Received: 26 February 2020 | Accepted: 31 March 2020

DOI: 10.1111/jfb.14338

REGULAR PAPER

JOURNAL OF FISH BIOLOGY fsbi

# Triploid Atlantic salmon *Salmo salar* have a higher dietary phosphorus requirement for bone mineralization during early development

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**Funding information**
This study was funded by the Fiskeri- og Havbruksnæringens Forskningsfond project (FHF, 900723) Solving Bottlenecks in Triploid Salmon Production – A Way to Strengthen the Sustainability of the Salmon Aquaculture Industry and a grant from the Research Council of Norway (NRC, 216197).

## Abstract

The effect of a dietary phosphorus regime in freshwater on vertebra bone mineralization was assessed in diploid and triploid Atlantic salmon, *Salmo salar*. Fish were fed either a low phosphorus (LP) diet containing 10.5 g $kg^{-1}$ total phosphorus or a normal phosphorus (NP) diet containing 17.4 g $kg^{-1}$ total phosphorus from ~3 to ~65 g (day 126) in body weight. Two further groups were fed the NP diet from ~3 g in body weight, but were then switched to the LP diet after 38 (~10 g in body weight) or 77 (~30 g in body weight) days. Growth, vertebral ash content (% ash) and radiologically detectable vertebra pathologies were assessed. Triploids were initially smaller than diploids, and again on day 77, but there was no ploidy effect on days 38 or 126. Vertebral ash content increased with increasing body size and those fish fed the NP diet had higher vertebral ash content than those groups fed the LP diet during the intervening time period, but this diet effect became less apparent as fish grew, with all groups having relatively equal vertebral ash content at termination. In general, triploids had lower vertebral ash content than diploids on day 38 and this was most evident in the group fed the LP diet. On day 77, those triploids fed the LP diet during the intervening time period had lower vertebral ash content than diploids. At termination on day 126, the triploids had the same vertebral ash content as diploids, irrespective of diet. There was a ploidy × diet interaction on vertebral deformities, with triploids having higher prevalences of fish with ≥1 deformed vertebra in all dietary groups except continuous NP. In conclusion, between days 0 and 77 (3–30 g body size), triploids required more dietary phosphorus than diploids in order to maintain similar vertebral ash content. A possible link between phosphorus feeding history and phosphorus demand is also discussed.

KEYWORDS

deformity, ploidy, radiology, sterile, vertebrae

## 1 | INTRODUCTION

In fish, phosphorus (P) is an essential mineral as it is a constituent in bone, scales, adenosine triphosphate, cell membranes and nuclei acids (Skonberg *et al*., 1997). As such, dietary P content can impact on mortality (Fjelldal *et al*., 2016), growth (Albrektsen *et al*., 2009; Åsgård & Shearer, 1997; Fjelldal *et al*., 2016; Ketola, 1975), sexual maturation (Fjelldal, Imsland, & Hansen, 2012), body lipid content (Albrektsen *et al*., 2009; Helland *et al*., 2005) and skeletal deformity development (Baeverfjord *et al*., 1998; Fjelldal *et al*., 2009) in farmed Atlantic salmon, *Salmo salar* (L. 1758). Due to a low content and inefficient absorption from water (Lall, 1991, 2002; Sugiura *et al*., 2004), inorganic P is often added to commercial fish diets to meet demand. However, unbalanced dietary P not only reduces fish health (Lall & Lewis-McCrea, 2007), but excess P waste is detrimental to the environment (Mente *et al*., 2006).

P nutrition in *S. salar* is important during all life stages for bone mineralization (Fjelldal *et al*., 2009; Fjelldal, Hansen, & Albrektsen, 2012; Fraser *et al*., 2019). If P supply is too low, osteoid deposition is normal, while the incorporation of minerals is hampered (Witten *et al*., 2019). As P crystalizes with calcium during mineralization, it cannot be replaced with other minerals in the process of bone mineralization. A low bone mineral content results in low mechanical strength and a soft bone structure (Fjelldal *et al*., 2006), and soft vertebrae are prone to compress (Fjelldal *et al*., 2007). Indeed, vertebra compression is the most problematic type of deformity in farmed *S. salar* (Fjelldal, Hansen, Breck, *et al*., 2012; Witten *et al*., 2005, 2009). As vertebral deformities have negative effects on growth, welfare (Hansen *et al*., 2010) and product quality (Michie, 2001), it is essential to assess P requirements during all the life stages under modern farming conditions.

Although sexually mature yet functionally sterile triploid *S. salar* males show spawning behaviour (Fjelldal *et al*., 2014), attempts to use triploids in Norwegian aquaculture (*e.g*., Sambraus, 2016) have been initiated to avoid hybrid introgression between escaped farmed and wild *S. salar* populations (Glover *et al*., 2017). Vertebral deformities are common in farmed (Fjelldal, Hansen, Breck, *et al*., 2012) and wild (Sambraus *et al*., 2014) *S. salar*, but have historically been a particular problem in farmed triploid *S. salar* (Fjelldal & Hansen, 2010). This has subsequently been found to be caused by an elevated dietary P requirement during the freshwater life stage in triploids that previously was not met by commercial diets designed for diploids (Fjelldal *et al*., 2016). Fjelldal *et al*. (2016) prevented deformities in triploids by feeding them a commercial diploid diet, but with extra P, during the entire freshwater period. However, such a lengthy period may be unnecessary as previous work has shown dietary P is most needed during rapid growth periods (Shearer, 1995). Therefore, to reduce the environmental load and production costs, knowledge about possible critical feeding periods in triploids is essential.

The present study investigated whether triploid *S. salar* require high dietary P during the entire freshwater life stage in order to maintain similar bone health parameters as diploid counterparts. To this end, we tested periodic feeding with low P (LP) and normal P (NP) diets in freshwater (3–65 g body weight) and assessed mortality, growth, radiology and vertebral ash content in triploid and diploid *S. salar*. The LP diet contained 10.5 g $kg^{-1}$ total P whereas the NP diet contained 17.4 g $kg^{-1}$ total P. The LP diet is expected to meet the P demands of diploids and the NP diet is expected to meet the P requirements of triploids (Fjelldal *et al*., 2016). As a secondary objective, we assessed vertebral ash content over time relating to dietary P history.

