

Phosphorus nutrition in farmed Atlantic salmon (*Salmo salar*): Life stage and temperature effects on bone pathologies

Thomas W.K. Fraser^{a,*}, P. Eckhard Witten^b, Sissel Albrektsen^c, Olav Breck^d, Ramon Fontanillas^e, Leo Nankervis^e, Tårn Helgøy Thomsen^e, Wolfgang Koppe^e, Florian Sembraus^a, Per Gunnar Fjellidal^a

^a Institute of Marine Research (IMR), Matre Research Station, NO-5984 Matredal, Norway

^b Ghent University, Biology Department, Ledeganckstraat 35, 9000 Ghent, Belgium

^c Nofima, Kjerreidviken 16, NO-5141 Fyllingsdalen, Norway

^d Marine Harvest Norway, Pb. 4102 Sandviken, 5038 Bergen, Norway

^e Skretting Aquaculture Research Centre, Sjøhagen 3, 4016 Stavanger, Norway

ARTICLE INFO

Keywords:

Fillet damage
Mineralization
Vertebral fusion
X-ray

ABSTRACT

Bone health is important for a viable and ethically sound Atlantic salmon aquaculture industry. Two important risk factors for vertebral deformities are dietary phosphorus and water temperature. Here, we explore the interplay between these two factors during a full production of Atlantic salmon. Salmon were fed one of three diets (low 4.4–5.0 g kg⁻¹, medium 7.1–7.6 g kg⁻¹, or high 9.0–9.7 g kg⁻¹ soluble phosphorus) from 3 to 500 g body weight, followed by a common diet of 7.3 g kg⁻¹ soluble phosphorus until harvest size at 4 kg. Additional groups were included to investigate the effects of water temperatures of 10 vs 16 °C (low and high diets only) and the switching of dietary phosphorus levels (from low to medium or high, from medium to low or high, from high to low or medium), starting at seawater transfer (~100 g body weight) and lasting for 4 months (~500 g body weight). During the experimental feeding period, the low phosphorus diet caused reduced bone mineralization and stiffness and a greater prevalence of vertebral deformities, compared to the medium and high phosphorus diets. However, the prevalence of severely deformed fish at harvest was reduced by switching from the low to either the medium or high phosphorus diets for 4 months after seawater transfer, followed by rearing on the standard commercial feed. Concurrently, switching from either the medium or high to a low phosphorus diet for the same period following seawater transfer had no effect on vertebral deformities at harvest. The higher water temperature for 4 months following seawater transfer increased the severity of deformities at harvest, irrespective of dietary phosphorus. Finally, low dietary phosphorus was associated with increased fillet damage, due to ectopic connective tissue around the spine, at harvest. In conclusion, dietary phosphorus levels of 5 g kg⁻¹ for the initial 4 months in seawater are more of a risk factor for vertebral pathologies if preceded by low, but not medium or high, dietary phosphorus in freshwater. However, dietary phosphorus levels may not play a role in temperature induced radiologically detectable vertebral pathologies. Under the reported growing conditions and diet compositions, a combination of 7.5–7.6 g kg⁻¹ soluble phosphorus during freshwater and 5.0 g kg⁻¹ for the first 4 months in seawater, was sufficient for normal bone health and growth in Atlantic salmon.

1. Introduction

A normal developing skeleton is a prerequisite for sustainable production and animal welfare, but vertebral column deformities are a persistent concern for farmed Atlantic salmon (*Salmo salar*) (Fjellidal et al., 2012a). Vertebral deformities reduce growth performance (Hansen et al., 2010), increase an individual's energy demand (Powell et al., 2009), and result in downgrading at harvest (Fraser et al., 2013).

In some cases, vertebra deformities may also result in problems during filleting due to tissue damage (Sullivan et al., 2007).

Dietary phosphorus supply has been highlighted as one of the key factors in the aetiology of vertebra deformities in farmed salmon (Bæverfjord et al., 2018), although a phosphorus deficient diet alone in some cases may not induce skeletal deformities (Gil-Martens et al., 2012; Witten et al., 2016). Phosphorus is an essential nutrient for fish with many metabolic functions as it is a constituent of bone, scales,

* Corresponding author at: Institute of Marine Research, Matre, 5984 Matredal, Norway.

E-mail address: Thomas.fraser@hi.no (T.W.K. Fraser).

adenosine triphosphate, cell membranes, and nucleic acids (Skonberg et al., 1997). Due to the low phosphorus salt content and inefficient absorption from water, phosphorus is supplied via the diet (Lall, 2003). If too low, dietary phosphorus leads to insufficient bone mineralization and a reduced mechanical stiffness of the vertebrae (Baeverfjord et al., 1998), which can result in the development of compressed vertebrae (Fjellidal et al., 2009). Although many papers have dealt with dietary phosphorus requirements during both freshwater (Fjellidal et al., 2012b; Helland et al., 2005; Ketola, 1975; Smedley et al., 2018; Vielma and Lall, 1998; Åsgård and Shearer, 1997) and seawater (Albrektsen et al., 2009; Baeverfjord et al., 1998; Fjellidal et al., 2009, 2012c) life stages separately, the combined effect of phosphorus nutrition in both environments has never been investigated.

Water temperature is also an important factor in the aetiology of vertebral column deformities in farmed salmon (Fraser et al., 2015; Grini et al., 2011; Ytteborg et al., 2010). However, the link between temperature and mineralization is not obvious. For example, elevated water temperature (16 °C) during the early seawater period resulted in more vertebra deformities than a lower seawater temperature (10 °C), but there was no temperature effect on mineralization (Grini et al., 2011). Therefore, the negative effect on normal bone development imposed by elevated temperature may be mediated via other mechanisms such as altered bone remodelling (Wargelius et al., 2010). So far, the combined effect of phosphorus nutrition and water temperature on the development of vertebra pathologies has not been studied.

Our aim was to determine whether dietary phosphorus requirements, primarily regarding bone health, can interact with life stage or water temperature. Therefore, we investigated growth, markers of bone health, and vertebral deformities in Atlantic salmon fed different dietary phosphorus levels from 3 to 500 g body weight, first in 13 °C freshwater (3–100 g body weight) and then either 10 or 16 °C in seawater (100–500 g body weight), and then followed up on one common diet at 8 °C until harvest size of 4 kg. The temperatures used throughout the study are based on those that are encountered during a typical production cycle. The dietary soluble (\approx digestible) phosphorus levels used ranged from 4.4–9.7 g kg⁻¹, a range that is above and below the official recommendation of 8 g kg⁻¹ available phosphorus for farmed Atlantic salmon (NRC 2011).

2. Materials and methods

2.1. Fish stock

All experiments were conducted in accordance with the laws and regulations of the Norwegian Regulation on Animal Experimentation 1996. Atlantic salmon larvae ($n = 2280$) with a mean weight of 2.8 g were provided by Marine Harvest (Mowi strain) and transported to Skretting ARC Lerang Research Station, from Vågafossen Settefisk AS, on the 13th March 2012 (day 0), whereupon they were kept on continuous light.

2.2. Diets

A commercial feed was produced according to the ingredient composition shown in Table 1. All chemical analyses described below were carried out in duplicate. A method for fractional extraction and analysis of NaOH soluble phosphorus was used as a predictor of digestible phosphorus in the diets (Albrektsen et al., 2018), based on the assumption that the chemical form and solubility of phosphorus are the main criteria for intestinal phosphorus absorption (Groote, 1986; Nordrum et al., 1997). The diets were produced to contain 3 different levels of phosphorus; low, medium, high that corresponded to 4.0–4.5 g, 7.0–7.5 g, and 9.0–9.5 g soluble phosphorus per kg of diet, respectively. Total phosphorus was determined by a spectrometric method (ISO 6491–1998, <https://www.iso.org/standard/12864.html>), while NaOH-extractable (soluble) phosphorus was determined

following incubation of duplicate ingredient samples (0.8 g) in 80 mL of 1 M NaOH for 16 h according to a procedure described in Ruban et al. (2001) and later modified by Hua et al. (2005). The method on soluble phosphorus was modified and validated by Hovde (2013). By using this method, it is not possible to differentiate between phytate phosphorus in the plant ingredients (unavailable) and other soluble and available forms of phosphorus. Therefore, digestible phosphorus levels in the diets (Table 1) may be slightly overestimated by about 15% due to variable phytate phosphorus levels in the plant ingredients. The chemical contents of the diets showed that the intended contents of nutrients and expected levels of phosphorus were largely achieved following the subtraction of phytate phosphorus. The low phosphorus diet fed in the period after transfer (100–500 g fish) contained 5 g kg⁻¹ soluble phosphorus, which is slightly higher than expected.

