


Mineral nutrition and bone health in salmonids

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Received 18 September 2017; accepted 15 April 2018.

Abstract

In the recent years, challenges faced in salmonid farming with regard to deformities have helped to improve our knowledge on skeletal biology and development of bone health in salmonids. Different nutritional, genetic and environmental factors are associated with skeletal deformities in salmonids and other fish species. Minerals are a group of essential nutrients having a vital role to play in skeletal development, growth and remodelling. The knowledge generated thus far on the structural and functional importance of minerals in salmonid bone health is largely restricted to phosphorus. A brief account of dietary phosphorus-related bone deformities encountered in salmonids, critical life stages for the development of bone deformities, recent developments in the understanding of their aetiology and dietary phosphorus levels required to improve bone health are presented. The effect of increased dietary level of phosphorus in reducing bone health disorders under conditions of improved feed conversion efficiency and use of triploids in salmon farming are illustrated with help of metadata analysis from literature. With regard to the role of other essential minerals (macro- and micro-) impacting bone health in salmonids, data available are extremely limited and hence information on other fish species and mammals is summarized in relation to bone development and incidence of deformities. The need to improve mineral bioavailability, utilization and reduce effluent mineral load is presented in brief with example from Norwegian salmon farming. Refinements in mineral requirement recommendations for salmonids and advanced methodologies for studying aetiology of skeletal anomalies, bone mineral status, skeletal development and deformities are also discussed.

Key words: Atlantic salmon, bone health, deformities, minerals, rainbow trout, requirement.

Introduction

Salmonids are an important group of farmed fish. The two major species of aquaculture importance are Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). Salmonid aquaculture has witnessed tremendous growth over the past few decades. The progress made in understanding of the nutrition and feeding of salmonids had a vital role to play in the development of the sector. Improving the feed conversion and nutrient retention

efficiencies in farmed salmonids are a few to mention. The rapid development also imposes challenges questioning the long-term sustainability and environmental safety of the farming activity. One of the challenges is the increasing incidence of bone deformities in farmed salmonids with adverse quantitative and qualitative implications. Bone deformities can reduce growth (Gjerde *et al.* 2005; Hansen *et al.* 2010), cause downgrading losses at harvest (Michie 2001) and impair fillet quality (Sullivan *et al.* 2007; Haugravoll *et al.* 2010). Further, there is an increased metabolic

cost in fish with bone deformities (Powell *et al.* 2009). In short, bone health has direct consequence on monetary, environmental and ethical aspects of salmon farming.

It can be generally argued that during the era of rapid development of salmonid farming, more emphasis is given to somatic growth while skeletal growth is compromised. Skeletal structure forms the basic framework for growth of a healthy animal, and dietary minerals are an important group of nutrients essential for proper skeletal development. The skeletal architecture of fish is comprised of an organic bone matrix, and inorganic minerals mostly comprised of collagen and hydroxyapatite (HAP), respectively. HAP is a hydroxylated polymer of calcium phosphate [$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$] embedded in a matrix of type I collagen fibres (Moss 1961). In fish, the bones represent a major and highly conserved reservoir of minerals, especially calcium (Ca), phosphorus (P), zinc (Zn) and manganese (Mn) (Watanabe *et al.* 1997). The concentration of these minerals in the vertebrae is considered to be most relevant indicators of the mineral status and to quantify dietary requirements of the aforesaid minerals in fish (Antony Jesu Prabhu *et al.* 2013, 2016b). Of all the minerals or even nutrients essential to fish, phosphorus is by far the most studied; nevertheless, the effect of dietary phosphorus on bone growth is still not well understood. The effect of dietary phosphorus deficiency or suboptimal supply affecting normal growth or mineralization in fast-growing fish has been well documented in salmonids (Sugiura *et al.* 2004). Even though the role of calcium in ensuring proper mineralization is understood, the significance of sufficient dietary calcium supply in salmonid feeds is still debated. In terms of the role of other minerals in bone health, our knowledge is very limited and requires further understanding of their role in bone formation and remodelling. Moreover, the challenges faced by the aquaculture industry in terms of sustainability of feed resources, novel feed formulations ensuring rapid growth, good health and less environmental impact have made mineral nutrition of salmonids ever more dynamic and vulnerable to changes. Recent compilations by NRC (2011) and Antony Jesu Prabhu *et al.* (2013, 2016b) have provided useful information on the present knowledge and highlighted the need for better understanding the mineral requirement of fish. One of the significance of dietary minerals in fish feeds is to ensure proper bone health. The role of nutrients in skeletal metabolism and physiology in fish has been reviewed by Cahu *et al.* (2003) and Lall and Lewis-McCrea (2007). A brief account on the role and functionality of fatty acids, vitamins and minerals in skeletal metabolism of fish was provided in the aforementioned reviews. In the past decade alone, a large body of literature has been generated in the context of minerals and their importance in fish bone health. The emphasis here was to review the recent developments on the role of

minerals in influencing bone growth, physiology and health with regard to salmonids.

Salmonid bone biology

Type of bone and vertebral development

Fish bones can be classified into two types, cellular (osteocytic) or acellular (anosteocytic) based on the presence or absence of osteocytes, respectively (Weiss & Watabe 1979). Acellular bones are formed by osteoblast cells, instead of osteocytes and are found in Cichlidae family. Cellular bones are found in fish species of the families Salmonidae, Cyprinidae and Clupeidae. In either case, the bones provide support to the structural integrity of the body for normal posture, development and locomotion. It provides a site for muscle attachment, protects vital organs and cells like the brain, and serves as a reservoir of ions (Lall & Lewis-McCrea 2007).

The vertebral column of Atlantic salmon is comprised of 57–60 vertebrae (Kacem *et al.* 1998), which starts to develop during early first feeding (Nordvik *et al.* 2005). The vertebral bodies are roughly cylindrical and consist of a biconoid amphicoelous core of compact bone surrounded by trabecular bone. The core of compact bone is mostly made up of collagen fibres oriented circularly, perpendicular to the cranial–caudal axis, while the trabecular bone has a more woven collagen matrix (Nordvik *et al.* 2005). The vertebrae are separated by a notochord-derived intervertebral tissue, which is running continuous throughout the vertebral column. The hydrostatic pressure of the notochord prevents direct contact between the vertebral bodies (Grotmol *et al.* 2003), which are interconnected via intervertebral ligaments (Nordvik *et al.* 2005). Bone tissue consists of an organic matrix, minerals and bone cells. The organic matrix is mostly made up of collagen, and the bone cells present in Atlantic salmon vertebrae are osteoblasts, osteocytes (Nordvik *et al.* 2005) and osteoclasts (Martens *et al.* 2006). Osteoblasts are anabolic cells located at the bone surface that secrete and deposit the organic matrix, whereas osteocytes are formed when osteoblasts are trapped during bone growth (Franz-Ondendaal *et al.* 2006). Osteoclasts are catabolic cells that resorb the organic matrix (Witten & Huysseune 2009b). In Atlantic salmon vertebrae, the density of osteoblasts is the highest at the cranial and caudal rims of the amphicoel, and at the distal ridges of the trabeculae, the number of osteocytes is ten times higher in trabecular than in compact bone (Nordvik *et al.* 2005), and osteoclasts are located in the trabecular bone (Martens *et al.* 2006). As the vertebrae increase in size, the organic matrix is first formed as osteoid, which subsequently mineralizes. The most abundant bone minerals are P and Ca, which are arranged in crystals of apatite. Teleost bone is generally thought to consist of Ca-deficient apatite and/or

Ca-rich apatite, both of which decompose at high temperatures to β -tricalcium phosphate (TCP) and HAP (Hamada *et al.* 1995; Mkukuma *et al.* 2004). Apatites consist of Ca, phosphate, hydroxide and carbonate (Mkukuma *et al.* 2004). In teleosts, various species have been shown to possess HAP or a combination of HAP and TCP after heating to 600 °C (Hamada *et al.* 1995). The molar Ca/P ratios of TCP and HAP are 1.5 and 1.67, respectively (Hamada *et al.* 1995). For Atlantic salmon, the molar Ca/P ratios in vertebral bone have been shown to range between 1.25 and 1.62 (Graff *et al.* 2002; Helland *et al.* 2005; Witten *et al.* 2005; Fjellidal *et al.* 2009; Grini *et al.* 2011), and there seems to be an increased ratio with age (Shearer *et al.* 1994; Graff *et al.* 2002).

Bone health in farmed salmonids

Over the years, salmonid farming has faced issues with defective bone formation and vertebral deformities. Although reported in a wide range of salmonid species, comprehensive reports are available only for the two most commercially important salmonids, rainbow trout and Atlantic salmon. In trout, a surveillance study was conducted in bone samples of over 400 farmed trout from 23 trout farms in France, and it was documented that vertebral anomalies were high in most trout farms and the quality of the vertebrae and bone tissue of these trout were generally poor (Aubin 2007). In Atlantic salmon, reports from salmon producers indicate that incidences of bone deformities were also high, but are relatively decreased in the present compared to the preceding decades. The disorder is however invariably present and sometimes at high incidence.

Mechanisms of development of bone deformities

The mechanism of development of bone deformities in salmonids is suggested to be through three different processes in skeletal physiology. All the three are reported in both rainbow trout and Atlantic salmon. (i) Osteoclasty, which is the demineralization and destruction of the organic matrix by osteoclasts (Sire *et al.* 1990; Witten & Hall 2003); (ii) periostenocytic osteolysis, leading to demineralization and destruction of the organic matrix by osteocytes (Lopez 1973; Kacem & Meunier 2000b); and (iii) halastasy, characterized by demineralization without destruction of the organic matrix with a slight decrease in bone mineral content (Lopez 1973; Kacem *et al.* 2000a; Kacem & Meunier 2003). In salmonids, the movement between adjacent vertebral bodies is mainly limited to lateral flexion during swimming. It is possible that the mechanical loading generated by the lateral musculature during swimming is strong enough to inhibit a normal arrangement of collagen fibrils as the bone grows if not enough minerals are embedded

into the collagen matrix. In accordance, vertebral deformities in Atlantic salmon seem to develop where the mechanical loading generated by the lateral musculature is the strongest (Fjellidal *et al.* 2009). On the other hand, it is also possible that a low mechanical stiffness and soft bone structure in the vertebral bodies damage the notochord-derived intervertebral tissue, which subsequently inhibits normal longitudinal growth of the vertebrae. A vertebral body with a low stiffness may get slightly anterior–posterior compressed when the lateral musculature contracts, and may return to its normal less compressed phenotype when the muscle relaxes. Such a nondeformational change in morphology may rupture the notochordal sheet or harm the notochord in other ways. Consequently, a damaged notochord may inhibit normal longitudinal growth of the vertebral bodies. Vertebral body compression and fusion are often characterized by the development of ectopic cartilage that replaces the notochord tissue (Kvellestad *et al.* 2000; Witten *et al.* 2005, 2006). Osteoblasts that are located at the growth zone of the vertebral body acquire a chondroblast-like phenotype and produce cartilage instead of bone, and the vertebral bodies increase in diameter but not in length and obtain a compressed phenotype with normal trabeculae (Witten *et al.* 2005). When adjacent vertebral bodies fuse, the cartilage that occupies the intervertebral region is remodelled into bone (Witten *et al.* 2006). Further, reduced elasticity, structural alterations and cellular changes in the intervertebral notochord tissue (Ytteborg *et al.* 2010a) and dorsoventral shifts and lack of intervertebral spaces between normal vertebrae (Fjellidal *et al.* 2007a) are probably involved in the development of vertebral fusions in Atlantic salmon. Fjellidal *et al.* (2007b) compared farmed Atlantic salmon postsmolts with a low and high mineral content, and found that the amount of organic matrix was equal while the amount of minerals was different. This indicates that the observed large variation in mineral content was a consequence of impaired mineralization rather than demineralization. Also the fact that low dietary P reduces vertebral bone tartrate-resistant acid phosphatase (TRACP) enzyme activity and MMP 13 (matrix metalloproteinase 13) mRNA expression (Fjellidal *et al.* 2012b) indicated that Atlantic salmon do not use vertebral bone as a reservoir for minerals under periods of low mineral supply. Indeed, in salmonids, resorption of scales form the primary means of meeting increased physiological demand for minerals, especially Ca (Persson *et al.* 1997, 2005).

