertebral column deformities. Trials lasting ten weeks and 17 weeks respectively tested animals with diets that had substantially reduced dietary P levels which led to the formation of extensive areas of non-mineralised, albeit regularly structured, bone at the vertebral column (Witten et al., 2016, 2019). Diet-related deformities did not occur, not even after 17 weeks of dietary P-deficiency. Experiments with zebrafish *Danio rerio* (Hamilton 1822) show similar results, this is the development of non-mineralised, albeit regular, bone structures as a results of low dietary P intake (Cotti et al., 2020).

Bone formation, but not mineralisation, continues in zebrafish and in seawater stages of Atlantic salmon but a lack of knowledge persists about freshwater stages of Atlantic salmon. This study provides analysis of the morphology, structures and microstructures of cells and connective tissue in vertebral bodies of Atlantic salmon parr. Vertebral centra compression and fusion, the most commonly observed deformity in Atlantic salmon (Fjelldal et al., 2007), arise within the intervertebral spaces (Witten et al., 2005; Ytteborg et al., 2010b). Therefore particular attention is paid to the effects of low and high dietary P on the intervertebral joints and ligaments. This 11-week lasting experiment uses diets with 3.5 g/kg (LP = low P), 5.6 g/kg (RP = regular P) and 9.3 g/kg (HP = high P) of soluble P. This study investigates how dietary P affects the animals' bone mineral and plasma inorganic phosphate (P*i*) levels. Consequences of these diets for vertebral body mineralisation, microanatomy, and cellular composition are described. The effect of the diets on the growth of the animals is studied. The curious findings that animals subjected to LP diets neither developed deformities nor growth retardation are discussed.

2. Materials and methods

2.1. Diet production and storage

Three isocaloric and isonitrogenous pre-smolt diets were formulated to contain different levels of total and soluble P. High and low P diets contained levels above and below the requirements for dietary P in farmed Atlantic salmon, respectively and an RP diet was designed to meet these requirements (8.8–11.0 g/kg of total P, 5.1–7.4 g/kg of soluble P) (Albrektsen et al., 2018). Soluble P was used as a predictor of digestible P in the diets (Albrektsen, 2015, Albrektsen et al., 2018; Fraser et al., 2019). This is based on the assumption that in teleost only soluble P is available for intestinal absorption (Morales et al., 2018) and the analysis of soluble P provides an accurate measure of available P content in the diet (Albrektsen et al., 2018). Regular P diet was based on the estimated requirement of 5.6 g/kg available P for juvenile stages of Atlantic salmon (Vielma and Lall, 1998a). Low P contained 6.8 g/kg of total P, 3.5 g/kg of soluble P, RP contained 10.0 g/kg of total P, 5.6 g/kg of soluble P, and HP contained 13.0 g/kg of total P, 9.3 g/kg of soluble P (Table 1). Dietary P levels were met by supplementation of inorganic phosphate in the form of mono-ammonium phosphate (MAP), while other ingredients, mainly wheat, were slightly modified to account for increasing MAP inclusion (Table 1). The diets were extruded to 2-mm pellets using a Wenger twin-screw extruder (TX57, Wenger, KS, USA) at an average temperature of 80 °C and a 1.8 mm diameter die hole. The

Table 1
Formulation and analysed composition of experimental diets.

	Low phosphorus (LP)	Regular phosphorus (RP)	High phosphorus (HP)
Formulation (g/kg)			
Soy protein concentrate	292.0	297.6	301.8
Wheat gluten	200.0	200.0	201.0
Fish meal	150.0	150.0	150.0
Wheat	134.5	104.5	75.1
Fish oil	192.0	199.6	207.0
Mono-ammonium phosphate	0.5	14.5	28.5
Premixes[a]	21.9	21.7	21.6
Water/ Moisture change[b]	9.2	12.1	15.0
Proximate and mineral composition analysis			
Moisture (%)	6.8	7.3	7.5
Crude protein (%)	45.5	46.5	47.4
Crude fat (%)	23.6	24.4	25.3
Ash (%)	4.9	5.3	6.6
Total P g/kg	6.8	10.0	13.0
Estimated available P g/kg[c]	2.6	5.9	9.1
Soluble P g/kg	3.5	5.6	9.3
Total Ca, g/kg	6.5	6.8	6.6
Total Ca:P ratio	1.1	0.7	0.5
Total Na, g/kg	1.7	1.9	1.8
Total Mg, g/kg	1.8	1.9	1.9
Total K, g/kg	8.9	8.8	8.6
Total Mn, mg/kg	36	32	31
Total Fe, mg/kg	152	172	166
Total Co, mg/kg	<1[d]	<1	<1
Total Cu, mg/kg	10	10	10
Total Zn, mg/kg	138	137	143

[a] Include vitamins and minerals (Trouw Nutrition) and amino acids (Skretting) estimated to cover requirements of Atlantic salmon according to the NRC (2011).
[b] Water added during feed processing to adjust for different moisture content of the raw materials in order to meet targeted moisture content in the final feed.
[c] Calculated based on content of total P, digestible P of raw material given in NRC (2011) and digestible P of mono-ammonium phosphate in Morales et al. (2018).
[d] Below detection limit.

extrudates were dried in a horizontal Wenger drier (Series III, 360, Wenger, KS, USA). Air flow, air temperatures and residence time were adjusted for each feed in order to reach the desired moisture level according to the diet formulation. The temperature in the different zones varied between 45 and 68 °C, and drying time between 8 and 9 min. The dried kernels were filled with oil in a 60 *l* rotating vacuum coater (Forberg, Oslo, Norway), cooled in a custom designed cooler (Skretting ARC, Stavanger, Norway) and packed in 25 kg plastic bags. The diets were produced four weeks prior to feeding, to allow time for analysis of total phosphorus levels. During this time, the feed was stored in an indoor storage facility under ambient temperature (≤12 °C) at the trial station.

2.2. Fish stock

The experiment took place at Skretting ARC Lerang Research Station (Forsand, Norway) using Atlantic salmon parr from Stofnfiskur (Iceland). The study was performed in agreement with Norwegian legislation and approved by the Norwegian Food Safety Authority (FOTS ID 19123). Eggs were fertilised in July 2018 and transported to Lerang Research Station at eyed egg stage. The eggs were incubated at 8 °C. Animals hatched in September and were reared at the same temperature. From start-feeding animals were kept in indoor tanks of 2000 *l* at a temperature of 12 °C. In January animals were moved to outdoor tanks with a volume of 5000 *l*. Temperature of water was 3 °C and reached 7.5 °C in mid-February. Animals were kept under 24:0 (light:dark) regime at a maximum density of 33 kg/m^3.

2.3. Experimental set-up

Animals were transferred to nine 100 *l* tanks at a density of 14.3 kg/m^3 to acclimatise for two weeks (Fig. 1). The water temperature was gradually raised to 12 °C with a 0.5 °C increase per day. The light regime was set to 12:12 (light:dark). Animals were fed a commercial diet, Nutra Olympic, pellet size 1.5 mm (Skretting, Norway) during the acclimation period. After two weeks acclimation all animals were anaesthetised with MS-222 (Tricaine methanesulphonate, Pharmaq, Norway) and graded to achieve an even weight within and between tanks at the start of the trial. Animals were anaesthetised, euthanised and sampled in the presence of accredited researchers certified by the Federation of Laboratory Animal Science Associations (FELASA). Four animals per tank were sampled for analysis at time point zero (T_0). At the start of the trial animals were returned to their respective tanks, three tanks per diet group, 50 animals per tank at a density of 9.5 kg/m^3 (Fig. 1). The average initial weight of the animals was 13.49 ± 1.50 g (Table 3). Importantly, animals were undisturbed throughout the experimental period without mechanical stress associated with handling, grading and vaccination.

The experimental trial lasted for 11 weeks. During the experiment animals were maintained under 12:12 (light:dark) regime to prevent premature onset of smoltification (Björnsson et al., 2000). Salinity (0.29 ± 0.12 ppt), oxygen levels (94.23 ± 6.84%) and temperature (12.40 ± 0.13 °C) were monitored daily. Diets were provided to the animals continuously using automatic feeders (Hølland Teknologi AS, Sandnes, Norway). Animals were fed for 14 s with 161 s intervals between feedings. Excess feed was collected three times a day by feed collectors with a mesh size of <2 mm to monitor feed intake.

