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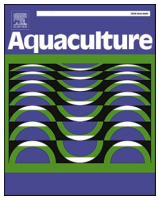

Phosphorus requirements in sea-cage farmed Atlantic salmon with an emphasis on bone health and digestibility

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ABSTRACT

Commercial fish feeds are supplemented with highly digestible inorganic phosphorus (P), a limited and expensive resource. As fish excrete excess dietary P, it is necessary to ensure they are fed the correct amounts to reduce costs and the release of phosphorus into the environment. The specific P requirements during the grow-out phase of farmed Atlantic salmon (*Salmo salar*, L.), which is when feeding intensity is highest, are unknown. In the current study, sea-cage reared salmon under natural light were fed one of six diets with increasing inorganic P levels (6.1-11.2 g/kg total P), from December 2022 (1.8 kg, sampling point I.), through April (2.8 kg, sampling II.), until July 2023 (4.2 kg, sampling III.). Response parameters were P digestibility, P retention, growth, vertebral deformities, vertebral mechanical strength and mineral content, bone microstructure, and microscopic location of bone minerals. Growth of the animals was lower between December-April (0.62 mm/day) with temperatures ranging from 5 to 9 °C compared with the period between April-July (0.98 mm/day, 7-14 °C). Phosphorus digestibility followed a similar trajectory with higher values in the second part of the study. Vertebral deformities were not affected by different dietary P levels. A regular somatic growth, bone mineralisation, and bone mechanical strength were achieved in animals fed 3.7 g/kg available P between December-April and in animals fed 4.6 g/kg available P between April-July. This shows the potential to reduce total dietary P content by 16 –24 % compared to current commercial feeds, without compromising bone mineralisation or skeletal health.

1. Introduction

The production of anadromous farmed Atlantic salmon (*Salmo salar*, L.) starts in land-based freshwater facilities where smolts are preadapted for the marine environment. Although some are kept on-land for the whole production cycle (Hersoug, 2022), most Norwegian salmon are transferred to sea-cages where they remain for about 1-2 years until they reach harvest size (e.g. Sigholt et al., 1995). Currently there are 989 sea-cage production sites in Norway (Pandit et al., 2023). Sea-cage production allows the continuous exchange of effluents with the surrounding water (Troell and Norberg, 1998; Wang et al., 2012) and represents the main source of phosphorus (P) emissions from salmon farming (Cho et al., 1994; Pandit et al., 2023). Phosphorus derived from fish farms is a pollutant that can increase the risk of eutrophication and the development of low oxygen zones (Correll, 1998; Boyd et al., 2019; Zohdi and Abbaspour, 2019; Akinnawo, 2023). In 2021, the Norwegian annual production of Atlantic salmon was 1.5 Mt (FAO, 2023) while the average P emission from salmon farming reached 16.3 kt, more than double the amount emitted in 2005 (Pandit et al., 2023).

Phosphorus is a key dietary component which provides energy for cells, it is essential for DNA and RNA molecules, protein phosphorylation, and for cell membranes (Wagner, 2024). Phosphorus is also one of the main components of vertebrate mineralised tissue. Together with calcium (Ca), P forms apatite, the mineralised part of the bone (Boskey, 2013). In farmed fish, Ca is absorbed by gills while the P requirement is met through the diet (Lall, 2002; Marshall, 2002). A shift to plant-based protein in salmon feeds where P is stored as relatively indigestible phytic acid (Ogino et al., 1979; Hua and Bureau, 2006; Kumar et al., 2019) has

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meant modern diets need supplementing with inorganic P in the form of monoammonium phosphate (MAP), monosodium phosphate (MSP), or monocalcium phosphate (MCP), which are all highly digestible (Morales et al., 2018).

Minerals provide bone with stiffness, the ability to resist bending and compression (Currey, 2003; Viguet-Carrin et al., 2006). As such, sufficient dietary P is imperative for bone development and mineralisation in farmed fish (Lall and Lewis-McCrea, 2007), particularly in the jaw and vertebral column (Bruno, 1990; Roberts et al., 2001; Witten et al., 2005; Fjelldal et al., 2012a; Bruno et al., 2013; Rauco et al., 2016). Collagen, the organic part of the bone matrix, provides bone with toughness, i.e. resistance to fractures (Currey, 2003; Ritchie et al., 2014). Vertebral deformities, especially the fusion of intervertebral joints, can relate to under and to over mineralisation of bone (Witten et al., 2006; Cotti et al., 2020). Multiple vertebral fusions reduce growth and welfare (Hansen et al., 2010) and cause down-grading during primary (Michie, 2001) and secondary (Fraser et al., 2019) processing in Atlantic salmon aquaculture, and should therefore be avoided by optimising dietary P supply.

Dietary P requirements in salmon depend on life stage and growth rate. For example, the P requirement for bone mineralisation appears to be higher during early compared to later life stages (Fjelldal et al., 2012a; Fjelldal et al., 2016; Fraser et al., 2019). Early freshwater stages of farmed Atlantic salmon experience high energy demand (Cho, 1992), fast relative growth (Austreng et al., 1987), and increased rates of oxidative stress associated with high metabolism (Hamre et al., 2022). The early requirements for dietary P are therefore high (9.2-11.4 g/kg total P, 7.9-9.8 g/kg available P) (Åsgård and Shearer, 1997). The initial phase in seawater has also been identified as critical in rapidly growing ~60 g post-smolts, whereby a suboptimal dietary mineral supply (10.4-12.2 g/kg total P, 5.4-6.3 g/kg available P) induced vertebral deformities (Fjelldal et al., 2009). However, tank-based studies show that larger post-smolts (100-200 g) can be fed relatively low P diets (4.7-8.4 g/kg total P) without compromising bone health (Witten et al., 2016, 2019; Fraser et al., 2019; Drábiková et al., 2023). This is because relative growth is lower later in life. The knowledge about the animals' dietary P requirements during grow-out in sea-cages where seasonal conditions may influence the dietary needs is still limited.

This study analysed the effect of six diets with graded levels of total P fed to sea-cage reared Atlantic salmon (1.8-4.2 kg) over the period of slow growth (December-April) with temperature ranging from 4.6 to 9.0 °C and fast growth (April-July) with temperature ranging from 6.7 to 13.9 °C. The diets (A-F) were formulated to contain total P levels according to their dry matter basis (DM) of either below (diets A-D, 6.1, 8.0, 8.7, and 9.5 g/kg, respectively), at (diet E, 10.4 g/kg), or above (Diet F, 11.2 g/kg) those recommended by the National Research Council (NRC, 2011). The range found in commercial feeds is between 9.4 and 13.0 g/kg total P (Chatvijitkul et al., 2017; Aas et al., 2019, 2022). The focus was on the analysis of bone and scale mineral content and vertebral centra mechanical properties as these are recognised as sensitive measures of mineral deficiency in Atlantic salmon (Davis and Gatlin, 1996; Lall, 2002; Fjelldal et al., 2004). Further, this study analysed the mineralisation pattern, and morphology of vertebral centra (x-ray images, series of histological sections, and micro-CT), and radiological analyses of vertebral centra deformities. The aim is to determine

Table 1
Diet formulation and composition specifying the three batches of diets A-F used to feed seawater stages of Atlantic salmon during the specified feeding dates.

Feeding dates	7.12.2022–16.2.2023						17.2. – 25.4.2023						26.4. – 3.7.2023					
Feed batch	I.						II.						III.					
Diet	A	B	C	D	E	F	A	B	C	D	E	F	A	B	C	D	E	F
Formulation (%)																		
Fish meal[1]	7.5						7.5						7.5					
Fish oil[1]	15.4						15.5						15.5					
Soy protein concentrate[2]	21.6						20.8						21.8					
Wheat gluten[3]	12.7						12.7						14.3					
Pea protein concentrate[4]	7.7						8.4						5.7					
Rapeseed oil[5]	16.0						15.6						15.7					
Wheat[6]	5.4	4.7	4.3	4.0	3.7	3.3	5.3	4.6	4.3	3.9	3.57	3.2	5.4	4.7	4.3	4.0	3.7	3.3
Dehulled beans[7]	10.0						10.0						10.0					
Vitamin mixes and astaxanthin	1.4						1.1						1.1					
Mineral premixes and inert marker	0.2						0.2						0.2					
Amino acid mixes	1.4						1.4						1.6					
Mono-ammonium phosphate	0.03	0.7	1.0	1.3	1.6	1.9	0.02	0.7	1.0	1.3	1.6	1.9	0.004	0.7	1.0	1.2	1.5	1.8
Water	1.0	1.1	1.1	1.1	1.2	1.2	1.4	1.5	1.5	1.6	1.6	1.7	1.2	1.3	1.3	1.4	1.4	1.5
Composition analysis																		
Moisture (%)	6.7	7.0	7.0	6.8	6.9	6.6	6.7	6.5	7.4	7.7	7.5	7.4	7.5	6.7	7.2	6.7	6.6	6.6
Crude protein (%)	38.9	39.2	39.4	39.8	40.6	40.4	39.1	39.4	39.4	39.2	39.5	39.7	38.0	39.0	39.3	40.1	40.0	40.4
Crude fat (%)	33.3	33.1	33.2	33.5	33.6	33.9	33.8	33.8	33.6	33.3	33.7	33.6	34.0	33.9	33.9	33.4	33.3	33.0
Ash (%)	3.7	3.9	4.0	4.1	4.3	4.5	3.7	3.8	3.9	4.1	4.2	4.4	3.7	3.9	4.0	4.1	4.3	4.5
Gross energy (MJ/ kg)[8]	25.4	25.3	25.3	25.5	25.7	25.6	25.7	25.6	25.5	25.2	25.4	25.4	25.8	25.8	25.9	26.0	25.7	25.6
Total P (g/kg (DM)[9])	6.5	8.1	8.8	9.5	10.7	11.6	5.9	7.9	8.5	9.1	10.0	10.8	6.1	7.7	8.5	9.2	10.2	10.9

[1] Supplied by Pelagia, Egersund, Norway.
[2] Supplied by CJ Selecta S.A., Brazil.
[3] Supplied by Roquette, Belgium.
[4] Supplied by Pinnlee Europe, The Netherland and Tulip Stars, China.
[5] Supplied by Emmelev A/S, Denmark.
[6] Supplied by Norgesmølene AS, Norway.
[7] Supplied by Soufflet Negoce.
[8] Calculated according to the following caloric values (MJ/kg): protein 23.6. lipid 39.5, and carbohydrate 17.1 (Albrektsen et al., 2018).
[9] Dry matter.

if an incremental reduction in dietary P supply has the potential to reduce P waste and increase P retention while maintaining growth without the development of skeletal deformities in Atlantic salmon reared in sea-cages under natural light.

2. Methodology

2.1. Diet

Six isocaloric and isonitrogenous diets were formulated by Mowi Feed (Bergen, Norway) to contain different levels of total dietary P (Table 1). Prior to feed formulation, all relevant raw materials were analysed for their proximate composition, including P. To provide an optimised nutrient profile and fresh feed to the fish, the feed production was organised in three batches (Table 1.). The control diet (E) was formulated according to the recommended level of total P (Lall and Bishop, 1977; NRC, 2011) and contained 10.0–10.7 g/kg total P. Diets with total dietary P below the recommended level (diets A-D) contained 5.9–6.1 g/kg (A), 7.7–8.1 g/kg (B), 8.5–8.8 g/kg (C), 9.1–9.5 g/kg total P (D), respectively. Diet F was formulated to have a total dietary P above the recommended level and contained 10.8 - 11.6 g/kg total P. Yttrium oxide (Y_2O_3) was used as an inert marker to determine digestibility of P. The three batches of feeds were fed to the fish for 72, 66, and 70 days, respectively.

Diet A contained the basal level of P. The surplus of P was supplied in a form of MAP, while the content of wheat was reduced to account for this inclusion (Table 1). Dietary Ca supplementation to compensate for decreasing Ca:P ratio was considered unimportant since diets were formulated not to exceed a total P of 17 g/kg, a content which can lead to reduced growth and lower zinc, and protein retention (Porn-Ngam et al., 1993; Satoh et al., 1996). Diets were produced by the Norwegian Food, Fisheries and Aquaculture Research Institute (Nofima) AquaFeed Technology Centre, extruded to 9 mm pellets and analysed. The content of ash (ISO 5984), crude protein (ISO 5983-2), fat (EC 152/2009), and moisture (ISO 6496) in the diets were analysed in duplicates by Nofima. Yttrium and total P were analysed according to NS-EN 15621:2017 using Agilent 5110 VDV ICP-OES. The diets were stored in a dark and dry indoor facility at ambient room temperature (10 °C). Animals were fed with an Auger-based feeding system using automatic feeders (Bettern S1 (Bio Marine AS, Skei, Norway) with a feeding pipe modified to have a single opening at the end only. Animals were fed once daily (7.12.2022–14.3.2023), twice daily (15.3.2023–26.4.2023), and thrice daily (27.4.2023–3.7.2023) with a target of 10 - 20 % overfeeding. The feed intake was calculated based on the daily collected uneaten feed with a plastic cone located inside the sea-cage. Uneaten material was collected by pumping up the pellets and weighing it. Subsequently, every day, 10 % of the collected uneaten pellets were stored at −20 °C. At the time of sampling, the waste feed was thawed, homogenised, and dried at 105 °C to calculate feed intake based on DM. The recovery rate of waste feed was calculated for each sea-cage based on the collection of feed within empty sea-cages.

The feed intake was then calculated based on the following formulation:

$$\textit{Feed intake}\ (\%BW) = \left(\frac{\left(\frac{\textit{total feed intake}\ (g)}{\textit{biomass gain}\ (g)} \right)}{\textit{Feeding days}} \right) x100$$

2.2. Experimental design

The study was conducted at the MOWI Feed Research Station (Aukan), Averøy, Norway (63°3′37″ N, 7°35′23″ E) between December 2022 and July 2023. The use of experimental animals was performed in strict accordance with the Norwegian Animal Welfare Act 2010. A veterinarian approved by the Norwegian Food Safety Authority was present on the site at all times during samplings. Salmon were reared under conditions comparable to standard commercial fish farming. The varying dietary P levels fed to the experimental fish could be considered within a regular natural fluctuation in food supply experienced by wild salmon (Kadri et al., 1995; Hendry and Beall, 2004; Thorstad et al., 2012). All measurements were done in fish anesthetised using MS-222 (0.003 % Finquel Vet., VESO Aqua, Oslo, Norway) in accordance with the supplier's instructions. Prior to any sampling, fish were euthanised using an overdose of MS-222 (0.02 % Finquel Vet.). Thus, in accordance with Norwegian and European legislation related to animal research, formal approval of the experimental protocol by the Norwegian Animal Research Authority (NARA) was not required. Animals hatched on 24.2.2021, started feeding on 19.4.2021, were vaccinated on 20.10.2021 (Alpha Ject micro 6, Alpha ERM salar, (Pharmaq, Oslo, Norway)), smoltified on 19.4.2022, and shipped to the Research Station on 27.4.2022. Prior to the experiment, animals were kept in two holding sea-cages (7 × 7 m) with an average stocking density of 11.2 kg/m^3.

In December (5.12.2022), seawater Atlantic salmon (Mowi strain) of a selected weight class (1.8 kg) were either euthanised and sampled for an initial sampling (100 animals) or anaesthetised, measured and randomly allocated into one of 24 sea-cages (5.2 × 5.2 × 6 m) after recovery (2160 animals). The study was done in quadruplicate with 90 animals per sea-cage. The stocking density remained below 2.1 kg/m^3 throughout the experimental period. An intermediate sampling was done in April (24.4.2023) while the final sampling was in July (3.7.2023) (Fig. 1).

