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# The Atlantic Salmon (*Salmo salar*) Vertebra and Cellular Pathways to Vertebral Deformities

Elisabeth Ytteborg<sup>1</sup>, Jacob Torgersen<sup>1</sup>,  
Grete Baeverfjord<sup>1</sup> and Harald Takle<sup>1,2</sup>

<sup>1</sup>Nofima AS, Norwegian Institute of Food,  
Fisheries and Aquaculture Research

<sup>2</sup>AVS Chile S.A.

<sup>1</sup>Norway

<sup>2</sup>Chile

## 1. Introduction

Stagnating fish stocks and a growing population demanding for aquatic food products have been the major driving forces behind the rapid increase in aquaculture production. Federation of European Aquaculture Producers (FEAP) estimates that 650 000 tons of fish are farmed in the EU annually (compared to 60 000 tons in 1970). Within Europe as a whole, the total production is more than 1.6 million tons (FEAP, 2009). Norway is a major contributor to Europe's aquaculture sector with over 860 000 tons of Atlantic salmon (*Salmo salar*) and trout produced each year, a production that has been more than doubled the last ten years (Directorates of fisheries, Norway 2009). Forecasts predict that production will need to increase for decades to come if demands are to be met (Brugere & Ridler 2004). To keep up with the growing demand, the aquaculture industry is constantly searching for new strategies to improve the rearing conditions and reduce production time and cost. However, as a relatively new industry, and as a consequence of intensified production regimes, the aquaculture sector faces growth constraints.

Farmed salmon is bred for rapid growth, and the industry aim at obtaining the optimal growth rate by optimizing both diets and environmental factors accordingly. However, intensive rearing conditions are linked to increased occurrence of production related diseases and malformations. Elevated temperature during the fresh water period was commonly used in the 90`ies to speed up developmental rate. An increasing number of fish developing manufacturing defects, such as skeletal abnormalities (figure 1), heart failure and jaw deformities was observed. Recommendations limiting temperatures to safe levels,  $\leq 8^{\circ}\text{C}$  during egg rearing and  $\leq 12^{\circ}\text{C}$  after fist feeding, led to substantial reductions in skeletal malformations (Baeverfjord et al., 1999). However, in the last few years, the start feeding temperature has been increased again, due to the stakeholders demand for reduced production time. Further, the growing need of replacing fish meal in commercial fish feeds have come into focus and deformities related to feed ingredient replacements, malnutrition and mineral deficiency are investigated.



Fig. 1. Deformed (top) and non-deformed Atlantic salmon and corresponding radiographic pictures. Photo: Grete Baeverford, Nofima.

In the present situation, fast growth in combination with unpredictable and potentially low bioavailability of nutrients is considered the main challenge for adequate skeletal development. Suboptimal supply of minerals (phosphorous, magnesium, zinc) and nutritional imbalances of fatty acids, vitamins (A, C and D) and amino acids are considered the main challenges in regard to skeletal malformations. The challenges related to bioavailability are further amplified with the introduction of vegetable meals, some of which are rich in antinutrients (e.g. phytic acid) that may further impair absorption. It is therefore important to completely understand the molecular and cellular events in bone development in salmon in order to deal with upcoming questions