Types of bone anomalies observed in farmed salmonids

The different anomalies encountered in the bones of fish can be categorized into few distinct groups based on their aetiology and clinical pathology. According to Lall and Lewis-McCrea (2007), bone disorders reported in fish

included kyphosis (humpback, hunchback), lordosis (saddleback, swayback), scoliosis (lateral curvature with rotation of the vertebrae) and platyspondyly (short tail, compressed vertebrae). Witten *et al.* (2009a) proposed 20 types of salmon vertebral column malformations that are repetitively observed under farming conditions: nine types of compression and fusion-related deformities, four types of abnormal radiotranslucent or radiopaque vertebral bodies, three types of spine curvatures, three types of symmetry deviations and displacement of vertebral bodies and one type of severe multiple malformations. Based on the suggested categories, we discuss the relationships between types of vertebral body malformations. A clear distinction between anomalies of vertebral axis and vertebrae was also made in the report of Witten *et al.* (2009a). Anomalies affecting the vertebral axis in Atlantic salmon can be divided into seven categories: spinal curvatures (three types: lordosis, kyphosis and scoliosis), symmetry deviations and displacement of vertebral bodies (three types: vertically shifted vertebrae, vertebral bodies with uneven internal structures and vertebrae centra shifted dorsally or ventrally), and severe co-occurring of multiple anomalies. The anomalies of vertebrae include dislocation, fusion, compression shortening, deformation or lack of the centra, lack or extra formation of the haemal and neural arches and apophysis, lack or separation of the ribs (Boglione *et al.* 2013b). Compressed vertebrae with intervertebral spaces are classified as platyspondyly, and compressed vertebrae without intervertebral spaces are classified as vertebral ankylosis and platyspondyly (Fjelldal *et al.* 2007a). Other deformities include 'neck-bend' or 'stargazer' and compressed snout (pugheadness), bent jaw (crossbite, hare-lip or front and downwards protuberance of jaw; reduction in lower jaw), short operculum and other defects (reduced or asymmetric fins, etc.). For more detailed information on skeletal anomalies in fish, the readers are referred to Witten *et al.* (2009a) and Boglione *et al.* (2013a,b).

Critical life stages and factors influencing bone health

Relevant life stages in the culture of the anadromous Atlantic salmon can roughly be divided into start feeding (freshwater), parr growth, smoltification, sea transfer, growth in seawater and reproduction after returning to freshwater. These life stages differ in environmental conditions and biological programming which require specific adaptations of the fish. General physiology changes profoundly when entering each new stage, with potential effects on energy demands, tissue deposition, growth and osmoregulation of the fish. At each stage, all of the above-mentioned factors combined are likely to affect the mineral requirement of the fish and in particular the periods of transitions from one stage to the next. Early stages of life cycle until the stage

of seawater transfer are considered to be the critical windows for the onset of bone deformities in salmonids. In Atlantic salmon, the different phases of life cycle have been associated with distinct anomalies, namely fusions (observed at all life stages), hyperdense vertebrae (freshwater phase) and compressed vertebrae (seawater phase). This information was generated through the monitoring programme of a salmon breeding company SalmoBreed AS. Of the 24 groups of Atlantic salmon examined by X-ray at relevant stages (between 20 and 150 g body weight), hyperdense vertebrae were detected in 14 (58% of groups). Compressed vertebrae were observed in nine of 20 groups (45% of groups). Not all cases were equally severe, but in the most extreme group, 70% of fish had typical compression of caudal vertebrae (short tails) when reaching brood fish age. In seawater, the incidence of compressed vertebrae increased with increasing fish size. The information obtained through this monitoring programme demonstrated that skeletal deformities at specific early life stages can have long-term effects.

Genetic, environmental and dietary factors are involved in the development of bone deformities in salmonids or fish in general. Aulstad and Kittelsen (1971) had suggested of a relation between inbreeding and the frequency of spinal deformities in a salmonid species, rainbow trout. Taniguchi *et al.* (1984) opined of genetic differences among parents to explain the incidence of vertebral malformation among progeny groups of red seabream. McKay and Gjerde (1986) described genetically inheritable spinal deformity in Atlantic salmon, with an estimated heritability index between 0.14 and 0.64. A complex form of vertebral deformity characterized by consecutive repetition of lordosis, scoliosis and kyphosis from the head to the caudal fin (referred to as LSK syndrome) was described in gilthead seabream (Afonso *et al.* 2000). The incidence of LSK syndrome was 0.2% in the whole population, and 6.5% within the affected family, which disagreed with the expected frequency of one gene hypothesis (simple Mendelian inheritance) and suggested a possibility of polygenic origin (Afonso *et al.* 2000). In Atlantic salmon, it was recommended not to select breeders from families with high incidences of deformed fish and more so for breeders showing deformities themselves. Although this strategy was not likely to significantly reduce the incidence of deformities, increase in the genetic susceptibility to vertebral deformities in the population can be reduced (Gjerde *et al.* 2005). In addition, genetic variation in apparent availability of minerals in seawater Atlantic salmon has also been reported (Thodesen *et al.* 2001). The apparent availability of P ranged between 24 and 55%, while that of Zn varied from 18 to 51% among 82 full-sib families of 4 kg (Thodesen *et al.* 2001); and individual variations in apparent availability of P between 30 and 49%, and of Zn from 32 to 58% have

been documented (Rydland 1998). It would be informative to study whether there is a correlation between the genetic variation in mineral availability and skeletal deformities. The knowledge on genetic factors influencing bone deformities is limited in salmonids, and the very few reports available indicate that the role of genetic inheritance in the onset of bone deformities requires more attention.

In the recent past, the use of a genetic intervention in production and the use of triploids in aquaculture have gained momentum in many countries. Among salmonids, triploids in commercial aquaculture production include rainbow trout in United States, Canada, France, Japan, UK, Korea, Iran, Turkey, Poland and Chile; brown trout in UK and France; brook trout in Canada and France; Atlantic salmon in Canada and Chile; Arctic charr in France, Canada, Iceland and Austria; and chinook salmon in Canada (Piferer *et al.* 2009). However, farming of triploid Atlantic salmon is yet to be practiced in Norway. The studies thus far taken up with triploid Atlantic salmon under-yearling smolts suggest a higher susceptibility to bone deformities than their diploid counterparts (Fjellidal & Hansen 2010). In the embryonic stage of the life cycle, the number of vertebrae developed is inversely related to the rate of development of the embryo and can also influence the incidence of deformed vertebrae (Garside 1966). Environmental factors play an important role in the onset of anomalies in the embryonic stage. The incubation conditions of the fertilized ova, especially temperature and photoperiod, play a major role in determining the healthy development of the notochord and thus influence bone health even before hatching (Canagaratnam 1959; Garside 1966). In the later stages of Atlantic salmon such as parr, presmolt, smolt and to certain extent in the post-smolt phase, the dietary factors play a decisive role in influencing the manifestation of the deformities. The first feeding and early juvenile stages of Atlantic salmon (from 0.18 to 20 g body weight) were particularly sensitive to dietary P restriction, with consequent adverse effects on vertebral and jaw development observed in harvest size fish (Figs 1 and 2, Baeverfjord *et al.* 2006). In seawater, the incidence of compressed vertebrae increased with increasing fish size with typical compression of caudal vertebrae (short tails) at harvest. This confirms the need to address skeletal deformities as a welfare issue in commercial production and that the deformities do appear along the whole production chain.

Mineral nutrition of salmonids – bone health perspective

Elemental stoichiometry, life stage and its relation to dietary minerals

Salmonids are characterized as low-P fish species which in general have soft rays with cycloid scales, elongated and

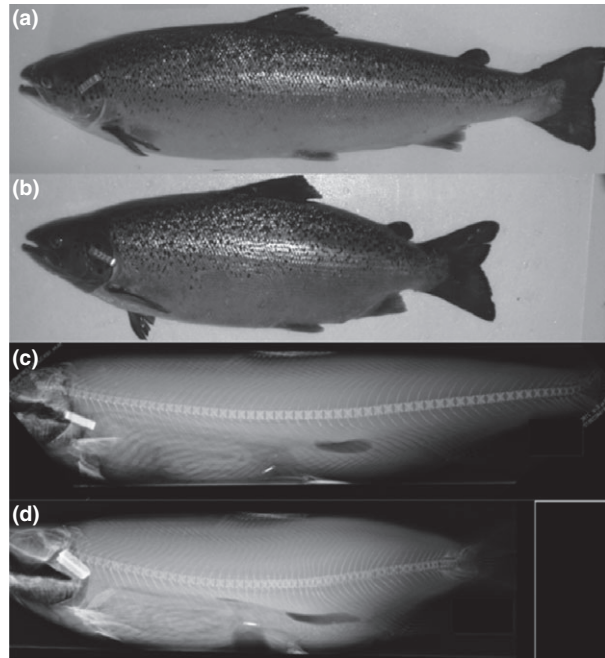


Figure 1 Atlantic salmon (harvest size) with normal body shape and spinal radiology (a and c, same fish) and severe shortening of body due to compressed vertebrae (b and d, same fish). From life cycle study with low P diets. Fish fed control diet (a and c, 16–17 g kg⁻¹ total P) or low-P diet (b and d, 10–12 g kg⁻¹ total P) in freshwater, followed by commercial diet in seawater (Baeverfjord *et al.* 2006).