Animals were subjected to ambient water temperature and natural light throughout (Fig. 1). The temperature ranged from 4.6 to 9.0 °C between December and April and from 6.7 to 13.9 °C between April to July (Fig. 1). Oxygen, temperature, and salinity were recorded by an Innovasea environmental station (Airmax 200WX RS422, InnovaSea Systems Inc., Bergen) at a depth of 5 m (Fig. 1). Data for day length was acquired using R library "Stream Metabolism" (Sefick Jr, 2016).

2.3. Sampling and growth analysis

All animals were measured (fork length [to the nearest cm]) and weighed [g]. Fulton's condition factor (K) was calculated according to Ricker (1975) following the eq. 100 × W/L^{-3}, where W is the animal's live body weight (g) and L is the fork length (cm).

The fork length increase (mm/day) was calculated for each individual sea-cage based on ($L_{(2\ or\ 3)} - L_1$)/ $t_{2\ or\ 3} - t_{1\ or\ 2}$, where L_1, L_2, and L_3 is average fork length (mm) in December, April, and in July at times t_1, t_2, and t_3, respectively. The specific growth rate (SGR, % body mass/ day) was calculated based on (e^q - 1) x 100 (Houde and Schekter, 1981), where q = [ln ($W_{2\ or\ 3}$)) ln ($W_{1\ or\ 2}$)]/ feeding days (Bagenal and Tesch, 1978) where W_1, W_2, and W_3 are average live body weight in December, April, and July. The feed conversion ratio (FCR) was calculated according to NRC (2011) where FCR = Total feed eaten (kg, DM)/ (Final biomass (kg) – Initial biomass (kg)).

2.4. The whole-body analysis

Animals were starved for 24 h prior to the samplings in December and April, and 48 h prior to the sampling in July. The whole-body content of ash, fat, and total P was analysed in duplicate from 10 individual animals in December, a pooled sample of 2 animals per sea-cage in April (4 samples/ diet group), and 3 individual animals per sea-cage in July (12 samples/ diet group) at BioLAb (Nofima). Samples collected in July were also analysed for the content of zinc (Zn). Samples in April

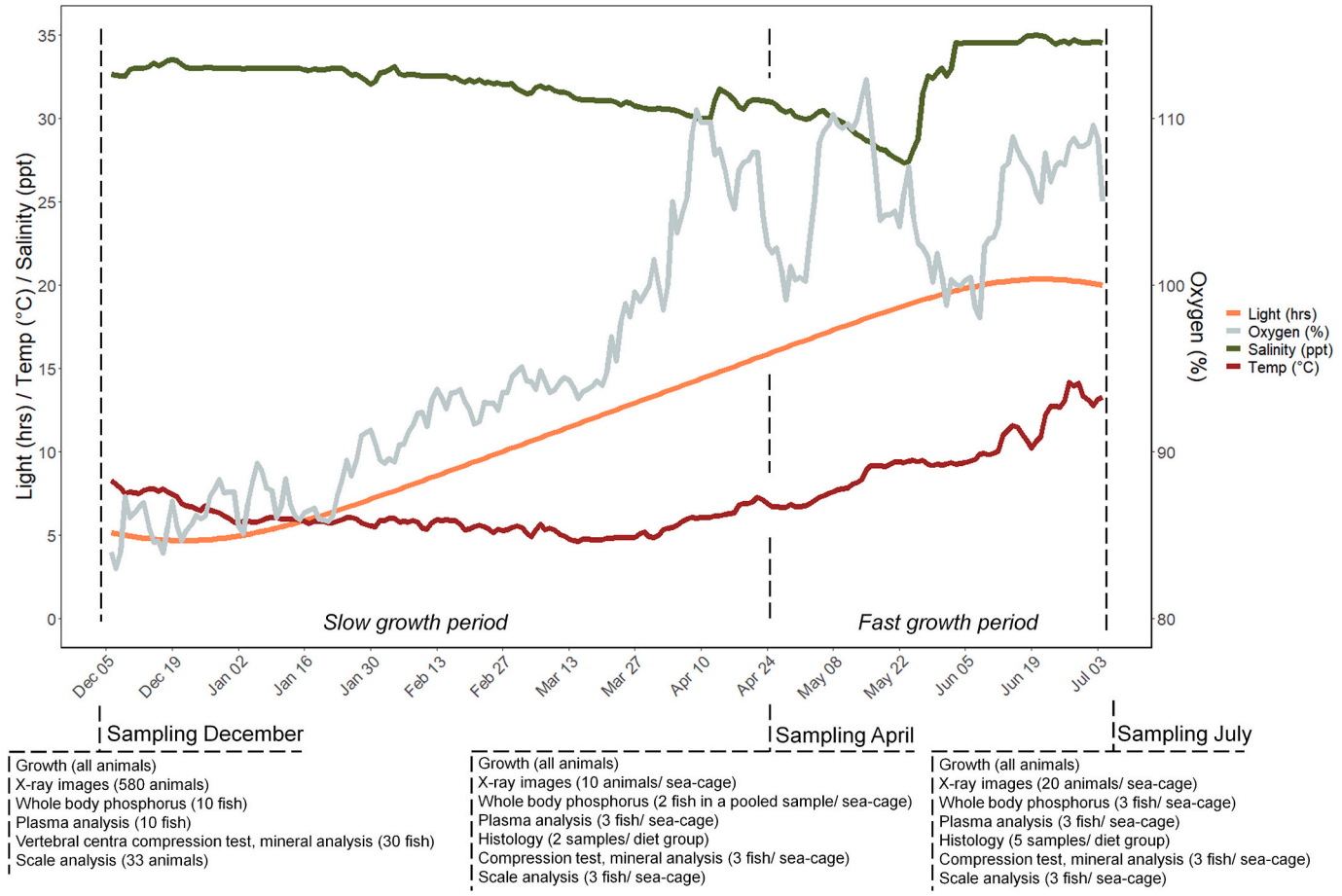

Fig. 1. Trial design specifying the length of the study, number and dates for sampling points, the analytical methods and number of samples analysed, as well as the environmental parameters (day length, water temperature, salinity, and oxygen) measured during the trial. Experimental diets were formulated to contain different levels of total dietary phosphorus (P) g/kg on a dry matter basis: 6.1 (diet A), 8.0 (diet B), 8.7 (diet C), 9.5 (diet D), 10.4 (diet E), and 11.2 (diet F). There were four sea-cages per diet group with 90 animals per sea-cage. The trial started in December (1st sampling point), the 2nd sampling point was in April, and the trial was terminated in July (3rd sampling point).

were freeze-dried prior to the analysis while samples in December and July were analysed on fresh matter. The analysis of ash and fat was according to ISO 5984 and EC 152/2009, respectively. Samples analysed for P and Zn were nitric acid digested using a Milestone UltraWave microwave (Milestone, Sorisole, Italy) and analysed with ICP-OES (a5110 VDV, Agilent, Oslo, Norway) using a reference method (NS-EN 15621).

2.5. Phosphorus digestibility

The apparent digestibility coefficient (ADC) of P was calculated according to NRC (2011):

$$\text{ADC}\ (\%) = \left(1 - \frac{\textit{Inert marker in feed}\left(\frac{g}{kg}DM\right)}{\textit{Inert marker in faeces}\left(\frac{g}{kg}DM\right)}\right) \times \left(\frac{\textit{P content in faeces}\left(\frac{g}{kg}DM\right)}{\textit{P content in diet}\left(\frac{g}{kg}DM\right)}\right) \times 100.$$

Feed and faeces samples were collected concurrently at the samplings in April and July. A pooled sample of 60-80 g (wet weight) of faeces per sea-cage was collected by stripping (Austreng, 1978). Available P was calculated based on a weighted total P (g/kg DM) average of the feed batches as follows:

$$\text{Available P} = \frac{\left(\textit{P ADC}\ (\%) \times \textit{weighted total dietary P}\left(\frac{g}{kg}, DM\right)\right)}{100}$$

The weighted total P (DM) from December 5 to April 24 was 6.1 g/kg, 8.0 g/kg, 8.8 g/kg, 9.5 g/kg, 10.5 g/kg, and 11.3 g/kg in diet A, B, C, D, E, and F respectively and from April 25 to July 3 it was 6.0 g/kg, 7.9 g/kg, 8.7 g/kg, 9.4 g/kg, 10.3 g/kg, and 11.1 g/kg total P in diets A-F.

2.6. The rate of P loss and retention

The rate of P loss and P retention was calculated for the whole experimental period (December-July). The content of whole-body P was

extrapolated to the sea-cage biomass. The dead biomass (dead and sampled fish) was accounted for. The gross P retention efficiency (%) was calculated based on Roy et al. (2024):

$$\text{P retention (\%)} = \frac{(\text{final body P content (\%)} \times \text{final biomass (incl.dead biomass) (g)}) - (\text{initial body P content (\%)} \times \text{biomass initial (g)})}{\text{total P in diet (DM)} \times \text{feed eaten (g)}} \times 100$$

The phosphorus losses were calculated based on Lellis et al. (2004):

$$\text{P lost (g P lost per kg fish weight gain)} = \frac{[(\text{total feed eaten (kg, DM)} \times \text{total P in diet (kg, DM)} - (\text{total P eaten (kg, DM)} \times \text{P retention (\%)})]}{\text{Final biomass gain (incl.dead biomass)(kg)}} \times 1000.$$

2.7. Plasma analysis

Blood was collected from 10 animals in December and three animals per sea-cage (12 samples/ diet group) in April and July by puncture of the ventral caudal peduncle using lithium heparanised Vacuette tubes (21G, REF 450062, VWR Avantor). Samples were kept on cooling elements. Plasma was obtained shortly after the blood collection by centrifugation at 4000 *g* at 4 °C for 10 min with a Universal 320 R Hettich Centrifuge (Tuttlingen, Germany) and stored at −20 °C until further analyses. The concentration of inorganic P (*Pi*) was measured based on Grini et al. (2011) with a phosphate assay kit (MAK488, Sigma-Aldrich, Merck, Darmstadt, Germany) using a microplate reader (Tecan Sunrise, Tecan Austria GmbH) according to the manufacturers' instructions. The dilution factor was 1:250 and samples were run in duplicates at Matre Research station (Institute of Marine Research). The ionised Ca^{2+} in plasma was measured using a radiometer (ABL90 FLEX PLUS, Brønshøj, Denmark).

2.8. Radiography

In December, 480 anaesthetised animals were radiographed and returned to the sea-cages after recovery. Additionally, 100 euthanised and sampled animals in December, 10 animals/ sea-cage, 40/ diet group in April, and 20 animals/ sea-cage, 80/ diet group in July) were filleted on the left side, placed on a digital tablet (Canon CXDI – 410C (43 × 43 cm), and radiographed with a portable x-ray unit GIERTH TR 90/20 (GIERTH X-Ray international GmbH, Riesa, Germany) by Aqua Kompetanse AS. Digital x-ray images were taken at 90 cm distance between the x-ray source and the tablet. The x-ray unit was set to 44 kV, 3.5 mA, and 0.18 s exposure time in December and 50 kV, 4.0 mA, and 0.2 s exposure time in April and July samplings. Images were digitised by a CANON Advanced Edge Enhancement software. The vertebral column was subdivided into post-cranial (vertebrae 1-2), abdominal (vertebrae 3-26), transitional (vertebrae 27-36), caudal (vertebrae 37-52), pre-ural (53-58), and ural (vertebrae 59-60) regions based on De Clercq et al. (2017); Sankar et al. (2024). X-ray images were assessed for the presence of vertebral deformities as DICOM files using Image J (Schneider et al., 2012). The specific types of deformities and deformity developmental pathways were assessed according to Witten et al. (2009) and Drábiková et al. (2022). Only animals with no deformed vertebra were used for compression testing and ash content analysis. For a better visualisation, a dual-energy X-ray absorptiometry with low energy (40 kV) and high energy (80 kV) using UltraFocus-DXA faxitron scan (Marlborough, MA, USA) was performed at INSERM U1132, Bioscar, Hopital Lariboisiére (Paris, France).

2.9. Vertebral body morphology

The transitional and caudal vertebral column regions were fixed in 10 % buffered formalin for 72 h. Samples were rinsed in tap water, gradually transferred to 70 % ethanol (EtOH) through steps of 30 % and 50 % EtOH. Vertebral columns were stored in 70 % EtOH.

2.10. Histology

2.10.1. Glycol metacrylate embedding

Vertebrae 32-34 (1 sample/ diet group in April and 2 samples/ diet group in July) and 35-37 (1 sample/ diet group in April and July) were dissected for demineralised and non-demineralised histological analysis, respectively. Vertebrae were gradually rehydrated through an EtOH series (50 % EtOH, 30 % EtOH, and demineralised water (dH_2O)) with 2 h per step. Vertebrae 32-34 were assigned for demineralised histology and were submerged into 25 % osteomol (Merck, Darmstadt) for 56 h with one solution change. Samples were rinsed overnight in running tap water then gradually dehydrated on a rocker with graded acetone steps (30 % and 60 %) for 2 h each. Samples were left overnight in 100 % acetone. For better embedding in glycol metacrylate (GMA), samples were chilled at −20 °C for 30 min prior to embedding. Embedding was done on ice according to Witten et al. (2001); Oralová et al. (2019). An automated microtome (Microm HM360, Prosan) was used to make serial parasagittal sections (4-5 μm). Additional samples, 1 sample/ diet group in July (vertebrae 33-34) were embedded in Technovit 7100 (Kulzer Technik GmbH, Wehrheim, Germany) according to the manufacturer's instructions. An automated heavy duty sectioning system Leica SM2500 (Leica microsystems Nussloch GmbH, Germany) was used to make parasagittal sections (3 μm) with a tungsten carbide knife. Sections were stretched in dH_2O, mounted on a slide, and dried at room temperature. Staining was done with toluidine blue and von Kossa/ Van Gieson (Presnell and Schreibman, 1998), coverslipped with DPX (Sigma-Aldrich), and observed using a Zeiss Axio Imager-Z compound microscope equipped with an Axiocam 503 colour camera.

2.10.2. Methyl metacrylate embedding

Vertebrae 33-35 (1 sample/ diet group in July) were embedded in Technovit 9100 (Kulzer Technik, Wehrheim, Germany) according to the manufacturers' instructions. Blocks were attached to plastic slides with Technovit EPOX. Cross-sections of vertebrae were grinded to the desired thickness (100 μm) with a metkon GEOFORM (Metkon Instruments Ltd., Bursa, Turkey) and subsequently polished with metkon FORCIPOL

(300–1 V) polisher and metkon DOSIONE using DIAPAT water-based diamond lubricant and subsequently DIAPAT-M water-based monocrystalline 3-μm diamond paste at Evolutionary Morphology of Vertebrates Group, Ghent University, Belgium. Ground sections were stained with Alizarin red stain and observed under a stereomicroscope. Images were taken with Zeiss Axio Zoom V16 Stereo Zoom Microscope equipped with a 5MP CCD for imaging and images were processed in Adobe Photoshop CS (Adobe Systems, San Jose, CA, USA).

2.10.3. μComputer Tomography scan (micro-CT)

Vertebrae 33 (1 sample/ diet group A, B, and E in July) and an 8 mm phantom rod of known bone mineral density (BMD) were scanned with SkyScan1272 (Bruker) at INSERM, Bioscar (Paris, France), installed with a Ximea xiRAY11 camera. Scans were taken at a distance of 173 mm between the camera and source and 96 mm between the object and source at 80 kV, 125 μA and 1900 ms exposure time. The final image pixel size was 10 μm. To allow a comparable analysis between the vertebral centra, cross sections with the smallest diameter of the notochord were picked manually in Dataviewer (Bruker), 69 sections were analysed anteriorly and posteriorly from this point to a total scan height of 1380 μm. The volume of interest (VOI) was selected manually and included the vertebral centra but excluded the neural and haemal arches. The BMD was calculated based on the 8 mm phantom.