2.4. Sampling and measurements

Intermediate and final samplings were carried out after seven and 11 weeks, respectively (Fig. 1). All animals were anaesthetised as described above. At the intermediate and final samplings, 15 animals per tank of each diet group were euthanised, while the remaining animals were bulk weighed and returned to their respective tanks. Sampled animals were measured (fork length) and weighed. Fulton's condition factor (K) was calculated according to the following equation:

$K = (W \times L^{-3}) \times 100$, where W is weight (g) and L is fork length (cm) (Busacker et al., 1990). The specific growth rate (SGR) was calculated as follows: SGR = (% body mass day^{-1}) = $[(W_{final} - W_{T0})^{1/Days} - 1] \times 100$, where W_{T0} and W_{final} represent the average animal weight (bulk and individual weight) per tank at T_0 and final (or intermediate) sampling (Gil Martens et al., 2006). The feed conversion ratio (FCR) was calculated on a dry matter basis (FCR = total feed consumed/ weight gain) (NRC, 2011). Feed intake (% of body mass day^{-1}) = total feed consumed (wet weight) / weight gain x SGR. Feed intake (g of feed day^{-1}) was recorded daily based on the quantity of recovered feed subtracted from the total daily ration. Excess feed collection was not completely accurate due to small size of the pellets. Values for FCR and feed intake are therefore slightly overestimated by about 15%. No mortalities occurred during the experimental period.

2.5. Blood plasma analysis

At the final sampling blood from nine animals per tank, 27 animals per diet was collected by puncture of the ventral caudal peduncle in lithium heparinised 4 m*l* Vacuette tubes (REF 454029, Austria) and placed on ice. Blood was collected with no prior starvation period (Albrektsen et al., 2009; Seternes et al., 2020) to analyse the effect of continuous feeding regime on plasma parameters and to limit the amount of stress and negative effect on the welfare of the animals (Santurtun et al., 2018; Waagbø et al., 2017). Blood from three animals was pooled. To obtain plasma, three pooled blood samples per tank (nine pooled blood samples per diet) were centrifuged at 4000*g* (4 °C) for 7 min with a VWR Mega star 600R centrifuge (Germany). Plasma was stored at −80 °C until further analyses. The activity of alkaline phosphatase (ALP) and concentrations of non-bound calcium (Ca^{2+}), chloride (Cl^-), sodium (Na^+) and P*i* were measured by the ARC (Stavanger, Norway) using a Konelab 30i chemistry analyser (Thermo Fisher

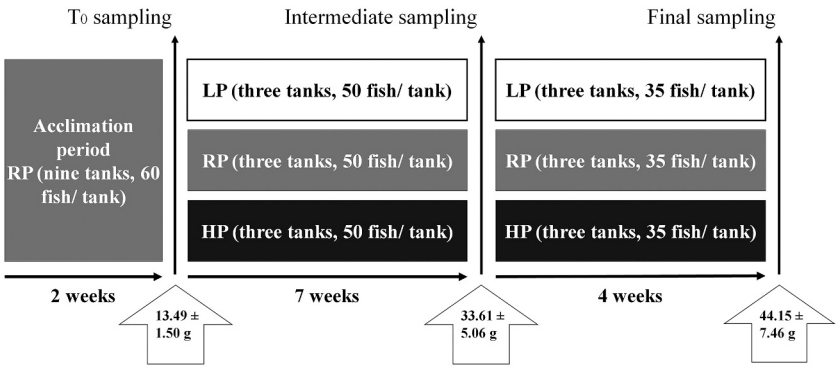


Fig. 1. Experimental set-up for the freshwater Atlantic salmon trial. Animals were fed a low phosphorus (LP), a regular phosphorus (RP) and a high phosphorus (HP) diet for a period of 11 weeks. Animals were sampled at T_0, intermediate and final sampling points.

Scientific, Switzerland) according to the manufacturers' instructions. Alkaline phosphatase activity was determined using *p*-nitrophenylphosphate (4-NPP) as a substrate in a 2-amino-2-methyl-1-propanol (AMP) phosphate-accepting buffer (art.#981833). The ALP levels were measured photometrically at 405 nm, with the activity expressed as enzyme units (U/*l*). The concentration of P*i* was measured using sulphuric acid and ammonium molybdate (art.#981891) and quantified photometrically at 340 nm. The non-bound Ca^{2+}, Cl^{-} and Na^{+} were measured by ion selective electrodes (art.#981595, 981596 and 981594 respectively).

2.6. Analysis of diets

Chemical analyses of the diets were done in duplicates. Proximate analysis (moisture, ash, crude protein and crude fat) and minerals (Ca, cobalt (Co), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), sodium (Na) and Zn) were analysed by Masterlab Analytical Services, Nutreco (Boxmeer, The Netherlands). Representative samples of the diets were taken after production to determine moisture (EC regulation 152/2009, Annex III A), ash (EC regulation 152/2009, Annex III M), crude fat (EC regulation 152/2009, Annex III H method A) and crude protein content (ISO 16634-1:2008). Representative diet samples for mineral analysis (Ca, Co, Cu, Fe, K, Mg, Mn, Na and Zn) were ground to 0.5 mm and measured by inductively coupled plasma mass spectrometry (ICP-MS, Thermo Fisher, Waltham, MA) after ashing and HCl extraction in accordance with NEN-EN 15510 (Bikker et al., 2017). Soluble and total P was determined by an accredited laboratory (BioLab, Nofima). Total P was measured spectrophotometrically (430 nm) after ashing and acid digestion in 6 M HCl (ISO 6491–1998). Soluble P was determined spectrophotometrically after incubation of a sample (0.8 g) in 80 m*l* 1 N NaOH for 16 h according to method of Ruban et al. (2001), Hua et al. (2005) and later modified and validated by Hovde (2013). Soluble NaOH extracted P was ashed and acid digested following the same method as for total P. These two methods differed solely in the first extraction step.

2.7. Mineral analysis - vertebrae and opercula

The calcium, P, Na, Mg and Zn content of vertebrae 1–19 (postcranial and abdominal regions only) and opercula collected at the final sampling was analysed by Eurofins Scientific (Barendrecht, The Netherlands). Samples were stored at −20 °C prior analysis. Vertebrae and opercula from three animals were pooled. To analyse the mineral content, two pooled vertebrae samples per tank and six pooled vertebrae samples per diet, while three pooled opercula samples per tank and nine pooled opercula samples per diet were digested by Multiwave 3000 (Anton Parr, Austria) based on (EN 13805:2014, 2014) standard and analysed by NexION ICP Mass Spectrometer (Perkin Elmer, USA). The mineral content is given in % or mg/kg of bone non-defatted wet weight.

2.8. Radiography

X-ray images were taken from animals filleted on both sides. There were 27 animals at T_0 and 45 animals per diet group at final sampling. Animals were placed on a 10 MP digital x-ray tablet and radiographed by a portable x-ray unit Gierth TR 90/30 peak (Germany) (www.gierth-x-ray.de). Digital x-rays were taken at 80 cm distance between the x-ray source and the tablet at 40 kV, 0.2 mA and 0.2 s exposure time. X-ray images were analysed as Dicom files using RadiAnt DICOM viewer (Medixant, Poland). To obtain higher resolution images 10 animals per tank, 30 animals per diet were also radiographed using AGFA Structurix NDT PbD2 film and a small animal radiation research platform (SARRP, XStrahl, Surrey, UK) at Ghent University hospital (INFINITY lab, Belgium). The apparatus was set to 40 kV with a tube current of 5 mA, 1 mm Al-filter, 10 x 10 cm collimator and the total exposure time of 30 s. The distance between the x-ray source and the film was 35 cm. Films were manually developed at the Center for Medical Genetics, Ghent University Hospital (Belgium) and digitised with an Epson Perfection V800 Photo transparency scanner at 2400 dpi. Adobe Photoshop V 6.0 was used for image processing.

2.8.1. Diagnosis of deformities

Deformities were analysed based on digital x-ray images. Vertebral column regions were identified following De Clercq et al. (2017). Types of vertebral deformities were recorded following Witten et al. (2009). The fusion of one or two vertebrae to the base of the skull (occipital region) or one or two vertebrae to the urostyle are a commonly observed non-pathological phenomenon that occurs in both wild and farmed osteichthyans (Arratia et al., 2001; Arratia and Schultze, 1992; Britz and Johnson, 2005; De Clercq et al., 2017; Johanson et al., 2005; Johnson and Britz, 2010; Witten et al., 2009). These types of fusions were therefore not included in the deformity count. Vertebral centra in Atlantic salmon parr regularly show a degree of phenotypic variability (Berge et al., 2009; Fjelldal et al., 2007; Gil Martens et al., 2006; Sambraus et al., 2020; Sullivan et al., 2007b; Vera et al., 2019; Ytteborg et al., 2010a) which was taken into consideration during the morphological analysis. Animals with one or more vertebral centrum deformity were classified as deformed.