2.3. Experimental design

For a schematic of the experimental design and environmental conditions see Fig. 1. Briefly, juveniles were divided equally between 3 dietary regimes; low (LP), medium (MP), and high (HP) phosphorus (8 tanks/diet, 95 fish/tank). Experimental feeding began on day 14 and all fish were vaccinated on day 98 with a standard 6 component vaccine (Alphaject micro 6, Pharmaq AS, Oslo, Norway). On day 155, the fish (approx. 100 g) were divided between 33 tanks (31–37 fish/tank) supplied with full strength seawater. Of these, 27 tanks were maintained at 16 °C, whereas 6 tanks were maintained at 10 °C. On day 156, the fish were divided into 11 groups; each original dietary group was divided into three with one group remaining on the freshwater diet while the other groups were fed one of the two alternate diets creating nine dietary groups (LP-LP, LP-MP, LP-HP, MP-LP, MP-MP, MP-HP, HP-LP, HP-MP, HP-LP) that were reared on 16 °C. A further two groups of the LP-LP and HP-HP combination were reared on 10 °C. All fish were tagged with a passive integrated transponder between days 266–267 to allow for individual recognition. On day 278, all groups were divided equally between 12 common garden tanks (63–66 fish/tank) where they were maintained on 8 °C and fed a common diet (Skretting AS Spirit Pluss, 9.1 and 7.3 g kg⁻¹ total and soluble phosphorus [analysed values, as above], respectively) until the end of the experiment on day 686. Fish were overfed by 20–30% throughout the experiment. Feeding was continuous from the start until day 174 whereupon it was switched to 3 feeds per day until day 234, when it was switched to 2 daily feeds until the end of the experiment.

2.4. Sampling

Individual fish weight and length were recorded on day 69, 266, 394, and at harvest on day 686. Body size data was used to calculate body condition (i.e. K factor) using the following equation; $K = \text{weight [g]} / \text{length}^3 [\text{cm}] \times 100$. Tank biomass was also recorded at the beginning of the experiment. Growth was calculated as the % mass gain/day (i.e. specific growth rate [SGR]) as follows; $(e^q - 1)100$ (Houde and Sheckter, 1981), where $q = [\ln(W_2) - \ln(W_1)](t_2 - t_1)^{-1}$ (Bagenal and Tesch, 1978), and W_2 and W_1 are average body mass at times t_1 and t_2 , respectively. The feed conversion ratio (FCR) was calculated on a dry matter basis per tank ($\text{FCR} = \text{feed consumed} / \text{weight gain}$) between days 69–100, 100–153, 153–278. Prior to the fish being 20 g, the FCR data was not calculated as the food pellets were too small to collect. The data for one MP diet tank was lost for the period June to August 2012.

The fish were anaesthetized with 0.1 g L⁻¹ of finquel (FINQUEL® vet.; Western Chemical Inc., Washington, DC, USA) before measurement. Externally visible deformities, sexual maturation (assessed by visual examination), and the occurrence of cataracts were recorded on day 266, 394, and at harvest on day 686. The cataract score was recorded at harvest and the scoring based on Wall and Bjerkås (1999). In brief, the scoring was done under darkened conditions by use of a slit

Table 1

Diet composition (LP = low phosphorus, MP = medium phosphorus, HP = high phosphorus) and proximate composition.

Parameter	Fresh water						Sea water		
	Diets from 3 to 20 g			Diets from 20 to 100 g			Diets from 100 to 500 g		
	LP	MP	HP	LP	MP	HP	LP	MP	HP
Diet ingredients (%)									
Fish meal (North Atlantic)	38.76	38.70	38.70	38.88	38.80	38.80	25.00	25.00	25.00
Soya protein concentrate (Selecta, Brasil)	15.50	15.50	15.50	15.00	15.00	15.00	19.35	19.30	19.30
Wheat gluten meal	15.89	16.27	16.55	14.91	14.91	14.91	18.56	18.76	18.96
Whole wheat	14.00	12.28	10.58	12.71	11.23	9.70	12.52	11.60	10.45
Fish oil (North Atlantic)	15.25	15.63	16.03	17.90	18.43	18.94	23.10	23.12	23.30
Monoammonium phosphate (Aliphos, The Netherlands)	0.00	1.02	2.04	0.00	1.02	2.04	0.87	1.63	2.39
Yttrium oxide	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Vitamin premix ^a	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Mineral premix ^b	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Diet composition (%)									
Protein	50.90	50.90	50.87	49.65	49.38	49.15	45.00	45.00	45.00
Lipid	21.70	22.04	22.40	24.31	24.79	25.25	28.00	28.00	28.10
Moisture	7.30	7.12	6.94	7.07	6.90	6.73	6.43	6.33	6.23
Ash	5.69	6.65	7.61	5.65	6.61	7.58	5.52	6.26	7.00
Diet P analysed (g kg⁻¹)									
Total P	8.60	11.10	13.90	8.40	10.90	13.10	9.80	11.70	13.10
Soluble P	4.60	7.60	9.70	4.40	7.50	9.40	5.00	7.10	9.00

^a Added per kg diet: vitamin D3, 3000 I.E., 160 mg; Vitamin E, 136 mg; thiamin, 20 mg; riboflavin, 30 mg; pyridoxine-HCl, 25 mg; vitamin C (stay-c), 200 mg; calcium pantothenate, 60 mg; biotin, 1 mg; folic acid, 10 mg; niacin, 200 mg, vitamin B12, 0.05 mg; menadione bisulphite, 20 mg.

lamp (Kowa SL-5 with 15× magnification). The severity was scored on a scale from 0 (no changes) to 4 (complete cataracts). The cataract scores per individual are given as the sum of both eyes, ranging from 0 to 8. Fish were sampled for radiology on days 153, 274, and 686. Vertebral ash content was measured on days 69, 155, 266, and 686. Vertebral stiffness was measured on day 69. Whole body phosphorus and the Ca:P ratio was measured on days 69, 155, and 266.

2.5. Radiology, total ash (mineral) content, and bone stiffness

Analysis of vertebral body deformities is based on the examination of radiographs. At sampling, whole fish were used for lateral radiographs and evaluated for vertebra deformities according to Witten et al. (2009). The vertebral columns were radiographed (Porta 100 HF; Eickemeyer Medizintechnik für Tierärzte KG, Tuttlingen, Germany) onto a 35 × 43 cm image plate in a rigid cassette (Dürr Medical, Bietigheim-Bissingen, Germany) with 40 kV and 10 mAs at a distance of 70 cm. The image plate was scanned (CR 35 VET; Dürr Medical) and the resulting image converted into a TIFF file (Vet-Exam Plus Software, version 4.14.0).

Vertebrae numbers 40–43 were dissected, the neural and haemal arches were removed, and the amphicoelus centra were used for mechanical testing and measurement of total ash (mineral) content. The vertebrae were compressed in the cranial-caudal direction using a texture analyser (TAXT2 Texture Analyser, Stable Micro Systems, Haslemere, UK) with a steadily advancing piston (6 mm min⁻¹). The resulting load-deformation data were continuously recorded and the stiffness calculated for each vertebra according to Fjellidal et al. (2004). After mechanical testing, vertebrae number 40–43 of each fish were pooled, defatted in acetone and chloroform baths, dried overnight at 100 °C, and then incinerated for 11.5 h in a muffle furnace (Mod. L40, Nabertherm GmbH, Bremen, Germany) (115 °C for 0.5 h, 540 °C for 5 h, and 750 °C for 6 h according to Kacem et al., 2000). The dry and total ash contents of each individual were weighed to the nearest 10⁻² mg. The total ash content was calculated as follows: Total ash content (% dry weight) = (total ash weight × 100) × (dry weight)⁻¹.

2.6. Mineral analyses in fish

Total phosphorus in pooled samples of whole-body were determined spectrophotometrically (430 nm) after ashing and treatment in 6 M HCl (ISO 6491-1998). Freeze dried samples of whole-body were analysed for total phosphorus (P) and calcium (Ca), and the whole-body Ca:P ratio calculated (whole-body Ca/whole-body P). Whole-body Ca was determined by atomic absorption spectrometry (ISO 6869-2000, <https://www.iso.org/standard/33707.html>).

2.7. Fillet damage

Macroscopic damage to the fillet, due to the occurrence of connective tissue, was scored as either present or absent at harvest. The fish were carefully filleted by hand and both fillets were evaluated for occurrence of excessive connective tissue. The structure of the excessive connective tissue allowed normal hand filleting without applying extra force. Excessive connective tissue was white and covered the area of the musculature closest to the vertebra column caudally to the anus (Fig. S1). Fish that had excessive connective tissue present in both fillets were classified as “damaged”.

2.8. Histological analysis

Based on the radiological examination, spine sections were selected for biopsies and histological examination. From each group, 4 fish with typical malformations and 2 fish with non-deformed spines (controls) were selected, 24 biopsies in total. Histological samples were decalcified and embedded in paraffin. Para-sagittal serial sections (periphery of the vertebral body to the mid line) of 5 µm thickness were stained with Masons trichrome as described in Presnell and Schreiber (1998). Sections were analysed with a Zeiss Axio Imager Z1 microscope and photographed with a Zeiss AxioCam.