## 2 | MATERIALS AND METHODS

### 2.1 | Ethical permission

The experiments were conducted at the Institute of Marine Research, Matre, Norway, approved by the Norwegian Animal Research Authority and performed according to prevailing animal welfare regulations under permit number 5283.

### 2.2 | Fish material

Diploid (63,250) and triploid (64,400) eyed eggs (365° days) were acquired from AquaGen (AquaGen AS, Trondheim, Norway) and received at the Matre Research Station on 20 December 2012. The eggs were transferred into hatching trays and incubated at an average temperature of 5.5 ± 0.3°C (mean ± S.D.). Peak hatching was on 10 and 20 January for triploids and diploids, respectively.

On 5 March, 6000 diploid and 6000 triploid *S. salar* fry were distributed into 24 covered fibreglass tanks (1.5 × 1.5 × 0.50 m) resulting in 12 tanks per ploidy and 500 fry per ploidy per tank. The fish were reared under continuous light and the water temperature was maintained at approximately 12°C (11.9 ± 0.6°C, mean ± S.D.) from stocking to termination of the experiment. With the commencement of first feeding on 6 May a commercial starter diet of 0.5 mm (15.0 g $kg^{-1}$ total P; Skretting, Nutra XP, Stavanger, Norway) was supplied throughout the day (24 h) and continued with 0.7 (15.0 g $kg^{-1}$ total P) and 1.0 mm (15.0 g $kg^{-1}$ total P) according to fish size.

### 2.3 | Experimental diet

Two experimental diets, differing in the level of total P (LP 10.5 ± 0.1% and NP 17.5 ± 0.1%) were formulated by Skretting AS (Stavanger, Norway) in 1.2, 1.5 and 2.0 mm pellet sizes (Table 1).

### 2.4 | Experimental design

The fish were switched to the experimental diets on 15 May (day 1) when the pellet size was 1.2 mm and the fish ~3 g in body weight. Eighteen tanks ($n$ = 9 per ploidy) were fed the NP diet, whereas six tanks ($n$ = 3 per ploidy) received the LP diet. At the second (day 38, ~10 g in body weight) and third (day 77, ~30 g in body weight)

**TABLE 1** Diet composition for the normal (NP) and high (HP) phosphorus diets in 1.2, 1.5 and 2.0 mm pellet size

| | HP 1.2 | HP 1.5 | HP 2.0 | NP 1.2 | NP 1.5 | NP 2.0 |
|---|---|---|---|---|---|---|
| *Ingredient (%)* | | | | | | |
| Wheat | 12.0 | 12.0 | 12.0 | 13.6 | 15.3 | 12.0 |
| Wheat gluten | 6.0 | 6.0 | 6.0 | 6.0 | 6.0 | 6.0 |
| Fishmeal | 56.6 | 50.5 | 50.0 | 51.0 | 49.7 | 47.2 |
| North American fishmeal (%) | 53.9 | 70.8 | 70.8 | 77.8 | 76.0 | 92.0 |
| South American fishmeal (%) | 46.1 | 29.2 | 29.2 | 22.2 | 24.0 | 8.0 |
| Fish oil | 9.4 | 9.7 | 10.5 | 9.5 | 9.6 | 10.5 |
| Soya protein concentrate | 7.1 | 11.7 | 11.0 | 15.0 | 15.0 | 15.0 |
| Mineral premix | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Vitamin premix | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Rapeseed oil | 4.0 | 4.2 | 4.5 | 4.1 | 4.1 | 4.5 |
| Sunflower meal | – | – | – | – | – | 4.4 |
| Monoammonium phosphate | 1.3 | 1.5 | 1.6 | – | – | – |
| *Chemical composition* | | | | | | |
| Crude protein (%) | 51.1 | 50.1 | 49.3 | 50.8 | 49.7 | 49.3 |
| Crude fat (%) | 21.0 | 21.0 | 22.0 | 21.0 | 21.0 | 22.0 |
| Total phosphorus (g/kg) | 17.7 | 17.4 | 17.4 | 10.8 | 10.7 | 10.5 |
| Ash (%) | 11.3 | 10.8 | 10.7 | 9.0 | 8.8 | 8.8 |
| Water (%) | 5.5 | 5.5 | 5.5 | 5.5 | 5.5 | 5.5 |
| Digestible energy (MJ/kg) | 19.1 | 19.0 | 19.2 | 19.2 | 19.1 | 19.1 |

*Note*. Digestible energy (DE) was calculated as DE = DE protein + DE fat + DE starch and measured using near infrared spectroscopy.

sampling points, six tanks ($n$ = 3 per ploidy) were switched from the NP to the LP diet, leaving six tanks ($n$ = 3 per ploidy) that were fed the NP diet throughout.

Fish length and weight was recorded from anesthetized fish (100 mg $l^{-1}$ Finquel) at the start of the experimental feeding (day 0), and days 38, 77 and 126. On day 77, the biomass in the tanks was reduced, leaving 200 randomly selected fish per tank. To simulate the production of underyearling smolts, the fish went from continuous light to a 12 h light:12 h dark regime on day 79 until the end of the experiment on day 126. At each sampling time, 30 fish per tank ($n$ = 90 per treatment per sampling) were randomly selected and euthanized with an overdose of anaesthesia (200 mg $l^{-1}$ Finquel), measured for fork length (to 1 mm) and weight (to 0.1 g), and frozen at −20°C for ash analysis. On day 126, a further 30 fish per tank were sampled for fork length and weight before being frozen for later radiology.