Most of the knowledge currently available on cellular mechanisms for bone development is adopted from studies using mammalian species. However, information from teleosts, like zebrafish (*Danio rerio*), sea bass (*Dicentrarchus labrax*), sea bream (*Sparus aurata*) and Atlantic salmon and related *in vitro* studies, is emerging. Molecular tools like *in situ* hybridization, microarray, real time quantitative PCR, immunohistochemistry and cell culture systems have allowed researchers working with teleosts to do more comparative and functional studies. Also, transgenic model organisms, such as zebrafish, and *in vitro* transfection with reporter gene constructs are now being more common and will provide valuable information on processes involved in bone metabolism. Relevant examples include studies on gene expression of several bone and cartilage associated marker genes, such as bone morphogenetic protein 2 (BMP-2) (Rafael et al., 2006), osteocalcin (Wargelius et al., 2009; Ytteborg et al., 2010b), osteopontin (Fonseca et al., 2007), vitamin D receptor (Lock et al., 2009), parathyroid related hormone (Flanagan et al., 2000) and proteoglycans (Pedersen et al. 2010; Conceição et al., 2008). The Sea bream vertebrae cell lines described by Braga et al. (2006) as well as two reports on zebrafish (DeLaurier et al., 2010; Kimmel et al., 2010) show the possibilities with fluorescent reporter gene constructs in bone research. The work developed during the last few years has provided clear evidence that fish can be adequate supplementary model systems to study bone and cartilage biology. Teleosts have been successfully used to analyze molecular and cellular mechanisms involved in different developmental pathways and revealed that the key genetic factors regulating lineage determination and differentiation of stem cells are conserved among vertebrates at the molecular level in both sequence and expression pattern (Kikuta et al., 2007; Shafizadeh et al., 2004; Nakashima et al., 2003; Aubin, 1998; Pinto et al., 2001; Renn et al., 2006; Wise et al., 2006; Ytteborg et al., 2010a). Due to the similar physiologic pathways and genetic

background of fish and mammals, this alternative system is also an interesting model to unveil some of the molecular determinants of human bone related diseases and malformations, like osteogenesis imperfecta, degenerated disc disease, persistent notochordal canal and scoliosis (Gorman and Breden, 2007; Nissen et al., 2006; Fisher et al., 2003). A number of animal models have been used to explore the pathology of spinal deformities and revealed that vertebral pathology presents a complex but comparable cross species etiology. With regard to complex disorders in humans, multiple models are critical for the investigation and manipulation of etiological factors.

Fish systems could be of benefit to vertebral research because they exhibit a diverse range of deformities, are free from skeletal appendices and substantial genomic resources have been developed for several species. Skeletal deformities in commercial salmon production have been recognized as a problem of obvious relevance to economy as well as animal welfare. Much effort has been put into understanding malformed development of Atlantic salmon vertebrae during the years due to the importance of this organism to the aquaculture industry. As a consequence, Atlantic salmon is emerging as an excellent model to study vertebral deformities and other relevant vertebral pathological states. In this review the current knowledge on the cellular and molecular mechanisms for skeletal homeostasis in the mature Atlantic salmon vertebrae is discussed. Further, the cellular mechanisms for differentiation and activation of osteoblasts and chondrocytes are described in relation to pathways for pathological development and discussed in the light of related pathological conditions in mammalian species.

## **2. Cellular and molecular mechanisms controlling bone formation**

Bone formation basically occur via two mechanisms in both mammals and teleosts: mesenchymal stem cells (MSC) either differentiate directly into bone producing osteoblasts (intramembranous ossification) or by first forming a cartilaginous template secreted by chondrocytes which is later replaced by bone (endochondral ossification) (Erlebacher et al., 1995). However, similarities and differences in tissue structure between teleost and mammalian bone have been described (Witten et al., 2009; Huysseune et al., 2000). In general, fish possess rather few long bones with growth plate-like arrangements exhibiting typical endochondral bone formation as seen in mammals. In the Atlantic salmon vertebrae, compact bone of the amphicoel and trabeculae is formed directly through intramembranous ossification, whereas the arch centra are modelled through endochondral ossification. Both mechanisms lead to the formation of mineralized extracellular matrix (ECM), consisting of fibers, mainly collagen embedded in a matrix of proteoglycans (PGs) and proteins. An overview of the two different processes in the Atlantic salmon vertebra is shown in figure 2a and b.