2.11. Compression testing – vertebral centra

Vertebral centra 32-34 from the transitional vertebral column region were used for compression testing according to Drábiková et al. (2023). At the time of sampling, a section of vertebral column immediately after the dorsal fin was dissected and stored at −20 °C. Prior to the compression testing, vertebrae were left to thaw at room temperature. Three vertebrae per animal were tested in December (30 animals), in April (3 animals/ sea-cage, 12 animals/ diet group), and in July (3 animals/ sea-cage, 12 animals/ diet group). Vertebrae were carefully dissected, and the neural and haemal arches, neural tubes, and notochord tissue (tissue within the intervertebral spaces) were removed. Vertebral centra anterior-posterior diameter (length), dorsal-ventral diameter (height), and lateral diameter (width) were measured by a Vernier calliper to the nearest 0.01 mm. Vertebrae were then placed in a 9 g/kg NaCl solution prior to compression testing to avoid drying and to mimic the regular osmolality of the extracellular fluids of the fish (Kültz, 2015). Vertebral centra were then compressed along the anterior-posterior axis using a texture analyser (TA-HD plus Texture Analyser, Stable Micro Systems Ltd., Surrey, UK) with a steadily advancing piston (0.01 mm/s). The test was terminated at 35 % compression of the vertebral centra. Calculations of the mechanical properties were based on a load-deformation curve and stress-strain curve either non-adjusted for vertebral centra size (stiffness, yield load, and resilience) (Fjelldal et al., 2004) or adjusted for vertebral centra size (elastic modulus, yield point, fracture point, and toughness) (Hamilton et al., 1981) according to Drábiková et al. (2022). Stiffness and elastic modulus determine rigidity of vertebrae and represent the gradient of the initial linear portion of the curves prior to the compressive force permanently damaging the vertebra (Fjelldal et al., 2024). The point at which vertebra begin to deform permanently under continuous compressive force is termed the yield point. Yield load and failure point define the level of load or stress which is required for the vertebra to fracture, and resilience and toughness are measures of energy absorptive capacity of the vertebra before such fracture happens.

2.12. Ash analysis – scales and vertebrae

Scales (approximately 40 scales/ animal, 3 animals/ sea-cage, 12 animals/ diet group) were collected in April and July from the left side of the fish, in the region between the adipose fin and lateral line (Niemelä et al., 2016). Samples were stored in plastic bags at −20 °C until further analyses. Scales and a pooled sample of three vertebrae per animal, previously used for compression testing, were thawed, weighed (wet weight [g]), and defatted in acetone: methanol bath (1:1, *v/v*) for 20 h with one solution renewal. Samples were then oven-dried (DRY-Line, VWR) for 24 h at 105 °C, placed in a desiccator, and weighed (dry weight [g]). Dried samples were incinerated in a muffle furnace (Mod. Controller P320; Nabertherm GmbH) (0.5 h at 115 °C, 4 h at 540 °C, and 6 h at 750 °C) (Grini et al., 2011). The relative ash content (%) was calculated according to Cavrois-Rogacki et al. (2021) as follows:

$$\text{Ash}\ (\%) = (\text{Ash weight}\ (g)/\text{Dry weight}\ (g))\ \text{x}\ 100.$$

2.13. Mineral analysis – scales and vertebrae

Incinerated scale and vertebrae samples were shipped to BioLAb (Nofima) and analysed for the quantity of Ca, P, and Zn. Vertebral samples of 100 mg and scale samples of 30 mg were nitric acid digested using a Milestone UltraWave microwave (Milestone, Sorisole, Italy) and analysed with ICP-OES (a5110 VDV, Agilent, Oslo, Norway).

2.14. Statistical analysis

The data were analysed in R version 4.4.1 (R Core Team, 2024, http://www.r-project.org). The level of statistical significance was set at $p < 0.05$. Data were checked for normality (histogram, q-q plots). Non-normally distributed data were log transformed. Linear mixed effects (LME) models with sea-cage and fish as random factors were used to assess the associations between dietary P, sampling point, and the combined effect of these two factors on vertebral centra measurements, vertebral centra mechanical properties, vertebral centra and scale mineral content. The final model was the model with the lowest Akaike information criterion (AICc) based on backwards model building. General linear models (GLM) were used to assess the effect of dietary P, sampling point, and the combined effect of these two factors on P ADC, available P, feed intake, FCR, SGR, fork length increase, body ash, and body fat; and the effect of dietary P on P loss, P retention, body Zn, and vertebral centra BMD. The significance of the main effects of the model were assessed by the "Anova" function from the "car" package. The phosphorus requirements were evaluated by broken-line models for whole-body P, plasma phosphate, and vertebral centra ash (%). The packages "ggplot2", "nlme", "MuMIn", "emmeans", and "segmented" were used for analysis and graphical presentation (Muggeo, 2008; Wickham, 2016; Pinheiro et al., 2021; Lenth, 2024). Non-parametric data that were not log-transformed (fork length, weight, and condition factor) were analysed by Kruskal Wallis test followed by a post-hoc Dunn's test with Bonferroni corrections.

3. Results

3.1. Mortality

The mortality rate between December and April was 2.5, 1.9, 3.3, 3.1, 6.1, and 4.5 % in animals fed diet A, B, C, D, E, and F, respectively. The mortality rate between April and July was 3.0, 0.3, 2.0, 2.0, 1.7, and 2.4 % in animals fed diet A, B, C, D, E, and F, respectively.

3.2. Phosphorus and digestibility

The full statistical output for the final models for the P digestibility and available P can be found in Supplementary Table 1. The P ADC increased in July compared with April with the most notable increase in diets B-D. At both samplings the P ADC was reduced in animals fed diet A compared with animals fed diets B-F (Table 2). The available P differed accordingly and showed the lowest values in animals fed diet A, followed by animals fed diets B-D, and animals fed diets E-F (Table 2).

Table 2
The apparent digestibility coefficient (ADC) of total P content in 6 diets (A-F) across two feeding periods (December-April and April-July) with corresponding calculated available *P* values.

Diet	A	B	C	D	E	F	A	B	C	D	E	F	*p*-values	
Sampling	April						July						Diet P	Sampling
P ADC (%)	37.4^{b}	46.6^{a}	47.2^{a}	48.4^{a}	53.6^{a}	51.3^{a}	42.2^{b}	52.9^{a}	53.5^{a}	53.7^{a}	55.7^{a}	55.5^{a}	<0.001	0.003
Available P (g/kg DM)	2.3^{d}	3.7^{c}	4.1^{bc}	4.6^{b}	5.6^{a}	5.8^{a}	2.5^{d}	4.2^{c}	4.6^{bc}	5.1^{b}	5.8^{a}	6.1^{a}	<0.001	<0.001

Data for P ADC and Available P are presented as treatment (diet groups) means ($n = 4$).
Different lowercase superscripts within row indicate significant differences between treatments within the same sampling point.

Table 3
Growth parameters showing the median and interquartile range of raw values for fork length, weight, and condition factor. The predicted values for fork length increase, specific growth rate (SGR), feed intake, total feed eaten, and feed conversion ratio (FCR) specify the least-square means and the lower and upper 95 % confidence intervals in brackets. Values for December show median and interquartile range. Different superscript lower-case letters indicate the significant differences among the diet groups within the sampling points. Different upper-case superscript letters indicate the significant differences between the sampling points of the same diet group. Samplings were done in December, April, and July. Animals were fed six different diets A-F with a mean available P (g/kg, (DM)) specified in brackets.

Diet (available P g/ kg (DM))/ parameter	Fork length (cm)	Weight (g)	Condition factor	Fork length increase (mm/ day)	SGR (% body mass/ day)	Feed intake (% of body mass/ day)	Total feed eaten (kg DM/ day)	FCR
December	49 (46-52)	1785 (1405-2165)	1.54 (1.39-1.70)					
April (slow growth period)								
A (2.3)	56 (53-59)^{cB}	2795 (2158-3432)^{B}	1.61 (1.43-1.78)^{aB}	0.54 (0.49-0.60)^{B}	0.33 (0.30-0.36)^{B}	0.33 (0.29-0.36)^{B}	0.69 (0.61-0.77)^{B}	1.08 (1.02-1.15)
B (3.7)	57 (53-61)^{bB}	2895 (2226-3564)^{B}	1.53 (1.35-1.71)^{abB}	0.62 (0.57-0.68)^{B}	0.35 (0.32-0.38)^{B}	0.29 (0.26-0.32)^{B}	0.69 (0.61-0.77)^{B}	0.99 (0.92-1.06)
C (4.1)	57 (54-60)^{abB}	2850 (2150-3550)^{B}	1.51 (1.33-1.69)^{abB}	0.61 (0.56-0.67)^{B}	0.33 (0.30-0.36)^{B}	0.33 (0.29-0.36)^{B}	0.67 (0.59-0.75)^{B}	1.07 (1.00-1.14)
D (4.6)	58 (55-61)^{abB}	2910 (2262-3558)^{B}	1.52 (1.35-1.69)^{bA}	0.62 (0.56-0.67)^{B}	0.35 (0.32-0.38)^{B}	0.29 (0.26-0.32)^{B}	0.70 (0.62-0.78)^{B}	1.00 (0.93-1.06)
E (5.6)	57 (54-60)^{abB}	2890 (2210-3570)^{B}	1.52 (1.35-1.69)^{abA}	0.61 (0.55-0.66)^{B}	0.34 (0.31-0.37)^{B}	0.31 (0.28-0.34)^{B}	0.65 (0.57-0.74)^{B}	1.07 (1.00-1.14)
F (5.8)	58 (54-62)^{aB}	2958 (2270-3642)^{B}	1.50 (1.33-1.68)^{cB}	0.64 (0.59-0.70)^{B}	0.35 (0.32-0.38)^{B}	0.30 (0.26-0.33)^{B}	0.67 (0.59-0.76)^{B}	0.98 (0.92-1.05)
July (fast growth period)								
A (2.5)	62 (59-65)^{bA}	3995 (3192-4798)^{bA}	1.70 (1.48-1.93)^{a}	0.77 (0.71-0.82)^{cA}	0.47 (0.44-0.50)^{bA}	0.50 (0.47-0.53)^{A}	1.24 (1.16-1.32)^{A}	1.07 (1.00-1.14)
B (4.2)	64 (60-68)^{aA}	4200 (3198-5202)^{aA}	1.59 (1.39-1.79)^{abA}	0.97 (0.91-1.02)^{abA}	0.52 (0.49-0.55)^{abA}	0.51 (0.48-0.55)^{A}	1.38 (1.30-1.46)^{A}	0.99 (0.92-1.05)
C (4.6)	65 (60-70)^{aA}	4200 (3292-5108)^{aA}	1.56 (1.39-1.74)^{bcA}	0.98 (0.93-1.04)^{abA}	0.55 (0.52-0.58)^{aA}	0.52 (0.49-0.55)^{A}	1.32 (1.24-1.40)^{A}	0.95 (0.88-1.02)
D (5.1)	65 (62-68)^{aA}	4170 (3335-5005)^{aA}	1.52 (1.33-1.72)^{cA}	1.01 (0.95-1.06)^{abA}	0.50 (0.47-0.53)^{abA}	0.49 (0.46-0.52)^{A}	1.26 (1.18-1.34)^{A}	0.98 (0.91-1.05)
E (5.7)	65 (61-69)^{aA}	4230 (3385-5075)^{aA}	1.55 (1.35-1.75)^{dA}	1.04 (0.99-1.10)^{aA}	0.53 (0.50-0.57)^{aA}	0.53 (0.50-0.56)^{A}	1.32 (1.24-1.40)^{A}	0.99 (0.92-1.06)
F (6.1)	65 (61-69)^{aA}	4250 (3274-5226)^{aA}	1.56 (1.38-1.74)^{bcA}	0.89 (0.84-0.95)^{bA}	0.51 (0.48-0.54)^{abA}	0.49 (0.45-0.52)^{A}	1.22 (1.14-1.30)^{A}	0.97 (0.90-1.04)

3.3. Growth and feed intake

The full statistical output for the final models for the growth and feed intake can be found in Supplementary Tables 2 and 3, respectively. From December to April, the increase in fork length (mm/day) was comparable among the diet groups (Table 3). This period was defined as a slow growth period. From April to July animals fed diets B-E grew 40 % faster and A and F fed animals 30 % faster compared with the slow growth period (Table 2). This period was defined as a fast growth period. The weight of the animals remained comparable among the diet groups at the end of the slow growth period and showed a significant reduction in diet A compared with the rest of the diet groups at the end of the fast growth period (Table 3). Feed intake was comparable among the diet groups (Table 3).

3.4. Phosphorus handling and whole-body analysis

The full statistical output for the final models for the P handling and

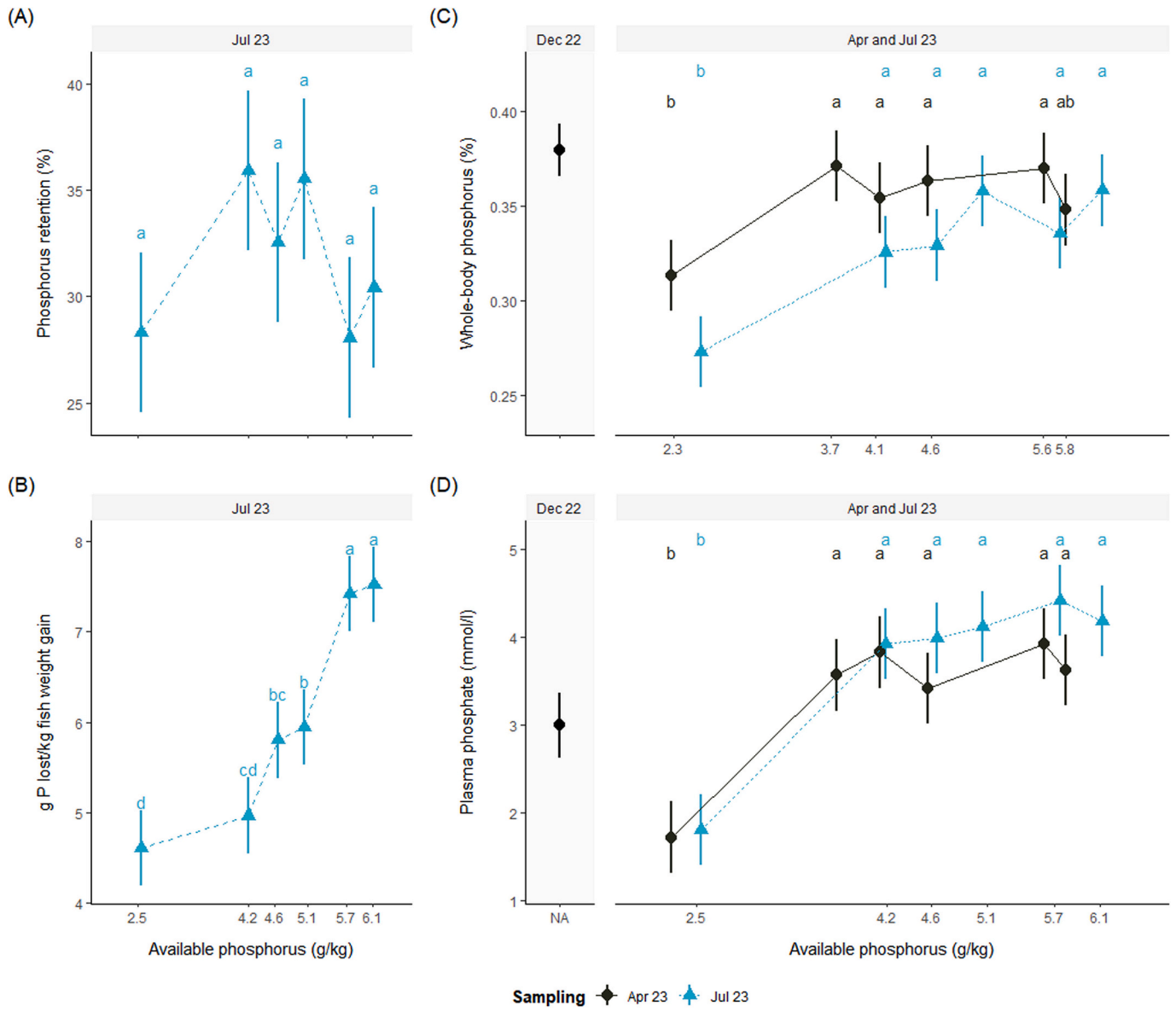

Fig. 2. Phosphorus (P) handling of Atlantic salmon in sea-cages fed different content of available P (g/kg on a dry matter basis). (A) Rate of P retention (%), (B) rate of P loss (g P not retained per kg of fish weight gain), (C) whole-body content of P (%), and (D) plasma P level (mmol/l). Different lower-case letters specify statistically significant differences between diet groups within the same sampling (black – April, blue – July). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