## 2.5 | Triploid induction and verification

Ploidy was determined based on red blood cell diameter (Benfey *et al*., 1984). On day 126, 120 blood smears of each putative ploidy were taken. After air drying, the blood smears were photographed at a resolution of 4.396 pixels $\mu m^{-1}$ using a Leica DMRE microscope (type 020–525.755, Wetzlar, Germany) at 40× magnification and a Scion camera (CFW-1312C, Frederick, Maryland). Three pictures were taken from each blood smear. From the pictures, 81–555 (average 286) blood cells from each fish were automatically size measured using ImageJ (Schneider *et al*., 2012) and the ObjectJ project 'Elliptical oocytes', as described in Thorsen and Kjesbu (2001). In the diploid group, the major axis blood cell diameter ranged from 15.2 to 17.1 μm, while the triploid group ranged from 18.2 to 20.9 μm. Hence, as there was no overlap in the red blood cell diameter between the groups, the induction of triploidy was considered 100% successful.

## 2.6 | Vertebra ash content

The whole vertebral column was carefully dissected, cleaned and evaluated for ash content for individual fish after residual neural and haemal arches and ribs were removed. The remaining vertebra bodies were 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) at 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)$^{-1}$.

## 2.7 | Radiology

Frozen whole fish were thawed and subsequently radiographed (40 kV, 10 mAs, 70 cm) using a portable X-ray apparatus Porta

100 HF (Eickemeyer Medizintechnik für Tierärzte KG, Tuttlingen, Germany) onto an image plate (10 × 6 cm, 16 × 48 cm) with a basic spatial resolution of 40 μm (Dürr Medical, Bietigheim-Bissingen, Germany). A CR 35 VET (Dürr Medical) was used to scan the image plate and regenerate the digital image. Vet-Exam Plus Software 4.14.0 was used to convert the image into a digital TIF file and adjust picture properties (brightness, contrast) when necessary. Deformity assessment was done according to the criteria in Witten *et al*. (2009).

### 2.8 | Statistical analyses

The data were transferred to R version 2.15.3 (R Development Core Team, http://www.r-project.org). Significance was assigned at $P < 0.05$. Specific growth rate (SGR) was calculated on a per group basis using the formula $(e^q - 1) \times 100$ (Houde & Scheckter, 1981), where $q = [\ln(W_2) - \ln(W_1)](t_2 - t_1)^{-1}$ (Bagenal & Tesch, 1978), and $W_2$ and $W_1$ are average body mass at times $t_1$ and $t_2$, respectively.

Data were checked for normality following visual examination of plots (*i.e*., histograms and/or q-q plots). Linear mixed effect models (LME) models were used for body mass, body length, body condition, vertebral ash content and the number of deformed vertebra/deformed fish within each time point separately. Ploidy (diploid *vs*. triploid) and diet plan (LP, NP-LP day 38, NP-LP day 77, and NP) were included as categorical independent variables, and tank was included as a random effect. The prevalence of fish with ≥1 deformed vertebra, SGR and mortality data were analysed using general linear models (GLMs) with tank averages and a GLM was also used to assess ash content on day 0 as only one fish per tank was sampled. For models in which more than one explanatory variable was investigated, all possible two-way interactions were allowed. The 'nlme' library within R was used for LME models and the 'Anova' command within the 'car' library was used to extract the results for the main effects. The 'lsmeans' command within the 'emmeans' library was used as a *post hoc* test to compare groups against one another while adjusting for the means of other factors within the model (Lenth, 2016). 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 R script (Sambraus *et al*.) used to analyse the data can be found in the Supporting Information Appendix S1.

## 3 | RESULTS

### 3.1 | Mortality

There was no ploidy effect on day 0 (pre-experiment mortality) and no ploidy, diet or ploidy × diet effects on days 38 or 128. On day 77 there was a ploidy × diet interaction (GLM, $\chi 2 = 6.8$, d.f. = 2, $P = 0.033$) as triploids had higher mean moralities than diploids when fed the NP diet (0.2 *vs*. 0.6% in diploids and triploids, respectively; least square mean (LSM), $P = 0.018$) and the NP-LP day 38 (0.2 *vs*. 1.2% in diploids and triploids, respectively; LSM, $P < 0.001$) diets.

### 3.2 | Body size and growth metrics

Body mass, fork length and body condition over time can be seen in Figure 1a–c and model output can be seen in Table 2. At the initiation of the study, diploids were significantly heavier and longer, with a higher condition (*K*) factor than triploids. Thereafter, triploids had a