more streamlined body and much more cartilage and less bone than many other bony fish. Ontogenetic differences are known to exist in whole-body mineral concentration of Atlantic salmon (Shearer *et al.* 1994; Ebel *et al.* 2015). The extent of mineralization in the endoskeleton and outer integument contributes to elemental stoichiometric patterns (Hendrixson 2002). Calculations using the mean data of Shearer *et al.* (1994) showed that the Ca:P mass ratio in Atlantic salmon varied with life stage and environment. The mass ratio of Ca:P was as low as 0.2–0.4 before first feeding (<0.1 g), reached to 0.86 at 0.25 g and steadily increased up to 1.04 in 64 g presmolt stage. During and after smoltification, the Ca:P ratio declined to 0.6 in 125 g smolt in seawater and thereafter ranged between 0.7 and 0.8 in adult Atlantic salmon (1–4 kg) in seawater. Moreover, juveniles (i.e. smolts) and post-spawn adults (i.e. kelts) migrating downstream to the ocean had a higher concentration of P compared to adults migrating upstream to spawn (by 20–35%). Hence, it could be inferred that Atlantic salmon moving to seawater require better mineralization and the P status of fish at the moment of seawater transfer has a role in performance in the seawater phase. This corresponds well with the observations cited in the previous paragraph that the impact of dietary mineral restriction in freshwater phase is manifested into skeletal

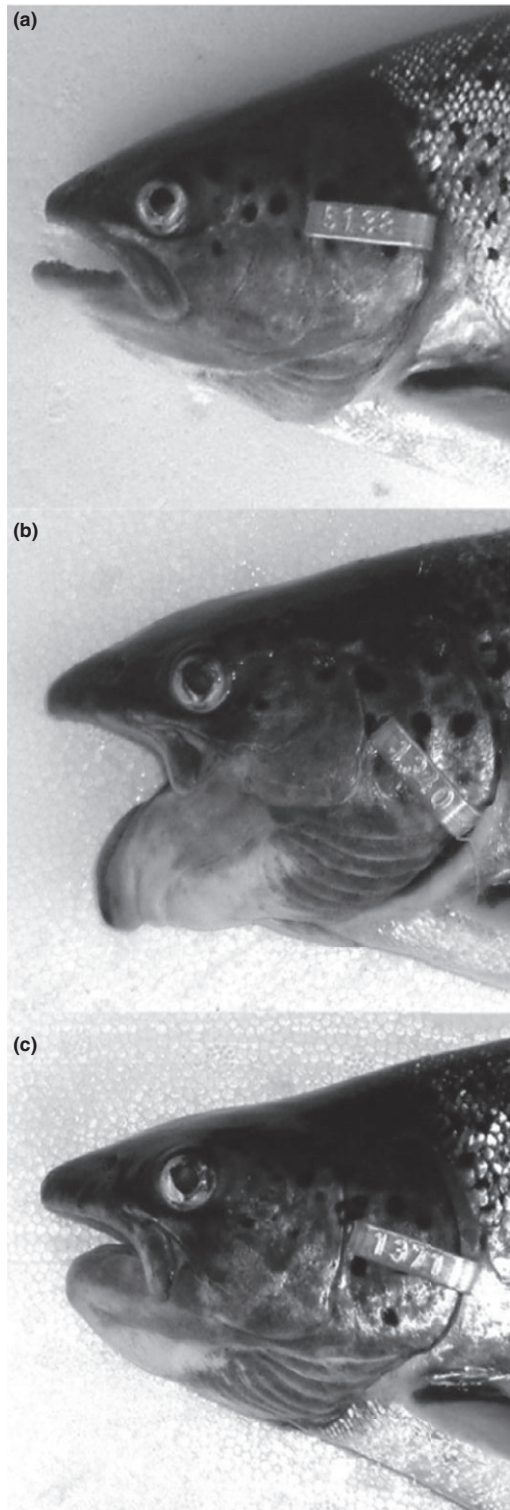


Figure 2 Morphology of lower jaw in harvest size Atlantic salmon in response to dietary P. (a) Normal mandible in control fish; (b) 'Dropped jaw' in fish fed low-P diet ($10\text{--}12\text{ g kg}^{-1}$ total P) throughout freshwater rearing, followed by commercial diet in seawater; (c) 'Box jaw' in fish fed low-P diet (same as b) in freshwater (Baeverfjord *et al.* 2006).

deformities in the seawater phase. Therefore, it becomes essential to assess not only the total P level in salmon at seawater transfer, but also the amount of P associated with structural bone matrix.

The Ca:P ratio of whole fish or tissues can be used as an indicator to the degree of P associated with bone matrix; higher the ratio, higher the proportion of Ca associated with P, as in the bones. The mass ratio of Ca:P in fish bones from 18 species was observed to be 2.14 (Hendrixson *et al.* 2007); the same for vertebrae, scale and operculum across species was estimated to be 1.98, 2.11 and 2.48 (calculated from Antony Jesu Prabhu *et al.* 2016b). Moreover, the mass ratios of Ca:P in vertebrae of Atlantic salmon and rainbow trout in freshwater were estimated to be 2.09 and 1.92, respectively. These values are lower than the mass ratio of Ca:P (2.17) in HAP (Russell *et al.* 1986), whereas the values of (Ca + Mg):P were slightly higher 2.12 and 1.95, which according to Hamada *et al.* (1995) is an indication that a proportion of Ca in HAP is replaced by Mg. Based on the nature of Ca:P stoichiometry in fish bones and assuming all fish Ca was associated with bones, the proportion of whole fish P associated with bones was estimated to be 72.7% (range: 46.3–81.3%) among 18 fish species, including two salmoniformes, namely rainbow trout and white fish (Hendrixson *et al.* 2007). Following the method of Hendrixson *et al.* (2007), it was estimated that 55.4% (95% CL: 46.9–67.1%) of P in whole fish (with mean body P of 3.95 g kg^{-1} fresh weight; 95% CL: 3.65–4.26) was associated with bone matter (Ca) in salmonids (Fig. 3; Atlantic salmon and rainbow trout, 0.1–4 kg body weight) using data from Shearer *et al.* (1994) and Antony Jesu Prabhu *et al.* (2013, 2016b). The estimated range of P associated with bone (Ca) also fits well in the lower range of the estimate reported by Hendrixson *et al.* (2007). Unlike in most vertebrates, it is also true that body-P pools in fish are characterized with high degree of stoichiometric flexibility which renders possibility for higher growth rates. The flexible body-P pools and significant sensitivity of growth rate to change in diet-P levels have direct consequence on bone mineral status, which represents a major P sink in fish (Benstead *et al.* 2014).

Phosphorus – the pivotal point of investigation

Effect of deficient or suboptimal supply of dietary phosphorus

Very limited data are available regarding the incidence of dietary mineral-related skeletal deformities in salmonid production; of which, most if not all are related to dietary phosphorus (Table 1). Phosphorus deficiency (Baeverfjord *et al.* 1998) and suboptimal phosphorus nutrition (Fjelldal *et al.* 2009) have shown to induce skeletal deformities in Atlantic salmon. Coordinated defect in mineralization of

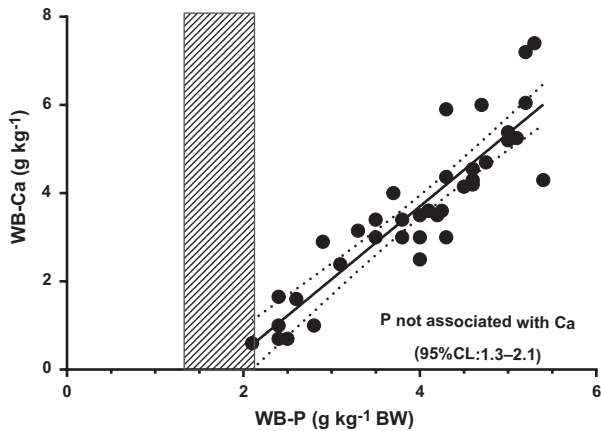


Figure 3 Estimation on the proportion of P associated with skeletal mass in salmonids as per Hendrixson *et al.* (2007). Whole-body Ca (in y-axis) and P (in x-axis) of well-growing Atlantic salmon and rainbow trout (as g kg^{-1} fresh weight). The linear regression with slope 1.65 ± 0.1 (mean, SE) and R^2 of 0.82 when extrapolated to zero Ca content gave an estimate of the P not associated with Ca (x-intercept, 1.76). The shaded area in the graph shows the 95% confidence interval of whole-body P not associated with Ca ($1.3\text{--}2.1 \text{ g kg}^{-1}$ wet weight) in salmonids from data reported in literature.

vertebral and mandibular structures in response to low dietary P was demonstrated by Helland *et al.* (2005, see Fig. 4). Further, inadequate P nutrition at the juvenile stage has shown to induce deformities later in life in Atlantic salmon (Fjelldal *et al.* 2012a). In rainbow trout, proportion of fish with radiological detectable vertebral deformities decreased with increasing dietary P content (Fontagné *et al.* 2009). Kacem *et al.* (2004) found that vertebrae from farmed rainbow trout had wide erosive lacunas, and suggested that this could characterize a physiological stress generated by mineral deficiency. Dietary P deficiency has also shown to induce vertebral deformities in Chum salmon, *Oncorhynchus keta* (Watanabe *et al.* 1980). In Atlantic salmon, the relation between vertebral deformities and diet-P level has been suggested to be linked to the structural integrity of the vertebral bodies (Baeverfjord *et al.* 1998; Fjelldal *et al.* 2009). Dietary P deficiency due to low availability of dietary P reduces vertebral mineral content (Albrektsen *et al.* 2009). There is a correlation between vertebral body mineral content and mechanical strength (Fjelldal *et al.* 2006, 2009), and between vertebral body mineral content and length–dorsoventral diameter (Fjelldal *et al.* 2007b). Vertebrae with a low mineral content are more compressed than vertebrae with normal mineral content in Atlantic salmon postsmolts. Besides reducing bone mineral content, low dietary P has also shown to increase alkaline phosphatase (ALP) and reduce TRACP enzyme activities, and reduce the MMP 13 mRNA expression of the vertebral bodies in Atlantic salmon (Fjelldal *et al.* 2012b). This

probably reflects compensatory mechanisms at mineral deficiency where increased ALP activity indicates elevated mineralization while reduced TRACP activity and MMP 13 expression indicate reduced tissue degradation and remodelling. Bone mineral content need not necessarily reflect a healthy skeletal development, compressed vertebrae formed due to inadequate P supply in an early stage may have equal mineral content and molar Ca/P ratio as normal vertebrae (Witten *et al.* 2005; Fjelldal *et al.* 2009). Some studies have shown that Atlantic salmon under-yearling smolts have a reduced vertebral bone mineral content during the first period in seawater (Fjelldal *et al.* 2006; Grini *et al.* 2011). Moreover, a short-term subclinical P deficiency in early life resulted in severe deformities of caudal vertebrae in seawater-adapted fish approaching harvest size (Fjelldal *et al.* 2012a). Several nutrients are suggested to play a role in the onset and/or manifestation of skeletal deformities (Lall & Lewis-McCrea 2007). Among minerals, phosphorus is the most studied mineral for its potential role in either preventing or reducing the severity of skeletal deformities in salmonids.