whole-body, and plasma Ca^{2+} analysis can be found in Supplementary Tables 4-6. Phosphorus retention rate (%) showed the highest values in animals fed diets B-D (Fig. 2A). Intrinsically, the P loss increased with the increased content of dietary P (Fig. 2B). The whole-body P content was reduced by about 16-20 % while plasma phosphate (P*i*) levels were reduced two-fold in animals fed diet A compared with animals fed diets B-F at both sampling points (Fig. 2C, D). The whole-body ash (g) showed a gradual increase over the course of the study (Table 4). The whole-body ash (%) analysis at the end of the slow growth period showed a significant 17 % reduction in animals fed diet A compared with animals fed diet C and D, but this was not significant in July when diet A was 13 % lower on average (Table 4). The broken-line model for whole-body P (%) and plasma P indicated a requirement of 3.75 and 4.2 g/kg available P in April and in July (Fig. S1A-B). At the sampling in April, plasma Ca^{2+} was 27 % higher and at the sampling in July it was 15 % higher in animals fed diet A compared with animals fed diets B-F (Table 4).

3.5. Skeletal deformities

3.5.1. Vertebral centra deformities

The proportion of deformed vertebral centra within the diet groups was comparable (Fig. 3A-C, Table 5). The prevalence of progressive vertebral fusion, a deformity with potential negative consequences on health of the animal and quality of the fillet, was low across the diet groups (Tabel 5).

3.5.2. Jaw deformity

At the end of the study in July, 2 out of 480 animals had a jaw deformity. One animal fed diet F had a lower and 1 animal fed diet D had an upper jaw deformity.

3.5.3. Vertebral centra measurements

The full statistical output for the final models for the vertebral centra measurements can be found in Supplementary Table 7. The vertebral centra length (anterior-posterior diameter) in animals fed diet A was

Table 4
The predicted values for whole-body ash (% wet weight (w.w.)), fat (% w.w.), zinc (Zn) (mg/kg w.w.), and plasma Ca^{2+} (mmol/l) showing the least-square means and the lower and upper 95 % confidence intervals in brackets. Values for December show median and interquartile range. Different superscript lowercase letters indicate the significant differences among the diet groups within the sampling points. Different upper-case superscript letters indicate the significant differences between the sampling points of the same diet group. Samplings were done in December, April, and July. Animals were fed six different diets A-F with a mean available P content (g/kg, (DM)) specified in brackets.

Diet (available P g/kg, (DM))/ parameter	Whole-body ash (g)	Whole-body ash (%)	Whole-body fat (%)	Whole-body Zn (mg/kg)	Plasma Ca^{2+} (mmol/l)
December	31.46 (27.65-35.26)	1.83 (1.77-1.86)	21.42 (20.57-22.27)	–	1.44 (1.42-1.47)
April (slow growth period)					
A (2.3)	42.2 (33.1-51.2)	1.51 (1.39-1.64)^{bA}	23.11 (22.36-23.86)^{aA}	–	1.18 (1.16-1.21)^{aB}
B (3.7)	43.9 (34.9-52.9)	1.75 (1.63-1.87)^{abA}	22.12 (21.37-22.87)^{abA}	–	0.87 (0.82-0.93)^{bB}
C (4.1)	49.2 (40.2-58.2)	1.79 (1.67-1.91)^{aA}	21.42 (20.65-22.18)^{bA}	–	0.91 (0.87-0.96)^{bB}
D (4.6)	52.8 (43.8-61.8)	1.84 (1.72-1.96)^{aA}	22.16 (21.41-22.91)^{abA}	–	0.88 (0.86-0.91)^{bB}
E (5.6)	49.4 (40.4-58.5)	1.76 (1.64-1.88)^{abA}	21.54 (20.78-22.29)^{abA}	–	0.86 (0.83-0.89)^{bB}
F (5.8)	50.4 (41.4-59.5)	1.72 (1.60-1.85)^{abA}	23.06 (22.31-23.81)^{aA}	–	0.86 (0.83-0.88)^{bB}
July (fast growth period)					
A (2.5)	55.0 (46.0-64.0)	1.43 (1.31-1.56)^{A}	23.68 (22.92-24.43)^{aA}	34.17 (31.10-37.23)^{a}	1.49 (1.48-1.51)^{aA}
B (4.2)	58.6 (49.6-67.7)	1.45 (1.33-1.57)^{B}	22.29 (21.54-23.04)^{abA}	30.75 (27.68-33.82)^{ab}	1.28 (1.24-1.31)^{bA}
C (4.6)	65.4 (56.4-74.5)	1.64 (1.52-1.76)^{A}	21.94 (21.19-22.69)^{bA}	30.71 (27.64-33.77)^{ab}	1.24 (1.22-1.26)^{bA}
D (5.1)	66.2 (57.2-75.3)	1.65 (1.53-1.77)^{B}	22.32 (21.57-23.07)^{abA}	29.92 (26.85-32.98)^{ab}	1.25 (1.23-1.27)^{bA}
E (5.7)	66.2 (57.1-75.2)	1.61 (1.49-1.73)^{A}	22.06 (21.31-22.81)^{bA}	27.25 (24.18-30.32)^{b}	1.19 (1.17-1.21)^{bA}
F (6.1)	60.5 (51.5-69.6)	1.55 (1.43-1.67)^{A}	22.47 (21.72-23.22)^{abA}	30.50 (27.43-33.57)^{ab}	1.26 (1.23-1.29)^{bA}

reduced by 0.5 mm at the end of the slow growth period and by 1.3 mm at the end of the fast growth period compared with the rest of the diet groups (Table 6). The vertebral centra length to height ratio in animals fed diet A was also significantly reduced at the end of both growth periods (Table 6).

3.6. Mineralisation

3.6.1. Radiography, non-demineralised histological sections, and micro-CT

Digital x-ray images showed a distinctly smaller mineralised area in vertebral centra in animals fed diet A (Fig. 4A) compared with animals fed diet B-F (Fig. 4B-F). The analysis of vertebral endplates on non-demineralised histological sections (rectangle in Fig. 5a) and edges of bone trabeculae on ground sections (rectangle in Fig. 5b) in animals fed diet B-F showed an osteoid seam at the edges of endplates (arrows in Fig. 5B-F) and bone trabeculae (arrows in Fig. 5H-L). These seams were broader in animals fed diet A (arrows in Fig. 5A and G).

The comparable analysis of BMD on micro-CT vertebrae scans (Fig. 6A-L) between the vertebral centra cross sections revealed a stepwise increase with the diet A sample showing the lowest, diet B intermediate, and diet E the highest BMD values (Fig. 6B). The diet A sample showed a reduced BMD (0.18 g/cm^3) compared with diet B (0.33 g/cm^3) and E sample (0.36 g/cm^3) (Fig. 6C).

3.6.2. Vertebral centra ash, Ca, and P content

The full statistical output for the final models for the vertebral centra mineral content can be found in Supplementary Table 8. The vertebral ash content (g and %), Ca, and P was comparable among diet B-F fed animals and significantly reduced in animals fed diet A at the end of both growth periods (Table 7). At the end of the fast growth period diet B fed animals showed a significant 2 % reduction in vertebrae ash (%) compared with animals fed C-F diets (Table 7). The broken-line model for vertebral ash (%) indicated a requirement of 3.75 and 4.8 g/kg available P in April and July, respectively (Fig. S1C). Independent from the degree of mineralisation no significant differences were detected concerning the vertebral Ca:P ratio (Table 7).

3.6.3. Scale ash, Ca, and P content

The full statistical output for the final models for the scale mineral content can be found in Supplementary Table 9. The scale ash content of animals fed diet A was reduced by 2 % at the end of the slow growth period and by nearly 5 % at the end of the fast growth period compared with the rest of the diet groups (Table 8). Scale P content was comparable among animals fed diets B-F at both samplings and reduced in animals fed diet A by 0.5 % at the end of the slow growth period and by 1.3 % at the end of the fast growth period (Table 8). The scale Ca:P ratio was significantly increased in the scales of animals fed diet A compared with the remaining diet groups (Table 8).

3.6.4. Mechanical strength of the vertebral centra

The full statistical output for the final models for the vertebral centra mechanical properties can be found in Supplementary Table 5 and 10. The stress strain curves for Atlantic salmon vertebrae define the amount of stress (MPa) required to strain (deform) the vertebra to 35 % of its original size (Fig. 7A). Stress required to deform vertebral centra of animals fed diet A was lower compared to animals fed diets B-F at the end of both growth periods (Fig. 7A) while vertebrae in diet B fed animals were more easily compressed at the end of the fast growth period compared with vertebrae of animals fed diet C-F (Jul 23 in Fig. 7A).

Vertebral stiffness and yield load of animals fed diet A were reduced by 25 % at the end of the slow and by 50 % at the end of the fast growth period compared with the rest of diet groups (Fig. 7B-C). Vertebral resilience remained comparable among the diet groups at the end of the slow growth period (Fig. 7D). Yield load and resilience were enhanced in animals fed diets B-E or remained constant in animals fed diet A between the sampling points.

The modulus of elasticity, yield point, failure point, and toughness demonstrated comparable results (Fig. 7E-H). Vertebrae from animals fed diet A showed a significant reduction in all parameters compared with the other diet groups with a further significant decrease as the study progressed. At the end of the fast growth period vertebrae from animals fed diet B had a tendency to show lower values compared with the other diet groups.

3.7. Morphology of the vertebral centra

The microscopical assessment of a series of histological sections of non-deformed vertebral centra in all diet groups showed well-developed vertebral bodies (Fig. 8A-F). The vertebral centra showed regular bone trabeculae (asterisk in Fig. 8A). The intervertebral joints were well-

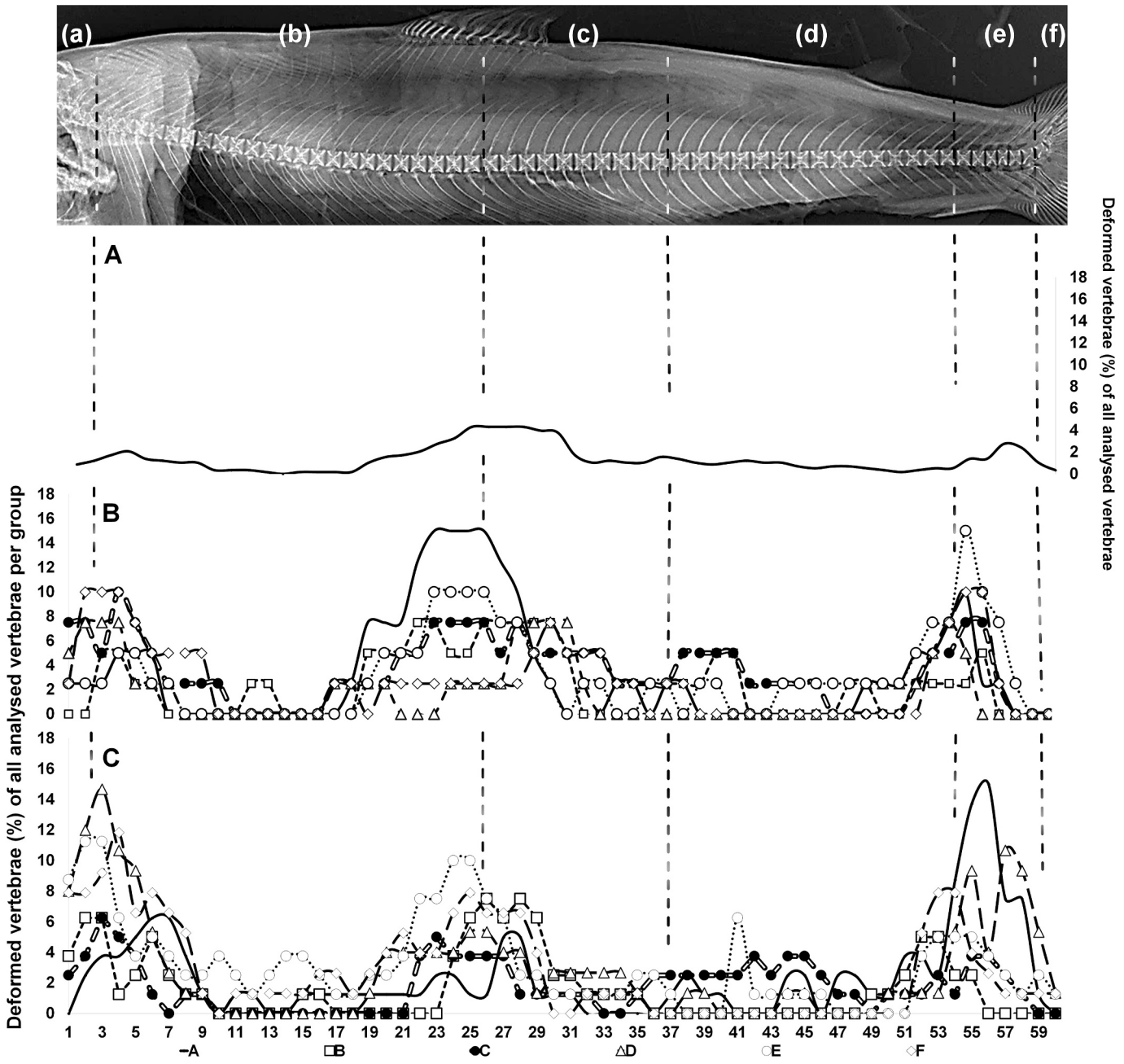

Fig. 3. The location and percentage of deformed vertebrae of all analysed vertebrae along the vertebral column (vertebrae 1-60). The x-ray image in the upper panel depicts six vertebral column regions of Atlantic salmon consisting of the post-cranial region (a) (vertebrae 1-2), abdominal region (b) (vertebrae 3-26), transitional region (c) (vertebrae 27-36), caudal region (d) (vertebrae 37-52), pre-ural region (e) (vertebrae 53-58), and ural region (f) (vertebrae 59-60). (A) The location and percentage of deformed vertebrae in 580 animals x-rayed in December. (B) The location and percentage of deformed vertebrae in 40 animals per diet group (A-F) x-rayed in April. (C) The location and percentage of deformed vertebrae in 80 analysed animals in diet group A-C, and E, 75 animals in diet group D, and 76 in diet group F x-rayed in July. Animals were fed diets A-F containing a mean available P on a dry matter basis of 2.3 g/kg (A), 3.7 g/kg (B), 4.1 g/kg (C), 4.6 g/kg (D), 5.6 g/kg (E), 5.8 g/kg (F) from December to April and 2.5 g/kg (A), 4.2 g/kg (B), 4.6 g/kg (C), 5.1 g/kg (D), 5.7 g/kg (E), 6.1 g/kg (F) from April to July.

developed and contained notochord sheath and its outer elastin layer, and collagen type I fibre bundles extending into the vertebral endplates in the form of Sharpey fibres (rectangle in Fig. 8A). The intervertebral spaces (triangle in Fig. 8A) were preserved and filled with chondrocytes (Fig. 8A-F).