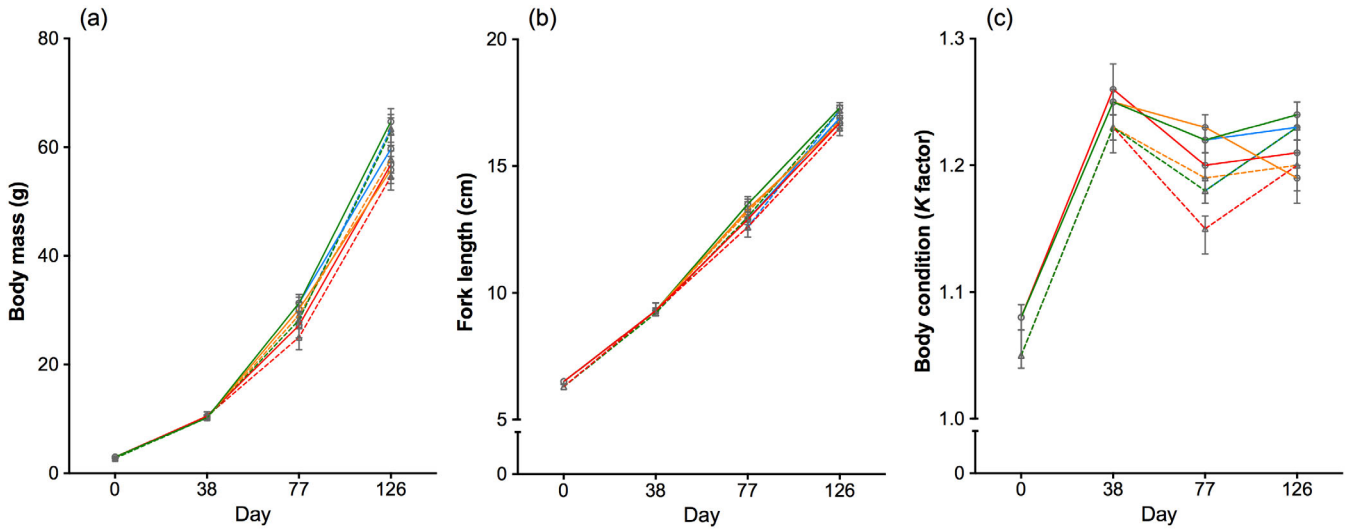


**FIGURE 1** Body size metrics in diploid and triploid Atlantic salmon *Salmo salar* fed either a low (LP) or normal (NP) phosphorus diet. Fish were fed either the LP or NP diets throughout or switched from the NP diet to the LP diet on day 38 (NP-LP day 38) or day 77 (NP-LP day 77). The full model output can be found in Table 2. Data are means ±95% C.I. *N* = 12 per ploidy on day 0 and 3–9 per group on all other days. (—○—) diploid LP; (—○—) diploid NP-LP day 38; (—○—) diploid NP-LP day 77; (—○—) diploid NP; (--△--) triploid LP; (--△--) triploid NP-LP day 38; (--△--) triploid NP-LP day 77; (--△--) triploid NP

**TABLE 2** Statistical output for LME models looking at body size metrics, SGR and vertebral ash content

| | | Body mass | | | Body length | | | Body condition | | | Vertebral ash content | | | SGR | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Day | Variable | $\chi^2$ | d.f. | *P* | $\chi^2$ | d.f. | *P* | $\chi^2$ | d.f. | *P* | $\chi^2$ | d.f. | *P* | $\chi^2$ | d.f. | *P* |
| 0 | Ploidy | 20.0 | 1 | <0.001*** | 17.1 | 1 | <0.001*** | 12.6 | 1 | <0.001*** | 0.09 | 1 | 0.766 | – | – | – |
| 38 | Intercept | 2347 | 1 | <0.001*** | 21599 | 1 | <0.001*** | 58285 | 1 | <0.001*** | 52919 | 1 | <0.001*** | – | – | – |
| | Ploidy | <0.01 | 1 | 0.993 | 0.08 | 1 | 0.777 | 4.11 | 1 | 0.043* | 11.0 | 1 | <0.001*** | 10.7 | 1 | 0.001** |
| | Diet | 0.45 | 1 | 0.504 | 0.40 | 1 | 0.527 | 0.93 | 1 | 0.334 | 48.8 | 1 | <0.001*** | 0.2 | 1 | 0.686 |
| | Ploidy × diet | <0.01 | 1 | 0.999 | 0.02 | 1 | 0.902 | 1.10 | 1 | 0.294 | 4.3 | 1 | 0.039* | 1.9 | 1 | 0.178 |
| 77 | Intercept | 1685 | 1 | <0.001*** | 11651 | 1 | <0.001*** | 53706 | 1 | <0.001*** | 56904 | 1 | <0.001*** | – | – | – |
| | Ploidy | 8.4 | 1 | 0.004** | 8.5 | 1 | 0.004** | 26.9 | 1 | <0.001*** | 1.5 | 1 | 0.217 | 7.7 | 1 | 0.006** |
| | Diet | 9.5 | 2 | 0.009** | 9.2 | 2 | 0.010** | 7.4 | 2 | 0.025* | 80.4 | 2 | <0.001*** | 19.1 | 2 | <0.001*** |
| | Ploidy × diet | 1.2 | 2 | 0.541 | 2.0 | 2 | 0.375 | 1.1 | 2 | 0.579 | 17.1 | 2 | <0.001*** | 4.1 | 2 | 0.131 |
| 126 | Intercept | 2939 | 1 | <0.001*** | 22996 | 1 | <0.001*** | 34717 | 1 | <0.001 | 10173 | 1 | <0.001*** | – | – | – |
| | Ploidy | 1.2 | 1 | 0.280 | 0.6 | 1 | 0.457 | 0.9 | 1 | 0.355 | 1.0 | 1 | 0.315 | 11.2 | 1 | 0.001*** |
| | Diet | 33.3 | 3 | <0.001*** | 18.8 | 3 | <0.001*** | 35.9 | 3 | <0.001*** | 12.6 | 3 | 0.006** | 13.4 | 3 | 0.004** |
| | Ploidy × diet | 8.9 | 3 | 0.030* | 6.2 | 3 | 0.103 | 3.3 | 3 | 0.343 | 5.8 | 3 | 0.119 | 5.1 | 3 | 0.162 |

*($P \leq 0.05$).
**($P \leq 0.01$).
***($P \leq 0.001$).

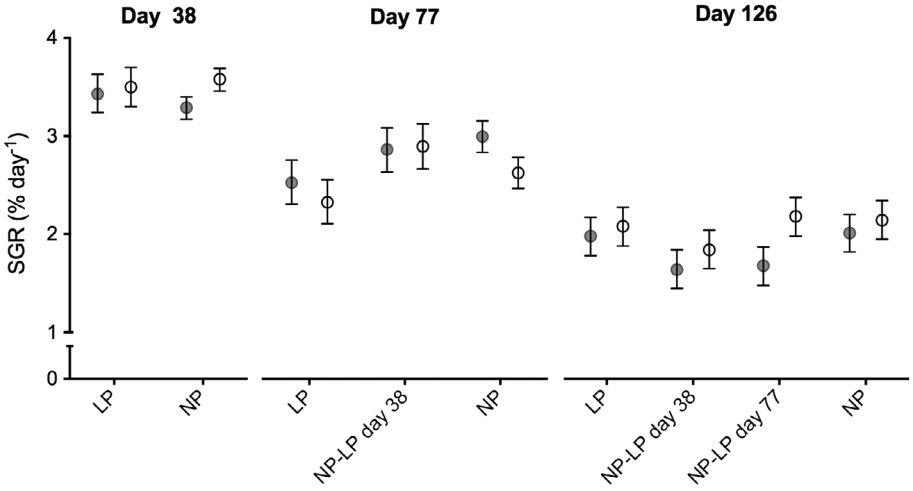


**FIGURE 2** Specific growth rate (SGR) in diploid and triploid Atlantic salmon *Salmo salar* fed either a low (LP) or normal (NP) phosphorus diet. Fish were fed either the LP or NP diets throughout or switched from the NP diet to the LP diet on day 38 (NP-LP day 38) or day 77 (NP-LP day 77). The full model output can be found in Table 2. Data are means ±95% C.I. *N* = 3–9 per group for each time point. (●) diploid; (○) triploid