### **2.1 Lineage determination and cellular differentiation**

The cellular lineage determination and differentiation of osteoblasts and chondrocytes from the MSC lineage are determined by a number of transcription factors, regulatory mechanisms, environmental conditions and mineral availability. The pathways are interconnected during vertebral formation and must be coordinated. In particular, the transcription factors Runx2, Osterix, Sox9, Twist and Mef2c have distinct functions both in the establishment of the vertebral bodies and later in the differentiation and maturation of

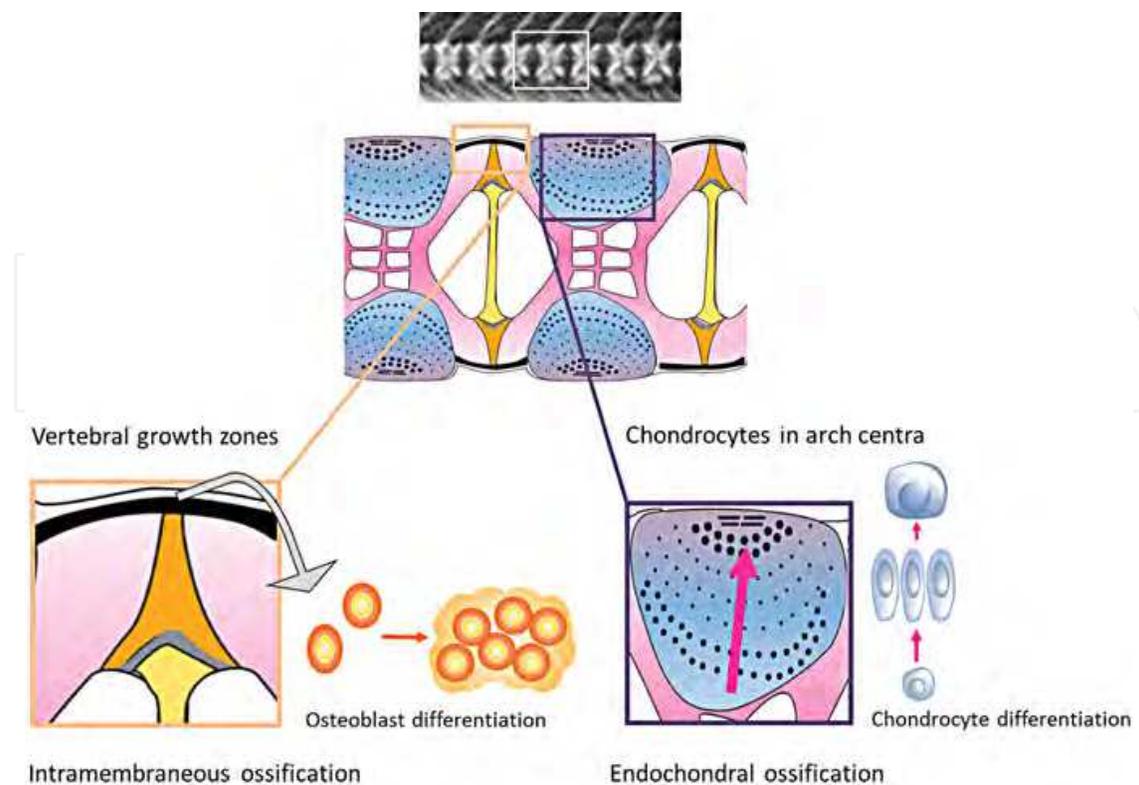


Fig. 2a. Overview of the intramembraneous and endochondral ossification in the Atlantic salmon vertebra. See the text below for details.