Vertebral endplates in animals fed diet A showed a characteristic inward bending (left brace in Fig. 8A) and the notochord sheath had a hemispherical shape (white asterisk in Fig. 8A) rather than conical as was the case in the rest of the diet groups (Fig. 8B-F). At the end of both growth periods, animals fed diet A displayed ectopic cartilage behind the vertebral endplates (red arrow in Fig. 8A, asterisks Fig. 8G), a characteristic sign of dietary P deficiency (Drábiková et al., 2021). By the end of the fast growth, ectopic cartilages behind vertebral endplates (red arrow in Fig. 8B-C) also developed in animals fed diets B-F (asterisks in Fig. 8G-L). Ectopic cartilages were observed to contain chondrocytes (black arrow in Fig. 8G) and mineralised cartilage like cells (white arrow in Fig. 8G) next to osteocytes (black arrow in Fig. 8H).

When analysing edges of the bones of the vertebral centra surrounding the bone marrow spaces (dotted rectangle in Fig. 9A) within bone trabeculae (white asterisk in Fig. 9B-G), the abundance of osteoblasts in animals fed diet A appeared increased at the end of the fast growth period (yellow arrow in Fig. 9B). In the same treatment, bone

Table 5
The raw values listing the proportion of deformed vertebra (V), prevalence of deformed animals (%), the number of animals >10 deformed V, the number of animals with progressive vertebral fusion, and the number of animals with a mild deformity. Animals were sampled in December (580 analysed animals), in April (40 analysed animals per diet group), and in July (80 analysed animals in diet group A-C, and E, 75 animals in diet group D, and 76 in diet group F).

		A	B	C	D	E	F
Prevalence of deformed V (%)	*December*					1.32	
	April	3.33	2.46	3.54	1.92	3.21	2.67
	July	2.48	1.67	1.81	2.96	3.25	2.89
Prevalence of deformed animals (%)	*December*					15	
	April	33	20	28	18	40	33
	July	53	26	21	41	39	38
Number of animals with >10 deformed V	*December*					10	
	April	2	2	3	1	1	2
	July	0	1	1	1	3	2
Progressive V fusion	*December*					31	
	April	6	3	8	2	5	3
	July	3	4	6	6	6	4
Mild deformity[a]	*December*					54	
	April	7	5	3	5	11	10
	July	39	17	11	25	25	25

[a] Mild deformity included: contained and stabilising vertebral fusion, homogenous and one-sided compression, decreased intervertebral space, and internal ventral shift

marrow spaces were found to be filled with a combination of adipose and connective tissue (triangles and black asterisk in Fig. 9B). Remnants of connective tissue (triangles in Fig. 9C), but mostly adipose tissue (black asterisk in Fig. 9C), could also be observed in specimens from animals fed diet B. Diet C-F animals had bone marrow spaces filled with adipose tissue only (black asterisks in Fig. 9D-G). Bone trabeculae of animals fed diet A showed an increased prevalence of lines of remodelling (black/ white arrows in Fig. 9B). The edges of the bone surrounding the bone marrow spaces were rough in animals fed diet A associated with a higher occurrence of osteoclasts (red arrow in Fig. 9B). The edge of the bone was smooth in the other treatment groups (Fig. 9C-G). At the edges of vertebral endplates (solid rectangle in Fig. 9A), osteoblasts were abundant and had a regular spindle shaped appearance in all diet groups (black arrows in Fig. 9H-M). Type I collagen fibre bundles (red arrowheads in Fig. 9H-M) and their extensions into the vertebral endplates were thick and clearly visible (black arrowheads in Fig. 9H-M).

Table 6
Predicted values for vertebral centra measurements showing the least-square means and the lower and upper 95 % confidence intervals in brackets. Values for December show median and interquartile range. Different superscript lower-case letters indicate the significant differences among the diet groups within the sampling points. Different upper-case superscript letters indicate the significant differences between the sampling points within the same diet group. Animals were sampled in December, April, and July. Animals were fed six different diets A-F with a mean available P (g/kg (DM)) specified in brackets.

Diet (available P g/ kg (DM))/ parameter	Vertebral length (mm)	Vertebral height (mm)	Vertebral width (mm)	Vertebral length: height ratio
December	6.75 (6.72-6.79)	6.93 (6.89-6.96)	7.34 (7.29-7.38)	0.97 (0.97-0.98)
April (slow growth period)				
A (2.3)	7.64 (7.45-7.82)^{bA}	8.33 (8.10-8.57)^{B}	9.08 (8.75-9.40)^{B}	0.91 (0.90-0.93)^{bA}
B (3.7)	8.13 (7.94-8.32)^{aB}	8.35 (8.10-8.59)^{B}	9.31 (8.99-9.64)^{B}	0.98 (0.97-0.99)^{aA}
C (4.1)	8.19 (8.00-8.38)^{aB}	8.34 (8.09-8.58)^{B}	9.37 (9.04-9.70)^{B}	0.98 (0.97-0.99)^{aA}
D (4.6)	8.13 (7.94-8.32)^{aB}	8.14 (7.90-8.39)^{B}	9.02 (8.69-9.34)^{B}	0.99 (0.97-1.00)^{aA}
E (5.6)	8.11 (7.92-8.31)^{aB}	8.30 (8.06-8.54)^{B}	9.22 (8.88-9.55)^{B}	0.98 (0.96-0.99)^{aA}
F (5.8)	8.02 (7.83-8.20)^{abB}	8.23 (8.06-8.54)^{B}	9.02 (8.70-9.35)^{B}	0.98 (0.97-0.99)^{aA}
July (fast growth period)				
A (2.5)	7.70 (7.51-7.89)^{bA}	9.39 (9.15-9.63)^{A}	10.62 (10.30-10.95)^{A}	0.82 (0.81-0.84)^{bB}
B (4.2)	8.98 (8.79-9.17)^{aA}	9.61 (9.37-9.86)^{A}	11.05 (10.72-11.38)^{A}	0.93 (0.92-0.95)^{aB}
C (4.6)	9.14 (8.95-9.33)^{aA}	9.74 (9.50-9.99)^{A}	11.15 (10.82-11.48)^{A}	0.94 (0.93-0.96)^{aB}
D (5.1)	8.81 (8.62-8.99)^{aA}	9.47 (9.22-9.71)^{A}	11.00 (10.67-11.32)^{A}	0.93 (0.92-0.95)^{aB}
E (5.7)	8.99 (8.79-9.18)^{aA}	9.45 (9.20-9.69)^{A}	10.98 (10.64-11.31)^{A}	0.95 (0.94-0.97)^{aB}
F (6.1)	8.86 (8.67-9.04)^{aA}	9.31 (9.06-9.55)^{A}	10.92 (10.60-11.25)^{A}	0.95 (0.94-0.97)^{aB}

4. Discussion

The present study determines the dietary P requirements in the grow-out phase of Atlantic salmon from 1.8 kg to harvest size (4.2 kg) reared from December until July in sea-cages under natural light and ambient temperatures. The results suggest that 3.7 g/kg available P (diet B) met the requirement of salmon during an average growth rate of 0.62 mm/day between December-April and a level of 4.6 g/kg available P (diet C) was sufficient for a regular growth and bone mineralisation during an average growth rate of 0.98 mm/day between April-July. The broken-line model for vertebral ash provided a comparable estimate for P requirements (3.75 and 4.8 g/kg available P for April and July, respectively). This creates the potential to reduce the total dietary P content by 16-24 % compared to the current recommendation (diet E, 10.4 g/kg (DM) total P) without compromising bone mineralisation and skeletal health.

4.1. Growth rate dictates the dietary P requirements

In Atlantic salmon aquaculture, sea-cage rearing represents the main growth period in terms of absolute weight gain and feed consumption (Dessen et al., 2017). Still, differences in water temperature alters both, growth and appetite. Atlantic salmon have reduced feed intake and reduced growth during cold periods with shorter day length (Simpson et al., 1996; Jobling, 2003). In contrast, prolonged day length and higher temperatures (until a temperature optimum of about 14 °C (Handeland et al., 2008)) increase both feed intake and growth (Weatherley et al., 1979; Forsberg, 1995; Jobling, 2003; Dumas et al., 2007; Hamre et al., 2022; Myklatun et al., 2023).

The increased growth in fork length, i.e. bone formation, was concomitantly associated with a reduced relative bone ash (%) across all diet groups in comparison with the initial values in December. Assuming the rate of bone mineralisation is constant irrespective of the rate of non-mineralised bone matrix formation (Arendth et al., 2001; Fjelldal et al., 2006), an elevation of growth results in a greater proportion of non-mineralised to mineralised bone matrix. Since Atlantic salmon display seasonal growth patterns with higher growth rates in spring and summer and reduced growth in autumn and winter (Forsberg, 1995), it is likely that the delayed mineralisation will be compensated for later in the development. It is important to note that the vertebral ash and mechanical properties in animals fed diet B-F show corresponding values to seawater Atlantic salmon of a similar size fed a commercial diet containing sufficient levels of dietary P (Drábiková et al., 2022) and similar values for bone P and Ca found in juvenile rainbow trout fed regular P diets (Hossain et al., 2020). Plasma Pi averaged 3.5 mmol/l which is

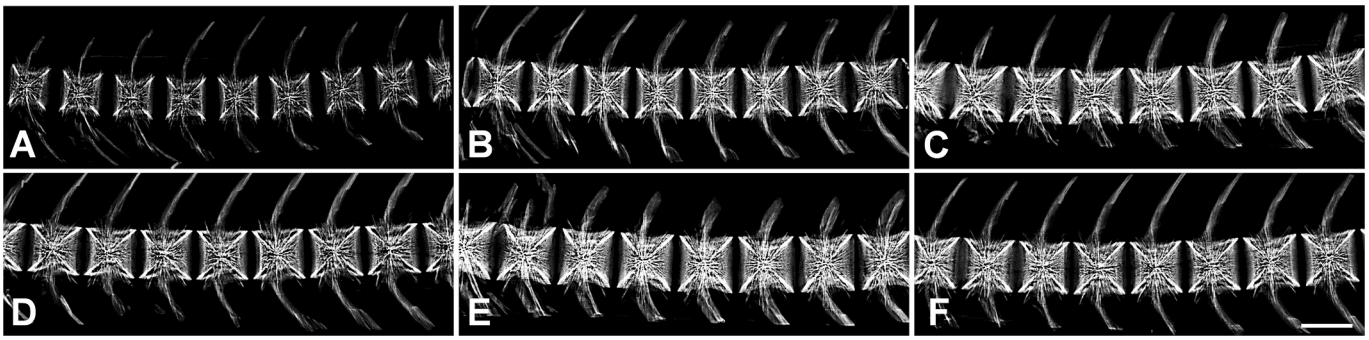

Fig. 4. Lateral x-ray images taken at the end of the trial in July showing quintessential vertebral centra of animals fed different levels of available phosphorus on a dry matter basis in g/kg: (A) 2.5 (diet A), (B) 4.2 (diet B), (C) 4.6 (diet C), (D) 5.1 (diet D), (E) 5.7 (diet E), (F) 6.1 (diet F). Specimens are oriented anterior to the left and dorsal to the top. Note that on radiographs only mineralised part of the bone is visualised while the non-mineralised part of the bone is radiolucent. Scale bar = 1 cm.

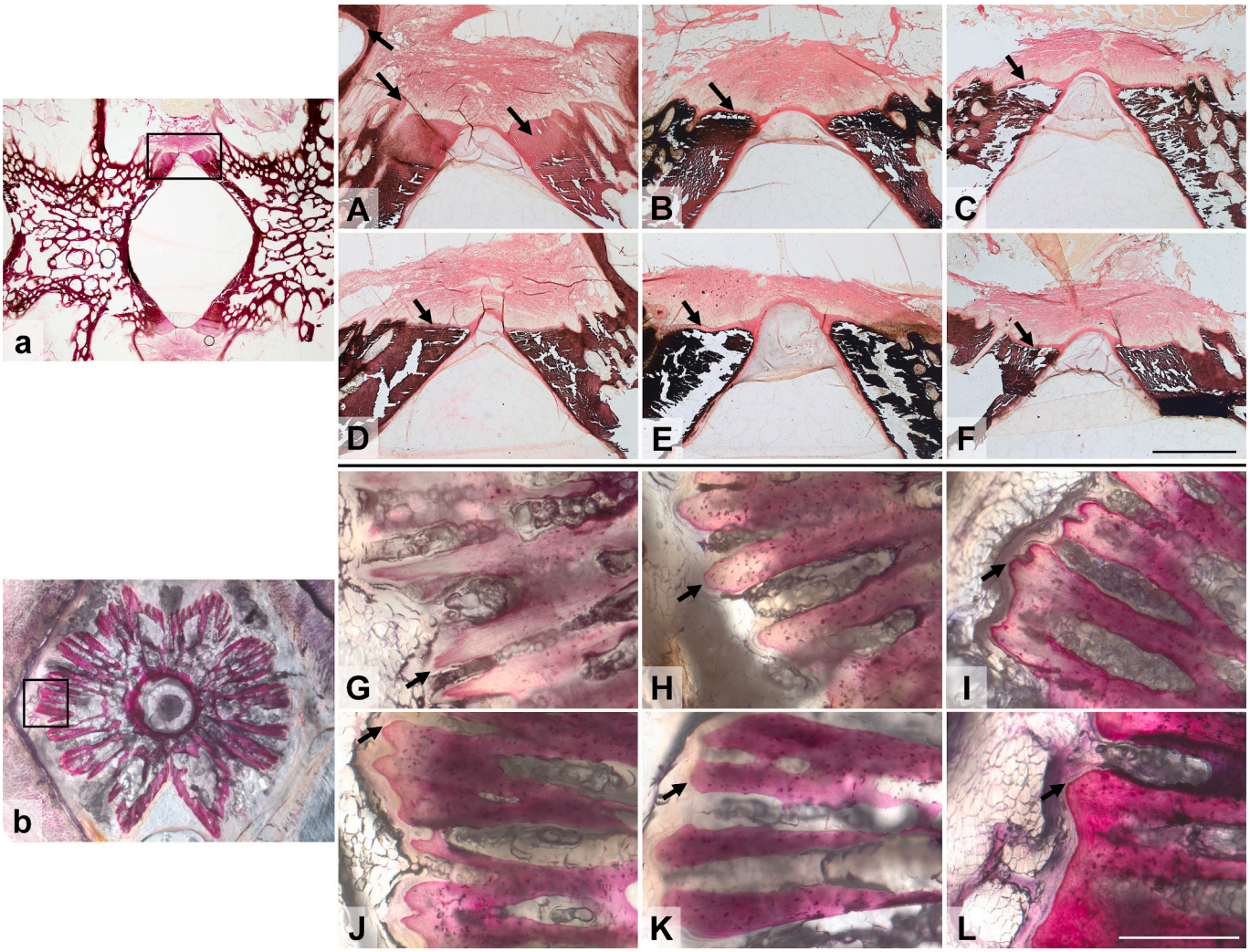

Fig. 5. Bone mineralisation at the end of the trial in July. Representative samples of non-demineralised parasagittal sections (A-F) of an area specified by the rectangle in (a) (von Kossa/ Van Gieson, scale bar = 1 mm) and non-demineralised ground cross-sections (G-L) of an area specified by rectangle in (b) (Alizarin red, scale bar = 500 μm). Samples originate from animals fed six different available phosphorus diets in g/kg on a dry matter basis containing 2.5 (diet A; A, G), 4.2 (diet B; B, H), 4.6 (diet C; C, I), 5.1 (diet D; D, J); 5.7 (diet E; E, K), or 6.1 (diet F; F, L). All sections are oriented with dorsal to the top (anterior to the left for parasagittal sections). Animals fed diet A developed extended areas of non-mineralised bone at the edges of vertebral endplates and bone trabeculae (arrows in A, G). Animals fed diets B-F show comparable extension of the osteoid seam representing regular delay between bone matrix formation and bone matrix mineralisation (arrows in B-F, H-L). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