Feed conversion ratio, growth rate and dietary phosphorus

The feed conversion ratio (FCR) of most salmon farming operations has been reduced from around 4 in the early 1970s to around 1.2 at present mainly due to high energy feeds (Wang *et al.* 2012; Tacon & Metian 2015). This has also greatly reduced overall environmental load of phosphorus, which is presently calculated to be less than 9 kg P per ton of salmon produced (Ytrestøyl *et al.* 2015). The improvement in FCR meant that the supply of structurally important nutrients especially phosphorus in the salmon feeds would be disproportionate to meet the physiological demand for growth. Shearer (1995) modelled a theoretical simulation for P requirement in salmonids, wherein dietary P requirement of rainbow trout increased with decreasing FCR. Although this hypothesis was not tested in an experiment *per se*, analysis of metadata from published P requirement studies in fish revealed a similar pattern of increase in dietary P required per unit weight gain with decreasing FCR (Fig. 5). This relation implies that, less dietary P is supplied to support a rather higher growth rate in fish fed diets with low FCR, thereby rendering the fish more susceptible to skeletal deformities over long term. In this scenario, expressing P requirements of salmonids as g P kg^{-1} weight gain will enable optimal supply of dietary P for faster growth without compromising on skeletal health. In wild fish populations, increased growth rate has a metabolic cost on skeletal development leading to delayed or compromised mineralization (Arendt & Wilson 2000; Arendt *et al.* 2001). Using data from published P requirement trials in

Table 1 Dietary phosphorus-induced skeletal deformities reported in Atlantic salmon and rainbow trout

Induced by deficient/low/high levels	Available P in diet	Associated factor	Type of deformity	Species and life stage	References
Low	0.34%		Soft opercle and gill arch, malformed ribs and spinal arches, scoliotic spine	Atlantic salmon parr and smolt	Baeverfjord <i>et al.</i> (1998)
Low	0.50%	Continuous light	Vertebral body compression	Atlantic salmon smolt	Fjelldal <i>et al.</i> (2009)
Low	0.40%		Vertebral body compressions	Atlantic salmon, under-yearling postsmolts	Fjelldal <i>et al.</i> (2012a)
Low	0.40%		Anterior and posterior trunk regions: type 8 (fusion centre), type 5 (one-sided compression) and type 2 (homogeneous compression)	Atlantic salmon, juvenile	Fjelldal <i>et al.</i> (2012b)
Low	0.40%		Tail and tail-fin regions: type 2 (homogeneous compression), type 5 (one-sided compression) and type 3 (compression and reduced intervertebral space)	Atlantic salmon, juvenile	Fjelldal <i>et al.</i> (2012b)
Low	0.40%		Externally visible lower jaw deformity	Atlantic salmon, seawater (SW) transfer and final harvest stage	Fjelldal <i>et al.</i> (2016)
Deficient	0.26%	Triploid	Type 1 (decreased intervertebral space)	Rainbow trout, SW transfer	Deschamps <i>et al.</i> (2014)
Low	0.40%	Triploid	Type 2 (homogeneous compression)	Atlantic salmon, smolt stage (SW transfer) and harvest	Fjelldal <i>et al.</i> (2016)
Deficient	0.26%	Triploid	Type 2 (homogeneous compression)	Rainbow trout, SW transfer and harvest	Deschamps <i>et al.</i> (2014)
Deficient	0.26%	Triploid	Type 3 (compression and reduced intervertebral space)	Rainbow trout, SW transfer and harvest	Deschamps <i>et al.</i> (2014)
Low	0.40%		Type 4 (compression without X-structure)	Atlantic salmon, harvest	Fjelldal <i>et al.</i> (2016)
Deficient	0.26%	Triploid	Type 4 (compression without X-structure)	Rainbow trout, SW transfer and harvest	Deschamps <i>et al.</i> (2014)
Low	0.40%		Type 8 (fusion centre)	Atlantic salmon, smolt stage (SW transfer) and harvest	Fjelldal <i>et al.</i> (2016)
Low	0.40%		Type 10 (widely spaced and undersized; caused by under mineralization)	Atlantic salmon, smolt stage (SW transfer)	Fjelldal <i>et al.</i> (2016)
Deficient	0.29%	Triploid	Type 10 (undersized and widely spaced vertebral body) and 11 (pronounced biconcave)	Rainbow trout, Juvenile	Le Luyer <i>et al.</i> (2014a,b)
Deficient	0.26%		Type 11 (pronounced biconcave)	Rainbow trout, SW transfer and harvest	Deschamps <i>et al.</i> (2014)
Low and High	1.21%	Diploid	Type 19 (internal dorsal or ventral shift)	Atlantic salmon, smolt stage (SW transfer)	Fjelldal <i>et al.</i> (2016)
High	1.21%	Triploid	Type 1 (decreased intervertebral space)	Atlantic salmon, smolt stage (SW transfer) and harvest	Fjelldal <i>et al.</i> (2016)
High	1.21%	Diploid	Type 6 (compression and fusion)	Atlantic salmon, harvest	Fjelldal <i>et al.</i> (2016)
High	1.21%	Triploid	Type 12 (hyper-radiodense, arrow)	Atlantic salmon, smolt stage (SW transfer)	Fjelldal <i>et al.</i> (2016)

Table 1 (continued)

Induced by deficient/low/high levels	Available P in diet	Associated factor	Type of deformity	Species and life stage	References
High	1.21%		Type 13 (hyper-radiodense with flat end plates, arrow)	Atlantic salmon, smolt stage (SW transfer)	Fjelldal <i>et al.</i> (2016)
Low		Triploid	Type 5 (one-sided compression)	Atlantic salmon, harvest	Smedley <i>et al.</i> (2016)
Low	0.40%	Triploid	Type 5 (one-sided compression)	Atlantic salmon, smolt stage (SW transfer)	Fjelldal <i>et al.</i> (2016)
Suboptimal for rapid growth and improved FCR (1.4% total P)		Transgenic, triploid	Major external skeletal disorder, unmarketable; shortened gill filaments, deformed operculum, twisted tail, twisted spine, scissor jaw, twisted jaw, protruding jaw, irregular body shape	Atlantic salmon, freshwater phase	Tibbetts <i>et al.</i> (2013)

fish, Benstead *et al.* (2014) also showed that fish that grew at a higher rate (measured as SGR) were more sensitive to low dietary P levels than fish that grew at a lower rate.

Apart from improving the FCR of feeds, effort to improve the growth rate of fish through manipulation of genetic and environmental variables might be indispensable for future development of salmonid aquaculture. The growth-enhanced transgenic Atlantic salmon grows at twice the rate and has a 10–20% improvement in gross feed conversion efficiency relative to nontransgenic salmon during presmolt and post-smolt stages (Cook *et al.* 2000; Tibbetts *et al.* 2013; Ganga *et al.* 2015). In the above-cited studies, body/bone mineralization indicators such as body ash, bone ash, Ca or P were lower in fast-growing transgenic salmon. High levels of P and Ca in the feed enabled to reduce external morphological deformities and improve body mineral content in GH-transgenic *Nile tilapia* (Lu *et al.* 2013). Tibbetts *et al.* (2013) also suggested that transgenic salmon might require higher dietary P supply to satisfy the requirement for faster growth and emphasized the need to assess dietary requirement for P in transgenic salmon. In the light of this, it is essential to revalidate the dietary requirements of structurally important minerals, especially P and Ca to decrease the incidence of skeletal deformities in fast-growing farmed salmonids.

Triploids may have higher requirement for dietary phosphorus

Several reports point to a higher prevalence of skeletal deformities in triploid Atlantic salmon and rainbow trout (O'Flynn *et al.* 1997; Sadler *et al.* 2001; Fjelldal & Hansen 2010; Leclercq *et al.* 2011; Taylor *et al.* 2013). Lower jaw deformities (LJD) seem to be a common form of skeletal

anomaly reported in triploid Atlantic salmon (O'Flynn *et al.* 1997; Sadler *et al.* 2001; Leclercq *et al.* 2011; Fjelldal *et al.* 2016). In triploid Atlantic salmon reared in freshwater, skeletal anomalies were not observed (Burke *et al.* 2010), whereas the contrary was observed in other reports (Sadler *et al.* 2001; Amoroso *et al.* 2016a,b). The tail region of the vertebrae (Smedley *et al.* 2016) and lower jaw (Fjelldal *et al.* 2016) are reported to be more susceptible to low dietary P-induced deformities in triploids. LJD previously reported in farmed Atlantic salmon (Bruno 1990; Roberts *et al.* 2001) were also induced by experimental P deficiency at start feeding and seawater phase of postsmolts (Fjelldal *et al.* 2016). Additional dietary P improved mineralization and decreased the incidence of skeletal deformity in triploid rainbow trout (Deschamps *et al.* 2014; Le Luyer *et al.* 2014b) and Atlantic salmon (Fjelldal *et al.* (2016). The prevalence of deformed vertebrae in triploid salmon fed low P diet (1%, total P) was threefold higher compared with diploids (Smedley *et al.* 2016). Increasing the level of dietary phosphorus (1.2–1.4%, total P) effectively reduced the incidence of these anomalies in triploid salmon (Fjelldal *et al.* 2016; Smedley *et al.* 2016). Correlation regression analysis of literature data revealed that the relation between dietary P and skeletal deformities is to a greater extent significant in triploids, than diploids, although the variation was high within triploids (Fig. 6). Even in transgenic Atlantic salmon reared from 100 to 300 g on a common grower diet (1.4% total P) at water temperatures ranging from 8 to 10 °C, triploids had significantly higher number of deformed individuals than diploids (Tibbetts *et al.* 2013). In this study, based on visual assessment, the triploid group had a higher percentage of fish with one or more skeletal disorders (2.9–3.7%) relative to diploids (0.3–0.9%), with transgenic triploids showing the highest (3.7%). Although

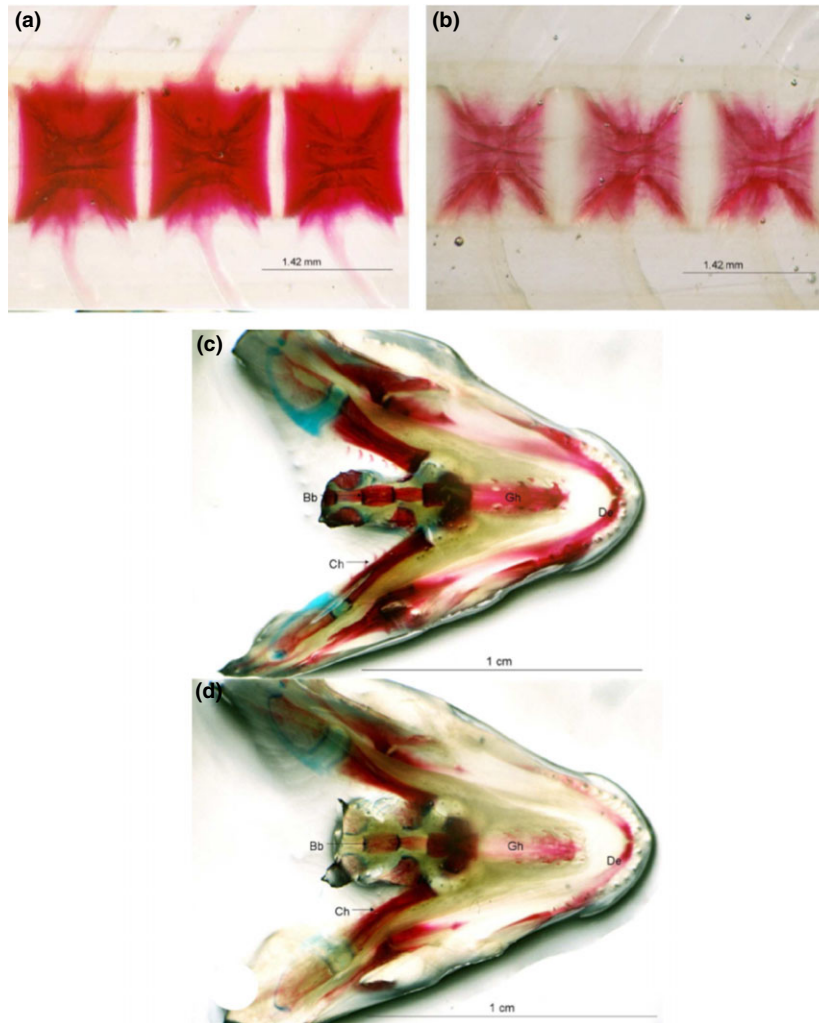


Figure 4 Impaired skeletal mineralization in response to low dietary P, demonstrated with Alizarin red whole mount staining. (a and b) Variation in ossification of vertebrae number 39–41 of Atlantic salmon parr, fed either a control diet with 14 g kg^{-1} total P (a) or a low-P diet with 9.5 g kg^{-1} total P (b) from 8 to 18 g size. (c and d) Difference in ossification of lower jaw (dissected, photograph is taken dorsally) of Atlantic salmon parr Alizarin Red fed control diet (c) and low-P diet (d). Cartilage is stained blue with Alcian Blue. Fish size of approx. 18 g. Adapted from Helland *et al.* (2005).