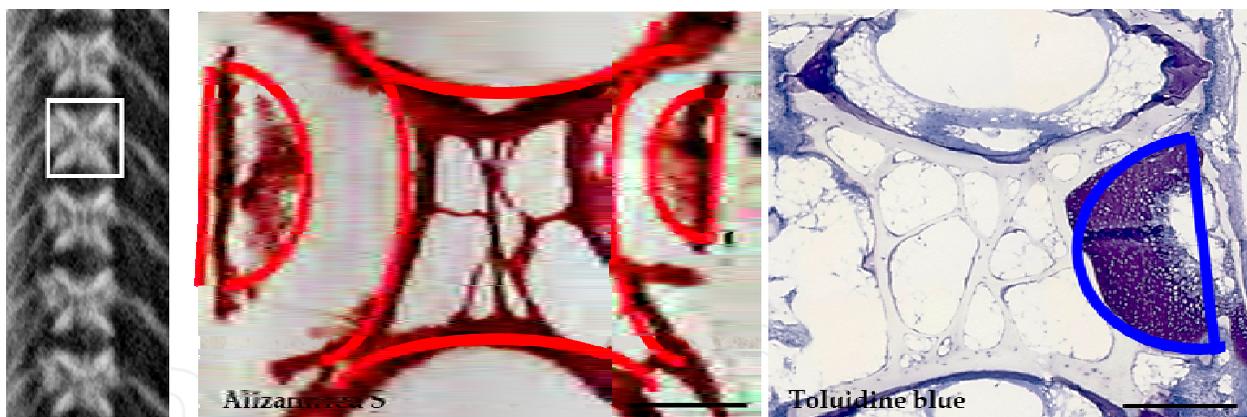


Fig. 2b. Bone and cartilage in the Atlantic salmon vertebra. Both endochondral and intramembraneous ossification leads to mineralized bone formation (red - Alizarin red S staining of bone in the centra and arches; blue - Toluidine blue staining of cartilage in the arches). See text below for further details on bone and cartilage formation. Vertebrae from 15g fish, scale bar= 200  $\mu$ m

specific skeletal cell types (Karsenty et al., 2009). Similarly, signaling molecules like bone morphogenetic proteins (Bmp2 and Bmp4) and hedgehog proteins (Ihh and Shh) play different roles both during cell differentiation and skeletal tissue ontogeny (Karp et al., 2000; Hogan et al., 1996; Spinella-Jaegle et al., 2001). Important signalling pathways that induce transcription of matrix producing and mineralizing genes in osteoblasts and chondrocytes include the downstream targets of Bmps; Runt-related transcription factor 2 (Runx2) the

zinc finger containing transcription factor Osterix and Sex determining region Y box 9 (Sox9). Whereas Runx2 and Osterix activates genes in the osteoblastic lineage (Karsenty et al., 1999; Otto et al., 1997; Nakashima et al., 2002), Sox9 regulates transcription of chondrocytic genes (Bell et al., 1997).

The differentiation of MSC into mature osteoblasts involves several phases, which may be divided into three subsequent stages; commitment, extracellular matrix production and mineralization. Estrogen and 1,25-dihydroxy vitamin D<sub>3</sub> are among the hormones shown to increase osteogenic differentiation via up-regulation of osteogenic growth factors, such as BMP2. Among the many transcription factors expressed early in osteogenesis, runx2 is noteworthy because it is required for bone formation and is an important early indicator of osteogenic capacity of cells. Downstream targets of Runx2 and Osterix include genes encoding both collagenous (e.g. Collagen 1 $\alpha$  and 1 $\beta$ ) and non-collagenous (e.g. Osteopontin, Osteocalcin, Osteonectin, Bone sialoprotein and Alp) proteins, which make osteoblasts capable of producing and mineralizing bone matrix (osteoid). In both teleosts and mammalian MSCs, alkaline phosphatase (Alp), col1a and osteopontin serve as useful markers of early osteogenesis and the expression of these genes usually increases throughout maturation. Col1 is the major structural component of bone, whereas the non-collagenous proteins binds inorganic minerals and are involved in the mineralization process (Cowles et al., 1998; Ikeda et al., 1992; Bolander et al., 1988; Termine et al., 1981). Upon maturation, osteoblasts start secreting osteoid and mineralizing components, leading to direct formation of bone via the intramembraneous ossification pathway. The key markers involved in osteogenesis are shown in Figure 3.