2.9. Statistical analysis

The data were transferred to R version 2.15.0 (R Development Core Team, <http://www.r-project.org>). Significance was assigned at $p < .05$. Data were checked for normality following visual

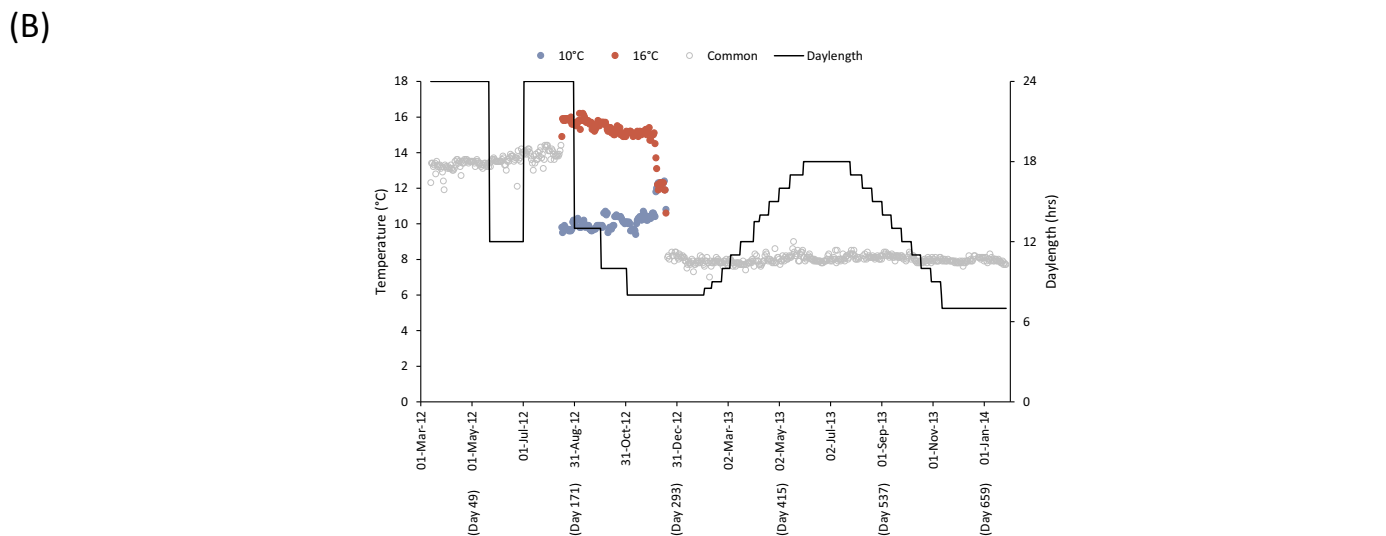
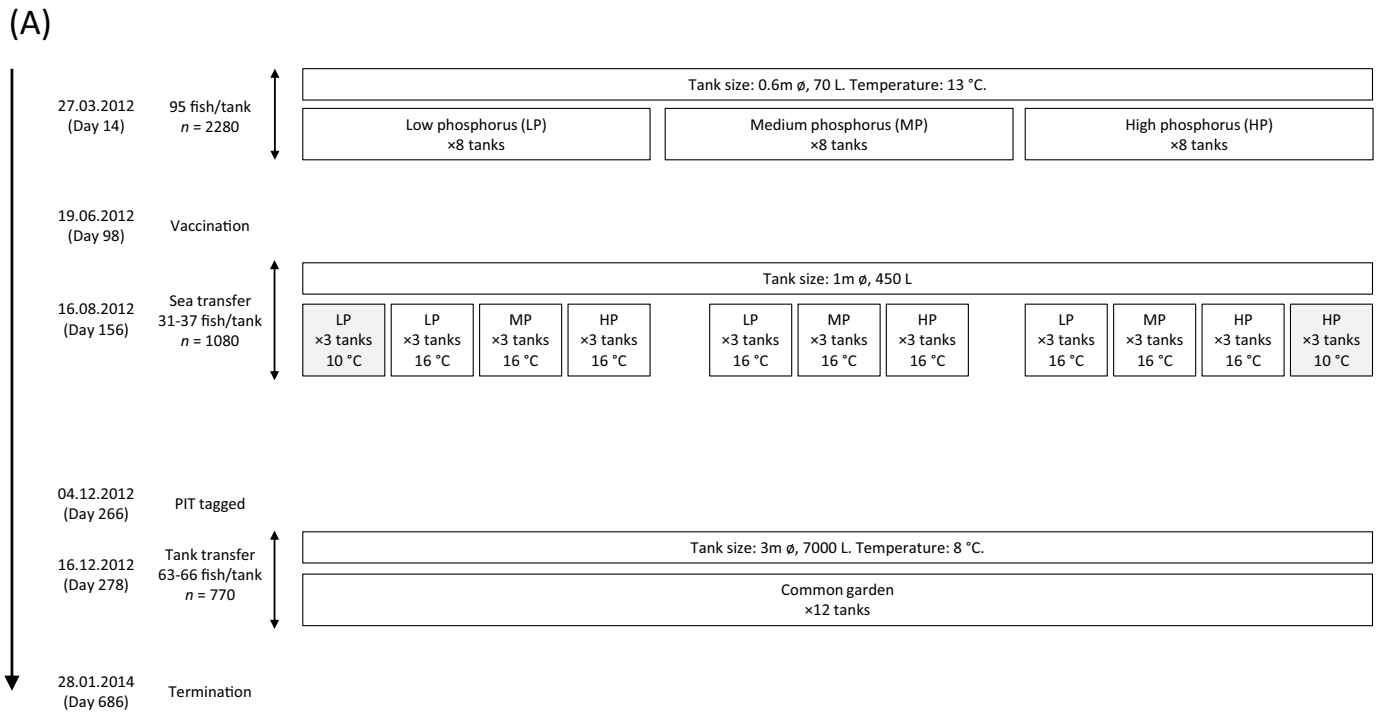


Fig. 1. Experimental design and environmental conditions. (A) Schematic of the experimental design. (B) The temperature and photoperiods used throughout. Note, the fish were given a “winter signal” to induce smoltification between the 22nd May 2012 and the 1st July 2012. In addition, the fish were maintained on simulated natural photoperiod (59° N, 6° E) from 2nd January 2013 onwards.

examination of plots (i.e. histograms and/or q-q plots). Linear mixed effect (LME) models were used for body mass, body length, and body condition. The random effect being the individual fish to account for repeated measures. LME models were used to assess associations between cataract score, sexual maturity or external deformities and body mass at harvest, whereas linear models (LM) were used to assess group effects on bone strength, vertebral mineral content, whole body phosphorus, the Ca:P ratio, and FCR. General linear models (GLM) with a binomial distribution were used to assess the prevalence of cataracts, sexual maturation, externally visible deformities, fish with ≥ 1 deformed vertebra, fish with ≥ 10 deformed vertebra, and fillet damage. For a comparison of sexual maturation in the temperature comparison, the exact binomial test was used. Cataract score was non-parametric, and therefore analysed using the Kruskal Wallis test. Post-hoc tests

included lsmeans for parametric data or the Dunns test for non-parametric data. The R libraries used were “nlme”, “MuMIn”, “multcomp”, “car”, “lsmeans”, and “dunn.test”. For models in which more than one explanatory variable was investigated, all possible 2-way interactions were initially allowed based on previous findings demonstrating that sex (Fraser et al., 2014), water temperature (Grini et al., 2011), and dietary phosphorus (Albrektsen et al., 2009; Deschamps et al., 2014; Fjellidal et al., 2016; Ketola, 1975; Prabhu et al., 2013; Åsgård and Shearer, 1997) can all influence growth in salmonids, and these effects can be transient. In addition, dietary phosphorus (Fjellidal et al., 2012d) and water temperature (Fjellidal et al., 2011) have been found to affect the level of early male sexual maturation leading to sex effects on growth. However, the final model was selected based on a comparison of all possible model combinations using the “dredge” command within

the “MuMIn” library, with the final model being the one with the lowest akaike information criterion (AICc), i.e. the best data fit weighted against the number of variables. The “Anova” command within the “car” library was used to extract the results for the main effects. Type II sum of squares were used for models without interactions, whereas main effects were calculated using type III sum of squares when interactions were present within the final model. The body mass and body length data were natural log transformed prior to analysis. All the raw data (Fraser et al.xlsx) and the R script (Fraser et al.R) used to analyse the data can be found in Supplementary material.

Due to the large number of groups within the experiment, the data was analysed in four ways; i) a comparison of being fed continuous LP, MP, or HP throughout the whole experiment, ii) a comparison of switching diet at seawater, iii) a comparison of being fed either the LP or HP throughout, but being exposed to different water temperatures at seawater transfer, and iv) general associations between harvest size and cataract score, sexual maturity, or external or radiologically identified deformities. This approach allowed for a more focused analysis and discussion of the research questions.

3. Results

The data on body size, condition, sexually maturity, and cataracts can be found in Table 2, while parameters regarding vertebral deformities can be found in Table 3. Significant explanatory variables for each parameter can be found within Tables 2 and 3. The full statistical output for the final models, as well as relevant post-hoc results can be found in Supplementary Tables S1–6. Data on specific growth rates can be found in Supplementary Table S7, but was not included for statistical analysis as only tank means were available prior to pit tagging and the body mass data indicated no major biological effects of diet.

3.1. Continuous LP, MP, or HP diet

There was no effect of dietary phosphorus or sex on body mass or length at any time point in freshwater or seawater, but MP fish had lower body condition than the LP fish in freshwater and the HP fish had lower condition factors than the MP and LP groups throughout the seawater period. Dietary phosphorus had no effect on FCR, cataracts, or the prevalence of sexual maturation.

Compared to the MP and HP groups, the vertebrae of the LP fed fish had a lower mechanical stiffness and whole-body phosphorus and Ca:P ratio, and the mineral content was lower at all time points except at harvest. Compared to the MP and HP groups, those fish fed the LP diet had a higher prevalence of externally visible deformities and fish with ≥ 1 and ≥ 10 deformed vertebrae at all time points. It was also noted that those fish fed the LP diet in freshwater had an increased number of deformed vertebrae in the tail fin region (v50–60) at day 266, whereas deformed vertebrae in this region were not as evident until harvest (day 686) in those fish fed the MP and HP diets (Supplementary Figs. S2–5). There was no difference in vertebra strength, mineral content, whole body phosphorus and Ca:P ratio, or the prevalence of deformed fish between MP and HP fed fish throughout. There was a tendency for the LP diet to increase the prevalence of fillet damage due to connective tissue, but this was not significant.