the assessment criteria used in the above study are subjective, this gives an indication and most importantly warrants dedicated attempts for further understanding. In vertebrates, high proportions of their body P mass are composed of bone; however, during the very early developmental stages of high specific growth rates, high quantities of P-rich RNA are required (Elser *et al.* 2006). Thus, metabolic P limitation during embryonic life could lead to developmental anomalies, which then manifest into deformities in a later stage. Therefore, the increased prevalence of skeletal deformities in triploids can be related to the ploidy itself. Further, the role of parental nutrition potentially influencing egg quality also needs to be better understood. These findings warrant the necessity to rethink the

strategy of using mineral requirement data generated from diploid individuals in formulating feeds for triploids. As skeletal deformities are likely to be a major constraint to further use of triploids in commercial salmonid production, this is a topic which warrants further studies.

Vaccination-induced anomalies and dietary phosphorus

Vaccination of salmon smolts before seawater transfer is a common practice in farming of Atlantic salmon. Vaccination too close in time to seawater transfer was suggested as a risk factor for the occurrence of vertebral deformities in later life stages (Larssen & Djupvik 2005). Vaccination-induced bone deformities are associated with abdominal

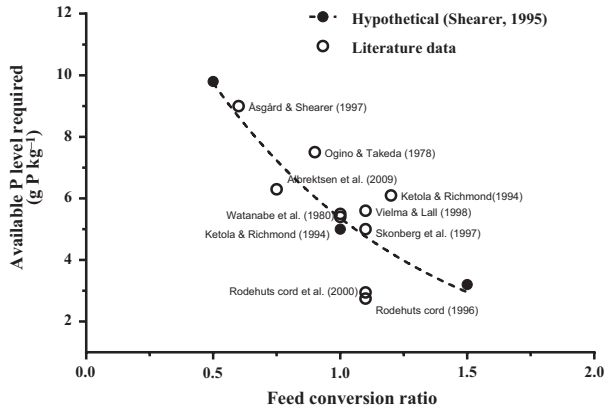


Figure 5 Impact of feed conversion ratio on the available phosphorus requirement of salmonids: Hypothetical response curve (dark circles), adapted from Shearer (1995), and literature data in salmonids (open circles) from published reports on P requirement studies in salmonids (in studies where P availability data were not available, nonphytic P provided by authors was assumed as available P).

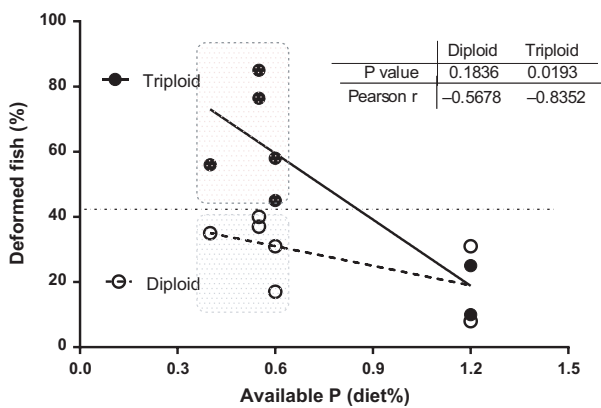


Figure 6 Correlation between available phosphorus levels in the diet and the prevalence of skeletal deformities in diploid (open circles and dotted line) and triploid (dark circles and solid line) Atlantic salmon. Data from Fjellidal *et al.* (2016) and Smedley *et al.* (2016). The percentage of deformed fish was considerably reduced by increased dietary P, the effect being significant in triploids ($P < 0.02$).

lesion scores and prevalence of spinal lesions in vaccinated fish (Berg *et al.* 2006; Aunsmo *et al.* 2008). Haugarvoll *et al.* (2010) reported proliferation and infiltration of connective tissue sheath into adjacent musculature the surrounding malformed vertebrae. Vaccinated salmon show a higher frequency of deformities in skull bones and vertebral column, suggesting a decrease in developmental stability caused by vaccination (Berg *et al.* 2012; Yurtseva *et al.* 2014). Gil Martens *et al.* (2010) identified an effect of injection *per se* on vertebral deformities suggestive of a localized triggering effect by injection-related processes on the development of spinal deformities. The studies on the role of

dietary phosphorus in ameliorating the effects of vaccination-induced deformities in Atlantic salmon are relatively less. High dietary phosphorus (9 g vs. 6 g kg⁻¹ available P) was ineffective in preventing bone malformations in Atlantic salmon injected with Freund's complete adjuvant leading to severe scoliosis in 7% of injected fish (Gil Martens *et al.* 2012). In another study, Atlantic salmon fed similar levels of dietary P showed no effects of vaccination or dietary phosphorus on the occurrence of externally detectable skeletal deformities. The present level of understanding precludes a conclusive evidence of the impact of dietary phosphorus on the incidence of vaccination-induced bone deformities in salmonids.

Recirculation aquaculture systems, bone health and phosphorus nutrition

Recirculating aquaculture systems (RAS) are being increasingly used in salmonid aquaculture, especially for production of rainbow trout and Atlantic salmon psmolts in freshwater. The water quality characteristics of RAS differ significantly from flow-through systems, with differences in soluble organic matter and minerals (Good *et al.* 2009; Martins *et al.* 2009). Apart from water quality, the abnormal nature of swimming termed as 'side-swimming' (Good *et al.* 2014) observed in salmonids reared in RAS could also impact bone health. In general, there is a lack of knowledge on how RAS specifically impacts skeletal development and bone health in fish, more so in salmonids. Abnormal swimming behaviour and increased deformities were observed in rainbow trout cultured in a near-zero water exchange RAS (Davidson *et al.* 2011). The authors attributed the incidence of deformities to the contrasting water quality characteristics especially high nitrate concentrations in the water. In common carp embryos, the concentration of ortho-phosphate-P, nitrate and the heavy metals arsenic and copper likely impaired the embryonic and larval development (Martins *et al.* 2009). Dissolved phosphorus concentration can accumulate up to 70-fold in a near-zero water exchange systems (Antony Jesu Prabhu 2015). Whether or not the increased P concentration of RAS water can contribute positively or negatively to the P requirement remain unanswered in salmonids. However, in freshwater *N. tilapia* (Eding *et al.* 2012), common carp (Antony Jesu Prabhu *et al.* 2017a) and marine turbot (van Bussel *et al.* 2013) there has been evidence of a positive effect on growth or body P balance.

Calcium and magnesium – overlooked minerals of bone health significance

Calcium is the most abundant mineral in the bones of fish comprising about 20–25% of the dry bone mass. The

significance of calcium as a structural mineral in fish bone relates to HAP, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, and TCP, the primary materials of the bone matrix with a Ca:P ratio of 1.6 to 2 in salmonid bones (Satoh *et al.* 1987d; Vielma & Lall 1998a). Although the concentration of Ca in the bones is twice that of phosphorus, the importance of dietary Ca in salmonid bone health has been largely neglected due to the unlimited access to Ca from water via branchial absorption. NRC (2011) recommends that Ca supplementation to fish feeds is required only when reared under Ca-free water conditions. However, it has been suggested that this can affect tissue mineral concentration in fast-growing salmonids (Vielma & Lall 1998b; Antony Jesu Prabhu *et al.* 2016b) and nonsalmonids (Lu *et al.* 2013), especially with more and more plant-based feed ingredients. In plant-derived feed ingredients, phytic acid is an antinutritional factor which reduces dietary mineral availability. Increasing the level of phytic acid in the diet did not affect P, Ca or Mg levels in vertebrae, but had negative effects on whole-body Ca, Mg and the Ca:P ratio in Atlantic salmon parr. Further, increasing level of sodium phytate inclusion in feeds for Atlantic salmon parr increased incidence of hyperdense vertebrae during later stage (Helland *et al.* 2006). Potential relationship between Ca metabolism and its interaction with other vital bone metabolic regulators like vitamin D₃, vitamin K, vitamin B₆, vitamin C, phosphorus and hormones in fish has been reviewed elsewhere (refer Lall & Lewis-McCrea 2007). Although the essentiality of dietary Ca supply in fish feeds is debated, recent developments indicate the necessity for a more objective view of its impact on bone health in fish (Lu *et al.* 2013; Hossain & Yoshimatsu 2014).

Similarly, effects of Mg deficiency are commonly cited (Lall 2002; NRC 2011) with reference to a rainbow trout study (Ogino *et al.* 1978) in which an abnormal curvature of the body was observed with Mg-deficient diets (0.05–0.15 g Mg kg⁻¹). Moreover, it was shown that inadequate magnesium supply may have a strong effect on whole-body concentration of calcium and phosphorus in salmonids (Shearer 1989), thereby exerting an indirect effect on bone mineralization. Mg deficiencies in rainbow trout also resulted in renal calculinosis, although no gross symptoms of skeletal deformities were observed (Covey *et al.* 1977). The only study reported thus far on Mg requirement in Atlantic salmon was conducted on parr (8 g) and reported 0.36 g Mg kg⁻¹ diet to be sufficient for normal bone formation (El-Mowafi & Maage 1998). However, the freshwater used was mixed with seawater, resulting in a final Mg concentration of 54 mg L⁻¹. Dietary Mg supply is more critical to fish during freshwater phase as aqueous Mg availability is limiting (Shearer & Åsgård 1992; Lin *et al.* 2013). As dietary Mg deficiency is very uncommon among practical diet formulation, Mg deficiency as potential causal

factor for skeletal deformities in salmonid production remains to be clarified.