significantly higher SGR between days 0 and 38, a significantly lower SGR between days 38 and 77 and a significantly higher SGR between days 77 and126, irrespective of diet (Figure 2 and Table 2). As such, there was no ploidy effect on body mass on day 38, but triploids were again significantly smaller than diploids on day 77, irrespective of diet. On day 126, there was a ploidy × diet interaction as triploids were significantly heavier than diploids when fed the NP-LP day 77 diet (LSM, d.f. = 16, $t = -2.2$, $P = 0.042$), but there was no ploidy effect in any other diet. For body condition, triploids had a significantly lower *K* factor on days 38 and 77 compared to diploids, irrespective of diet. For dietary effects irrespective of ploidy, the LP diet had a significantly lower SGR than all other diets on day 77 (LSM, <0.001; see Supporting Information Appendix S1, lsmeans M26 for all group comparisons), and on day 126 the NP-LP day 38 diet had a significantly lower SGR than the LP and NP diets (LSM, $P < 0.05$; see Supporting Information Appendix S1, lsmeans M27 for all group comparisons). As such, there was a negative association between the amount of time fed the LP diet on body mass and *K* factor, and by day 126 those fed the LP diet and the NP-LP day 38 diet were significantly smaller with lower *K* factors than those fed the NP or NP-LP day 77 diets, irrespective of ploidy (LSM, $P < 0.01$; see Supporting Information Appendix S1, lsmeans M4 and M12 for all group comparisons).

## 3.3 | Vertebra ash content

At the initiation and the end of the study (day 126), there were no ploidy effects on vertebral ash content, but on days 38 and 77 there were ploidy × diet interactions (Figure 3 and Table 2). On day 38, although triploids had a lower vertebral ash content in both diets, the ploidy difference was 5% in the LP diet compared to 2% in the NP diet. On day 77, triploid vertebral ash content was 5% lower than in diploids when fed the LP

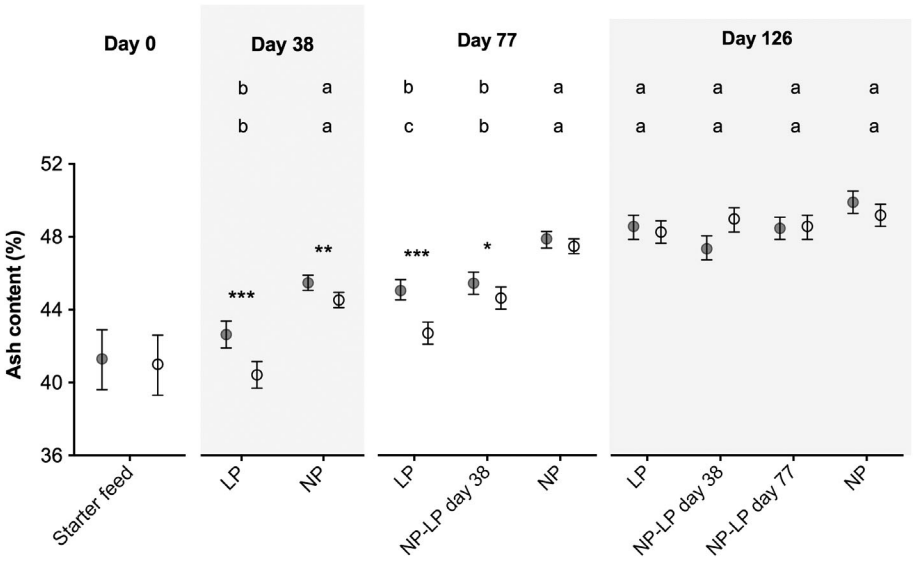


**FIGURE 3** Mineral content in the vertebral column of diploid and triploid Atlantic salmon *Salmo salar* fed either a low (LP) or normal (NP) phosphorus diet during early development. Fish were fed either the LP or NP diets throughout or switched from the NP diet to the LP diet on day 38 (NP-LP 10 g) or day 77 (NP-LP 30 g). The full model output can be found in Table 2. An asterisk indicates a significant ploidy effect within a given timepoint and diet (LSM *post hoc*, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.01$). Different lowercase letters indicate a significant dietary effect within a time point for each ploidy (LSM, $P < 0.05$). Data are means ±95% C.I. $N = 12$ per ploidy on day 0 and 3–9 per group on all other days. (●) diploid; (○) triploid