3.2. Switching dietary phosphorus at seawater transfer

There were some transient effects of switching diet at seawater transfer on body mass and condition, but not length. The only dietary effect at harvest was that the LP-MP fed fish had a lower body condition than the MP-MP fed fish, with the MP-HP fish being intermediary. For both the MP and the HP diets, there was a significant drop in FCR when switched to LP vs those that were on MP or HP. Generally, the early seawater diet had no effect on cataract prevalence or severity, with the exception that those fish fed the HP-MP diet combination had a

significantly lower prevalence of cataracts than those fed HP-LP. There was a trend for reduced levels of grislings at harvest in those fish fed the HP diet in seawater, but this was not significant (linear model, $p = .06$). Sex had only minor transient effects on body condition within the HP and LP dietary phosphorus groups (data not shown).

Switching the diet for the first 4 months in seawater had no effect on the prevalence of fish with ≥ 1 deformed vertebra. However, switching those fish fed the LP diet in freshwater to either the MP or HP diet in seawater led to a significant reduction in the prevalence of fish with ≥ 10 deformed vertebra. Switching from the HP diet to the LP diet for the initial 4 months in seawater led to a significant reduction in vertebral ash content while in those fish fed the LP diet in freshwater, switching to MP and HP diets in seawater led to an increase in the whole-body Ca:P ratio compared to those fish fed the LP diet continuously. There was no effect of switching diet at seawater transfer on fillet damage due to connective tissue, although there was a tendency (GLM, $p = .05$) for a negative association with dietary phosphorus in those fish fed the HP diet during freshwater.

3.3. Water temperature and dietary phosphorus following seawater transfer

Irrespective of diet, those fish maintained on 10 °C for the initial 4 months of seawater rearing weighed significantly less on day 266, but became significantly heavier at termination of the experiment, than those fish kept at 16 °C. Furthermore, the fish maintained at 10 °C had lower body condition on day 394 and at harvest, but not on day 266. There was also an interaction between diet and temperature on body condition, but not mass, with the HP fed fish having a significantly lower body condition than the LP fish at 16 °C, but not at 10 °C. Those fish maintained on 16 °C had a significantly higher prevalence of cataracts and sexual maturation than those kept on 10 °C, as well as a higher cataract score. There was no effect of dietary phosphorus on cataracts or the prevalence of sexual maturation.

Water temperature at seawater transfer had no effect on the whole-body phosphorus or the Ca:P ratio, ash content, or the prevalence of fish with one or more deformed vertebrae. However, those fish raised at 16 °C did have a higher prevalence of externally visible deformities and fish with ≥ 10 deformed vertebra, irrespective of diet. Here, it was also observed that those fish fed the LP diet had significantly more fillet damage than those fed the HP diet, irrespective of temperature.

3.4. Vertebral deformities

In total, 13 different deformity types were recorded. Here, it was notable that homogeneous compressions were more evident at termination, whereas compression with reduced intervertebral space was more evident on day 266 (500 g fish). Otherwise, those fish given the LP diet were the only groups to show compressed vertebrae without the X structure (type 4, Witten et al., 2009) whereas compressed and fused vertebrae were observed in all groups (see Supplementary Fig. S6).

3.5. Histopathology

The variety of bone cell types and structural components identified on bone sections are shown in Fig. S7, based on control material from this study. Osteoblasts are abundant, osteocytes are present in low numbers, and bone resorption by multinucleated osteoclasts is common. The bone is rich in Sharpey fibres.

To choose samples for histological examinations, a sub sample of x-rays were analysed to locate and dissect deformed and non-deformed vertebral bodies, respectively. In freshwater, 8 ($\times 7$ LP, $\times 1$ MP) out of 22 fish were diagnosed for a low mineralised spine phenotype based on X-rays. The histological analysis shows that all fish with this phenotype developed ectopic cartilage adjacent to the vertebral body growth zone of the vertebral body endplate. Ectopic cartilage is located dorsal and ventral (Fig. S8). Fig. S9 shows the regular, non-pathological situation

Table 2
Data on feed conversion ratio, body size, cataracts, and sexual maturation in Atlantic salmon fed one of three diets, low (L), medium (M), and high (H) phosphorus (P), from 3 g body size, before either continuing on the same diet or being switched to one of the other two diets at seawater transfer. Significant model effects are provided. Statistical output and post hoc results can be found in Tables S1–6. Data are means ± SE (n).

Parameter	Temp.		16 °C		16 °C		16 °C		16 °C		16 °C		16 °C		
	Day	Temp.	FW diet		MP		HP		LP		MP		HP		
			LP	LP	LP	LP	LP	LP	LP	LP	LP	LP	LP	LP	LP
Food conversion ratio	100		0.64 ± 0.02 (8)	0.63 ± 0.02 (8)											
	153		0.69 ± 0.01 (8)	0.67 ± 0.01 (7)											
	278		0.73 ± 0.01 (3)	0.75 ± 0.01 (3)										0.77 ± 0.01 (3)	
	69		22 ± 0.5 (136)	22 ± 0.6 (137)											
	266		525 ± 175 (63)	568 ± 177 (67)											
	394		1267 ± 326 (63)	1302 ± 333 (67)											
Mass (g)	686		3848 ± 895 (63)	3894 ± 1098 (67)											
	69		11.4 ± 0.1 (136)	11.4 ± 0.1 (137)											
	266		32.1 ± 2.9 (62)	32.8 ± 2.7 (65)										32.9 ± 2.9 (58)	
	394		41.9 ± 3.8 (62)	42.8 ± 3.5 (65)										42.9 ± 3.7 (58)	
	686		61.2 ± 5.4 (62)	61.9 ± 6.4 (65)										62.7 ± 5.5 (58)	
	69		1.46 ± 0.01 (136)	1.42 ± 0.01 (137)											
Condition	266		1.53 ± 0.17 (62)	1.57 ± 0.16 (65)										1.54 ± 0.18 (58)	
	394		1.70 ± 0.20 (62)	1.64 ± 0.21 (65)										1.65 ± 0.15 (58)	
	686		1.68 ± 0.22 (62)	1.64 ± 0.25 (65)										1.60 ± 0.19 (58)	
	266		58 (80)	60 (82)										49 (77)	
	394		91 (69)	85 (74)										86 (69)	
	686		96 (69)	96 (73)										97 (66)	
Cataract score	686		4.5 ± 0.13 (66)	4.0 ± 0.16 (70)										3.8 ± 0.16 (64)	
	266		1 (80)	4 (82)										6 (77)	
	686		11 (80)	12 (82)										3 (77)	
	Sexual maturation (%)	100		0.65 ± 0.01 (8)	0.67 ± 0.01 (8)										
		153		0.68 ± 0.01 (8)	0.67 ± 0.01 (3)										
		278		0.76 ± 0.01 (3)	0.70 ± 0.01 (3)										
69			23 ± 0.5 (138)	—											
266			534 ± 140 (57)	486 ± 98 (60)											
394			1284 ± 309 (57)	1099 ± 251 (60)											
Food conversion ratio	686		3788 ± 1035 (57)	4405 ± 1000 (60)											
	69		11.6 ± 0.1 (138)	—											
	266		33.0 ± 2.4 (56)	31.4 ± 2.2 (58)											
	394		43.1 ± 3.4 (56)	42.3 ± 3.3 (58)											
	686		62.4 ± 6.2 (56)	65.9 ± 5.2 (58)											
	69		1.43 ± 0.01 (138)	—											
Mass (g)	1.54 ± 0.17 (61)		1.58 ± 0.16 (44)	1.56 ± 0.11 (58)											
	1.60 ± 0.17 (61)		1.62 ± 0.21 (44)	1.43 ± 0.11 (58)											
	1.55 ± 0.21 (61)		1.60 ± 0.30 (44)	1.53 ± 0.21 (58)											
	65 (82)		45 (60)	0 (80)											
	90 (69)		84 (50)	35 (69)											
	100 (68)		94 (50)	65 (69)											

(continued on next page)

Table 2 (continued)

Parameter	16 °C		10 °C		Significant variables				
					Diet		SW		Temperature
	HP	LP	HP	LP	HP	LP	SW	Diet	Temp
Cataract score	4.2 ± 0.18 (68)	4.1 ± 0.17 (63)	4.1 ± 0.17 (63)	2.4 ± 0.2 (60)	2.3 ± 0.3 (45)				Temp
Sexual maturation (%)	2 (82)	5 (60)	3 (73)	0 (83)	1 (80)				Temp
	6 (82)	7 (60)	10 (73)	0 (83)	0 (80)				

of bone formation without the presence of ectopic cartilage. A less commonly observed pathology was the development of ectopic cartilage in the region of the bone trabeculae that connects the vertebral body endplates.

In post-smolts, 6 (×5 LP, ×1 MP) out of 18 fish showed a low mineralised spine phenotype based on x-rays. These fish displayed ectopic cartilage adjacent to the vertebral body growth zone, but more of this cartilage has been developed. The cartilage also acquires a more hyaline phenotype. Fig. S10 compares growth zones in a regular (non-deformed) and a malformed vertebral body. In fish without malformations, only bone is formed in the vertebral body growth zone. In fish with radiological signs of malformations, abundant ectopic cartilage was present.