2.9. Vertebral body morphology, mineralisation pattern and microanatomy

Nine vertebral columns from T_0 were used for whole mount Alizarin red S staining. At final sampling, six vertebral columns per tank, 18 vertebral columns per diet were used for whole mount Alizarin red S staining and two samples per diet were used for histological analysis. Samples were fixed in 10% buffered formalin for 72 h for subsequent histological and whole mount staining analyses. Vertebral columns were rinsed overnight with tap water, stepwise transferred from 30% to 70% ethanol and stored in 70% ethanol until further analysis.

2.9.1. Whole mount Alizarin red S staining

Whole mount Alizarin red S staining for mineralised bone was performed according to Witten et al. (2019) with the application of 5% KOH instead of 1% KOH in the steps using glycerol solutions. Observations of the vertebral column were made using oblique illumination on a Zeiss Axio Zoom V16 Fluorescence Stereo Zoom Microscope equipped with a 5MP CCD camera for imaging.

2.9.2. Histology

According to Witten et al. (2019) vertebral bodies 32–35 and 36–39 were dissected for decalcified and non-decalcified serial sections and embedded in GMA (glycol metacrylate). Vertebral body samples were gradually rehydrated through ethanol series, each step lasting 1 h (50%, 30% and dH_2O). Vertebrae allocated for the decalcified sections were submerged in 4% PFA with 0.2 M EDTA in 1 x PBS, pH 7.2 for three weeks. The solution was renewed every two days. Samples were subsequently rinsed with running tap water for two hours and gradually dehydrated on a rocker for one hour per acetone solution (30%, 60% and 100%). The GMA embedding protocol was performed according to Witten et al. (2001). An automated microtome (Microm HM360, Prosan) was used to make serial parasagittal sections of 2 μm. Sections were stretched on dH_2O mounted and dried at RT. Decalcified sections were stained with toluidine blue, and haematoxylin and eosin, and non-decalcified sections with Von Kossa - van Gieson (Presnell and Schreibman, 1998). Sections were cover slipped with DPX and observed using a Zeiss Axio Imager-Z compound microscope equipped with an Axiocam 503 colour camera.

2.10. Vertebral body measurements

2.10.1. Vertebral centra

Measurements of vertebral centra from final sampling were taken on images of digitised radiographic films (10 animals per tank, 30 animals per diet) with ImageJ software (National Institute of Health, USA). The length, height (radiopaque area) and the radiolucent area (R) (Fig. 2A') were measured on vertebral centra 32–41 following Witten et al. (2016). The radiolucent area is located between the borders of the two mineralised vertebral body end plates, as opposed to the intervertebral space located between the borders of the non-mineralised bone of the vertebral body end plates (Fig. 3A'). The length:height ratio was calculated to determine the shape of the vertebral centrum, i.e. square (regular) or rectangular (decreased length:height ratio).

2.10.2. Vertebral bodies

The distance between the consecutive post-zygapophyses and pre-zygapophyses on the neural side of the vertebral bodies and distance between consecutive neural and haemal arches was measured (Fig. 3C', C). Measurements were taken on images of whole mount Alizarin red S stained vertebral bodies using ImageJ software. The distance between 10 successive vertebral bodies (vertebral bodies 32 to 41 according to section 2.10.1) were taken from three animals per tank, thus nine animals per diet group. The distance was consistently measured between the borders of the non-mineralised bone.

2.11. Vertebral centrum compression tests

Vertebral centrum compression tests were carried out at the Institute of Marine Research, Matredal, Norway. At final sampling, vertebral centra 31–33 from the vertebral region with the highest stiffness and yield load (Fjelldal et al., 2004, 2005), were dissected from six animals per tank, thus 18 animals per diet group and stored at −20 °C. Neural and haemal arches were removed. The centra from three individual vertebra were tested with a texture analyser (TA-T2, Texture Analyser; Stable Micro Systems, Haslemere, UK) by compressing a single vertebral centrum in a cranial-caudal axis of the vertebra with a steadily advancing piston (0.01 mm/s). Stiffness and yield load of vertebral centra was calculated based on a continuously recorded load-deformation data (Fjelldal et al., 2004).

2.12. Statistical analysis

All data are given as mean ± standard deviation. The data was analysed with a one-way ANOVA followed by Tukey's post-hoc test (Bonferroni corrected). Distance between post-zygapophyses and pre-zygapophyses was analysed by a Kruskal-Wallis test, with pairwise post-hoc comparisons (Bonferroni corrected). Pearson's correlation analysis was used to examine possible relationships between the values of mineral content analysed in opercula and values of mineral content analysed in vertebral columns. Statistical analysis was done using SPSS (Version 26, IBM Corp., USA). Tanks were considered statistical replicates and the level of statistical significance was $P < 0.05$.

3. Results

3.1. Diets

The proximate, macro and micromineral and soluble P analysis is shown in Table 1. The analysis showed that the desirable content of soluble P (3.5, 5.6 and 9.3 g/kg in LP, RP and HP diets) was largely achieved. The estimated available P content was calculated to be 2.5, 5.9 and 9.1 g/kg in LP, RP and HP diets. Digestibility of P is most effective at low dietary P levels (Albrektsen et al., 2018). Based on Albrektsen et al., 2018) it is estimated that about 85, 75 and 65% of dietary soluble P will be digested by increasing the dietary P levels as applied in the current study. Thus the level of soluble P available for absorption is very close to the value of estimated available P in LP diet while it is lower than the estimated values for available P in RP and HP diets. The proximate composition and content of the remaining macro and microminerals were comparable among the diets (Table 1).

3.2. Growth data

At the end of the experiment there were no significant differences in the final weight of animals compared among the three diet groups (Table 2). The average final weight for all diet groups was 44.15 ± 7.46 g and the average fork length measured 14.98 ± 0.80 cm. Similarly, there were no significant differences among diet groups concerning condition factor K with an average value of 1.31 ± 0.06. The average specific growth rate for all diet groups was 1.57 ± 0.12% body mass day^{-1}, the average FCR was 1.14 ± 0.08, the average feed intake was 1.93 ± 0.13% body mass day^{-1} and the feed intake (g day^{-1}) averaged 23.19 ± 3.25 with no significant differences among the diet groups (Table 2).

3.3. Blood plasma analysis

At final sampling P*i* plasma levels in LP animals (1.75 ± 0.20 mmol/l) were three times lower compared to HP and RP diet groups ($P < 0.05$) (Table 3). High P and RP animals had similar P*i* plasma levels of 4.76 ±

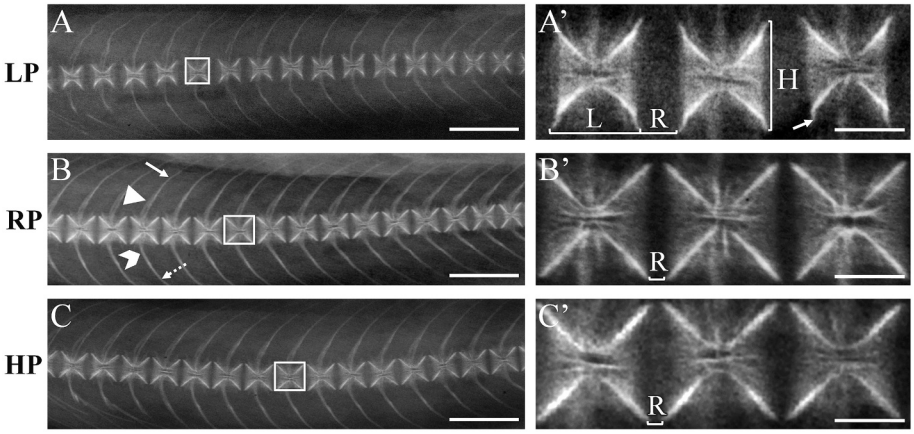


Fig. 2. Radiography for vertebral body morphology and vertebral centra measurements. Animal representatives for the phosphorus (P) diet groups: low P, LP (A–A'), regular P, RP (B–B′) and high P, HP (C–C′). Specimens are oriented anterior to the left and dorsal to the top. Vertebral body end plates are recognisable as typical X-shaped structures. Neural spine (block arrow), neural arch (arrowhead), haemal arch (chevron) and haemal spine (dotted arrow) are shown in (B). (A'–C′) Magnifications of (A-C) showing a more detailed image of the vertebral centra. Vertebral centra measurements as indicated in (A'): height (H), length (L) and radiolucent area (R) were taken on digitised x-ray films. Notice the distal zones of the vertebral body end plates in the LP animal representative (arrow) with a slight inward bending (A'), i.e. away from the intervertebral space. The vertebral body end plates are straight in both RP animal (B′) and HP animal (C′). Scale bars (A–C) = 5 cm, (A'–C′) = 1.25 cm.