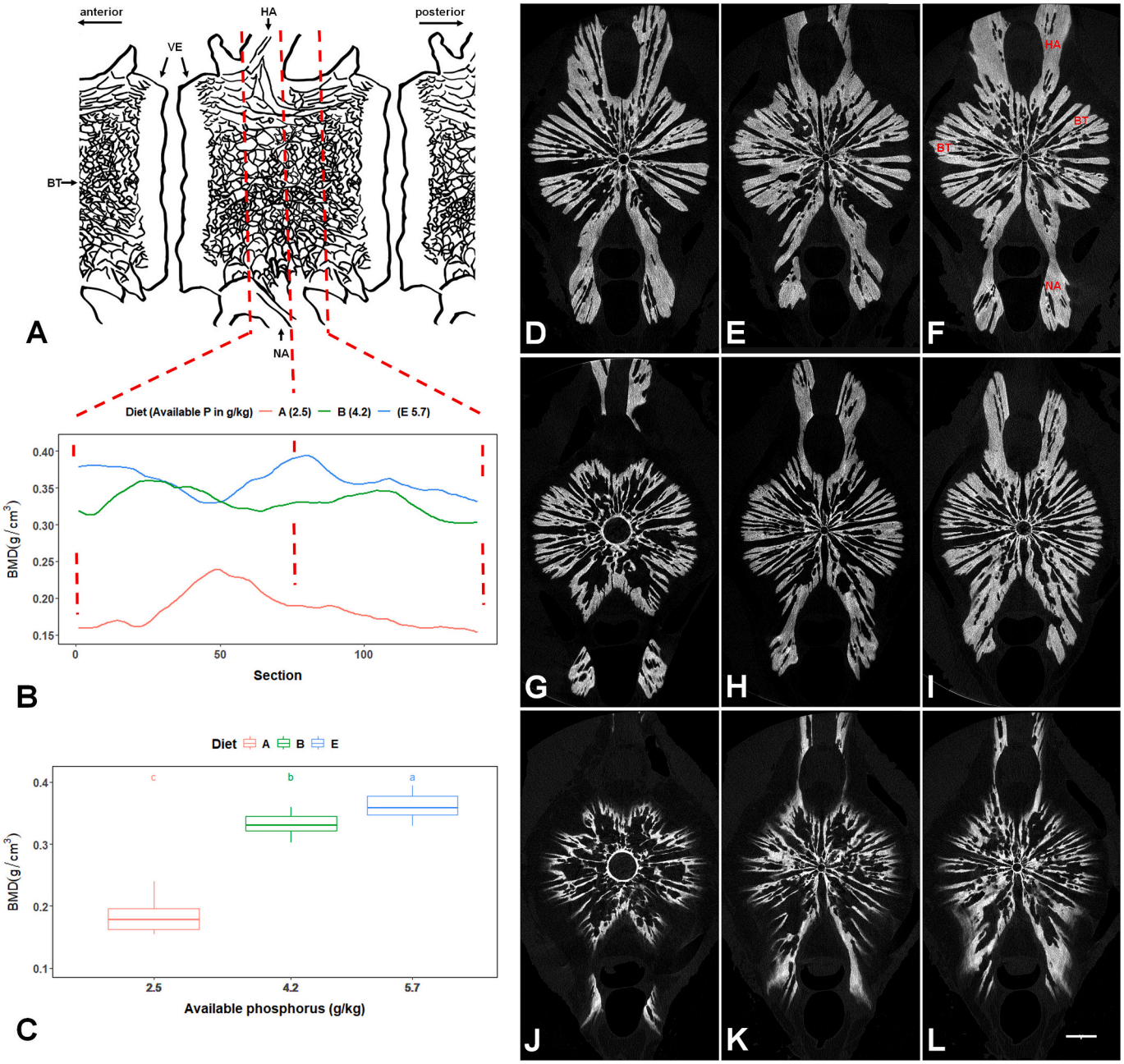

Fig. 6. Micro-CT at the end of the trial in July. (A) A graphical depiction of vertebral centra in a parasagittal plane with red lines indicating the anterior most point, the centre of the vertebral centra, and the posterior most point that were included in the analysis of bone mineral density (BMD). Haemal arches (HA), vertebral endplates (VE), bone trabeculae (BT), and neural arches (NA) of the vertebral centra are indicated in (A, F). (B) Graph showing the differences in BMD along the analysed width of the vertebra among samples from different diet groups. (C) Box plots display the predicted values for vertebral centra BMD showing least-square means and the lower and upper 95 % confidence intervals. Different letters refer to the statistically significant differences between the diet groups. Micro-CT scans show cross-sections of vertebral centra oriented dorsal to the top from animals fed: (D, E, F) 5.7 g/kg (diet E), (G, H, I) 4.2 g/kg (diet B), and (J, K, L) 2.5 g/kg (diet A) available phosphorus (P) on a dry matter basis (scale bar = 3 mm). (D, G, J) Show cross-sections of vertebrae with the lowest measured BMD across the diets. (E, H, K) Show cross-sections of the centre of the measured vertebrae across the diets. (F, I, L) Show cross-sections of vertebrae with the highest measured BMD across the diets. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

within the range described for Atlantic salmon according to Vielma and Lall (1998b); Prabhu et al. (2013); Drábiková et al. (2023). Thus, under the current experimental conditions, a 24 % reduction in dietary P (diet B, 8 g/kg (DM) total P, 3.7 g/kg available P) compared to the P requirement published by NRC (comparable to diet E with 10.4 g/kg (DM)) allowed normal bone development and mineralisation during the period of 0.62 mm/day growth. Subsequently, a 16 % reduction in dietary P (diet C, 8.7 g/kg (DM) total P, 4.6 g/kg available P) supplied during the period of 0.98 mm/day growth ensured good somatic growth and bone ash comparable to values measured in animals fed diets D-F. Accordingly, a supplementation of P in >1.7 kg farmed Atlantic salmon above 3.7 g/kg available P during slow growth periods and feeding >2.9 kg farmed salmon P above 4.6 g/kg available P during fast growth periods in sea-cages will not improve somatic growth or bone mineralisation. A period of accelerated growth from August until October with reported growth rates of 1.07-1.5 mm/day (Fjelldal et al., 2016; Myklatun et al., 2023) was not included in the present study. Future studies can assess if the dietary P inclusion needs to be further adjusted.

Table 7
Predicted values for vertebral centra ash and mineral content showing the least-square means and the lower and upper 95 % confidence intervals in brackets. Values for December show median and interquartile range. Different superscript lower-case letters indicate the significant differences among the diet groups within the sampling points. Different upper-case superscript letters indicate the significant differences between the sampling points of the same diet group. Animals were sampled in December, April, and July. Animals were fed six different diets A-F with a mean available P content (g/kg (DM)) specified in brackets.

Diet (available P g/kg (DM)) / parameter	Ash (g)	Ash (%)	Ca (%)	P (%)	Ca:P ratio	Ca:P molar ratio
December	0.19 (0.17-0.21)	33.72 (33.05-35.59)	10.55 (10.26-11.24	5.21 (5.11-5.60)	2.00 (1.99-2.03)	1.55 (1.54-1.57)
April (slow growth period)						
A (2.3)	0.24 (0.22-0.26)^{bA}	26.39 (25.42-27.36)^{bA}	10.86 (10.44-11.28)^{bA}	5.28 (4.99-5.57)^{cA}	2.06 (2.01-2.11)^{A}	1.59 (1.55-1.63)^{A}
B (3.7)	0.32 (0.29-0.35)^{aB}	32.08 (31.09-33.07)^{aA}	13.08 (12.65-13.51)^{aA}	6.40 (6.11-6.69)^{abA}	2.05 (2.00-2.10)^{A}	1.58 (1.54-1.62)^{A}
C (4.1)	0.32 (0.29-0.36)^{aB}	31.87 (30.88-32.86)^{aA}	12.84 (12.41-13.27)^{aA}	6.30 (6.01-6.59)^{abA}	2.04 (1.99-2.09)^{A}	1.58 (1.54-1.62)^{A}
D (4.6)	0.31 (0.28-0.34)^{aB}	32.67 (31.69-33.66)^{aA}	13.12 (12.69-13.55)^{aA}	6.28 (5.98-6.57)^{bA}	2.09 (2.04-2.14)^{A}	1.62 (1.58-1.66)^{A}
E (5.6)	0.33 (0.30-0.36)^{aB}	32.71 (31.72-33.70)^{aA}	13.36 (12.93-13.79)^{aA}	6.91 (6.62-7.20)^{aA}	1.95 (1.90-2.00)^{B}	1.51 (1.47-1.55)^{B}
F (5.8)	0.32 (0.29-0.35)^{aB}	33.46 (32.47-34.45)^{aA}	13.51 (13.08-13.94)^{aA}	6.59 (6.30-6.89)^{abA}	2.05 (2.00-2.10)^{A}	1.59 (1.55-1.63)^{A}
July (fast growth period)						
A (2.5)	0.25 (0.23-0.28)^{bA}	21.17 (20.19-22.14)^{cB}	8.85 (8.43-9.27)^{bB}	4.30 (4.01-4.59)^{bB}	2.06 (2.01-2.11)^{A}	1.59 (1.55-1.63)^{A}
B (4.2)	0.46 (0.42-0.51)^{aA}	29.14 (28.15-30.13)^{bB}	12.35 (11.92-12.78)^{aB}	6.02 (5.72-6.31)^{aB}	2.05 (2.00-2.10)^{A}	1.59 (1.55-1.63)^{A}
C (4.6)	0.52 (0.47-0.57)^{aA}	31.03 (30.04-32.01)^{abA}	12.88 (12.45-13.31)^{aA}	6.33 (6.04-6.63)^{aA}	2.03 (1.98-2.08)^{A}	1.57 (1.53-1.61)^{A}
D (5.1)	0.51 (0.47-0.57)^{aA}	31.71 (30.72-32.70)^{aA}	13.18 (12.75-13.61)^{aA}	6.45 (6.16-6.74)^{aA}	2.04 (1.99-2.09)^{A}	1.58 (1.54-1.62)^{A}
E (5.7)	0.49 (0.45-0.54)^{aA}	31.26 (30.27-32.24)^{aB}	13.05 (12.62-13.48)^{aA}	6.43 (6.14-6.72)^{aB}	2.03 (1.98-2.08)^{A}	1.57 (1.53-1.61)^{A}
F (6.1)	0.49 (0.44-0.53)^{aA}	31.30 (30.32-32.29)^{aB}	13.08 (12.65-13.51)^{aA}	6.41 (6.12-6.70)^{aA}	2.04 (1.99-2.09)^{A}	1.58 (1.54-1.62)^{A}

Table 8
Predicted values for scale ash and mineral content showing the least-square means and the lower and upper 95 % confidence intervals in brackets. Values for December show median and interquartile range. Different superscript lower-case letters indicate the significant differences among the diet groups within the sampling points. Different upper-case superscript letters indicate the significant differences between the sampling points of the same diet group. Animals were sampled in December, April, and July. Animals were fed six different diets A-F with a mean available P content (g/kg (DM)) specified in brackets.

Diet (available P g/ kg (DM))/ parameter	Ash (%)	Ca (%)	P (%)	Ca:P ratio	Ca:P molar ratio
December	28.82 (28.19-29.57)	12.14 (11.81-12.51)	6.38 (6.21-6.52)	1.91 (1.90-1.92)	1.48 (1.47-1.48)
April (slow growth period)					
A (2.3)	25.49 (24.73-26.28)^{bB}	11.10 (10.71-11.49)^{bA}	5.58 (5.39-5.78)^{bA}	1.99 (1.98-2.00)^{aB}	1.54 (1.53-1.55)^{aB}
B (3.7)	27.39 (26.55-28.25)^{aB}	11.61 (11.19-12.02)^{abA}	6.04 (5.83-6.24)^{aB}	1.92 (1.91-1.94)^{bB}	1.49 (1.48-1.50)^{bB}
C (4.1)	27.89 (27.04-28.76)^{aB}	11.99 (11.59-12.39)^{aA}	6.24 (6.04-6.44)^{aA}	1.92 (1.91-1.93)^{bB}	1.48 (1.48-1.49)^{bB}
D (4.6)	28.28 (27.42-29.17)^{aA}	12.08 (11.66-12.49)^{aA}	6.30 (6.09-6.51)^{aB}	1.92 (1.91-1.93)^{bB}	1.48 (1.47-1.49)^{bB}
E (5.6)	27.66 (26.82-28.53)^{aB}	11.99 (11.57-12.40)^{aA}	6.25 (6.04-6.46)^{aA}	1.92 (1.91-1.93)^{bB}	1.48 (1.47-1.49)^{bB}
F (5.8)	28.55 (27.68-29.45)^{aA}	12.27 (11.87-12.66)^{aA}	6.41 (6.21-6.61)^{aA}	1.91 (1.90-1.92)^{bB}	1.48 (1.47-1.49)^{bB}
July (fast growth period)					
A (2.5)	23.68 (22.97-24.42)^{bA}	10.42 (10.05-10.80)^{bA}	5.11 (4.92-5.29)^{bB}	2.04 (2.03-2.05)^{aB}	1.58 (1.57-1.59)^{aA}
B (4.2)	28.80 (27.92-29.70)^{aA}	12.45 (12.07-12.83)^{aA}	6.41 (6.22-6.60)^{aA}	1.94 (1.93-1.95)^{bB}	1.50 (1.49-1.51)^{bA}
C (4.6)	29.15 (28.27-30.07)^{aA}	12.76 (12.38-13.14)^{aA}	6.57 (6.38-6.76)^{aA}	1.94 (1.93-1.95)^{bB}	1.50 (1.49-1.51)^{bA}
D (5.1)	28.89 (28.02-29.80)^{aA}	12.55 (12.17-12.93)^{aA}	6.49 (6.30-6.68)^{aA}	1.93 (1.92-1.95)^{bB}	1.50 (1.49-1.50)^{bA}
E (5.7)	29.01 (28.12-29.92)^{aA}	12.61 (12.23-12.99)^{aA}	6.51 (6.32-6.70)^{aA}	1.94 (1.93-1.95)^{bB}	1.50 (1.49-1.51)^{bA}
F (6.1)	28.90 (28.02-29.81)^{aA}	12.30 (11.90-12.69)^{aA}	6.38 (6.19-6.57)^{aA}	1.93 (1.92-1.94)^{bB}	1.49 (1.48-1.50)^{bA}

Considering that the animals fed diet A received up to 46 % less estimated available P compared with the newly recommended levels in diet C it is surprising that their growth was only subtly, although significantly, reduced while the negative effect on bone ash content was obvious. Under conditions of low dietary P intake, bone matrix formation and bone matrix mineralisation can be uncoupled. Bone formation continues but new bone is no longer mineralised (Witten et al., 2019; Cotti et al., 2024). Phosphorus requirements for bone mineralisation are thus higher than the requirements for bone formation (growth) (Prabhu et al., 2013; Rodehutscord, 1996). A multitude of studies on freshwater (Åsgård and Shearer, 1997; Fjelldal et al., 2012a; Fraser et al., 2019; Drábiková et al., 2021) and seawater stages of Atlantic salmon (Gil Martens et al., 2012; Fjelldal et al., 2012b) fed P deficient diets observed reduced bone mineralisation while growth remained unaffected. Bone ash has been recognised as an effective marker for P utilisation in salmonids (Ketola, 1975; Åsgård and Shearer, 1997; Vielma and Lall, 1998a). The broken-line model for vertebral ash demonstrated a more sensitive estimate for P requirements in July compared with broken-line model for whole-body and plasma P. This along with other studies, provides further support towards identifying bone (vertebral) ash as one of the most sensitive methods to recognise suboptimal dietary P levels (Gil Martens et al., 2012, Fjelldal et al., 2012a, 2012b; Fraser et al., 2019; Drábiková et al., 2021).