At harvest, we selected 4 fish with skeletal malformations, and 2 without, from each of the 11 groups. Fish with deformities showed primarily different compression and fusion phenotypes (Fig. S11). There was a strong anterior posterior gradient, as only few fish had deformities in the anterior part of the spine whereas deformities in the posterior part of the spine were more abundant (Fig. S3–5). The majority of diagnosed deformities related to low mineralised vertebral body endplates (x-ray diagnosis based). Typical was the development of ectopic cartilage, the replacement of regular (notochord-based) intervertebral tissue by ectopic cartilage, and the extension of ectopic cartilage as chondroid into adjacent musculature. Only few fish with low mineralised vertebral body endplates (x-ray diagnosis based) showed alterations of the intravertebral body bone spongiosa. Fig. S12 shows examples in comparison to regular intravertebral body bone spongiosa: Hyper-dense bones with a large amount of chondroid bone and soft, undulating, intervertebral bone trabeculae.

In fish with pronounced vertebral body compression, ectopic cartilage completely replaced the notochord tissue in the intervertebral spaces (Fig. S13). The vertebral body growth zone displayed the transition of bone forming cells (osteoblasts) into cartilage forming cells (chondroblasts). This transition suggests that the cells from ectopic (pathological) cartilage derive from osteoblasts that undergo osteoblastic to chondroblastic transdifferentiation. Accordingly, a gradient from bone to cartilage tissues was recorded. This tissue is not present in regular vertebral bodies.

Ectopic cartilage extends from compressed and fused vertebral bodies into a tissue that is intermediate between cartilage and connective tissue: chondroid (Fig. S14). In areas of vertebral fusion, fibrous chondroid tissue has an amorphous extracellular substance with staining properties similar to extracellular cartilage matrix. This tissue is present in the musculature where it replaces regular muscle fibres to a large degree (Fig. S14).

3.6. Associations between deformities, cataracts, and sexual maturation on body size parameters

In models with all groups pooled, there was a significant negative association between cataract score, external deformities, and sexual maturation on body mass at harvest. Generally, body mass increased with cataract score up until a score of 4, but then decreased between scores of 5–8. Here, those fish with a score of 8 were the only group significantly smaller (LME: $\chi^2 = 67$, $df = 8$, $p \leq .001$; -1050 ± 321 g) than those fish without cataracts at harvest. Fish that had matured as either post-smolts or grilse were significantly smaller (LME: $\chi^2 = 35$, $df = 1$, $p \leq .001$; -860 ± 144 g) than those fish that remained immature. Finally, fish with an externally visible spinal deformity were significantly smaller (LME: $\chi^2 = 62$, $df = 1$, $p \leq .001$; -653 ± 72 g) than those fish with no externally visible deformity.

4. Discussion

Our objective was to study the effects of dietary phosphorus level on vertebral column bone health in response to feeding regime and water

Table 3
Data on vertebra deformities, bone, and fillet damage in Atlantic salmon fed one of three diets, low (L), medium (M), and high (H) phosphorus (P), from 3 g in body size, before either continuing on the same diet or being switched to one of the other two diets at seawater transfer. Significant model effects are provided. Statistical output and post hoc results can be found in Tables S1–6. Data are means ± SE (n).

Parameter	Day	Temp.					
		16 °C			10 °C		
		FW diet		SW Diet		MP	
	LP	HP	LP	HP	LP	MP	
Vertebral stiffness (N/mm)	69	40.9 ± 8.3 (12)	–	–	–	91.4 ± 11.5 (12)	–
Vertebral total ash (%)	69	34.8 ± 3.1 (32)	–	–	–	46.0 ± 1.0 (32)	–
	155	38.2 ± 4.2 (12)	–	–	–	51.1 ± 2.1 (12)	–
	266	54.6 ± 2.3 (9)	53.3 ± 4.4 (9)	55.4 ± 2.1 (9)	55.9 ± 1.1 (9)	56.6 ± 1.0 (9)	56.5 ± 1.3 (9)
	686	55.0 ± 1.6 (9)	55.4 ± 1.1 (9)	54.7 ± 2.4 (9)	56.0 ± 0.9 (9)	55.5 ± 1.7 (9)	55.3 ± 1.7 (9)
Whole body P (mg kg ⁻¹)	69	3020 ± 91 (8)	–	–	–	3583 ± 55 (8)	–
	155	3063 ± 72(6)	–	–	–	3738 ± 66 (6)	–
	266	3615 ± 95 (3)	3789 ± 54 (3)	3714 ± 146 (3)	3557 ± 127 (3)	3786 ± 48 (3)	3792 ± 92 (3)
	69	0.41 ± 0.09 (8)	–	–	–	0.56 ± 0.13 (8)	–
Whole body Ca:P	155	0.43 ± 0.10 (6)	–	–	–	0.66 ± 0.06 (6)	–
	266	0.73 ± 0.02 (3)	0.82 ± 0.02 (3)	0.90 ± 0.03 (3)	0.82 ± 0.03 (3)	0.78 ± 0.05 (3)	0.86 ± 0.07 (3)
External deformities (%)	153	30 (80)	17 (66)	27 (74)	4 (80)	1 (82)	0 (77)
	266	34 (80)	30 (66)	41 (74)	19 (80)	24 (82)	17 (77)
	686	53 (80)	30 (66)	51 (74)	28 (80)	33 (82)	22 (77)
≥1 deformed vertebra (%)	153	95 (42)	–	–	–	5 (42)	–
	274	50 (24)	38 (24)	50 (24)	25 (24)	21 (24)	17 (24)
	686	86 (69)	81 (59)	78 (68)	60 (73)	63 (73)	56 (66)
≥10 deformed vertebra (%)	153	86 (42)	–	–	–	0 (42)	–
	274	13 (24)	13 (24)	21 (24)	0 (24)	4 (24)	0 (24)
	686	72 (69)	54 (59)	51 (68)	42 (73)	36 (73)	39 (66)
Fillet damage (%)	686	31 (29)	43 (28)	53 (30)	40 (35)	23 (31)	33 (24)

Parameter	16 °C	Significant variables					
		10 °C			10 °C		
		LP		HP		MP	
	LP	HP	LP	HP	MP	Temperature	
Vertebral stiffness (N/mm)	–	–	–	–	–	na	na
Vertebral total ash (%)	–	–	–	–	–	MP - Time	Diet × Time
	56.2 ± 0.7 (9)	57.2 ± 0.4 (9)	54.7 ± 1.4 (9)	56.0 ± 1.0 (9)	56.0 ± 1.0 (9)	HP - Diet + Time	Diet × Time
	54.3 ± 2.1 (9)	56.0 ± 1.3 (9)	56.0 ± 0.9 (9)	56.1 ± 1.0 (9)	–	–	–
Whole body P (mg kg ⁻¹)	–	–	–	–	–	–	–
	3798 ± 22 (3)	3678 ± 84 (6)	3821 ± 30 (3)	3506 ± 84 (3)	3720 ± 51 (3)	LP - Diet	Diet
Whole body Ca:P	–	–	–	–	–	–	–
	0.75 ± 0.03 (3)	0.79 ± 0.02 (3)	0.79 ± 0.02 (3)	0.73 ± 0.04 (3)	0.76 ± 0.05 (3)	LP - Diet × Time	Diet + Temp + Time
External deformities (%)	2 (82)	4 (73)	5 (60)	12 (83)	0 (80)	–	–
≥1 deformed vertebra (%)	15 (82)	21 (73)	18 (60)	6 (83)	3 (80)	MP - Time	Diet + Temp + Time
	34 (82)	25 (73)	30 (60)	23 (83)	11 (80)	HP - Time	Diet + Temp
	–	14 (42)	–	–	–	LP - Time	Diet + Temp
	0 (24)	21 (24)	13 (24)	54 (24)	29 (24)	MP - Time	Diet + Temp
	66 (68)	52 (66)	46 (50)	78 (74)	38 (68)	HP - Diet × Time	Diet + Temp + Time

(continued on next page)

Table 3 (continued)

Parameter	Significant variables										
	16 °C					10 °C					
	HP		MP		HP	LP		HP		10 °C	
									Diet	SW Diet	Temperature
≥10 deformed vertebra (%)	0 (24)	4 (24)	2 (42)	17 (24)	0 (24)	–	–	–	Diet × Time	LP - Diet	Diet + Temp
Fillet damage (%)	43 (68)	36 (50)	4 (24)	34 (74)	15 (68)	–	–	–	Diet	–	Diet
	43 (28)	32 (22)	14 (28)	24 (33)	8 (25)	–	–	–	Diet	–	Diet

temperature. The LP diet significantly affected indicators of bone health and increased the prevalence of severe vertebral body deformities. However, this could be partially rescued by switching the diet to a higher ($\geq 7.1 \text{ g kg}^{-1}$ soluble phosphorus) phosphorus content for 4 months following seawater transfer. However, high dietary phosphorus could not prevent the increase in vertebral body deformities observed in fish reared at high water temperatures for 4 months following seawater transfer, suggesting dietary phosphorus may not be a risk factor for water temperature induced vertebral body deformities.