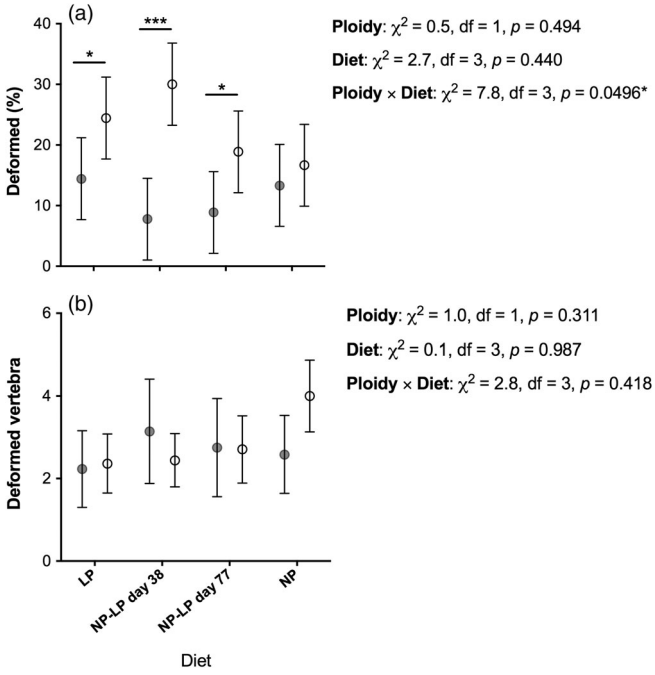


**FIGURE 4** Radiologically detectable deformities in diploid and triploid Atlantic salmon *Salmo salar* fed either a low (LP) or normal (NP) phosphorus diet. Fish were fed either the LP or NP diets throughout or switched from the NP diet to the LP diet on day 38 (NP-LP day 38) or day 77 (NP-LP day 77). The statistics are from (a) a general linear model and (b) an LME model. Data are means ±95% C.I. $N = 3$ per group. (●) diploid; (○) triploid

diet, 2% lower than diploids when fed the NP-LP day 38 diet, but there was no ploidy effect in the NP diet. Dietary effects independent of ploidy were evident on days 38 and 77, with a positive association between the length of time fed the NP diet and vertebral ash content (Table 2). On day 126 there was a main dietary effect, but multiple comparisons between groups revealed no significant differences between dietary groups (LSM, $P > 0.05$; see Supporting Information Appendix S1, lsmeans M16 for all group comparisons).

In a model comparing vertebral ash content over time in the LP and NP diets only for each ploidy, day 38 had significantly lower values than day 77 (LSM, $P < 0.001$) and day 77 had significantly lower values than day 126 (LSM, $P < 0.001$) in all diets (see Supporting Information Appendix S1, lsmeans M33 for all group comparisons).

### 3.4 | Radiology

Triploids had significantly higher prevalences of fish with ≥1 deformed vertebra in all diets except the NP diet (Figure 4a), but there was no ploidy effect on the number of deformed vertebra per deformed fish (Figure 4b). In both ploidy, deformities were located primarily in the vertebra below the dorsal fin (Figure 5a,b) and were mainly related to compression and fusion of vertebra or increased radiodensity (Figure 5c,d).

## 4 | DISCUSSION

Our primary aim was to determine whether triploid *S. salar* required elevated dietary P throughout the freshwater life stage in order to maintain bone ash content at the same level as diploids. We found triploids had lower vertebra ash content during the earlier stages, but not the later stages of development, irrespective of diet. However, triploids recovered to diploid levels earlier in development when fed the NP diet. Therefore, triploid *S. salar* may require more dietary P from days 0 to 77 (3–30 g in body size) in order to maintain a bone ash content equal to diploids. Our secondary aim was to assess vertebral ash content over time with respect to diet. Here, both ploidy were found to require more dietary P during the early compared to the later stages of the freshwater phase in order to achieve higher bone ash content.