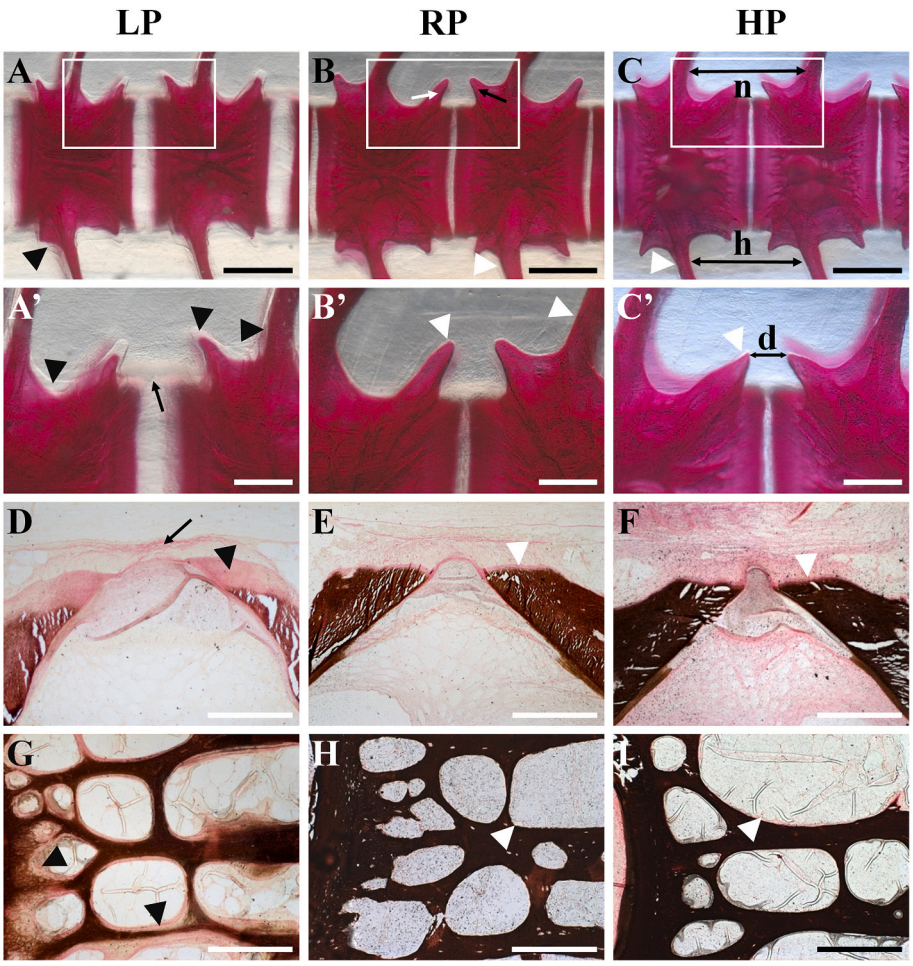


Fig. 3. Mineralisation pattern. The whole mount Alizarin red S stained vertebral bodies (scale bars (A–C) = 1 cm, (A'–C′) = 0.5 cm) and non-decalcified histological sections (Von Kossa-Van Gieson staining, scale bars (D–I) = 250 μm) of the animal representatives of the phosphorus (P) diet groups: low P (LP) (A, A', D, G), regular P (RP) (B, B′, E, H) and high P (HP) (C, C′, F, I). Specimens are oriented anterior to the left and dorsal to the top. (B) shows a post-zygapophysis (white arrow) and a pre-zygapophysis (black arrow). (C) shows the distance between neural (n) and haemal arches (h). (A'–C′) Magnifications of the white boxes in (A–C). The true intervertebral space is shown in (A') (arrow). (C′) Shows the distance between the post- and pre-zygapophysis of the adjacent vertebral bodies (d). Measurements (d, h and n) were consistently taken between the borders of a non-mineralised bone. (A', D, G) Show the enlarged areas of non-mineralised bone in zygapophyses, neural and haemal arches, vertebral body end plates and bone trabeculae of LP specimens (black arrowheads). (B–C, B′–C′, *E*-F, H–I) Show a thin non-mineralised bone tissue layer (osteoid) at the apical edges of the zygapophyses, neural and haemal arches, vertebral body end plates and bone trabeculae in RP and HP specimens (white arrowheads). Mineralised bone in (D–I) can be observed in black. Note the smooth outer surface of the non-mineralised bone of trabeculae in LP specimen (G) with no indication of bone resorption associated with an increased metabolic demand of the animal. Black arrow in (D) shows well-developed collagen fibre bundles in LP animal. (Note: the apparent tears in the bone tissue are a commonly observed artefact in non-decalcified histological sections). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Growth data measurements. Growth measurements, specific growth rate (SGR) and feed conversion ratio (FCR) of animals fed by a low phosphorus (LP), regular phosphorus (RP) and high phosphorus (HP) diet. There were no significant differences among diet groups.

Growth data	LP	RP	HP
Initial weight (g)	13.64 ± 0.97	13.70 ± 1.57	13.12 ± 1.50
Final weight (g)	43.33 ± 7.48	45.28 ± 8.24	43.84 ± 6.67
Initial fork length (cm)	10.46 ± 0.26	10.41 ± 0.43	10.30 ± 0.42
Final fork length (cm)	14.80 ± 0.71	15.16 ± 0.92	14.98 ± 0.73
Condition factor (K) final sampling	1.33 ± 0.07	1.29 ± 0.07	1.30 ± 0.05
SGR intermediate sampling (% body mass day^{-1})	1.94 ± 0.11	1.90 ± 0.18	1.89 ± 0.09
SGR final sampling (% body mass day^{-1})	1.56 ± 0.13	1.57 ± 0.13	1.57 ± 0.09
FCR	1.15 ± 0.09	1.06 ± 0.05	1.21 ± 0.10
Feed intake (% of body mass day^{-1})	1.93 ± 0.20	1.79 ± 0.11	2.05 ± 0.08
Feed intake (g of feed day^{-1})	23.39 ± 3.38	21.90 ± 4.15	24.06 ± 2.58

0.26 mmol/*l* and 4.72 ± 0.96 mmol/*l*, respectively. The levels for ALP, Ca^{2+}, Cl^{-} and Na^{+} were comparable in animals from all three diet groups (Table 3).

Table 3
Blood plasma analysis. Listed values for calcium (Ca^{2+}), inorganic phosphorus (P*i*), sodium (Na^{+}), chloride (Cl^{-}) and alkaline phosphatase (ALP) in plasma of Atlantic salmon parr fed low phosphorus (LP), regular phosphorus (RP) and high phosphorus (HP) diets. The level of P*i* was about 60% lower in LP animals relative to RP and HP animals. The statistical significance ($P < 0.05$) is denoted by a lowercase superscript (within a column).

Diet	Ca^{2+}	P*i*	Na^{+}	Cl^{-}	ALP
	mmol/*l*				(U/*l*)
LP	1.18 ± 0.09	1.75 ± 0.20^{a}	156.50 ± 1.62	118.26 ± 0.99	170.37 ± 13.65
RP	1.19 ± 0.04	4.72 ± 0.96^{b}	157.88 ± 5.64	118.48 ± 3.30	191.51 ± 38.25
HP	1.12 ± 0.05	4.76 ± 0.26^{b}	154.73 ± 2.40	118.74 ± 3.71	169.71 ± 20.62

3.4. *Vertebral centra deformities*

Radiological analysis of T_0 samples showed that three out of 27 animals had a vertebral centrum deformity, 0.25% of all examined vertebrae were deformed. There was a single hyper-radiodense vertebral centrum in the abdominal region present in two animals. One animal had a contained complete fusion of the preural centra 4–3.

