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Impact of dietary phosphorous in diploid and triploid Atlantic salmon (*Salmo salar* L.) with reference to early skeletal development in freshwater

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ABSTRACT

In order to assess the effect of dietary phosphorus (P) in reducing vertebral malformations and improving freshwater (FW) performance in triploid Atlantic salmon (*Salmo salar*), both triploid and diploid Atlantic salmon were fed three different dietary P inclusion levels (low: 4.9, medium: 7.7, and high: 9.7 g available P kg^{-1}) from first feeding until smolt. Somatic and skeletal response was assessed at fry (~0.5 g), parr (~5 g) and smolt (~45 g) stages. Triploid parr initially grew faster on the high P diet, while groups fed low P resulted in a significantly higher weight at smolt. Image analysis of double stained Alcian blue and Alizarin red S fry revealed that low P fed triploid fish presented less well mineralised vertebrae, and significantly more malformed vertebrae in both parr and smolt stages following x-ray radiographic assessment. Triploid parr fed high and medium P had similar numbers of malformed vertebrae relative to their diploid counterparts but greater numbers than at smolt. Low P fed triploids had the highest prevalence of jaw and vertebral malformations as well as the highest number of deformed vertebrae in the central caudal vertebral region, which was more pronounced at parr than at smolt. Shorter vertebrae dorso-ventral lengths were observed throughout the spinal column (R1–R4) in parr fed low P and only in the caudal region (R3) at smolt. In parr, both ploidies showed reduced phosphate homeostasis protein *fgf23 gene* expression in vertebrae when fed low P diets, while triploids showed greater down-regulation of osteogenic factors (*alp*, *opn* and *igf1r*) between diets relative to diploids, suggesting possible greater active suppression of mineralisation and reduced osteogenic potential in triploids. No effects of diet or ploidy on gene expression were evident at smolt. Comparisons between development stages suggest early P supplementation in triploids is crucial for skeletal development. Ultimately, reducing vertebral deformities observed at smolt with higher P supplementation in triploids could contribute towards improving skeletal performance and welfare of the stocks in the marine phase.

1. Introduction

Recent years have seen increased commercial interest in the use of artificially induced triploid Atlantic salmon (*Salmo salar*). Artificial triploid induction, assuming use of all female stocks, in Atlantic salmon has been proposed to eliminate adverse impacts of farmed escapees breeding with wild populations (Glover et al., 2013), improve potential for greater harvest weights (Fjelldal et al., 2015; Smedley et al., 2016) and have been suggested to widen windows of seawater transfer (Taylor et al., 2012). Awareness of suboptimal culture conditions relative to diploids including mixed ploidy rearing (Taylor et al., 2014) and high environmental temperature (Atkins and Benfey, 2008) combined with low oxygen levels (Hansen et al., 2015; Sambraus et al., 2017) have improved production prospects. However, recurrence of vertebral and jaw malformations have hindered wider commercial adoption due to concerns over welfare (Fraser et al., 2012a). Aetiology of skeletal deformities in farmed triploids are largely associated with higher suboptimal egg incubation temperature (Fraser et al., 2014c), accelerated growth (Leclercq et al., 2011; Taylor et al., 2012; Taylor et al., 2014) in association with dietary deficiencies (Fjelldal et al., 2015). In particular, high prevalence of skeletal deformities that are typically observed in triploid Atlantic salmon at harvest, have been shown to be associated with vertebral deformities already present at sea transfer (Fjelldal and Hansen, 2010; Fjelldal et al., 2015; Smedley et al., 2016). Hence, environmental and particularly nutritional requirements in freshwater rearing of triploid Atlantic salmon must be addressed.