Microminerals in bone health – prospective research domain in salmonids

Essential microminerals such as Zn, Mn and Cu are needed for the growth and development of healthy bones in fish (Lall 2002). However, the understanding on the role of microminerals in skeletal development has received limited attention in studies of fish. In terrestrial animals, the role of Zn, Cu, Mn, Se, Br, Si, Sr and Fl in promoting either bone formation or mineralization has been documented (Beattie & Avenell 1992). Moreover, certain essential microminerals at higher concentrations have adverse effects on skeletal tissue metabolism of vertebrates (Lall & Lewis-McCrea 2007). This section attempts to shed light on the prospective role of less-studied essential microminerals in skeletal metabolism of salmonids from the growing knowledge in higher vertebrates or model fish species.

Dietary zinc, bone deformities and role in osteology

Zinc depletion has negative effects on bone mineralization in rainbow trout (Satoh *et al.* 1987a) and Atlantic salmon (Maage & Julshamn 1993). Impact of zinc deficiency on bone health in rainbow trout was well characterized by compressed vertebra throughout the vertebral column, termed ‘short-body dwarfism’ (Satoh *et al.* 1987b,c). In Atlantic salmon, Zn deficiency-induced vertebral deformities in early life stages were conspicuous even at 1 kg body weight. These show that dietary zinc has a critical role to play in skeletal development even from early life stages, as shown in red seabream larvae (Nguyen *et al.* 2008). Zinc is the most important and highly studied micromineral of significance to bone health in salmonids or fish in general. Zinc is the most abundant micromineral in the vertebrae of many teleost fish species (Antony Jesu Prabhu *et al.* 2016b) and has functional importance in the activity of ALP, implicated in processes related to mineralization of bone. In the bone health perspective, dietary zinc deficiency can lead to reduced growth, short-body dwarfism, compressed vertebrae, and demineralization of bones leading to loss of P and Ca in juvenile and adult fish (Lall 2002; Witten *et al.* 2009a). Dietary Zn deficiency is reported to retard growth and induce dwarfism in animals and fish (MacDonald 2000). With increasing incorporation of plant-derived ingredients in salmonid feeds, ensuring sufficient supply and improving the availability of dietary Zn might be critical in ensuring proper bone health in farmed salmonids. Increasing the level of phytic acid in the diet of Atlantic salmon in freshwater had a dose-

dependent reduction in vertebral Zn concentration, which later correlated with increasing incidence of hyperdense vertebrae in seawater phase.

In osteology, zinc has an anabolic effect on osteogenesis by stimulating cell proliferation, ALP activity and collagen synthesis in osteoblastic cells, along with signs of improving Ca deposits in the extracellular bone matrix (Seo *et al.* 2010). The nutritional and physiological control of zinc homeostasis in animals is achieved by the coordinated activity of two families of Zn transport proteins, namely ZIPs (SLC39s) and ZnTs (SLC30s) (Lichten & Cousins 2009; Hogstrand 2013). Few of these Zn transport proteins have been identified and characterized in salmonids (Zheng *et al.* 2014). Mammalian studies provide strong evidence for involvement of Zn signalling in bone and cartilage formation. According to Fukada *et al.* (2013), mammalian bone formation is controlled by Zn signals affecting both chondrocyte and osteoblast functions through Zn transporters ZIP13, ZIP14 and ZnT5. Apart from these, ZnT1, ZIP4 and ZIP6 have also been involved in skeletal deformed phenotypes in mice (see Hogstrand 2013). In zebrafish, silencing of ZIP6 resulted in a phenotype referred to as 'dwarfed embryo' by Hogstrand (2013), wherein longitudinal migration of stem cells and extension of body axis during embryonic development were blocked resulting in shortened anterior–posterior axis (Yamashita *et al.* 2004). In salmonids, the mechanism driving the phenotype 'short-body dwarfism' reported under dietary Zn deficiency has not been studied. As these are observed in juvenile fish, it is less likely to be mediated through migration of cells during embryogenesis; nevertheless, the role of zinc in bone formation and development is very clear and needs to be better understood in salmonids and fish in general.

Selenium, selenoproteins and teratogenic deformities in salmonids

Selenium, as a nutrient, is required in fish diet at concentrations of about 0.1–0.5 $\mu\text{g g}^{-1}$ dry weight (Hodson & Hilton 1983; NRC 2011). In fish nutrition, selenium is largely viewed as a nutrient involved in antioxidant mechanisms and redox reactions in liver and blood cells; the role of Se in bone metabolism remains unexplored in farmed fish, more so in salmonids. Recent findings show that selenium in the form of selenoproteins plays a vital role in bone metabolism in higher vertebrates (Pietschmann *et al.* 2014; Zhang *et al.* 2014). About 25 selenoproteins in mammals and more than 32 selenoproteins in fish have been identified, the functions of many are yet to be understood (Lobanov *et al.* 2008). Among the different selenoproteins, mRNA expression of selenoprotein (SePP) in rainbow trout has been studied in response to dietary Se levels and forms (Fontagné-Dicharry *et al.* 2015; Pacitti *et al.* 2015, 2016).

However, these studies were not focused on the responses in bone metabolism. In mammals, SePP concentration in serum is associated with markers of bone turnover and bone mineral density (Hoeg *et al.* 2012) and is the essential Se transporter in bones (Pietschmann *et al.* 2014). Selenoproteins are important in the regulation of inflammation and bone cell proliferation/differentiation and the prevention of the bone resorption through the inactivation of osteoclasts (Zeng *et al.* 2013).

Selenium (Se) is known to have a narrow margin of safety between deficiency and toxicity in fish (Hilton *et al.* 1980; Lemly 1997). Teratogenic deformities in fish are congenital malformations due to excessive Se deposited in developing eggs, originating from parental diet. The embryo during development utilizes the Se-contaminated yolk which results in deformed hard and soft tissues due to substitution of Se for sulphur in structural and functional proteins. In short, teratogenic deformity is a permanent marker of Se toxicity in fish occurring due to excess maternal transfer of Se into eggs (Lemly 1997). Se toxicity-induced teratogenic deformity in fish is shown to have an exponential relation to egg selenium concentration (Lemly 1997). Analysis of data from three salmonid species (Holm *et al.* 2005; Hardy *et al.* 2010) suggests a similar relation to be true up to egg Se concentration of 10 $\mu\text{g g}^{-1}$. Moreover, species-specific variations in the sensitivity were also observed among the salmonid species (Fig. 7). In zebrafish, hypoactive locomotion and compressed spine of larvae were related to increase in maternal Se transfer (Penglase *et al.* 2014).

On the other hand, increased replacement of marine ingredients by plant ingredients reduces selenium levels in fish feeds (Fontagné-Dicharry *et al.* 2015; Antony Jesu Prabhu *et al.* 2017b), while current EU legislation limits Se supplementation beyond a total Se concentration of 0.5 mg kg^{-1} in complete feed. Recent findings, however, show that supranutritional levels of Se in diets of seabream larvae improved the transcriptional expression of bone morphogenic protein 4 (BMP4), ALP, osteocalcin, osteonectin, osteopontin, and matrix gla protein, suggesting improved skeletal development (Saleh *et al.* 2014). This is of significance in Atlantic salmon as extracellular matrix (ECM) components like col1a1, osteocalcin and osteonectin; BMPs, namely BMP2 and BMP4; and transcription factors like runx are involved in maturation and mineralization of osteoblasts (Ytteborg *et al.* 2010a). Overall, the role of selenium as a nutrient or a toxicant affecting bone metabolism in salmonids seems to be more directed through impact on maternal transfer of Se to eggs, embryogenesis and larval development (Holm *et al.* 2005). The possibility of Se interacting with other nutrients or environmental conditions to impact bone health during these critical life stages is also to be explored. Therefore, the

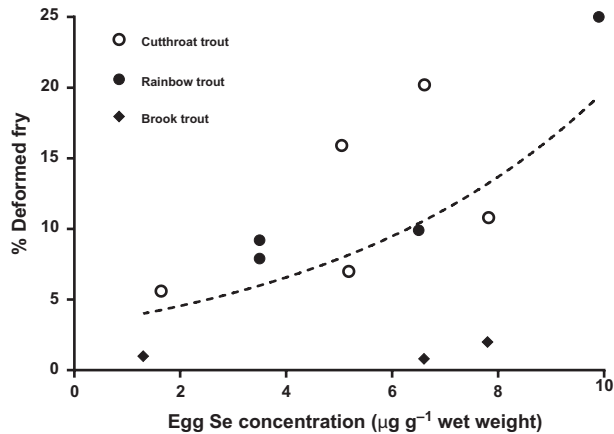


Figure 7 Selenium concentration of eggs and the associated prevalence of teratogenic deformities in three salmonid species, namely cutthroat trout (open circles) from Hardy *et al.* (2010), rainbow trout (dark circles) and brook trout (dark diamonds) from Holm *et al.* (2005).

knowledge on Se influencing bone health in mammals and modal fish species (Kupsco & Schlenk 2016) needs to be exploited to expand the understanding in salmonids.

Impact of iron on bone metabolism

Iron deficiency anaemia and pro-oxidant toxicity of excess iron are well known in animals and in fish. In salmonids, the nutritional significance, requirement and utilization of dietary iron are better studied in Atlantic salmon (Andersen *et al.* 1996; Maage & Sveier 1998; Naser 2000), whereas the knowledge on physiology of iron absorption and metabolic regulation is well understood in rainbow trout (Desjardins *et al.* 1987; Kwong & Niyogi 2008; Kwong *et al.* 2010, 2013). Nevertheless, the functional and physiological importance of iron in influencing bone metabolism in fish is far from recognized. In mammals, deficiency and excess of dietary iron play a vital part in bone formation and resorption, respectively (Medeiros *et al.* 2004; Katsumata *et al.* 2009; Tsay *et al.* 2010; Jia *et al.* 2012). Iron deficiency-induced retardation in bone formation was associated with downregulation of runt-related transcription factor 2 (*runx2*), osteocalcin and type I collagen in rat femur (Katsumata *et al.* 2016). Similar defects in bone formation characterized by reduced number of calcified vertebrae and downregulation of genes involved in bone formation (*runx2a*, *coll1a1a*, *bmp2a* and *bmp2b*) are reported in an iron-deficient model (*weh^{tp85c}*) of zebrafish (Bo *et al.* 2016). On the other hand, iron overload increases bone resorption (Tsay *et al.* 2010) through increased osteoclastogenesis and osteoclast differentiation as a consequence of ROS production (Jia *et al.* 2012; Xiao *et al.* 2015). In mammals, the cellular mechanism of iron overload affecting bone metabolism is proposed to be through

dysregulation of the BMP/SMAD signalling pathway, leading to low hepcidin levels, thus accumulation of iron in tissues (Parrow & Fleming 2014). Bo *et al.* (2016) also suggested that iron deficiency anaemia affects bone formation, potentially through the BMPs signalling pathway in zebrafish. It is therefore likely that iron deficiency or excess could have a fairly similar effect on bone metabolism in salmonids, as reported in zebrafish or mammals.