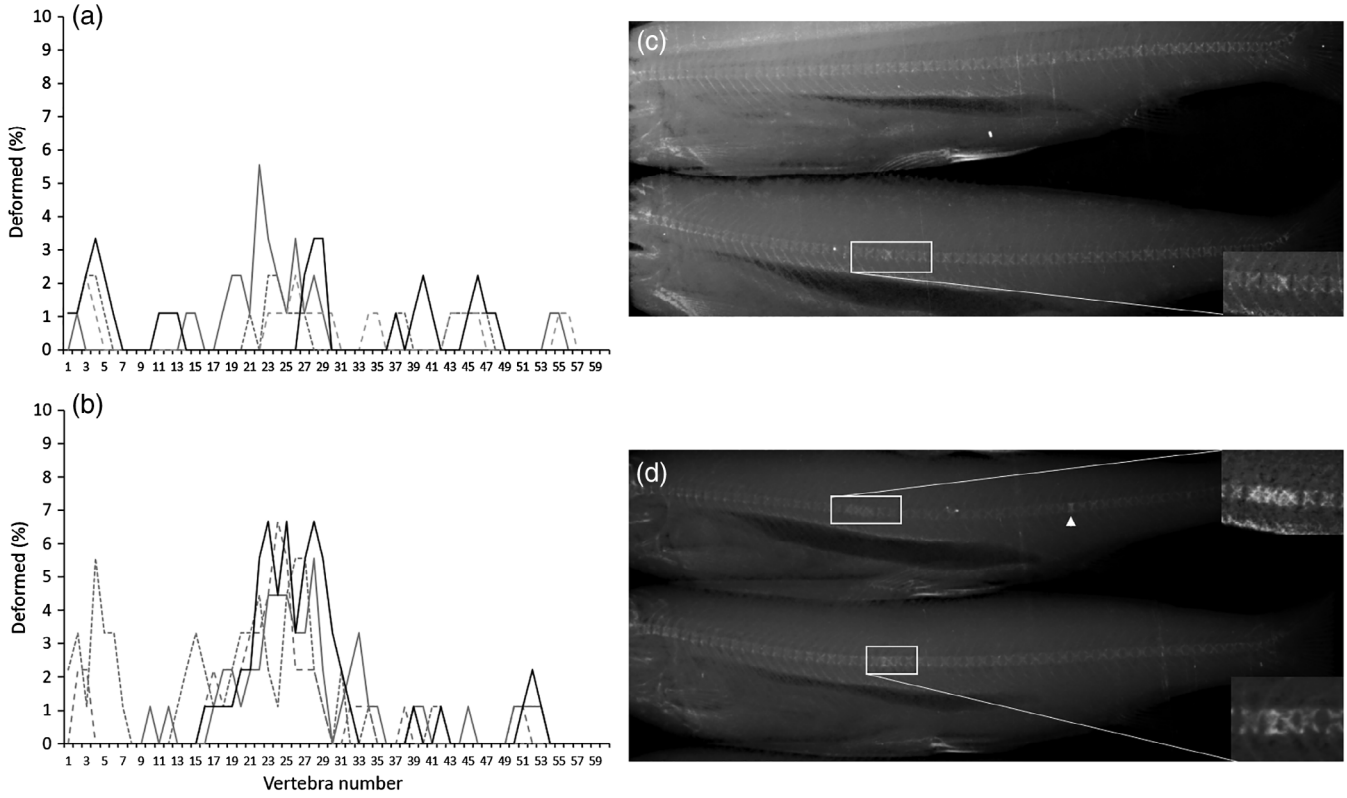


**FIGURE 5** Deformity location and radiographs of diploid and triploid Atlantic salmon *Salmo salar* fed either a low (LP) or normal (NP) phosphorus diet. Fish were fed either the LP or NP diets throughout or switched from the NP diet to the LP diet on day 38 (NP-LP day 38) or day 77 (NP-LP day 77). The prevalence of deformed vertebra in (a) diploid and (b) triploid *S. salar* ($N$ = 90 fish per group). Lateral radiographs of (c) diploid and (d) triploid *S. salar* from the LP groups. Observed vertebral deformities included compression and fusion (enhanced areas of the vertebral column found within the white boxes), as well as hyper-radiodense vertebra (white arrowhead). (——) LP; (– – –) NP-LP day 38; (– – –) NP-LP day 77; (——) NP

### 4.1 | Triploids require higher dietary P from early feeding to maintain vertebral ash content

We found triploids could have lower vertebral ash content compared to diploids, but this was time and diet related. For example, the ploidy effect was only apparent on days 38 and 77. In addition, the triploids recovered to diploid values by day 77 when fed continuous NP, but still showed lower values at this time when fed either continuous LP or when switched to the LP diet on day 38. The lack of a ploidy effect on day 126, irrespective of diet, indicates that triploids may only need additional dietary P up to day 77 in order to show similar vertebral ash levels over time as in diploids. Therefore, there may be a critical window with regard to vertebral ash content that was not identified in previous studies that only assessed larger fish at the end of the freshwater life stage (*e.g.* Burke *et al.*, 2010; Fjelldal *et al.*, 2016; Smedley *et al.*, 2018).

### 4.2 | Ploidy effect evident in NP diet

It was notable that when fed the NP diet triploids still had a lower vertebral ash content than diploids on day 38. Initially, this was surprising, as in Fjelldal *et al.* (2016) a diet of 16.3 g $kg^{-1}$ total P resulted in equal vertebral ash content between ploidies in fish of 55–60 g body size, and in Smedley *et al.* (2018) diets of 13, 17 or 20 g $kg^{-1}$ total P also led to no ploidy differences in 45 g smolts. Here, although factors that will influence dietary P requirements, such as P digestibility, feed conversion ratio or feed intake, were not measured and as such cannot be compared across studies, it is noticeable that neither of these latter studies assessed ploidy effects in smaller fish, and we found our current ploidy effect relatively early in development when fish were around 10–30 g in body size.

The early ploidy effect in the NP diet was closely associated with growth differences, as triploids had a much higher SGR between days 0 and 38 compared to diploids when fed the NP diet, and high growth is associated with increased P requirements in *S. salar* (Shearer, 1995). At later life stages, NP fed triploids either grew more slowly or at the same rate as diploids, and this was associated with similar vertebral ash content between triploids and diploids. Similarly, on day 0, triploids were smaller than diploids and this reduced growth may explain the lack of a ploidy effect on vertebral ash content prior to being fed the experimental diets. However, further work with higher dietary P diets is required to determine whether the dietary P requirement is higher

or whether triploids simply have a lower maximum vertebral ash content during the early life stages.

### 4.3 | Ploidy interaction with diet on vertebra ash content

The vertebral ash content increased in both ploidy throughout the study, with fish fed the NP diet during the corresponding time period having higher ash content than those fed the LP diet. However, in all groups the ash content at the end of the experiment was comparable to levels found in wild *S. salar* smolts (*i.e.*, around 46–50%, Fjelldal *et al.*, 2006). In both ploidy, the switching from NP to LP on day 38 resulted in a plateauing ash content until day 77. This suggests that the bone that was deposited between days 38 and 77 had an equal ash content to the bone before day 38. In contrast, the fish that continued with the NP diet had an increasing ash content between days 38 and 77. Hence, for these fish the bone that was deposited between days 38 and 77 had a higher ash content than the bone deposited before day 38. With the LP to NP diet shift on day 38, diploids had a reduced ash content on day 126 when compared to continuous NP, while triploids did not. Thus, in the later part of the experiment diploids tended to require more dietary P than triploids in order to support maximum vertebral ash content. Why triploids were able to utilize P better in the later feeding periods and increase their bone ash content more than diploids is unknown. This could be related to P digestibility, but no ploidy effect has been observed on this parameter in *S. salar* smolts (Burke *et al.*, 2010; Peruzzi *et al.*, 2018). Similarly, no effect of ploidy on feed intake was observed at the temperature used in the current study (12°C) in post-smolts (Sambraus *et al.*, 2017) or harvest size fish (Sambraus *et al.*, 2018). However, feed conversion ratio has been found to be lower in triploids (Fraser *et al.*, 2013; Smedley *et al.*, 2018), but not always (Burke *et al.*, 2010; Hansen *et al.*, 2015; Oppedal *et al.*, 2003), therefore further work in this area is required.