Repeated observation of lower condition factors in triploid Atlantic salmon (Fjelldal and Hansen, 2010; Taylor et al., 2011; Taylor et al., 2012) may suggest a lower total body lipid content (Herbinger and Friarrs, 1991). Alternatively this could suggest an increased deposition

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of skeletal components over muscle, which alongside increased skeletal malformation prevalence, illustrate the potential for higher mineral requirement in triploid bone formation. Dietary phosphorous (P) deficiency is a primary nutritional risk factor for skeletal development in fish (Lall and Lewis-McCrea, 2007) including Atlantic salmon (Asgard and Shearer, 1997; Fjelldal et al., 2012a) and is also an essential structural component of nucleic acids, ATP and cell membrane phospholipids. Alongside calcium (Ca) it is the main mineral component of bones, teeth and scales and, unlike calcium, must be met by diet in fish (Lall, 2003). The minimum recommended requirement for P in salmonids is 8 g total P kg^{-1} (NRC, 2011). Long term effects of impeded growth, poor vertebral mineralisation and increased prevalence of vertebral malformations have been observed in diploid Atlantic salmon juveniles fed levels below these recommendations (Fjelldal et al., 2009; Fjelldal et al., 2012a). Skeletal malformations observed in diploid Atlantic salmon at harvest are shown to originate from nutritional P deficiency in freshwater (Fjelldal et al., 2012a), which has also been implicated in triploid Atlantic salmon (Smedley et al., 2016). Fjelldal et al. (2015) clearly showed fewer vertebral and jaw malformations at seawater transfer and harvest in triploid Atlantic salmon fed higher P inclusion in freshwater (9.4 vs. 7.1 g total P kg^{-1}). Results suggest freshwater developmental stages in triploids require higher P inclusion for correct skeletal development.

One of the factors that may influence the observed differences in skeletal development and associated nutritional requirement between triploids and diploids is growth rate. Vertebral mineralisation can be compromised during periods of accelerated growth (Hansen et al., 2010). Elevated rearing temperatures for Atlantic salmon juveniles result in faster growth accompanied by poor vertebral mineralisation through disrupted bone and cartilage formation (Ytteborg et al., 2010). Instances of faster growth alongside higher incidence of vertebral deformities in triploid Atlantic salmon have been reported (Leclercq et al., 2011; Taylor et al., 2012) and may indicate dietary requirements could be greater for triploids than their diploid counterparts. Higher P and protein diets have been shown to stabilise severity of malformations and sustain faster growth in seawater for triploid Atlantic salmon (Smedley et al., 2016). Faster growth in diploids coincide with increased bone density and expression of *igf-I receptor* in bone (Wargelius et al., 2005), where local expression may be associated with initiating extracellular matrix (ECM) production. Hence, accelerated growth factors anticipated in triploid freshwater growth may impact regulation of mineralisation of the ECM. Environmental factors that induce accelerated growth in diploid Atlantic salmon such as high temperature, have also led to vertebral fusions and associated upregulation of *matrix metalloproteinase 13* (*MMP-13*) (Wargelius et al., 2010) and downregulation of osteogenic marker *collagen typ Iα1* (*Col1a1*) (Ytteborg et al., 2010). MMP-13 is a matrix metalloproteinase which is involved in the degradation of the extracellular matrix (ECM) associated with chronic inflammatory responses characterised by bone remodelling and leading to deformity. Collectively, given the known differences in growth performance between diploids and triploids these osteogenic biomarkers may be useful in elucidating mechanisms for triploid associated skeletal deformity and the role of dietary P in homeostatic mechanisms of bone growth.

Hydroxyapatite, the key mineral component of bone, synthesis is dependent on phosphate (PO_4^{3-}) and calcium (Ca^{2+}) homeostasis which is systemically regulated primarily through circulating parathyroid hormone (PTH), 1,25-dihydroxyvitamin D [1,25(OH)2D] and fibroblast growth factor 23 (FGF23; Martin et al., 2012). These factors regulate PO_4^{3-} intestinal absorption, remodelling in bone and excretion in kidney, through sodium-phosphate cotransporter (Npt2a) activity, according to demand and availability. Increased kidney Npt2a activity is observed under periods of dietary PO_4^{3-} restriction in rainbow trout (Sugiura et al., 2003), however, little research has been conducted on these P-bone homeostasis pathways in salmonids, let alone triploid fish (Fjelldal et al., 2015). In other vertebrates, FGF23 secretions are known to be directly induced through expression of *fgf23* from osteoblasts and osteocytes, the mineralising osteogenic cells in bone, and promote renal PO_4^{3-} resorption with coordination of [1,25(OH)2D] (Martin et al., 2012). In addition, alkaline phosphatase (ALP) and osteopontin (OPN) are both markers for extracellular mineralisation around osteoblasts. ALP provides osteoblasts with PO_4^{3-} by dephosphorylating exogenous β-glycerophosphate (Beck et al., 2000; Pombinho et al., 2004). In turn, the presence of free PO_4^{3-} stimulates OPN secretions to the osteoblast ECM to promote osteogenic function (Beck et al., 2000). As such, ALP and OPN and their corresponding transcription factors, are collectively strong indicators of osteogenic activity in the presence of free PO_4^{3-} and may also be useful markers to determine ploidy differences in response to dietary P.