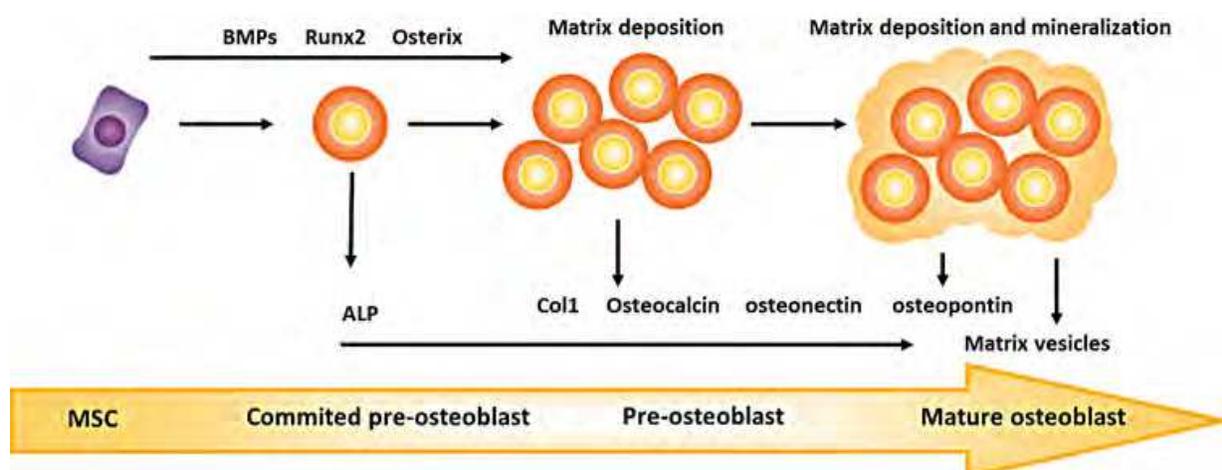


Fig. 3. Osteoblast differentiation, maturation and key factors involved. After commitment to the osteoblast lineage, matrix deposition starts. Mature osteoblasts are responsible for both osteoid production and mineralization. See text for details.

The chondrocytes undergo a more synchronized process of proliferation, differentiation and maturation so that three pronounced zones can be identified in the growing cartilage: resting, proliferating and hypertrophic zones (Hunziker et al., 1994). Chondrocytes in the resting zone are irregularly scattered in cartilage matrix, whereas chondrocytes in the proliferating and hypertrophic zones are arranged in columns. The chondrocytes in the resting zone serves as stem-like cells in the growth plate, stimulated by e.g. growth hormone (GH) and insulin like growth factor (IGF). The proliferating zone is the region for active cell

replication and chondrocytes in this zone are mostly devoted to cell cycle processes. Chondrocyte hypertrophy is the final step of chondrocyte maturation, regulated by the transcription factors Myocyte enhancer factor 2c (Mef2c) and Runx2 (Arnold et al., 2007; Kim et al., 1999). Parathyroid hormone related protein (PTHrP) and Ihh appear to play important roles in proliferating chondrocytes by maintaining cells in a proliferative condition, hence preventing chondrocyte hypertrophy. After commitment to the hypertrophic state, chondrocytes start expressing Col10 (Ytteborg et al., 2010b; Arnold et al., 2007), a unique component of the matrix produced by hypertrophic cells and extensively used as a marker for chondrocyte hypertrophy (Iyama et al., 1991). Once hypertrophy is reached, endochondral ossification can be initiated (Mackie et al., 2008). Hypertrophic chondrocytes induce angiogenesis by secreting angiogenetic factors, such as the Matrix metalloproteinases (Mmps) and Vascular endothelia growth factor (VEGF) so that osteoblasts and osteoclasts may enter via newly formed blood vessels (Blavier et al., 1995). The key markers involved in chondrogenesis are shown in Figure 4.