At final sampling, nine out of 45 animals were diagnosed with mostly minor vertebral centrum deformities in the RP diet group, 1.15% of all examined vertebrae. In LP and HP animals, respectively six and four out of 45 animals were diagnosed for mostly minor vertebral centrum deformities, 0.41% and 0.30% of all examined vertebrae. Deformities

typically affected two vertebrae. The deformity types were compression, compression and fusion, contained vertebral centrum fusion, decreased intervertebral space, and hyper-radiodense vertebral centra. A single RP animal suffered from a deformity which affected 17 consecutive vertebrae composed of a fusion centre and compressed vertebrae. Deformities were located in the abdominal region in 80% of the cases. Animals in LP and HP diet groups did not develop any specific type of a deformity compared to RP animals. There was neither an increase nor a decrease of deformities in LP or HP animals compared to RP animals and T_0 samples.

3.5. Vertebral body morphology

X-rays only show the mineralised parts of the vertebral bodies. On radiographs HP and RP animals show fully mineralised vertebral centra, arches and spines (Fig. 2B–C) with vertebral body end plates recognisable as typical X-shaped structures (Fig. 2). In contrast, the mineralised parts of the vertebral body end plates in LP animals (radiopaque area) retained the same size that was present prior to the experiment (Fig. 2A). Still, bone growth in LP animals continued with the developed non-mineralised (radiolucent) vertebral body end plates (as evidenced by whole mount Alizarin red S staining and histological examination, see section 3.7.2). The apparent space between vertebral body end plates on x-ray images (radiolucent areas) extended proportionally to the length of the animal. The distal zones of low mineralised vertebral body end plates appear slightly bent inwards, i.e. away from the intervertebral space (indicated by arrow, Fig. 2A').

3.6. Vertebral body measurements

The average length of vertebral centra 32–41 in RP and HP diet groups was 1.93 ± 0.15 mm, the average height of the vertebral centra for both diet groups was 2.12 ± 0.13 mm. The average length of the radiolucent area (non-mineralised bone + intervertebral space) was 0.29 ± 0.03 mm in animals from both groups. The vertebral centra in the LP diet group measured 1.47 ± 0.10 mm in length and 1.83 ± 0.15 mm in height. Radiolucent areas (non-mineralised bone + intervertebral space) measured 0.74 ± 0.16 mm (Fig. 4). Low P animals had a slight but significantly decreased vertebral centrum length:height ratio (radiopaque area only) compared to RP animals due to the reduced length of the vertebral centra (Fig. 4). The sum of the vertebral length (radiopaque area) and the space between vertebral centra (radiolucent areas) was not significantly different among the three diet groups (Fig. 4). The increased distance between the bone of the post- and pre-zygapophyses of the consecutive centra in LP specimens compared to both HP and RP specimens ($P < 0.05$) (Table 4) (data from Alizarin red S whole mount stained specimens) indicates an increase of intervertebral spaces (Fig. 3A-A').

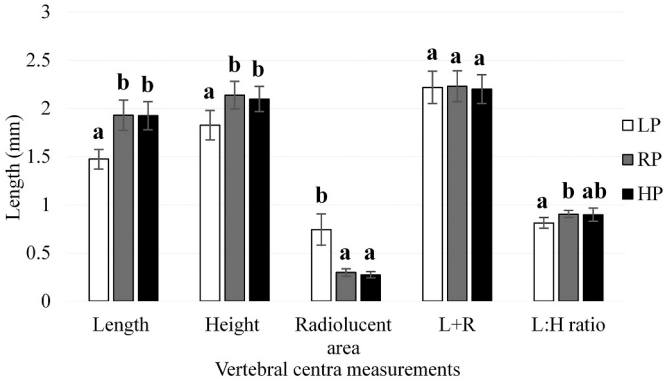


Fig. 4. Vertebral centra measurements. Significant differences between diet phosphorus (P) groups (low P (LP), regular P (RP) and high P (HP)) are denoted by a different letter ($P < 0.05$). Note that the vertebral centra length and height (radiopaque area) was decreased and the radiolucent area between the vertebral centra was enlarged in the LP animals relative to the RP and HP animals.

Table 4
Vertebral body measurements. Measurements of the distance between post-zygapophyses and pre-zygapophyses and between neural and haemal arches of the vertebral bodies taken from the whole mount Alizarin red S specimens. The distance between post-zygapophyses and pre-zygapophyses was significantly increased in low phosphorus (LP) animals relative to regular phosphorus (RP) and high phosphorus (HP) animals ($P < 0.05$) (denoted by a lowercase superscript letter (within a row)).

	LP	RP	HP
Distance between post-zygapophyses and pre-zygapophyses (mm)	0.47 ± 0.07^a	0.36 ± 0.06^b	0.33 ± 0.06^b
Distance between neural arches (mm)	1.70 ± 0.11	1.73 ± 0.11	1.70 ± 0.12
Distance between haemal arches (mm)	1.66 ± 0.10	1.70 ± 0.11	1.68 ± 0.11

3.7. Mineralisation

3.7.1. Mineral analysis

Mineral analysis of vertebrae showed no significant differences between animals fed HP and RP diet (Table 5). In the LP diet group, Ca and *P* values were reduced by about 50%, Mg values decreased by 35% ($P < 0.05$) (Table 5). The Ca:P ratio and the values for Na and Zn were not significantly different between the diet groups (Table 5). Mineral analysis of Zn in opercula showed significant differences among all three diet groups in the following order: LP < RP < HP (Table 5). The remaining minerals measured in opercula were not significantly different between RP and HP diet groups. In the LP diet group Ca, P and Mg values were reduced by about 50% (Table 5). There was a strong positive correlation between the mineral content of operculum and vertebral column ($r(53) = 0.94$, $P < 0.001$ (Fig. 5) among the three diet groups.

3.7.2. Mineralisation pattern

Alizarin red S stained specimens and non-decalcified parasagittal histological sections in LP animals demonstrate large areas of non-mineralised bone on all skeletal surfaces especially at the distal zones of vertebral body end plates (Fig. 3.A–A', D, G). In contrast, skeletal elements in HP and RP animals only show a thin layer of non-mineralised bone (osteoid) (Fig. 3B–C, B'C', *E*–F, H–I). The distribution of minerals in bone of HP and RP animals is comparable. Smooth trabecular bone surfaces indicate that there is no increased bone resorption rate associated with the metabolic demands of the animals

Table 5
Mineral analysis. Listed values showing the differences of macromineral (calcium (Ca), phosphorus (P)), micromineral (sodium (Na), magnesium (Mg) and zinc (Zn)) content (% or mg/kg of bone non-defatted wet weight) in vertebrae and operculum of animals fed a low phosphorus (LP), regular phosphorus (RP) and high phosphorus (HP) diet. The significant differences ($P < 0.05$) among diet groups (within a column) are illustrated by a lowercase superscript letter.

Tissue	Diet	Ca (%)	P (%)	Ca:P	Na (%)	Mg (mg/kg)	Zn (mg/kg)
Vertebrae (V1–19)	LP	0.77 ± 0.10^b	0.68 ± 0.05^b	1.13	0.08 ± 0.00	258.33 ± 24.51^b	80.33 ± 10.84
	RP	1.42 ± 0.08^a	1.20 ± 0.08^a	1.19	0.08 ± 0.01	378.50 ± 16.26^a	77.00 ± 11.31
	HP	1.58 ± 0.28^a	1.19 ± 0.08^a	1.33	0.09 ± 0.01	415.50 ± 39.36^a	69.00 ± 13.67
Operculum	LP	1.75 ± 0.16^a	0.82 ± 0.07^a	2.12	0.15 ± 0.00^a	309.22 ± 23.22^a	60.89 ± 4.51^a
	RP	3.42 ± 0.20^b	1.72 ± 0.09^b	1.98	0.16 ± 0.02^{ab}	694.67 ± 35.75^b	71.22 ± 3.82^b
	HP	3.59 ± 0.22^b	1.78 ± 0.08^b	2.01	0.17 ± 0.01^b	730.78 ± 39.24^b	79.11 ± 5.14^c