Manganese – essential for extracellular matrix formation

Manganese deficiency was demonstrated to affect skeletal development by resulting in short-body dwarfism in juvenile rainbow trout (Ogino & Yang 1980), but could not be observed in larger fish (Knox *et al.* 1981). In Atlantic salmon, Maage *et al.* (2000) observed developmental abnormalities of salmon fry fed Mn-deficient diets. The knowledge available thus far in salmonids or fish in general on Mn deficiency affecting bone health is restricted to gross phenotypic consequences. Studies with the objective of understanding the basic mechanistic role of Mn in bone metabolism are absent in fish. The mineralized ECM of salmon vertebrae is characterized by consisting of fibres, mainly collagen embedded in a matrix of proteoglycans (PGs) and proteins (Ytteborg *et al.* 2012). In mammals, Mn-dependent hydrolase and transferase enzymes, namely glycosyltransferase, xylosyltransferase, phosphohydrolase and phosphotransferase, are essential for synthesis of matrix proteoglycans (Dermience *et al.* 2015). Mn deficiency reduced the PG and mucopolysaccharide content in chick bones (Leach & Muenster 1962; Leach *et al.* 1969). Moreover, reduction in Mn-SOD activity also contributes to defective bone formation as osteoblasts are sensitive to free radicals generated by osteoclast activities. Although fish bones are different from that of chick bones, longitudinal growth retardation in chick tibia during Mn deficiency (Wang *et al.* 2015) appears to correlate with the dwarf phenotype observed in salmonids. Moreover, Mn deficiency also induces bone resorption by upregulating the expression of RANKL (receptor activator of nuclear factor kappa B ligand), an upstream regulator of genes (e.g. TRAP and cathepsin K) directly involved in osteoclast functioning (Liu *et al.* 2015). In summary, Mn deficiency can lead to impaired formation of ECM, retardation of chondrocyte differentiation and increased osteoclast activity, thereby resulting in stunted growth and resorption of bones.

Methodological considerations

Better understanding of mineral requirements

In mineral requirement studies of fish, the major response criteria are growth, target tissue element status, and biochemical enzyme activities responsive to the element. These

criteria are very effective but are relatively late in responding. During the last decade, an array of sensitive markers and approaches are made available and this gives the opportunity to re-examine mineral requirements, similar to evaluations of upper tolerable concentrations of the elements (Waagbø 2006). Despite the fact that Atlantic salmon is among the major farmed species, mineral requirement studies are relatively few, especially in the present era of dynamic changes in dietary composition and culture conditions. Dietary phosphorous requirement represents a good example in Atlantic salmon with risk of marginal feed P levels resulting in poor mineralization and bone deformities in both controlled experiments and commercial farm operations. Based on the analysis of the meta-data from over 70 different P requirement studies covering about 40 fish species, it was found that vertebral or bone mineral concentration was the most appropriate criteria for determining the requirement of P, Ca, Zn and Mn (Antony Jesu Prabhu *et al.* 2013). It is interesting to note that all the four minerals (P, Ca, Zn and Mn) play a critical role in bone metabolism and skeletal deformities of one form or other have been associated from deficient or suboptimal dietary supply, except for Ca.

Mineral requirement data available with salmonids often include the use of semi-synthetic diets based on refined ingredients, which normally do not support growth equivalent to practical diets, thereby limiting practical applicability of data (Antony Jesu Prabhu *et al.* 2016a,b). A meaningful application of such experimental data for practical intensive conditions was discussed by Tacon (1995). Moreover, the fact that knowledge on mineral requirements of fish, which are more often than not based on dietary concentration, also limits the validity of the data when dietary composition changes. Nutrient requirement of an animal is directly related to its growth, be it somatic or gonadal. In aquaculture production, somatic growth is of prime interest and hence expressing mineral requirements per unit weight gain would enable requirement estimates to be more objective. As weight gain is dependent on feed intake and efficiency which in turn is governed by the digestible energy (DE) of the feeds, expressing mineral requirements per unit DE has also been proposed (Rodehutsord 1996). Further, the relation between weight gain and the absolute content of nitrogen (N) or phosphorus (P) in salmonid is relatively well conserved and found to be linear (Bureau *et al.* 2003). This relationship between weight gain, P and N content could also be exploited to express mineral requirements based on unit N gain. Expressing mineral requirements on g kg^{-1} weight gain or g MJ DE , or mg g^{-1} N gain, on available basis would be more appropriate to avoid the variations arising due to difference in diet composition, feed efficiency and growth rate (Antony Jesu Prabhu *et al.* 2013).

Although knowledge on mineral requirements of salmonids is available, studies aimed at determining the optimal inclusion or supplemental level of minerals to practical formulations in order to ensure proper mineralization are required; at present, such studies are very limited. Recently, few such studies have been conducted to re-evaluate the mineral requirements of rainbow trout when fed diets completely devoid of fishmeal (Read *et al.* 2014; Antony Jesu Prabhu *et al.* 2015, 2017b). These studies report changes in basic metabolism and utilization of minerals when fed complete plant-based diets, thereby altering dietary requirements (Antony Jesu Prabhu *et al.* 2014, 2015, 2016a; Fontagné-Dicharry *et al.* 2015). In animal feeds, practical recommendations should cover safe levels (sufficient but not toxic) and at the same time consider variable farming conditions. The mineral requirement recommendations by NRC (2011) were inadequately updated, and hence, it becomes important to establish or re-evaluate mineral requirements for salmonids, especially Atlantic salmon in seawater.

Methods for studying aetiology of bone disorder in salmonids

The research interest in fish skeletal biology has grown tremendously in the past couple of decades with the emergence of skeletal malformations as a welfare issue in fish farming. This has resulted in a comprehensive insight into bone matrix and cell biology; moreover, functional -omic studies have identified critical players in normal bone formation and remodelling (Le Luyer *et al.* 2014a). The preferred method for fish skeletal deformity diagnostics has for long been radiography. Radiography allows the evaluation of calcification level and for the identification of pathology in the bones. Repeated X-ray of individually tagged fish has provided insight into the pathogenesis, as well as the potential for healing. It is important to detect bone pathology as early as possible in the fish ontogeny. However, radiography has its limitations in diagnosing early stages of malformation leading to visible deformity. Mechanical competence of bone and hence its risk of fracture and deformities are dependent on the bone mass, matrix architecture and mineral content, mineral crystallinity and collagen cross-link ratio (Currey 2003). Histological staining techniques and mineral content have commonly been used to analyse morphology and mineral status in teleost bone, respectively. However, histological staining (e.g. von Kossa or Alizarin red S staining) has been shown to be unsuitable for the detection of small quantities of mineralized matrix (Bonewald *et al.* 2003). Thus, important information may be ignored. For example, the composition of HAP crystals and calcium phosphates in salmon bone formed during a period of mineral deficiency seems to be different from that formed when mineral supply is adequate (Ytteborg *et al.* 2012); however, chances of detecting these changes through

general staining are very limited. Various techniques have been applied for evaluating the quality of bone, including densitometry for assessment of bone mass and density, computed tomography for evaluation of bone microarchitecture, serum biochemistry for measurement of bone remodelling and histomorphometry for assessment of cellular activity. Grotmol *et al.* (2006) used electron microscopy to study bone quality during development and Totland *et al.* (2011) employed microcomputed tomography and compression to analyse bone mineral content and mechanical properties after exercise in the salmon vertebra. In humans, Fourier transform infrared (FTIR) spectroscopic imaging has been a useful tool in the characterization of disease-related changes in mineralized tissues (Boskey 2003; Faibish *et al.* 2005). Analyses using FTIR spectroscopic imaging of salmon bone to detect architectural changes are in progress, providing a strong basis for expanding the scope of FTIR to studies of skeletal pathology related to dietary mineral supply in teleost. Molecular markers and gene expression techniques are commonly used to study bone remodelling in mammals and teleost. The correlation between gene expression responses, histochemical observations and incidences of deformities exemplifies the potential of using molecular markers as early warning signals for understanding different pathogenesis. Immunohistochemistry gives valuable information about the distribution and localization of proteins involved in bone and cartilage development, and TUNEL, ALP, TRAP and other enzymatic assays have been successfully applied to study bone resorption and metabolism (Witten 1997; Ytteborg *et al.* 2010a,b,c; Andrade *et al.* 2011).

Recently, several potential biomarker genes, directly involved in bone and cartilage development and ossification pathways, were identified in salmon (Ytteborg *et al.* 2010a,b,c). Gene markers and mRNA-based techniques (qPCR and *in situ* hybridization techniques) have further been used to describe transcription profiles of osteoblasts and chondrocytes involved in developing vertebral fusions (Ytteborg *et al.* 2010b) and mechanisms involved in vitamin deficiency and temperature-induced deformities (Ornstrud *et al.* 2009; Ytteborg *et al.* 2010b), and to survey normal bone development in general (Krossøy *et al.* 2009; Ytteborg *et al.* 2010a). The use of *in vitro* studies is an alternative method applied to further unravel the underlying mechanisms of bone development (Le Luyer *et al.* 2014a). Some obvious advantages of such methods over experiments conducted *in vivo* include the ability to have more control on the cells being assessed and their environment, the relative ease of sampling and analysing changes in the parameters applied under investigation and the ability to reduce animal experiments. Teleost *in vitro*-based osteoblast systems are being developed, where cellular differentiation and lineage determination can be studied in more

controlled environments (Miyake & Hall 1994; Rafael *et al.* 2010; Ytteborg *et al.* 2010d). *In vitro* systems based on teleost scales have also been developed and used to study, for example osteogenesis and hormonal regulation (Yoshikubo *et al.* 2005). Similar systems are currently being developed for salmon. These systems may be further developed and used for quick and inexpensive screening of dietary components as well as for more advanced studies, like fluorescence marked reporter gene constructs and incorporation of radiolabelled isotopes to follow minerals from cellular uptake to secretion of matrix-producing vesicles and mineralization. However, a thorough understanding of the model systems employed is required to gain better understanding from the laboratory experiments. Studying the specific roles of particular components in the processes will provide novel and important information on the mechanisms involved in skeletal formation in fish and help develop strategies to prevent bone deformities.

Novel methods for determination of fish mineral status

Mechanical strength of single vertebrae. There is a close correlation between the mechanical yield load and stiffness and total ash content of Atlantic salmon vertebrae (Fjelldal *et al.* 2006, 2009). The mechanical strength of teleost vertebrae is tested in a compression jig wherein a load is applied along the cranial–caudal axis of the vertebrae, and the resulting deformation is continuously recorded (Fjelldal *et al.* 2004). These data can be used to calculate the mechanical stiffness, yield load and resilience. Of these measures, the stiffness is the easiest to calculate, and in Atlantic salmon postsmolts also the most closely associated with mineralization when different fish groups are compared (Fjelldal *et al.* 2006). The first authors that measured biomechanical properties of teleost vertebrae recorded it as stress and strain (Hamilton *et al.* 1981), where stress is the force per unit area and strain is the percentage change in length. This approach is better adopted for uniform specimens than for whole bones (Cullinane & Einhorn 2002) and later authors have not used this conversion when whole vertebrae are tested (Fjelldal *et al.* 2004, 2006, 2009). Unfortunately, reference values on normal biomechanical properties of Atlantic salmon vertebrae at different fish sizes are lacking; the ‘unconverted’ mechanical strength of the vertebrae will increase with fish size. With good reference stiffness values for properly mineralized and morphologically normal and undeformed vertebrae at different fish sizes, the compression test could be used as a quick, cheap and accurate tool to evaluate the mineral status of farmed Atlantic salmon.