The present study aims to investigate dietary P supplementation in diploid and triploid Atlantic salmon siblings with an emphasis on impacts at three freshwater life stages: fry, parr and smolt. Growth performance, skeletal malformation, mineral composition and mRNA expression of key bone homeostatic genes were analysed.

2. Materials and methods

2.1. Fish stock

On January 19, 2012, sibling groups of diploid and triploid Atlantic salmon eggs (20 dams & 5 sires, Atlantic QTL-innOva® IPN) were supplied from AquaGen (Norway) to Howietoun Fish Farm, Stirling (56°N, 4°W) at 395 degree-days post-fertilisation (°DPF). Triploidy was induced using a hydrostatic pressure shock of 9500 psi applied 300 °min post-fertilisation for 50 °min at 8 °C (Taylor et al., 2011). From fertilisation to point of shipping ova were incubated in upwelling silos at temp range 3.2–8.0 °C. Prior to shipping, equal numbers per family (1500/family) and ploidy were pooled. Eyed eggs (5000/tank, 250/family) were equally split between 12 × 250 L tanks (6 per ploidy) and reared under constant darkness at 8.7 ± 1.0 °C until first feeding (929 °DPF; March 26, 2012). Fry were transferred at 1387 °DPF (~0.43 g) to the Niall Bromage Freshwater Research Facility (NBFRF), Stirling (56°N, 4°W) where they were maintained in 12 × 980 L covered circular tanks. Fish numbers were periodically reduced by randomly netting and removing 50% of the population (average fish no. per tank: 5000 eyed ova; 2500 @ 0.43 g; 1250 @ 5 g; 625 @ 30 g; 414 at smolt) to maintain stocking density < 30 kg m^{-3}. Fish were reared under continuous light (LL) until August 31, 2013 followed by simulated natural photoperiod and ambient water temperature (Fig. 1) until smoltification (~45 g, 3321 °DPF, April 24, 2013). Smoltification was verified using smolt index scoring (all tanks scoring mean 4.0 on 24th April) according to Sigholt et al. (1995) following 400 degree days of increasing daylength post-winter solstice. All fish were vaccinated with PHARMAQ Alphaject 2.2 on February 26, 2013. Triploid rate was verified through erythrocyte measurements. Red blood smears were prepared from blood collected from the caudal vein using heparinised syringes from fish at 5 g (n = 100/ploidy, 16–17 fish per tank (n = 6/ploidy), representing 1.3% of total population number (7500 at 5 g). After air drying, slides were fixed in 100% methanol and then placed into Giemsa stain for 10 min. Erythrocyte length and diameter were measured at 100× magnification using image capture (ImagePro Software). A total of 30 randomly chosen nuclei per slide were measured to the nearest 0.01 μm. Diploid control groups had significantly smaller nuclear lengths with no overlaps with pressure shock triploid groups (2N: 7.3–7.5 μm; 3N: 9.2–10.1 μm) confirming that all fish that were subjected to hydrostatic pressure shock were likely to be triploids.

2.2. Experimental setup and sampling

Duplicate groups of diploid and triploid fish were fed from first feeding until smoltification, one of three different diets using clockwork belt feeders according to manufacturer's tables in relation to biomass