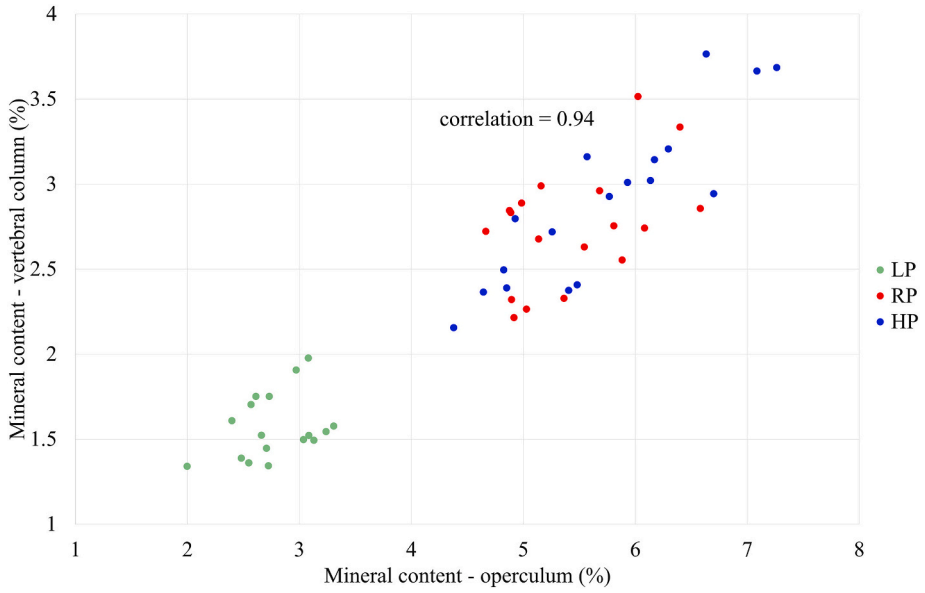


Fig. 5. Correlation between total mineral content of opercula and total mineral content of vertebral column. Each point represents an individual animal. Atlantic salmon were fed low phosphorus (LP), regular phosphorus (RP) and high phosphorus (HP) diet.

fed LP diet (Fig. 3G).

3.8. Structural alterations of vertebral columns in LP animals

On histological sections, the non-mineralised peripheral vertebral body end plates in LP animals appear slightly bent inwards, i.e. away from the intervertebral space (Fig. 6A, D), compared to vertebral body end plates of HP and RP animals which remain straight (Fig. 6B–C, E–F). The notochord-derived collagen type II based inner part of the intervertebral ligaments are thicker in LP animals. The length of the entire intervertebral ligament appears increased (Fig. 6A) compared to HP and RP animals (Fig. 6B–C). Apart from lengthening and thickening, animals from all three diet groups display intact structures of the intervertebral ligaments and of the notochord cells that populate the intervertebral discs (Fig. 6A–F). No pathological alterations in the intervertebral disc that would precede the development of vertebral centrum compression or fusion were detected (Fig. 6G–I). Low P animals show small amounts of ectopic cartilage; not present in the HP or RP animals; located at the interface between the vertebral body end plates and the trabecular bone (Fig. 6J–L). The extend of collagen type I fibre bundles, perforating the vertebral body end plates, Sharpey''s fibres, were well-developed in all diet groups including LP animals (Fig. 6M). Sections from animals of the LP diet group show well-developed abundant, spindle-shaped osteoblasts in the vertebral body end plate growth zone, which indicates active bone formation independent from bone mineralisation (Fig. 6N).

3.9. Compression tests

The mechanical testing of vertebral centra showed no significant differences in vertebral centra stiffness between RP animals (106.45 ± 15.33 N/mm) and HP animals (109 ± 17.13 N/mm) (Fig. 7). The stiffness of vertebral centra in LP animals was decreased (17.34 ± 8.63 N/mm) compared to vertebral centra stiffness in HP and RP animals (Fig. 6) ($P < 0.05$). The yield load for vertebral centra of LP animals was reduced by about 50% (12.67 ± 1.09 N) relative to vertebral centra of HP animals (28.88 ± 3.71 N) and RP animals (29.00 ± 3.49 N) (Fig. 6) ($P < 0.05$).

4. Discussion

This study examines how dietary P as a single factor influences the structure of the vertebral bodies in Atlantic salmon parr. Remarkably, on a gross morphological (radiographic) level, no increase and no specific type of vertebral column deformity were associated with any of the three experimental diets. Vertebral centra in RP and HP group animals were virtually identical. Vertebrae from LP group animals, though undermineralised, did not show any particular deformities. The animals developed large areas of non-mineralised bone thus bone and opercula mineral content was reduced. Given the low plasma P*i* levels in LP animals it was, however, surprising that LP animals did not show any growth retardation. This warrants discussion as continuous feeding applied in this experiment appears to be more important to maintain growth and the animals'' health than the total dietary P level. This study further discusses the necessity to use diets with high P content in the freshwater stages of Atlantic salmon. Concerning the structural alterations in the intervertebral ligaments observed in this study, the lengthening of the intervertebral ligaments appears to compensate the slight inward bending of the soft and non-mineralised vertebral body end plates in LP animals. It is discussed whether the thickening of the intervertebral ligaments possibly mechanically compensates for the lack of minerals in the bone.

4.1. Preserved structural integrity of the vertebral column in LP diet group animals

The number of deformed animals on a gross morphological level was low in all diet groups (LP = 6, RP = 9, HP = 4) with an average of two deformed vertebrae per deformed animal, predominantly located in the abdominal region. According to Hansen et al. (2010) such rate of deformed vertebrae per animal is neither a health nor a welfare concern. Two affected vertebrae per deformed animal in the abdominal region were also detected in wild Atlantic salmon (Sambraus et al., 2014). Frequencies of deformities recorded in the current study therefore likely represent a background deformity level.

In previous studies deficiency in dietary P has been associated with the development of vertebral deformities in Atlantic salmon (Sugiura et al., 2004; Sullivan et al., 2007a). A study by Baeverfjord et al. (1998) investigated the impact of P-deficiency in an Atlantic salmon parr trial lasting 12 weeks (initial weight 4.7 g) and in a 15 weeks long seawater study (initial weight 112 g). These studies determined low P as the main causative factor for vertebral column deformities. Likewise, Fjelldal et al. (2016) and Smedley et al. (2018) reported that diets insufficient in

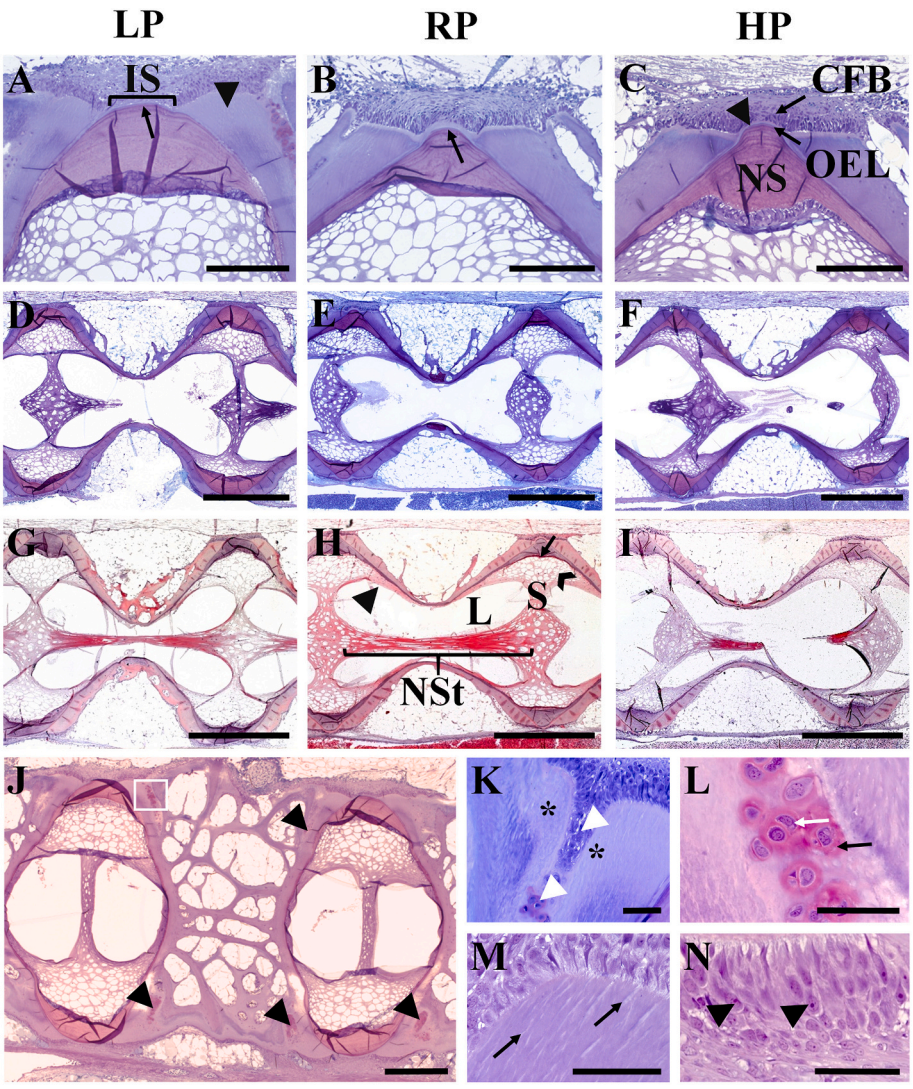
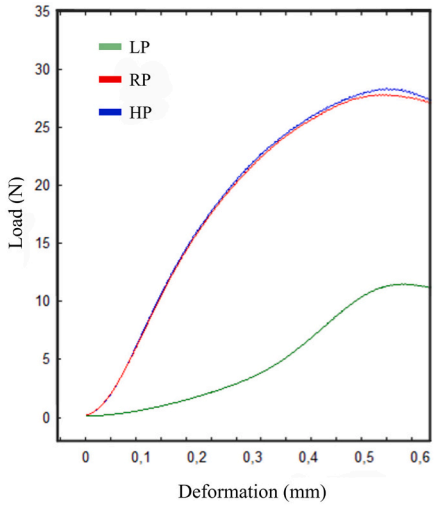