Fish fed from 3 to 500 g body weight the $4.4\text{--}5.0 \text{ g kg}^{-1}$ (LP) soluble phosphorus diet, which is below the recommended level of 8 g kg^{-1} available phosphorus for Atlantic salmon (NRC 2011), had an elevated prevalence of vertebral deformities as typically found in salmonids (Baeverfjord et al., 1998; Deschamps et al., 2014; Fjellidal et al., 2012b, 2016; Taylor et al., 2015), although not always (Gil-Martens et al., 2012; Witten et al., 2016). The LP diet was associated with numerous markers of impaired bone mineralization such as reduced vertebral ash content (Albrektsen et al., 2009; Deschamps et al., 2014; Fjellidal et al., 2012b; Helland et al., 2005; Witten et al., 2016), reduced whole-body phosphorus (Baeverfjord et al., 1998) and Ca:P ratio (Albrektsen et al., 2009), and the induction of markers of bone mineralization (Fjellidal et al., 2012c, 2016; Ytteborg et al., 2016). However, switching LP fish to either the MP or HP diet at seawater transfer for 4 months did mitigate the prevalence of fish with severe vertebral deformities. This is similar to previous work where adequate dietary phosphorus at seawater transfer was found to mitigate deformities in a stock already suffering from a high prevalence of tail deformities as post-smolts compared to a low phosphorus diet (Fjellidal et al., 2009). However, we found that switching the diet for 4 months after seawater transfer had no effect on the prevalence of vertebral deformities at harvest in the MP-LP or HP-LP fish, despite a reduction in whole-body phosphorus to those levels found in the LP-LP fish. In another study, bone and scale mineral content and alkaline phosphatase (ALP) also indicated early phosphorus deficiency signs in smolts fed 5 g kg^{-1} soluble phosphorus (Albrektsen et al., 2018). The dietary phosphorus requirement of salmon is expected to be lower at later life stages due to a decrease in the utilisation of dietary energy as the growth rates decline (Shearer, 1995). Evidence of this may be found in the mineral content of the bone, as we found that although the LP diet had consistently lower values throughout the study, there was no significant difference between the LP and MP groups at the end of the different feeding regimes (day 266). This could also be explained by the slightly higher soluble phosphorus content in the LP diet during the seawater compared to freshwater phase, $5.0 \text{ vs } 4.4\text{--}4.6 \text{ g kg}^{-1}$, respectively. However, Deschamps et al. (2014) found that a phosphorus deficient diet (0.5% total phosphorus) reduced ash content and bone mineralization in small rainbow trout (*Oncorhynchus mykiss*), to a greater extent than in larger trout.

We found a combination of $7.5\text{--}7.6 \text{ g kg}^{-1}$ (MP) soluble phosphorus during freshwater and 5.0 g kg^{-1} (LP) during the early seawater phase, was sufficient for bone health in Atlantic salmon under the current conditions. Similarly, a dietary requirement of 8 g kg^{-1} soluble phosphorus was needed to reach the highest whole-body mineral reservoirs in pre-smolts (Ytteborg et al., 2016) in accordance with the requirement for available phosphorus set by NRC (2011), whereas the soluble phosphorus requirement for normal bone development was met at 6.5 g kg^{-1} (Ytteborg et al., 2016). In fast growing 0+ smolts, the phosphorus requirement for normal bone development was met between $5.1 \text{ and } 7.4 \text{ g kg}^{-1}$ soluble phosphorus (Albrektsen et al., 2018). In seawater, Fjellidal et al. (2012c) found that post-smolts (230 g) fed 4 g kg^{-1} available dietary phosphorus for 49 days had poorer bone health, as assessed by bone ALP expression, bone tartrate resistant acid phosphatase (TRACP), matrix metallo-proteinase 13 (*mmp13*) gene expression, and vertebral mineral composition and stiffness. In contrast, we found salmon on the MP-LP diet had no differences in vertebral ash, whole body phosphorus or Ca:P ratios, or vertebral deformities,

compared to those salmon fed the MP diet throughout.

Increasing the water temperature at seawater transfer led to an increase in the prevalence of severely deformed fish. This is similar to a previous report whereby an increased prevalence of vertebral deformities, particularly in the tail region, was observed after keeping Atlantic salmon on higher (16 °C) vs lower (10 °C) temperatures at seawater transfer (Grini et al., 2011). Previous work in salmon has shown that serum phosphorus drops during seawater transfer, suggesting fish may be deficient for phosphorus during this period (Grini et al., 2011). However, we found no significant interaction between diet and water temperature on the level of vertebral deformities, suggesting that either dietary phosphorus is not a factor in the effect of water temperature at seawater transfer, or the dietary phosphorus levels used were not high enough to mitigate the temperature effect. Previous work has found that high water temperature (16 vs 10 °C) alters the dorsal ventral diameter of vertebra (Grini et al., 2011). Therefore, temperature induced bone remodelling/growth together with the higher growth rates achieved by the 16 °C group during the early seawater phase, could explain the increase in severity of deformities observed in the 16 vs 10 °C groups rather than insufficient dietary phosphorus.

Fish with low-mineralised vertebral bodies were diagnosed for the presence of ectopic cartilage. Freshwater fish and post-smolts showed ectopic cartilage adjacent to the vertebral growth zones before compression became visible on x-rays. In contrast, at harvest ectopic cartilage and fibrocartilage was present in the intervertebral space in vertebral bodies that are visibly compressed on x-ray, and fibrocartilage extended into the musculature. Pauwels model (Pauwels, 1960) could explain the development of cartilage and fibrocartilage. Here, mechanical forces determine the nature of skeletal cells (Hall, 2015); strain favours the formation of ligaments and intramembranous bone and compression favours the development of cartilage. Combinations of strain and compression generate intermediate tissues, such as fibrous connective tissue and fibrocartilage (Weinans and Prendergast, 1996). One can well imagine that the bending of under-mineralised vertebral body endplates transmits compression to adjacent structures and this compression triggers the development of ectopic cartilage and fibrocartilage cartilage. Ectopic cartilage in the intervertebral space of compressed Atlantic salmon vertebral bodies has been reported previously (Kvellestad et al., 2000; Witten et al., 2005) and is known to be caused by compression of vertebra body spongiosa trabeculae (Helland et al., 2006).

We found fibrocartilage to extend from deformed vertebral bodies into the musculature; a pathology that affects animal welfare and production. Previous studies about compressed vertebral bodies only reported cartilage in intervertebral spaces. Whether this more severe pathology relates to the size and age of the fish, or if particular risk factors exist that promote muscle infiltration, needs to be evaluated. Inflammation is a factor that has been associated with vertebral fusion and alterations of the musculature because large numbers of granulocytes can occur in the vicinity of the vertebral column (Kvellestad et al., 2000; Munday et al., 2017). Other studies have found no indications for inflammation (Witten et al., 2005). Granulocytes that surrounded the spinal cord and thus appear in the vicinity of vertebral bodies are indeed common in farmed Atlantic salmon, but also in fish that have no deformities (Boglione et al., 2013). Using a potent local inflammation inducer, Gil-Martens et al. (2012) did not observe a connection between vertebral body compression and inflammation. Similarly, no connection was observed between vertebral body compression, phosphorous deficiency, and inflammation. This suggests that mechanical forces according to Pauwels (1960) model rather than inflammation are causative for the occurrence of ectopic fibrocartilage.

Although not consistent across all comparisons, low dietary phosphorus tended to be associated with an increased occurrence of connective tissue within the fillet. This condition reduces the value of the fillet and in severe cases decreases processing efficiency, as these fish cannot be machine filleted (Sullivan et al., 2007). The occurrence of

such connective tissue has been linked to compressed vertebrae/compressed or missing inter-vertebral space (Haugarvoll et al., 2010), therefore one would expect the low dietary phosphorus group to show the highest prevalence of fillet damage as this group had the highest prevalence of deformed vertebra. However, it was noted that there were fish with no deformed vertebra that still had connective tissue within the fillet, and fish with up to 48 deformed vertebra that had no occurrence of connective tissue. Therefore, further work is required into the aetiology of this condition.

There was a notable effect of temperature on growth and cataract development, but no effect of diet. Higher temperatures following seawater transfer can lead to an increase in growth, but also have a negative effect on long-term performance (Grini et al., 2011). Indeed, the optimum temperature for growth in post-smolts has been found to be between 12.8 (Handeland et al., 2008) and 13 °C (Handeland et al., 2003), whereas salmon reared at 10 and 16 °C were predicted to grow at similar rates (Handeland et al., 2008). We found the 16 °C group were larger after 4 months in seawater, but smaller at harvest size, a similar finding to Grini et al. (2011). The reasons behind this result are unknown, but sexual maturation, skeletal deformities, and cataracts are likely to reduce long-term growth rates and were higher/more severe in the 16 vs 10 °C groups. Indeed, all 3 factors were negatively associated with harvest weight. High water temperature around seawater transfer (i.e. 16 °C) is a known risk for cataract development (Bjerkås et al., 2001). The causes of this may be multifactorial, but include a deficiency of dietary histidine, an important amino acid for cataract development, and/or osmoregulatory stress around smoltification (Bjerkås and Sveier, 2004). In terms of diet, low (Albrektsen et al., 2009; Deschamps et al., 2014; Ketola, 1975; Prabhu et al., 2013; Åsgård and Shearer, 1997) or even high (Fjelldal et al., 2016) dietary phosphorus levels have been associated with reduced growth, however, salmonids can maintain growth at pathologically low dietary phosphorus levels (Baeverfjord et al., 1998; Fjelldal et al., 2012b; Helland et al., 2005) as found in the current study.