Radiography. Radiography (X-ray) is the classical method for evaluation of skeletal pathology. A correct radiographic diagnosis will, however, rely on image quality as well as

diagnostic skills. Fish radiography suffers from low contrast of ossified tissues in fish, as well as the small size of the individual structures. Witten *et al.* (2009a) identify technical X-ray as the method giving the highest quality images. Practical experience demonstrates that use of a mammography source is preferable in fish smaller than 100 g since is better suited to image low contrast tissues in small and soft structures like bones of small fish. In fish larger than 100 g, standard equipment sufficient adjustment of key settings may be adequate (Hjelde & Baeverfjord 2009). While both analogue and digital set-ups are in use, digital set-ups allow for image enhancement to improve image quality and evaluation, as well as allowing for morphometric measurements and analyses. In farmed Atlantic salmon, twenty different types of bone pathology are suggested, of which many have been observed in studies related to mineral deficiencies (Witten *et al.* 2009a). Further development and refinement of diagnostic criteria for skeletal pathology associated with low mineralization and specific deficiency conditions are strongly warranted. MicroCT is a highly specialized instrument based on X-ray principles, which provides high-quality images in 3D (Totland *et al.* 2011). MicroCT The method is expected to remain limited to specialized scientific purposes, due to both availability and cost and time required per analysis. For practical purposes, fish radiographies rarely are of a quality which allows for quantitatively reliable judgement of mineral saturation of bone. Bone densitometry, commonly used in human medicine to measure mineral status of bone, is based on X-ray technology. Similar to standard radiography, a need for special adaptation of such methods and instruments is expected if used on fish.

Resource use efficiency of minerals in salmonid farming

The utilization efficiency of dietary minerals (especially P) in salmon feeds has been under critical scrutiny over the years. The situation has been even more relevant in the recent years with the challenge of transition from marine ingredients. Phosphorus is a nutrient of bone health and environmental significance, with reduced availability of plant-derived feed ingredients. Supplementation of inorganic phosphorus to salmonid feeds has been inevitable to meet the dietary requirement of fish for maximal weight gain as well as proper bone mineralization. The challenge however has been to 'improve bone health with minimal environmental impact'. Phosphorus from feed loss, faeces and urinary excretion is the major component in the effluents from aquaculture industry, which contributes to local eutrophication (Einen *et al.* 1995; Temporetti & Pedrozo 2000). The metabolic loss of P in salmonids is 0.02–0.07 g P kg⁻¹ fish day⁻¹ (Bergheim *et al.* 1982), which

includes 5–10% of endogenous P loss (Antony Jesu Prabhu *et al.* 2015). A theoretical estimation using dietary input of 1% feed (with 15 g P kg⁻¹ feed) of the body weight day⁻¹ for a 1 kg salmon and a P digestibility of 40% means that available P from diet per day is 0.06 g P. This is in the range of the reported daily excreted amounts, indicating that the metabolism of P is under a close homeostatic regulation (Vielma & Lall 1998a). The understanding of phosphorus utilization in salmonid feeds is well studied and mathematical models are available to predict and thereby limit P discharge (Bureau & Cho 1999; Bureau *et al.* 2003; Hua & Bureau 2006). Phosphorus is a vital yet limited and nonrenewable resource for life on earth. The ever-increasing global population and the need to feed the billions had put global P availability at stake (Cordell *et al.* 2011). As regard to the use of phosphorus resources, about 89% of the global phosphorus is used for food production, 7% in animal feeds and 82% as fertilizers (GPRI, 2010). The overall environmental load derived from aquaculture is comparatively small when compared to runoff from communities, industries, agriculture and forestry. At least in Norway, phosphorus levels in salmon feeds and in effluents are being gradually reduced (Fig. 8). For instance, release of phosphorus from salmon farms in Norway has decreased from 10 kg P ton⁻¹ of fish produced in 1990 (Ackefors & Enell 1994) to 8.45 kg P ton⁻¹ of fish produced in 2012 (ca. from Ytrestøyl *et al.* 2015), which is about 15%. Nevertheless, about 12 046 and 15 011 tons of phosphorus were used in salmon feeds in 2010 and 2012, respectively, of which 72% was lost to the environment and an additional 15–16% was lost in post-harvest trimmings (Ytrestøyl *et al.* 2015). These data warrant the necessity for improving the phosphorus use efficiency in salmon farming through increase in bioavailability, retention of dietary phosphorus and maximizing phosphorus recovery from effluents and trimmings.

Detailed quantitative information on the budgeting of minerals other than phosphorus is lacking in fish farming. However, level of microminerals in Norwegian fish feeds have generally decreased during the decade from 2000 to 2010 without compromising on the dietary levels needed to meet the requirement of fish (Sissener *et al.* 2013). This indicates that the micronutrient load into the environment would have also decreased. Moreover, more use of organic sources of microminerals is anticipated in the future, in view of a better relative bioavailability over inorganic forms. Organic minerals have demonstrated their potential in livestock and poultry; however, their practical utility and superior bioavailability over inorganic forms are far from convincing in fish. An empirical analysis of metadata from published reports showed large variations in the bioavailability of organic forms, except for Se (Antony Jesu Prabhu *et al.* 2016b). The metadata included responses such as

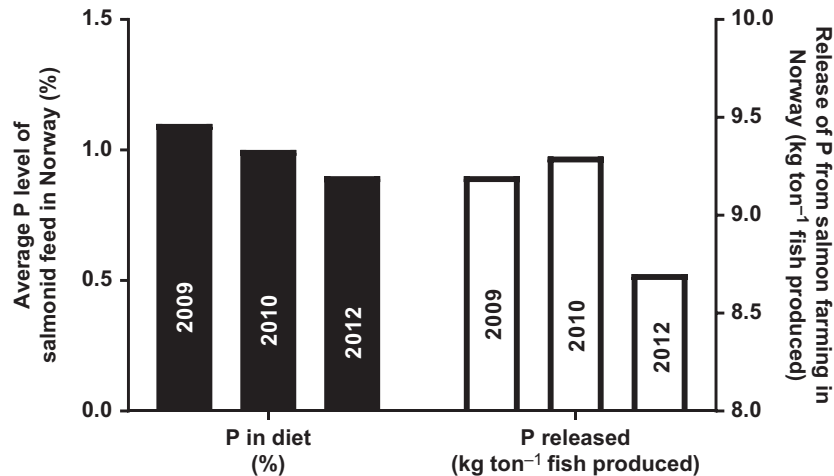


Figure 8 Average dietary phosphorous level of salmon feeds (black bars; % of diet, left y-axis) and the rate of effluent phosphorus released from salmon production (white bars; kg P ton⁻¹ fish produced, right y-axis) in Norway during 2009 (Wang et al. 2012), 2010 (Ytrestøyl et al. 2011) and 2012 (Ytrestøyl et al. 2015).

apparent availability and retention; however, analysis on absorption pathways, physiological and cellular mechanisms at sites of absorption, cellular trafficking and target delivery will be more pertinent in deciding the relative efficacy of different mineral sources. Improving the bioavailability of minerals in fish feeds is the most sustainable and eco-friendly approach to ensure proper bone health and reduce environmental impact of aquaculture. In terms of improving dietary mineral availability, use of phytase can not only improve the availability of phytate-P found in plant ingredients, but also of other microminerals like Zn. However, the limitation of phytase in salmon feeds has been their efficiency at low temperatures; hence, a low-temperature-adapted phytase could be of better use in salmonids. Genetic selection and transgenesis can be potentially employed to improve mineral availability and utilization efficiency in fish. *Nile tilapia* and Japanese medaka transgenic to *Aspergillus niger* phytase gene showed better utilization of dietary phytate phosphorus (Kemah 2004; Hostetler et al. 2005). On the other hand, attempts to recycle P lost in post-harvest trimmings are underway though research aiming at developing P supplements from fish bones (Ytteborg et al. 2016). Further research should focus on improving availability of ingredient bound minerals and use low levels of mineral supplements of better availability with less environmental impact.

Concluding remarks

In the present era and more so in the future, the key to sustainability of salmonid aquaculture rests on, how well, healthy animal production and environmental safety are managed without compromising faster growth. It is

indispensable to realize that dietary nutrient levels required by fish will be altered with the dynamic changes occurring in feed composition, genetic improvement of stock, fast-growing transgenic varieties, etc. Lall and Lewis-McCrea (2007) suggested that attempts to characterize skeletal deformities in fish will be adversely affected by limited consideration for nutrient deficiencies. In the literature, among dietary factors, dietary phosphorus has been of major focus in understanding the aetiology of skeletal deformities in Atlantic salmon and rainbow trout production. However, how much of available P should be supplied to obtain harmonious skeletal and muscular growth still remains unanswered. On the other hand, the effect of increasing dietary P levels also results in increased excretion of P emphasizing the need to identify strategies to improve P utilization in salmonids. Efforts are already underway and it is evident that the amount of P released per unit quantity of fish produced has decreased over the years, although the total quantity released from salmon farming is increasing due to increasing production. It is of utmost importance that phosphorus use efficiency is improved through increase in bioavailability and retention of dietary phosphorus in salmonid farming and fish farming in general.

The effect of other macro- and microminerals for commercial fish production appears less clear, and data available at present are weak and needs to be strengthened. Few studies on dietary requirement for essential minerals in salmonids of more recent date are conducted. The earlier estimates are lacking in relevance to counter new challenges faced. Moreover, transition of requirements estimated from short-term and small-scale experiments with semi-purified diets mostly on juvenile fish to operational practical recommendations for entire life cycle is needed. Major shifts in

fish feed ingredient profiles during recent years call for increased attention to mineral contents and availability in diets. In particular, the increase in use of plant ingredients is expected to increase the need for balancing the dietary levels of certain minerals. Similar consideration applies to high ash raw materials of animal origin. More knowledge on how to treat and use relevant raw materials in order to improve availability of minerals is strongly warranted, in particular how to use novel ingredients safely over long term. Maximum limits have been established in the EU for a range of essential microminerals in fish feeds. There is a need to assess the potential benefits of improving bioavailability through organic sources or using higher levels of certain essential microminerals to improve bone health in salmonids, especially towards better fish welfare in seawater phase. In recognition of the complexity of fish mineral nutrition through the production cycle, there is a need to update methodology to record mineral requirements, fish mineral status and their biological functions, like skeletal development across life stages. The potential of developing and using *in vitro* methods and biochemical (molecular, proteomic, metabolomic, and ultrastructural tools) tools needs further validation for a clear demonstration on their usefulness to study the main issues related to 'bone deformities'.

Acknowledgements

Funding for this work was supported by two research grants (i) Norwegian Seafood Research Fund (FHF), grant no. 900661; (ii) Norwegian Research Council, grant no. 244490. The constructive comments of the anonymous reviewers is also acknowledged.

Author contributions

All authors contributed equally in manuscript writing. The corresponding author in addition contributed towards overall structuring and content editing.

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