Fig. 6. Structural alterations of the vertebral column in LP animals. Decalcified histological sections representing specimens from Atlantic salmon parr fed low phosphorus (LP) (A, D, G, J–L), regular phosphorus (RP) (B, E, H) and high phosphorus (HP) (C, F, I). Note that (D–F, J) are parasagittal sections positioned further away from the middle of the vertebral centrum while (G-I) are sagittal sections closer to the mid-line of the vertebral centrum. The orientation of the specimens is anterior to the left and dorsal to the top. (A, D) Depict vertebral body end plates in LP animals that developed a slight inward bending, i.e. bending away from the intervertebral space. The vertebral body end plates of RP and HP animals remained straight (B–C, E–F) (toluidine blue staining, (A–C) scale bars = 250 μm, (D–F) scale bars = 1000 μm). (A) Shows the true intervertebral space (IS) extended in the LP animal group relative to the IS in the RP and HP animal groups. (C) The intervertebral ligament consists of collagen type I fibre bundles (CFB), the external collagenous ligament (black arrowhead), the notochord sheath (NS) and its outer elastin layer (OEL). The notochord sheath in LP vertebral centra was thickened (A) in comparison to the notochord sheath of the intervertebral ligament in RP animal (B) and HP animal (C). (D–I) Display intact vertebral body growth zone, the intervertebral ligaments and the intervertebral space in all diet groups. There was no sign of osteogenic tissue transformation into chondrogenic tissue at the vertebral body growth zones nor cartilage replacing notochord tissue in the peripheral intervertebral space that would foreshadow the development of vertebral centrum compression or fusion (haematoxylin and eosin staining, scale bars = 1000 μm). (H) Shows the intervertebral disc or the inner part of the intervertebral joint. The intervertebral disc consists of the notochord epithelium (basal cell layer) (arrow), vacuolated notochord cells (chevron), non-vacuolated and flattened cells (arrowhead) surrounding the extracellular lacunae (L), intervertebral septum (S) and notochord strand (NSt). (J) Depicts LP vertebral centrum with areas of ectopic cartilage (arrowheads and white square) located at the interface between the vertebral body end plates and the trabecular bone. (K) Ectopic cartilage was present as a gradient of cells with different cellular morphology (arrowheads) embedded in the non-mineralised part of the trabecular bone (asterisks). (L) Magnification of the white square in (J). (L) Ectopic cartilage composed of chondrocytes within isogenic groups (white arrow) surrounded by extracellular matrix (black arrow). (M) Depicts the extend of collagen type I fibre bundles, Sharpey's fibres, inside the bone of the vertebral body end plates in LP animal (arrows). (N) Shows active, spindle-shaped osteoblasts (arrowheads) located at the vertebral body growth zone. The sections were stained with toluidine blue, scale bars (J) = 500 μm; (K) = 400 μm; (L–N) = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

P, fed to Atlantic salmon from start feeding until smoltification (50 g), increase rates of vertebral column deformities. Fjelldal et al. (2012a) reported that Atlantic salmon (initial weight 1.3 g) fed a blue whiting fishmeal-based P-deficient diet for 11 weeks in freshwater stages has long-term consequences for the deformity development in the seawater. Therefore, changes on a tissue and cellular level could be already expected to appear in the P-deficient freshwater stage of Atlantic salmon of this study.

The most common types of vertebral centra deformities in Atlantic salmon, i.e. vertebral compression and fusion, do not start as bone pathologies but by alterations of tissues in the intervertebral space. Vertebral centra compression is characterised by the transformation of osteogenic tissue into chondrogenic tissue at the vertebral body growth zone. Cartilage replaces the notochord in the peripheral intervertebral space (Kvellestad et al., 2000; Witten et al., 2005). Also, when vertebral bodies begin to fuse the notochord tissue in the intervertebral space transforms into a cartilaginous tissue. This cartilage is subsequently replaced by bone which leads to centra fusion (Witten et al., 2006; Ytteborg et al., 2010b). Nonetheless, apart from lengthening and thickening of the intervertebral ligament in LP animals, all the components of the intervertebral joint were present and intact in all three diet groups. This includes the collagen type I fibre bundles that extend between the bone of the vertebral body end plates, the intervertebral ligament and components inside the intervertebral space e.g. the notochord epithelium, vacuolated notochord cells, intervertebral septum and notochord strand. Vertebral centra from LP animals show abundant and active osteoblasts in the vertebral body growth zone (Fig. 6N) and well-developed Sharpey's fibres in all diet groups including LP animals (Fig. 6M). The fact that tissues and structures in the intervertebral space of LP animals do not show any principal alterations indicates that low dietary P intake is not a primary or the single cause of vertebral centra compression and fusion in the freshwater parr stage of Atlantic salmon.

This study provided the animals with stable and controlled environmental conditions. There was no change in temperature, and there were no stressors present, such as grading, mechanical handling or vaccination. Other studies that investigated the relationship between dietary P and the development of deformities conducted experiments that included increased water temperature (Smedley et al., 2018) and handling associated with grading or vaccination on multiple occasions



	LP	RP	HP
Vertebral centrum stiffness (N/mm)	17.34 ± 8.63[b]	106.45 ±15.33[a]	109.66 ± 17.13[a]
Yield load (N)	12.67 ± 1.09[b]	29.00 ± 3.49[a]	28.88 ± 3.71[a]

Fig. 7. Mechanical properties - vertebral centra. Load deformity graph shows the extent of deformation (millimetres) on the x-axis in relation to the load (Newton) on the y-axis in vertebral centra from Atlantic salmon parr fed low (LP) regular (RP) and high phosphorus (HP) diets. The lines represent the mean values for each diet group. Lowercase superscript letters in the table show the significant differences ($P < 0.05$) in vertebral stiffness (N/mm) and yield load (N) between diet groups.

(Fjelldal et al., 2016; Fraser et al., 2019; Smedley et al., 2018). Increased temperature and handling stress represent factors that can induce the development of deformities (Fraser et al., 2015, 2019; Grini et al., 2011; Maria Poli, 2009; Ytteborg et al., 2010a). The exclusion of stressors may further explain the equally low prevalence of deformities in animals from all diet groups in this study. Still, long-term consequences necessitate further analysis. The ongoing follow-up study will therefore provide details about structure and morphology of the vertebral bodies in the seawater Atlantic salmon following P-deficiency in the freshwater.

4.2. How to maintain a functional vertebral column under dietary LP conditions

This study recognises that LP animals lengthen and thicken their intervertebral ligaments through detailed observations of serial histological sections. These findings are supported by the measurements of the distance between post- and pre-zygapophyses on whole mount Alizarin red S stained specimens. Possibly, lengthening and thickening of the intervertebral ligament represent functional adaptations of the vertebral column to compensate for low mineralised vertebral body end plates. The intervertebral joint (the intervertebral ligament and intervertebral disc (see Fig. 6C, H)) (Schmitz, 1995) is subjected to lateral bending stresses and compressive forces during undulatory swimming (Long, 1992; Symmons, 1979). The joint functions as (a) an elastic storage of energy while it moves from a bent to an unbent position and (b) as a muscle antagonist that dissipates energy when the joint moves from an unbent to a bent position (Long, 1992).