It is important to note that in this study rearing conditions included natural photoperiod while salmon farmers often use continuous light on sea-cages to postpone maturation and to promote growth (Hansen et al., 1992). The continuous light is applied in the period from mid-January to the summer solstice (20-22 June) (Fjelldal et al., 2005; Oppedal et al., 2006). The here reported growth rates under natural light (0.62-0.98

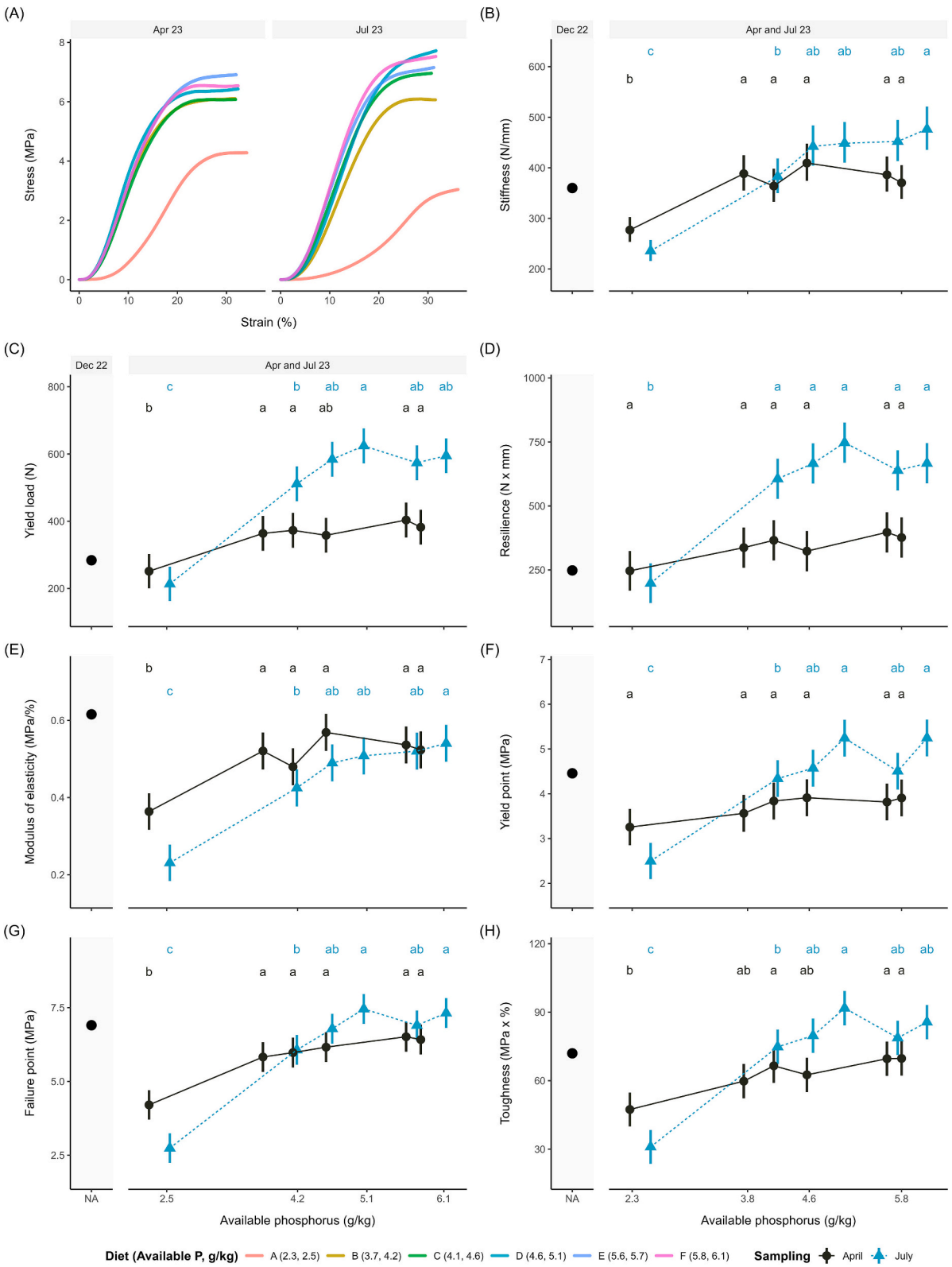

Fig. 7. Compression test analysis of vertebral centra from animals fed different levels of available phosphorus (P) on a dry matter basis. (A) Stress-strain curve for vertebral centra sampled in April and July (A). (B-D) Parameters not adjusted for the vertebral centra size, i.e. vertebral centra stiffness, yield, and resilience. (E-H) Parameters adjusted for the vertebral centra size, i.e. modulus of elasticity, yield point, failure point, and toughness. Base-line data analysed in animals sampled in December are indicated. Each graph shows the predicted values for specific mechanical property showing the least-square means and the lower and upper 95 % confidence intervals for each diet groups at both sampling points. Letters at the top of the graph show the statistical differences among the diet groups in April (black letters) and in July (blue letters). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

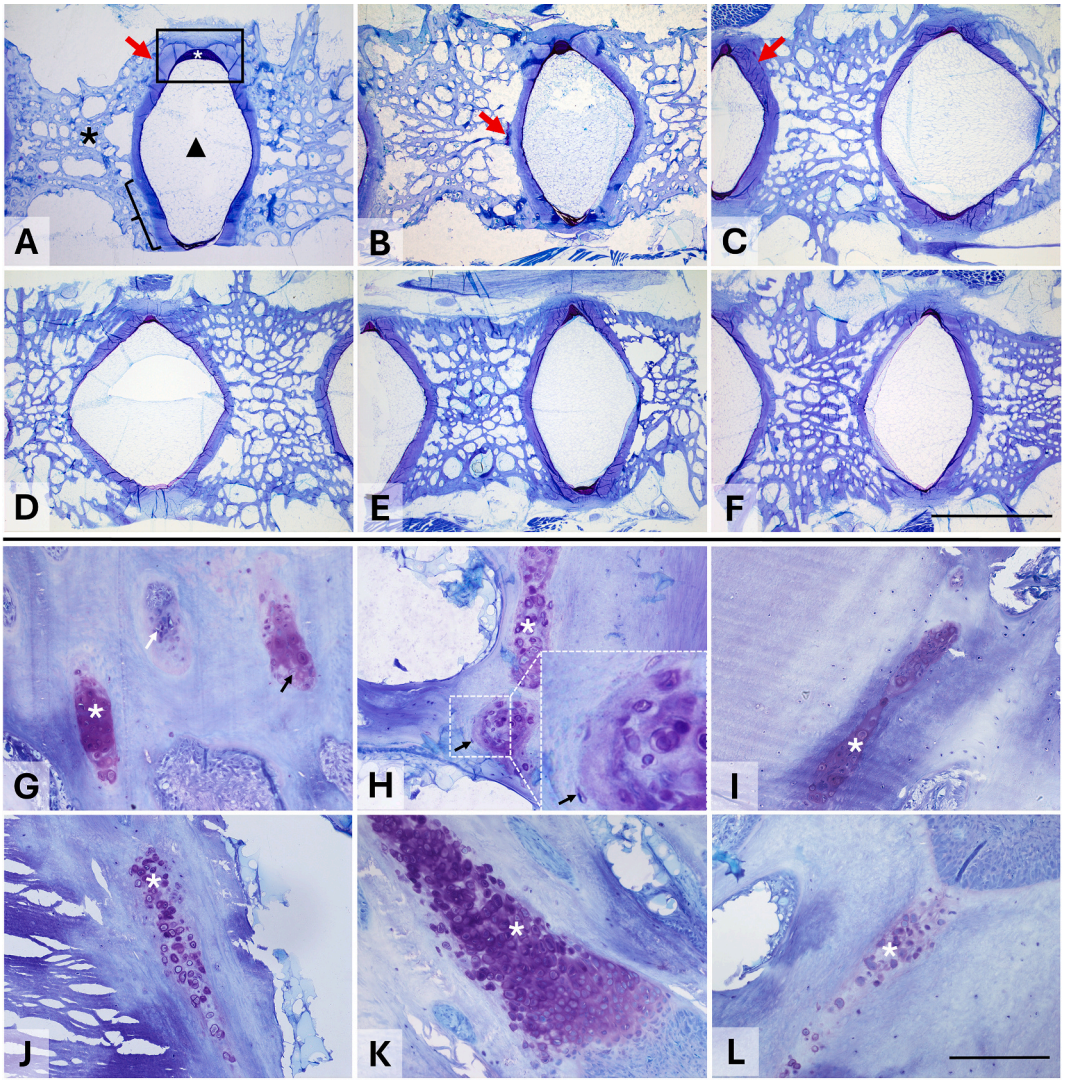

Fig. 8. Vertebral centra morphology at the end of the trial in July. (A-F) Representative samples of demineralised histological sections (toluidine blue) of vertebral bodies (scale bar = 5 mm) specifying vertebral centra (black asterisk), intervertebral spaces (triangle), intervertebral joints (rectangle), and a common location of ectopic cartilage behind the vertebral endplates (red arrows in A-C). (G-L) Detailed images of ectopic cartilages located behind the vertebral endplates (scale bar = 250 μm). Specimens originate from animals fed six different phosphorus (P) diets containing 2.5 g/kg (A, G), 4.2 g/kg (B, H), 4.6 g/kg (C, I), 5.1 g/kg (D, J); 5.7 g/kg (E, K), or 6.1 g/kg (F, L) available P on a dry matter basis. Specimens are oriented anterior to the left and dorsal to the top. Intervertebral spaces and intervertebral joints were intact and did not show signs of fibrocartilage which would indicate development of compressed or fused vertebrae. Specimens from animals fed diet A showed characteristic signs of P deficiency manifested through bent vertebral endplates (brackets in A) while vertebral endplates in animals fed diet B-F were straight (B-F). Note that the notochord sheath (white asterisk) acquired a characteristic shape of a hemisphere in animals fed a diet low in P (A) relative to the conically shaped notochord sheath in animals fed a diet sufficiently high in dietary P (B-F). Ectopic cartilages developed behind the vertebral endplates in all diet P groups (asterisks in G-L). The observed combination of chondrocyte forming isogenic groups (black arrow in G) surrounded by extracellular matrix, which stained positive for the presence of proteoglycans (purple), within mineralised bone matrix, and mineralising cartilage like cells (white arrow in G) in proximity to osteocytes (black arrows in H) indicate similarities between the observed ectopic cartilages and chondroid bone. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mm/day, 0.35-0.55 % body mass/day) were within the expected range for salmon subjected to natural light and similar temperatures e.g. 0.3-0.6 % body mass/day reported in Austreng et al. (1987); 0.2-1.0 % body mass/day in Forsberg (1995); 0.5 % body mass/day in Warren-Myers et al. (2022); 0.78 mm/day in Kråkenes et al. (1991); 0.98 mm/day in Myklatun et al. (2023, 2025). But indeed, relative to growth at similar latitudes under continuous light the currently observed values were lower compared to other studies, e.g. a mean growth of 1.1 % body mass/day throughout the year in a study by Forsberg (1995) and growth between 0.9 and 1.0 % body mass/day measured in Endal et al. (2000). The present results showed decreasing bone ash content related to the faster growth induced by rising water temperature and increased day length. Comparably, Fjelldal et al. (2005, 2006) observed reduced vertebral mineral content in fast growing smolts reared under continuous light. Fjelldal et al. (2012b) showed a reduced vertebral length to dorsal-ventral diameter (height), in other words vertebral compression, in post-smolts kept at an elevated temperature (16 °C) and continuous light compared to smolts under 12 h light/dark per day. Further studies can assess if the continuous light influences the dietary P demand during the grow-out phase of farmed Atlantic salmon in sea-cages.