There was no effect of dietary phosphorus on the level of sexual maturation, in contrast to a previous report of a negative association (Fjelldal et al., 2012d). However, a lower water temperature at seawater transfer was associated with reduced levels of early sexual maturation. This is of particular interest to those wishing to produce larger smolts through temperature manipulation as high-water temperature around seawater transfer has recently been identified as a risk factor for the incidence of post-smolt maturation, particularly in males (Fjelldal et al., 2011; Imsland et al., 2014). The exact mechanism is unknown, but is expected to be related to energy reserves at or prior to specific life history events (Adams and Thorpe, 1989). Here, it is noted that body condition (a.k.a. condition factor), a proxy for energy reserves (Herbinger and Friars, 1991), was much higher in LP fish that were reared at 16 vs 10 °C, but no such pattern was evident in the HP fish. Therefore, further work is required into the role of energy reserves and temperature manipulation on the prevalence of early sexual maturation. Similar to recent work (Fraser et al., 2019), we found that those fish that sexually matured as post-smolts survived in seawater, but were significantly smaller at harvest than fish that remained immature throughout.

5. Conclusions

Atlantic salmon suffer from a higher risk of developing of vertebral deformities when fed 4.4–4.6 g kg⁻¹ soluble phosphorus during the freshwater life stage compared to 5.0 g kg⁻¹ during the first 4 months in seawater. However, the effects of the LP diet could be partially mitigated by feeding fish ≥7.1 g kg⁻¹ soluble phosphorous in seawater. Fish fed the LP diet also tended to have more fillet damage than those fed the HP diet, irrespective of water temperature. Furthermore, high water temperatures for 4 months following seawater transfer resulted in an increase in vertebral deformities, but this temperature effect appears

independent of dietary phosphorus levels. Therefore, further work is required into understanding the mechanisms behind temperature induced vertebral deformities.

Funding

This project was funded by the Fiskeri- og Havbruksnæringens Forskningsfond ([FHF] Fisheries and Aquaculture Industry Research Fund), project number 900798.

Acknowledgements

We thank Britt Sværen for help with bone analyses.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aquaculture.2019.734246>.

References

- Adams, C.E., Thorpe, J.E., 1989. Photoperiod and temperature effects on early development and reproductive investment in Atlantic salmon (*Salmo salar* L.). *Aquaculture* 79, 403–409.
- Albrektsen, S., Hope, B., Aksnes, A., 2009. Phosphorus (P) deficiency due to low P availability in fishmeal produced from blue whiting (*Micromesistius poulassou*) in feed for under-yearling Atlantic salmon (*Salmo salar*) smolt. *Aquaculture* 296, 318–328.
- Albrektsen, S., Lock, E.-J., Bæverfjord, G., Pedersen, M., Krasnov, A., Takle, H., Veiseth-Kent, E., Ørnsrud, R., Waagbø, R., Ytteborg, E., 2018. Utilization of H₂SO₄ hydrolysed phosphorus from herring bone by-products in feed for Atlantic salmon (*Salmo salar*) 0+ postsmolt. *Aquac. Nutr.* 24, 348–365.
- Åsgård, T., Shearer, K.D., 1997. Dietary phosphorus of juvenile Atlantic salmon, *Salmo salar* L. *Aquac. Nutr.* 3, 17–23.
- Bæverfjord, G., Åsgård, T., Shearer, K.D., 1998. Development and detection of phosphorus deficiency in Atlantic salmon, *Salmo salar* L., parr and post-smolts. *Aquac. Nutr.* 4, 1–11.
- Bæverfjord, G., Prabhu, A.J.P., Fjelldal, P.G., Albrektsen, S., Hatlen, B., Denstadli, V., Ytteborg, E., Takle, H.R., Lock, E.-J., Berntssen, M., Lundebye, A.-K., Åsgård, T.E., Waagbø, R., 2018. Mineral nutrition and bone health in salmonids. *Rev. Aquac.* 1–26. <https://doi.org/10.1111/raq.12255>.
- Bagenal, T.B., Tesch, F.W., 1978. Age and growth. In: Bagenal, T.B. (Ed.), *Methods for Assessment of Fish Production in Fresh Waters*. Blackwell Scientific Publications, Oxford, pp. 101–136.
- Bjerkås, E., Sveier, H., 2004. The influence of nutritional and environmental factors on osmoregulation and cataracts in Atlantic salmon (*Salmo salar* L.). *Aquaculture* 235, 101–122.
- Bjerkås, E., Bjørnstad, E., Breck, O., Waagbø, R., 2001. Water temperature regimes affect cataract development in smolting Atlantic salmon, *Salmo salar* L. *J. Fish Dis.* 24, 281–291.
- Boglioune, C., Gisbert, E., Gavaia, P., Witten, P.E., Moren, M., Fontagné, S., Koumoundouros, G., 2013. A review on skeletal anomalies in reared European fish larvae and juveniles. Part 2: Main typologies, occurrences and causative factors. *Rev. Aquac.* 5, S121–S167.
- Deschamps, M.-H., Poirier Stewart, N., Demanche, A., Vandenberg, G.W., 2014. Preliminary study for phenotypic description of vertebral abnormalities in triploid trout subjected to prolonged deficiency in phosphorus. *J. Appl. Ichthyol.* 30, 833–839.
- Fjelldal, P.G., Grotmol, S., Kryvi, H., Gjerdet, N.R., Taranger, G.L., Hansen, T., Porter, M.J.R., Totland, G.K., 2004. Pinelectomy induces malformation of the spine and reduces the mechanical strength of the vertebrae in Atlantic salmon, *Salmo salar* L. *Pineal Res.* 36, 132–139.
- Fjelldal, P.G., Hansen, T., Breck, O., Sandvik, R., Waagbø, R., Berg, A., Ørnsrud, R., 2009. Supplementation of dietary minerals during the early seawater phase increase vertebral strength and reduce the prevalence of vertebral deformities in fast-growing under-yearling Atlantic salmon (*Salmo salar* L.) smolt. *Aquac. Nutr.* 15, 366–378.
- Fjelldal, P.G., Hansen, T., Huang, T., 2011. Continuous light and elevated temperature can trigger maturation both during and immediately after smoltification in male Atlantic salmon (*Salmo salar*). *Aquaculture* 321, 93–100.
- Fjelldal, P.G., Hansen, T., Breck, O., Ørnsrud, R., Lock, E.-J., Waagbø, R., Wargelius, A., Witten, P.E., 2012a. Vertebral deformities in farmed Atlantic salmon (*Salmo salar* L.) – etiology and pathology. *J. Appl. Ichthyol.* 28, 433–440.
- Fjelldal, P.G., Hansen, T., Albrektsen, S., 2012b. Inadequate phosphorus nutrition in juvenile Atlantic salmon has a negative effect on long-term bone health. *Aquaculture* 334–337, 117–123.
- Fjelldal, P.G., Lock, E.-J., Hansen, T., Waagbø, R., Wargelius, A., Gil Martens, L., El-Mowafi, A., Ørnsrud, R., 2012c. Continuous light induces bone resorption and affects vertebral morphology in Atlantic salmon (*Salmo salar* L.) fed a phosphorus deficient diet. *Aquac. Nutr.* 18, 610–619.
- Fjelldal, P.G., Imsland, A., Hansen, T., 2012d. Vaccination and elevated dietary phosphorus reduces the incidence of early sexual maturation in Atlantic salmon (*Salmo salar* L.). *Aquaculture* 364–365, 333–337.
- Fjelldal, P.G., Hansen, T.J., Lock, E.-J., Wargelius, A., Fraser, T.W.K., Sambras, F., El-Mowafi, A., Albrektsen, A., Waagbø, R., Ørnsrud, R., 2016. Increased dietary phosphorus prevents vertebral deformities in triploid Atlantic salmon (*Salmo salar* L.). *Aquac. Nutr.* 22, 72–90.
- Fraser, T.W.K., Hansen, T., Skjæraasen, J.E., Mayer, I., Sambras, F., Fjelldal, P.G., 2013. The effect of triploidy on the culture performance, deformity prevalence, and heart morphology in Atlantic salmon. *Aquaculture* 416–417, 255–264.
- Fraser, T.W.K., Hansen, T., Mayer, I., Skjæraasen, J.E., Glover, K.A., Sambras, F., Fjelldal, P.G., 2014. The effect of triploidy on vaccine side-effects in Atlantic salmon. *Aquaculture* 433, 481–490.
- Fraser, T.W.K., Hansen, T., Fleming, M., Fjelldal, P.G., 2015. The prevalence of vertebral deformities is increased with higher egg incubation temperatures and triploidy in Atlantic salmon *Salmo salar* L. *J. Fish Dis.* 38, 75–89.
- Fraser, T.W.K., Fjelldal, P.G., Schulz, R.W., Norberg, B., Hansen, T.J., 2019. Termination of puberty in out-of-season male Atlantic salmon smolts. *Comp. Biochem. Physiol. A* 232, 60–66.
- Gil-Martens, L., Fjelldal, P.G., Lock, E.-J., Wargelius, A., Wergeland, H., Witten, P.E., Hansen, T., Waagbø, R., Ørnsrud, R., 2012. Dietary phosphorus does not reduce the risk for spinal deformities in a model of adjuvant-induced inflammation in Atlantic salmon (*Salmo salar*) post-smolts. *Aquac. Nutr.* 18, 12–20.
- Grimi, A., Hansen, T., Wargelius, A., Fjelldal, P.G., 2011. The effect of water temperature on vertebral deformities and vaccine-induced abdominal lesions in Atlantic salmon, *Salmo salar* L. *J. Fish Dis.* 34, 531–546.
- Groote, G.D., 1986. Biological Availability of Phosphorus in Feed Phosphate for Broilers. *Zootec International*.
- Hall, B.K., 2015. *Bones and Cartilage. Developmental and Evolutionary Skeletal Biology*, 2nd edition. Academic Press, London (892 pages).
- Handeland, S.O., Björnsson, B.Th., Arnesen, A.M., Stefansson, S.O., 2003. Seawater adaptation and growth of post-smolt Atlantic salmon (*Salmo salar*) of wild and farmed strains. *Aquaculture* 220, 367–384.
- Handeland, S.O., Imsland, A.K., Stefansson, S.O., 2008. The effect of temperature and fish size on growth, feed intake, food conversion efficiency and stomach evacuation rate of Atlantic salmon post-smolts. *Aquaculture* 283, 36–42.
- Hansen, T., Fjelldal, P.G., Yurtseva, A., Berg, A., 2010. A possible relation between growth and number of deformed vertebrae in Atlantic salmon (*Salmo salar* L.). *J. Appl. Ichthyol.* 26, 355–359.
- Haugarvoll, E., Bjerkås, I., Szabo, N.J., Satoh, M., Koppang, E.O., 2010. Manifestations of systemic autoimmunity in vaccinated salmon. *Vaccine* 28, 4961–4969.
- Helland, S., Refstie, S., Espmark, Å., Hjelde, K., Bæverfjord, G., 2005. Mineral balance and bone formation in fast-growing Atlantic salmon parr (*Salmo salar*) in response to dissolved metabolic carbon dioxide and restricted dietary phosphorus supply. *Aquaculture* 250, 364–376.
- Helland, S., Denstadli, V., Witten, P.E., Hjelde, K., Storebakken, T., Bæverfjord, G., 2006. Occurrence of hyper dense vertebrae in Atlantic salmon (*Salmo salar* L.) fed diets with graded levels of phytic acid. *Aquaculture* 261, 603–614.
- Herbinger, C.M., Friars, G.W., 1991. Correlation between condition factor and total lipid content in Atlantic salmon, *Salmo salar* L., parr. *Aquac. Res.* 22, 527–529.
- Houde, E.D., Scheckter, R.C., 1981. Growth rates, rations and cohort consumptions of marine fish larvae in relation to prey concentration. In: *Rapports et Proces-verbaux des Réunions du Conseil International pour l'Exploration de la Mer*. 178. pp. 441–453.
- Hovde, G., 2013. Validation of a method for analysis of soluble phosphorus by use of alkaline extraction and spectrophotometric determination. In: *Nofima Research Report*. <https://nofima.brage.unit.no/nofima-xmlui/handle/11250/284039>.
- Hua, K., Liu, L., Bureau, D.P., 2005. Determination of phosphorus fractions in animal protein ingredients. *J. Agric. Food Chem.* 53, 1571–1574.
- Imsland, A.K., Handeland, S.O., Stefansson, S.O., 2014. Photoperiod and temperature effects on growth and maturation of pre- and post-smolt Atlantic salmon. *Aquac. Int.* 22, 1331–1345.
- Kacem, A., Gustafsson, S., Meunier, F.J., 2000. Demineralization of the vertebral skeleton in Atlantic salmon *Salmo salar* L. during spawning migration. *Comp. Biochem. Physiol. A* 125, 479–484.
- Ketola, H.G., 1975. Requirement of Atlantic salmon for dietary phosphorus. *Trans. Am. Fish. Soc.* 104, 548.
- Kvellstad, A., Høie, S., Thorud, K., Tørud, B., Lyngøy, A., 2000. Platyspondyly and shortness of vertebral column in farmed Atlantic salmon *Salmo salar* in Norway - description and interpretation of pathological changes. *Dis. Aquat. Org.* 39, 97–108.
- Lall, S.P., 2003. *The Minerals in Fish Nutrition*. pp. 259–308.
- Munday, J.S., Perrott, M.R., Symonds, J.E., Walker, S.P., Lovett, B., Preece, M.A., Davie, P.S., 2017. Unilateral perivertebral fibrosis associated with lordosis kyphosis and scoliosis, (LKS) in farmed Chinook salmon in New Zealand. *Dis. Aquat. Org.* 121, 211–221.
- Nordrum, S., Åsgård, T., Shearer, K.D., Arnesen, P., 1997. Availability of phosphorus in fish bone meal and inorganic salts to Atlantic salmon (*Salmo solar*) as determined by retention. *Aquaculture* 157, 51–61.
- NRC - National Research Council, 2011. *Nutrient Requirements of Fish and Shrimp*. National Academy Press, Washington, D.C.
- Pauwels, F., 1960. A new theory on the influence of mechanical stimuli on the differentiation of supporting tissue. The tenth contribution to the functional anatomy and causal morphology of the supporting structure. *Zeitschrift Fur Anatomie Und Entwicklungsgeschichte* 121, 478–515.
- Powell, M.D., Jones, M.A., Ljalad, M., 2009. Effects of skeletal deformities on swimming performance and recovery from exhaustive exercise in triploid Atlantic salmon. *Dis. Aquat. Org.* 85, 59–66.