### 4.4 | Ploidy effect on vertebra deformities

Many studies have shown that triploids develop more deformed vertebrae, as assessed by radiology, than diploids (*e.g.*, Fjelldal & Hansen, 2010). This ploidy effect is generally already observed in freshwater and is expected to be related to low vertebral mineralization (Fjelldal *et al.*, 2016; Smedley *et al.*, 2018). We observed more fish with ≥1 deformed vertebra in triploids compared to diploids fed all diets except continuous NP. However, there was no ploidy effect on the number of deformed vertebra per deformed fish. Although the deformities observed were relatively minor in the current study, based on the premise that effects on growth have been observed in fish with ≥5 deformed vertebra (Hansen *et al.*, 2010), deformities that occur in freshwater may develop chronically and affect other vertebra during later life stages (Grini *et al.*, 2011). Furthermore, negative long-term effects of suboptimal P nutrition in freshwater on bone health during seawater grow-out have been observed in both diploid (Fjelldal, Hansen, & Albrektsen, 2012; Fraser *et al.*, 2019) and triploid *S. salar* (Fjelldal *et al.*, 2016). These pathologies surfaced as vertebra compressions in the tail region of the vertebral column at harvest size, the most problematic deformity in farmed *S. salar* (Witten *et al.*, 2005). Triploid deformities peaked in those vertebra found under the dorsal fin, which is a common observation in triploids (Fjelldal & Hansen, 2010; Fraser *et al.*, 2015; Peruzzi *et al.*, 2018).

### 4.5 | Ploidy effects on growth and condition

Triploids were smaller at the start of the experiment but equal or larger than diploids on day 126. It is not uncommon for triploids to show slower development at early life stages, but then equal or improved growth rates come the end of the freshwater life stage (*e.g.*, Taylor *et al.*, 2011). Similarly, many studies have reported ploidy effects on body condition, with triploids being fatter, thinner or no different to diploids (*e.g.*, Sambraus *et al.*, 2018). To date, these inconsistent ploidy effects on growth and body condition remain unexplained, but likely reflect differences in rearing conditions, such as water temperature, for which triploid *S. salar* have different optima (Fraser *et al.*, 2015; Sambraus *et al.*, 2017, 2018). However, triploids can regularly match or surpass diploids for growth up until smoltification (*e.g.*, Fjelldal & Hansen, 2010; Taylor *et al.*, 2011).

### 4.6 | Increase in vertebral ash content over time with the NP diet

We observed a gradual increase in vertebra ash content over time. This reflects that newly laid down bone develops a higher ash content than the total mass of older and inner bone. Moreover, mineralization of *S. salar* vertebra may resume deep inside the bone (Witten *et al.*, 2019). However, that is a more likely path of mineralization when previously laid down bone is undermineralized after a period of phosphorous deficiency (Witten *et al.*, 2019). During vertebra growth, new bone (osteoid) is deposited mainly at the cranial and caudal rims of the amphicoelous and at the distal ridges of the trabeculae (Nordvik *et al.*, 2005). Whether the observed increase in ash content over time with the NP diet is restricted to the amphicoelous core bone or the bony trabeculae, or is uniform in both compartments, is unknown and requires further investigation. Whether or not the increased ash content is associated with a change in ash composition, such as apatite type and calcium content (Hamada *et al.*, 1995), is also unknown. Furthermore, the present analysis was based on all vertebrae pooled per individual and regional differences in mineralization may exist (Solstorm *et al.*, 2016). Why the normal osteological ontogeny in freshwater is reflected by a positive development in ash content, and when this elevation eventually plateaus, is unknown, as is the reason for this change with age and size. Clearly, an increase in ash content comes with the cost of developing more negative buoyancy, and teleosts without a swim bladder have a lower bone mineral

content than those with a swim bladder (Casadevall *et al*., 1990). Hence, there must be a strong driver in the functional anatomy of the vertebral column in *S. salar* that allows for the ontological change in mineralization rate. Increased mineral content will increase the mechanical strength of the vertebra (Fjelldal *et al*., 2006) and one may expect the chronic mechanical stress on the vertebrae caused by muscular contractions during swimming to be different in bottom-dwelling parr and constantly swimming pelagic post-smolts. Therefore, the current observation of increased ash content of vertebra in freshwater may be a pre-adaption for a life in seawater. Similarly, the morphology of the vertebral column changes in *S. salar* during smoltification and this has been suggested to reflect a pre-adaption to changes in swimming mode when entering seawater (Fjelldal *et al*., 2006). Whether wild Atlantic salmon show an equal increase in vertebra bone ash content in freshwater remains to be assessed.

### 4.7 | Does the dietary P history impact on P demand for bone mineralization?

Looking at diploids only, one may conclude that the phosphorus nutrition history impacts on vertebra ash content. Between days 0 and 38, diploids on LP developed lower ash content, but on day 77 diploids fed on the NP-LP day 38 regime developed equal ash content to those on continuous LP by day 77. Hence, the new bone deposited between days 38 and 77 must have had a more effective mineralization in the LP than the NP-LP day 38 fish. Therefore, diploids with different P feeding histories showed different degrees of mineralization between two sampling points even though they were on the same diet between the sampling points. One possible explanation for this is that prolonged feeding with LP stimulated a reduction in the individual's P demand. This may reflect a compensatory mechanism, where domesticated *S. salar* under prolonged suboptimal P nutrition upregulate their capacity to mineralize newly formed bone. Thus, the individual's P history may impact on its P demand, and suboptimal P nutrition may be especially demanding for fish that have received adequate dietary P earlier on and would make for an interesting study. Previous work in rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) has also found the dietary P history of the individual can influence its nutritional P requirement, with P-deficient fish retaining a higher percentage of available P compared to previously starved and sufficient P fed fish (Sugiura *et al*., 2010).

#### ACKNOWLEDGEMENTS

We thank the technical staff, in particular Lise Dyrhovden, at the Matre Research Station for providing fish husbandry.

#### AUTHOR CONTRIBUTIONS

Study ideas and funding are attributed to P.G.F. and T.H. Data generation was done by F.S., P.G.F., T.H., B.S.D., A.T. and R.S. Data analysis was done by T.W.K.F., F.S. and P.G.F. All authors contributed to manuscript preparation.

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**How to cite this article:** Sambraus F, Hansen T, Daae BS, et al. Triploid Atlantic salmon *Salmo salar* have a higher dietary phosphorus requirement for bone mineralization during early development. *J Fish Biol*. 2020;97:137–147. https://doi.org/10.1111/jfb.14338