The cost of feed represents about 50 % of the total cost of Atlantic salmon production (Asche and Oglend, 2016). The grow-out phase of salmon farming is the most significant in terms of feed consumption (Cowey, 1995) and presents the greatest potential to reduce P waste, even with a marginal MAP reduction. Among the groups fed MAP supplemented diets, animals fed diets B-D showed the highest (33-36 %)

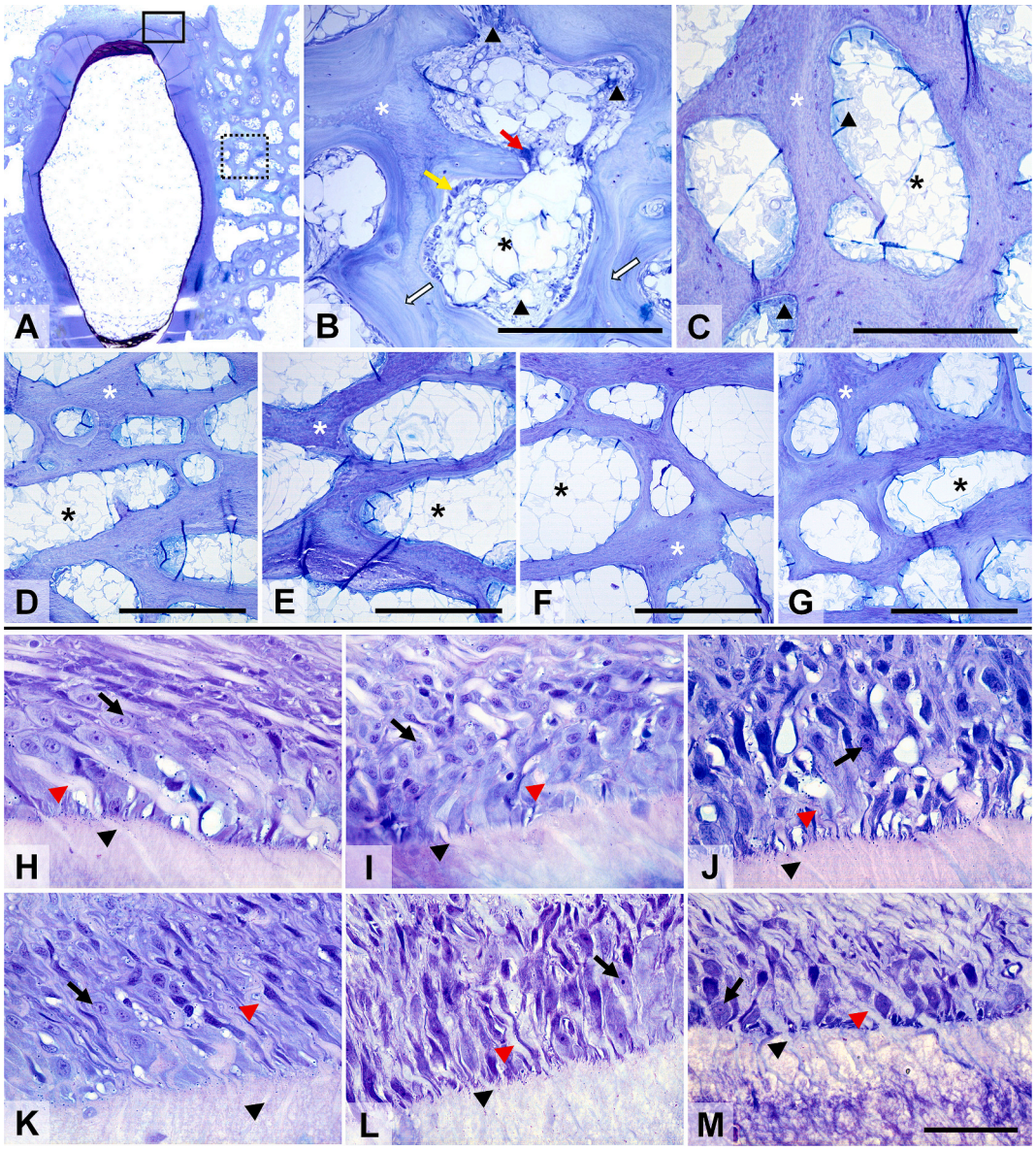

Fig. 9. Microstructure of the vertebral centra at the end of the trial in July. (A) Parasagittal demineralised section specifying vertebral centra growth zone (solid line rectangle) and bone trabeculae with bone marrow spaces (dotted line rectangle). Representative samples of bone trabeculae with bone marrow spaces (B-G) and vertebral centra growth zone (H-M) on demineralised histological sections (toluidine blue, (B-G) scale bar = 200 μm; (H-M) scale bar = 50 μm). Specimens are oriented anterior to the left and dorsal to the top. Specimens originate from animals fed six diets with a different total dietary phosphorus content in g/kg on dry matter basis: 2.5 (diet A; A-B, H), 4.2 (diet B; C, I), 4.6 (diet C; D, J), 5.1 (diet D; E, K); 5.7 (diet E; F, L), or 6.1 (diet F; G, M). A detailed observation of the bone trabeculae (white asterisks in B-G) surrounding the bone marrow spaces filled with adipose tissue, the regular tissue of bone marrow spaces in teleost fish (black asterisks in B-G). The vertebral centra in animals fed diet A showed the presence of connective tissue (triangles in B). Remnants of connective tissues were also observed in the bone marrow spaces of vertebral centra in animals fed diet B (triangles in C). The bone trabeculae in animals fed diet A were frequently observed with lines of resorption and lines of newly laid bone (black/ white arrows in B). The prevalence of osteoblasts at the edges of bone surrounding the bone marrow spaces was higher in diet A (white arrow in B) compared with the rest of the groups. The edges of the bone in specimens from animals fed diet A appeared rougher seemingly due to a higher occurrence of osteoclasts (red arrow in B). The surfaces of the bone trabeculae in specimens from animals fed B-F were smooth. (H-M) At the vertebral centra growth zone, osteoblasts were present in active spindle shaped form (black arrows), type I collagen fibres (white arrowheads) and their extensions into the bone (Sharpey fibres) were intact (black arrowheads). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

while diet E-F fed animals had the lowest (28-30 %) P retention rates. In all cases, the P retention rates represented an improvement compared to the generally reported values (18-29 %) (Aas et al., 2022). The P retention rate in animals fed diet A was also relatively high (28 %). This is especially noteworthy since diet A was supplied by a negligible concentration of MAP (0.02 %) meaning that the dietary P was acquired from raw materials consisting mostly of plant protein (57.4 % inclusion rate) and to a lesser extent of fish meal added at an inclusion rate of 7.5 %. Indeed, dietary P deficiency had previously been observed to increase the active intestinal P uptake and renal P reabsorption in Atlantic salmon and rainbow trout (Avila et al., 2000; Sugiura et al., 2003; Sugiura and Ferraris, 2004; Martin et al., 2012; Fjelldal et al., 2016; Smedley et al., 2018; Drábiková et al., 2023). Conversely, a higher total P content increased relative P loss in rainbow trout (Ketola and Harland, 1993; Rodehutscord et al., 2000; Lellis et al., 2004) and in Atlantic salmon of the present study in which the P loss increased from 4.6 to 7.5

g P per kg fish weight gain in those fed diet A and F, respectively.

4.2. Temperature dependent P apparent digestibility coefficient

Rising temperatures have previously been shown to increase the ADC of protein, lipid, starch, and energy in rainbow trout (Azevedo et al., 1998; Hua and Bureau, 2009a, 2009b). A study by Azevedo et al. (1998) shows that ADC of P increases in rainbow trout reared at 9 °C and 12 °C compared with 6 °C and again decreases when animals are kept at 15 °C. The increase in ambient water temperature between the slow and fast growth period in the present study also showed a positive effect on P ADC and concurrently on the level of available P. This improvement was most notable in diets B-D which contained reduced available P levels (3.7–5.1 g/kg) compared to the current recommendations of 10 g/kg total P, 9 g/kg available P (Lall and Bishop, 1977; NRC, 2011) but sufficiently high to support regular mineralisation in the on-growing stages of salmon as demonstrated in the current study. These updated P recommendations are within the range of estimated requirements of 5.1-7.4 g/kg available P for freshwater and seawater stages of Atlantic salmon reported by Vielma and Lall (1998a); Prabhu et al. (2013); Albrektsen et al. (2018). The achieved total P ADC of 37.4-55.5 % is located between previously reported values of 24 % in 270 g Atlantic salmon (Greiling et al., 2019), 30-41 % in 700 g salmon (Albrektsen et al., 2018), and 59.8 % in 120 g rainbow trout (Morales et al., 2018). The observed differences between this and studies by Albrektsen et al. (2018); Morales et al. (2018); and Greiling et al. (2019) are likely associated with the interspecies differences, fish size, feed formulation, and type and concentration of dietary P used (NRC, 2011; Hua and Bureau, 2006).

4.3. The effect of dietary P on growth

In agreement with Witten et al. (2016, 2019) and Drábiková et al. (2021, 2023) Atlantic salmon fed diet A continued to grow and synthetise bone matrix without the incorporation of minerals. Vertebral centra of diet A fed animals showed a reduced vertebral centra length to height ratio indicating an increased likelihood of vertebral compression development (Fjelldal et al., 2009). As the study progressed, the growth in length of animals fed diet A was reduced and the condition factor significantly increased with respect to the other diet groups. Reduced weight in animals fed diet A appeared at the end of the fast growth period. While whole-body and plasma P in animals fed diet A were consistently reduced, the level of whole-body ash was lowered in April and comparable between the diet groups at the sampling in July. One explanation for this mismatch is the increased gain in weight (i.e. muscle mass) relative to growth in fork length (i.e. vertebral column) manifested through the increased condition factor at the end of the study in July relative to April. The reported values for vertebral ash content ranges from 30 to 47 % (Vielma and Lall, 1998a; Fjelldal et al., 2016; Drábiková et al., 2022) while the whole-body ash content fluctuates between 1.61 and 2.35 % (Davidson et al., 2017; Albrektsen et al., 2018). Therefore, in the present study, the faster growth of muscles over bone potentially masked the effect of reduced dietary P intake on whole-body ash content. Alternatively, the comparable whole-body ash content could be due to an increased dissolved Ca uptake. With a concentration of about 400 mg of Ca/l and 0.003-0.027 mg of P/l, seawater is a good source of dissolved Ca but a poor one for P (Sverdrup et al., 1942; Jones and Spencer, 1963; Lall and Kaushik, 2021). Indeed, plasma Ca values had elevated levels in animals fed diet A (1.5 mmol/l in July) compared with levels found in animals fed diets B-F (1.2-1.3 mmol/l) but also compared with the values ranging between 0.7 and 1.2 mmol/l reported in Vielma and Lall (1998b); Drábiková et al. (2021). Animals fed diet B showed a regular growth rate throughout the study indicating that these animals obtained sufficient dietary P. Although an increased feed intake could compensate for the reduced dietary P content (Shearer, 1995) the feeding rates in all diet groups were comparable. As shown in Atlantic salmon (Åsgård and Shearer, 1997), channel catfish (*Ictalurus punctatus*) (Eya and Lovell, 1997), and rainbow trout (Rodehutscord, 1996), P requirements for bone mineralisation are higher than for bone formation (growth). Thus, depressed growth occurs only after reduced bone mineral content as was the case in the current study.

4.4. Bone dynamics in response to growth and P deficiency

Mature vertebral centra bone marrow spaces of teleost fish are filled with adipose tissue (Witten et al., 2001; Witten and Huysseune, 2009). Bone of animals fed diet A showed an abundance of osteoblasts, bone resorption lacunae, and connective tissue in bone marrow spaces (Fig. 9B) indicating active bone remodelling common for periods of increased growth (Witten and Villwock, 1997; Fig. 3F and 4A in Nordvik et al., 2005). Remnants of connective tissue were also found in samples of diet B fed animals (triangles in Fig. 9C). Similarly in studies by Fjelldal et al. (2012b, 2016) analysing Atlantic salmon at sea transfer and in seawater, a P deficient diet (7.1 g/kg total P, 4.0 g/kg available P) significantly increased bone alkaline phosphatase activity, a marker of increased osteoblast activity (bone forming cells) and an enzyme stimulating bone mineralisation (Liu et al., 1987; Vimalraj, 2020). Cotti et al. (2020, 2024) showed an increased bone matrix formation in P-deficient zebrafish (*Danio rerio*) likely due to an increased mechanical stimulation of bone cells inside the soft non-mineralised bone. An increased rate of bone matrix synthesis and remodelling in diet A fed animals is therefore plausible. The presence of connective tissue inside bone marrow spaces as an indicator of vertebral deformity development is dubious. As described in Witten and Huysseune (2009), the connective tissue is later likely to be replaced by adipose tissue, the regular tissue of teleost bone marrow spaces. Moreover, vertebral deformities affecting bone structures within the vertebral centra are potentially reversible under favourable conditions while deformities of the intervertebral joints are not (Drábiková et al., 2022). Microscopically, the intervertebral spaces remained intact with no signs of fibrocartilage formation while macroscopically, radiography showed a low prevalence of animals with >10 deformed vertebrae across all diet groups. An increased deformity prevalence (>10 affected vertebrae per animal) would indicate an adverse impact on animal welfare (Hansen et al., 2010). The absence of fibrocartilage at the intervertebral joints is essential, as its presence indicates a pathological fusion of the vertebral centra. This condition may progress to fibrotic (white) tissue formation within the surrounding musculature, potentially leading to a reduction in fillet quality (Witten et al., 2005, 2006; Fraser et al., 2019). Further studies may analyse if occurrence of connective tissue within bone marrow spaces also represents a risk factor for the development of fibrotic tissue in the muscles. Importantly, reduced dietary P levels did not lead to de-novo development of deformities in >1.7 kg Atlantic salmon in sea-cages.

A singular anomaly observed across all diet groups at the end of the fast growth period was ectopic cartilage behind vertebral endplates, a characteristic previously described in P-deficient Atlantic salmon only (Witten et al., 2016, 2019; Fraser et al., 2019; Drábiková et al., 2022). In the current study, the development of such cartilage can be indicative of an adaptation in response to the increased growth rate, as it is the case in chondroid bone (Witten and Hall, 2002). The presence of both chondrocytes and osteocytes provides further evidence that the observed ectopic cartilage resembles chondroid-like bone (Meunier and Huysseune, 1992: Hall, 2005; Witten et al., 2010). A typical characteristic of chondroid bone is its fast development. As an example, chondroid bone develops during the dramatic alteration of the lower jaw in male Atlantic salmon during maturation, i.e. the fast growth of a kype (hook) (Witten and Hall, 2002, 2003, 2015; Gillis et al., 2006).

4.5. Bone mechanics in salmon vertebrae

As shown here and in other studies on Atlantic salmon (Fjelldal et al., 2005; Drábiková et al., 2021, 2022) stiffness, yield load, and resilience

of a mineralised vertebra increased proportionally to its size. The vertebral centra modulus of elasticity, yield point, failure point, and toughness were reduced at the end of the slow growth period and subsequently, at the end of the fast growth period they returned to the baseline values taken in December. Uniquely, vertebral centra of animals fed diet A showed distinctly reduced values for all mechanical properties, comparable to earlier studies by Fjelldal et al. (2012b); Witten et al. (2016); Drábiková et al. (2021, 2023). This supports the current findings that diet A was not sufficient to mineralise the vertebral bodies of >1.7 kg Atlantic salmon in sea-cages neither during the slow nor fast growth periods. The comparable results of all mechanical properties in vertebral centra of animals fed diet B and C and the other diet groups support the assumption that 3.7 g/kg (DM) of available P was sufficient to mineralise the vertebral centra throughout the slow growth period. An available P content of 4.6 g/kg (DM) facilitated a regular bone mineralisation and bone mechanical properties during the fast growth period.

5. Conclusions

This study evaluated the potential to reduce phosphorus (P) content in salmon diets containing 7.5 % fish meal and 57 % plant-based ingredients during the sea-cage grow-out phase. Salmon were reared under natural photoperiods, with water temperatures ranging from 5 to 9 °C between December and April. During this period, fish exhibited an average growth rate of 0.62 mm/day and required 3.7 g/kg dry matter (DM) of available P to support normal somatic growth, adequate bone mineralisation, and proper bone mechanical properties. From April to July, as temperatures increased to 7–14 °C and growth rates accelerated to 0.98 mm/day, dietary requirements rose to 4.6 g/kg (DM) of available P.

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CRediT authorship contribution statement

Lucia Drábiková: Data curation, Investigation, Conceptualization, Validation, Writing – review & editing, Writing – original draft, Methodology, Visualization, Formal analysis. **Saskia Kröckel:** Project administration, Conceptualization, Funding acquisition, Investigation, Formal analysis, Writing – review & editing, Data curation. **P. Eckhard Witten:** Writing – review & editing, Supervision, Formal analysis, Investigation, Conceptualization, Validation, Funding acquisition. **Guido Riesen:** Funding acquisition, Resources, Conceptualization, Validation. **Paul Morris:** Validation, Resources, Funding acquisition. **Agnés Ostertag:** Investigation, Data curation, Methodology, Writing – review & editing. **Martine Cohen-Solal:** Methodology, Writing – review & editing, Investigation. **Thomas W.K. Fraser:** Software, Writing – review & editing, Visualization, Data curation, Methodology. **Per Gunnar Fjelldal:** Project administration, Conceptualization, Writing – review & editing, Resources, Investigation, Supervision, Methodology, Software, Validation, Funding acquisition.

Author statement

During the preparation of this work the authors have not used any tools to generate scientific writing used in this research paper. The authors take full responsibility for the content of the published article.

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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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Data availability

Data will be made available on request.

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