- Prabhu, A.J.P., Schrama, J.W., Kaushik, S.J., 2013. Quantifying dietary phosphorus requirement of fish – a meta-analytic approach. *Aquac. Nutr.* 19, 233–249.
- Presnell, J.K., Schreiber, M.P., 1998. *Humason's Animal Tissue Techniques*. The Johns Hopkins University Press, Baltimore (572 pages).
- Ruban, V., López-Sánchez, J.F., Pardo, P., Rauret, G., Muntau, H., Quevauviller, 2001. Harmonized protocol and certified reference material for the determination of extractable contents of phosphorus in freshwater sediments – a synthesis of recent works. *Fresenius J. Anal. Chem.* 370, 224–228.
- Shearer, K.D., 1995. The use of factorial modeling to determine the dietary requirements for essential elements in fishes. *Aquaculture* 133, 57–72.
- Skonberg, D.I., Yogev, L., Hardy, R.W., Dong, F.M., 1997. Metabolic response to dietary phosphorus intake in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 157, 11–24.
- Smedley, M.A., Migaud, H., McStay, E.L., Clarkson, M., Bozzolla, P., Campbell, P., Taylor, J.F., 2018. Impact of dietary phosphorus in diploid and triploid Atlantic salmon (*Salmo salar* L.) with reference to early skeletal development in freshwater. *Aquaculture* 490, 329–343.
- Sullivan, M., Hammond, G., Roberts, R.J., Manchester, N.J., 2007. Spinal deformation in commercially cultured Atlantic salmon, *Salmo salar* L.: a clinical and radiological study. *J. Fish Dis.* 30, 745–752.
- Taylor, J.F., Martínez-Rubio, L., del Pozo, J., Walton, J.M., Tinch, A.E., Migaud, H., Tocher, D.R., 2015. Influence of dietary phospholipid on early development and performance of Atlantic salmon (*Salmo salar*). *Aquaculture* 448, 262–272.
- Vielma, J., Lall, S.P., 1998. Control of phosphorus homeostasis of Atlantic salmon (*Salmo salar*) in fresh water. *Fish Physiol. Biochem.* 19, 83–93.
- Wall, T., Bjerkås, E., 1999. A simplified method of scoring cataracts in fish. *Bull. Eur. Assoc. Fish Pathol.* 19, 162–165.
- Wargelius, A., Fjellidal, P.G., Grini, A., Gil-Martens, L., Kvamme, B.-O., Hansen, T., 2010. MMP-13 (Matrix MetalloProteinase 13) expression might be an indicator for increased ECM remodeling and early signs of vertebral compression in farmed Atlantic salmon (*Salmo salar* L.). *J. Appl. Ichthyol.* 26, 366–371.
- Weinans, H., Prendergast, P.J., 1996. Tissue adaptation as a dynamical process far from equilibrium. *Bone* 19, 143–149.
- Witten, P.E., Gil-Martens, L., Hall, B.K., Huysseune, A., Obach, A., 2005. Compressed vertebrae in Atlantic salmon *Salmo salar*: evidence for metaplastic chondrogenesis as a skeletogenic response late in ontogeny. *Dis. Aquat. Org.* 64, 237–246.
- Witten, P.E., Gil-Martens, L., Huysseune, A., Takle, H., Hjelde, K., 2009. Towards a classification and an understanding of developmental relationships of vertebral body malformations in Atlantic salmon (*Salmo salar* L.). *Aquaculture* 295, 6–14.
- Witten, P.E., Owen, M.A.G., Fontanillas, R., Soenens, M., McGurk, C., Obach, A., 2016. Primary phosphorus-deficiency in juvenile Atlantic salmon: the uncoupling of bone formation and mineralisation. *J. Fish Biol.* 88, 690–708.
- Ytteborg, E., Vegusdal, A., Witten, P.E., Berge, G., Takle, H., Østbye, T.K., Ruyter, B., 2010. Atlantic salmon precursor cells from white muscle differentiate into osteoblasts *in vitro*: polyunsaturated fatty acids and temperature influence gene expression and PGE2 production. *Biochim. Biophys. Acta* 1801, 127–137.
- Ytteborg, E., Beaverfjord, G., Lock, E.-J., Pedersen, M., Takle, H., Ørnstrud, R., Waagbø, R., Albrektsen, S., 2016. Utilization of acid hydrolysed phosphorus from herring bone by-products in feed for Atlantic salmon (*Salmo salar*) start-feeding fry. *Aquaculture* 459, 173–184.