In this study LP animals showed 50% reduction in bone mineral content. Both the reduced bone mineral content and the presence of extended areas of non-mineralised bone matrix apparent especially at the distal zones of vertebral body end plates resulted in reduced vertebral centrum stiffness. As hypothesised by Witten et al. (2019), the compressive loads of muscles exerted on the intervertebral joint are likely to be transmitted to the non-mineralised trabecular bone through the soft, non-mineralised vertebral body end plates. In line with studies by Fraser et al. (2020) and Witten et al. (2019), also in this study LP animals showed a slight inward bending of the vertebral body end plates. Likewise, this slight inward bending of the vertebral body end plates coincided with the presence of ectopic cartilage at the interface between the vertebral body end plate and the trabecular bone of the vertebral centrum. The thickening and lengthening of the intervertebral ligament has, however, not been reported in the above mentioned studies. The lengthened intervertebral ligaments compensate for the loss of the intervertebral space as a consequence of the vertebral body end plates that are bent away from the intervertebral space. Indeed, LP animals developed regular size and the distance between vertebral centra was the same in all diet groups.

It is likely that the thickened intervertebral ligaments in P-deficient animals of this study mechanically compensate for soft and non-mineralised vertebral body end plates. Despite the lack of bone mineralisation, collagen type I based bone matrix production continues. Possibly the collagen type I production is even enhanced. Interestingly, Cotti et al. (2020) describe P-deficient zebrafish with highly active osteoblasts, indicated by the enlarged endoplasmic reticulum cisternae and increased bone matrix production in comparison to RP and HP zebrafish. One of the components of the intervertebral ligament are fibre bundles composed of collagen type I. It is difficult to differentiate between the fibre bundles of the intervertebral ligament and the non-mineralised collagen type I fibres in the bone matrix, e.g. in the whole mount stained Alizarin red S specimens. In this study, a thickening of the intervertebral ligament in LP animals is observed but it is difficult to evaluate whether bone matrix production is increased. The functional role of the collagenous matrix in bone is to absorb energy and to provide toughness (Viguet-Carrin et al., 2006). Similarly, the naked notochord of the extant Atlantic hagfish *Myxine glutinosa*, L. (Long et al., 2002) and the white sturgeon *Acipenser transmontanus* Richardson, 1836 (Long, 1995) absorb energy. Both species retain a continuous notochord throughout life surrounded by a strong and thick notochord sheath (Cole, 1905; Leprévost et al., 2017; Schmitz, 1998; Welsch et al., 1998). Interestingly, also in this study a thickening of the notochord sheath, i.e. the intervertebral ligament, in low mineralised vertebral bodies was observed.

As described by Witten et al. (2019) the non-mineralised vertebral body end plates which bend inwards restore their mineral content following a refeeding period with a regular dietary P which likely relates to their subsequent resumed angle i.e. bending outwards. Thus, the inward bending has no lasting consequences that would lead to the development of vertebral deformities. Likewise, ectopic cartilage that developed in LP animals at the interface between the bone of the vertebral body end plate and the trabecular bone becomes mineralised and resorbed if animals are fed a P-sufficient diet again. It is therefore expected that the lengthening and thickening of the intervertebral ligament observed in this study can also be restored under regular dietary P supply conditions.

4.3. Is continuous feeding more important than the dietary P level?

In this study, an increased dietary P-content had no influence on the animals' plasma P*i* levels. In contrast, the low P diet resulted in a dramatic, three-fold, decrease of plasma P*i*. Similarly, Vielma and Lall (1998a) reported a three-fold plasma P level decrease in Atlantic salmon fed P-deficient diet for 16 weeks.

Curiously, LP group animals in this study did not show any growth retardation, despite the reduction of bone mineralisation and plasma P*i* levels. Besides dietary P level, feed intake is one of the factors that determines the provision of dietary P in an animal (Shearer, 1995). The feed intake was however comparable among all three diet groups and animals fed LP showed distinct signs of P deficiency on macro and microscopic level.

The continuous growth observed in this study is more likely related to the continuous feeding regime. Likewise, several studies that examined the effect of dietary P-deficiency under continuous feeding conditions did not register any growth reduction. Comparable to the results obtained here, early freshwater stages of Atlantic salmon continuously fed a diet with low dietary P levels for nine weeks had a comparable weight gain with animals fed a diet with regular dietary P (Åsgård and Shearer, 1997). Similarly in a study on Atlantic salmon parr by Fjelldal et al. (2012a) where the animals were continuously fed a P-deficient diet for 11 weeks, bone mineralisation was arrested but not growth. Regular growth was also reported for P-deficient seawater stages of Atlantic salmon continuously fed for 14 weeks (Gil Martens et al., 2012) and likewise, for Atlantic salmon seawater stages continuously fed for a period of 11 weeks (Fjelldal et al., 2012b).

In contrast, a study with low P diets on Atlantic salmon parr fed twice a day observed growth reduction after 15 weeks (Vielma and Lall, 1998b). Similarly, a study by Witten et al. (2019) on early-seawater stages of Atlantic salmon fed a P-deficient diet twice a day noted a significant decrease in growth of the LP animals after seven and 17 weeks. Rainbow trout provided with a P-deficient diet twice a day had a reduced growth after 11 weeks (Coloso et al., 2003), 16 weeks (Koko et al., 2010), and 18 weeks (Shearer and Hardy, 1987). Hardy et al. (1993) reported a growth decrease in rainbow trout after four weeks of feeding a diet extremely low in P three times a day.

Hossain et al. (2020) and Antony Jesu Prabhu et al. (2014) investigated the post-prandial changes in plasma mineral levels of rainbow trout. The plasma P levels showed a peak about six and four hours post feeding respectively and started to decrease thereafter. It can be postulated that animals reared under an interval feeding strategy involving fasting of more than six hours experience a decrease in plasma P levels before the next feeding event. In contrast, the continuous feeding regime provides the animal with a steady P supply and leads to stable blood plasma P levels. Indeed, dietary P requirements for optimal growth are lower than the requirements for maximum bone mineralisation (Antony Jesu Prabhu et al., 2013; Rodehutscord, 1996). This can explain why in the current study LP animals subjected to a continuous feeding regime maintained growth while large areas of new formed bone remained non-mineralised.

Still, it is remarkable that the here observed low plasma Pi levels (1.75 mmol/*l*), also observed in a study by Vielma and Lall (1998a) (1.09 mmol/*l*), are able to maintain growth of Atlantic salmon parr. Similarly, a study by Antony Jesu Prabhu et al. (2014) where rainbow trout fed a plant based diet containing 6.1 g/kg of total P had plasma Pi levels of 1.70 mmol/*l* and comparable growth to animals fed a plant based diet supplemented with di-calcium phosphate containing 10.3 g/kg of total P. In humans, hypophosphatemia (low plasma phosphate) (Pappoe and Singh, 2010) causes enhanced cellular P uptake (Elisaf and Siamopoulos, 1997) and thus a shift of P from extracellular (plasma) to P intracellular (Amanzadeh and Reilly, 2006). A similar extra- to intracellular shift of P may have occurred in Atlantic salmon, a shift that maintained cell function and growth.

The current study and studies by Fjelldal et al. (2012a, 2012b) and Gil Martens et al. (2012) provide evidence that a continuous feeding strategy of a P-deficient diet can supply a steady P intake. Even if dietary P content is low, continuous supply apparently maintains regular growth of Atlantic salmon parr.

5. Conclusion

A diet with a high P level (9.3 g/kg of soluble P), in comparison to a regular P level diet (5.6 g/kg of soluble P), had no beneficial effects on Atlantic salmon parr concerning bone formation, bone mineralisation, growth and prevention of vertebral centra deformities. Likewise, a diet low in P (3.5 g/kg of soluble P) did not cause the development of vertebral column deformities in the freshwater stage of Atlantic salmon. Aside from the lengthened and thickened intervertebral ligaments, discussed in the context of vertebral column adaptations to the low mineralised bone matrix, the intervertebral joints remained intact. The finding that continuous feeding, even with a LP diet, maintained growth of Atlantic salmon parr for a minimum of 11 weeks bears potential to reduce the dietary P content without negative consequences for animal growth and welfare. Ongoing studies examine the long-term consequences of dietary P excess and deficiency during the animals freshwater stage on animals' growth and on the health of the vertebral column.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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