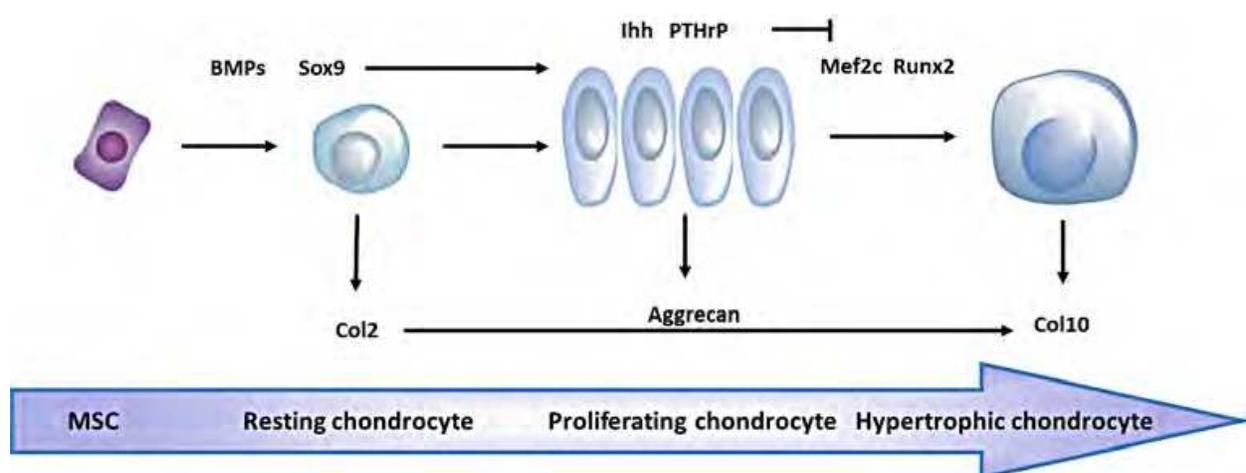


Fig. 4. Chondrocytic differentiation, maturation and key factors involved. Resting, proliferating and hypertrophic chondrocytes are clearly visible as zones in the growth plate. See text for details.

Osteoclasts are cells involved in removing damaged bone, repair mechanisms, mineral homeostasis and replacement of cartilage with bone, both in mammals (review in Boyle et al., 2003) and teleosts (reviewed in Witten et al., 2009). Osteoclasts provide an acidic environment where mineralized matrix may be dissolved through secretion of cathepsins, mmps and tartrate resistant acid phosphatase (TRAP) (Delaisse et al., 2003; Motyckova et al., 2001; Ortega et al., 2003; Engsig et al., 2000). As in mammals, osteoclasts in Atlantic salmon are multinucleated and the mechanisms involved in activation and differentiation of osteoclasts are conserved (review in Witten et al., 2009). Mononuclear cells respond to macrophage colony stimulating factor (M-CSF) produced by nearby stromal cells and osteoblasts, through activation of c-fms, the receptor for M-CSF (Wiktorjedrzejczak et al., 1990; Yoshida et al., 1990). The other signaling system essential for osteoclast differentiation is triggered when receptor activator of nuclear factor kappa ( $\kappa$ ) B ligand (RANKL), a member of the tumor necrosis factor (TNF) family, activates its receptor RANK (reviewed in Collin-Osdoy et al., 2004). Among the downstream genes of RANKL are genes directly involved osteoclast function (e.g. TRAP and Cathepsin K). The key markers involved in

osteoclastogenesis are shown in Figure 5. In addition, mononucleated osteoclasts are also found in both mammals and teleosts and are considered to participate in minor, fine tuning bone resorption (Witten et al., 2009). However, since teleost lack haemopoietic tissue in bone marrow, the question of the origin of these cells remains unknown. In the vertebrae of Atlantic salmon, multinucleated osteoclasts have been identified in the arch centra and trabeculae but not in the compact bone of the amphicoel (Witten et al., 2009).

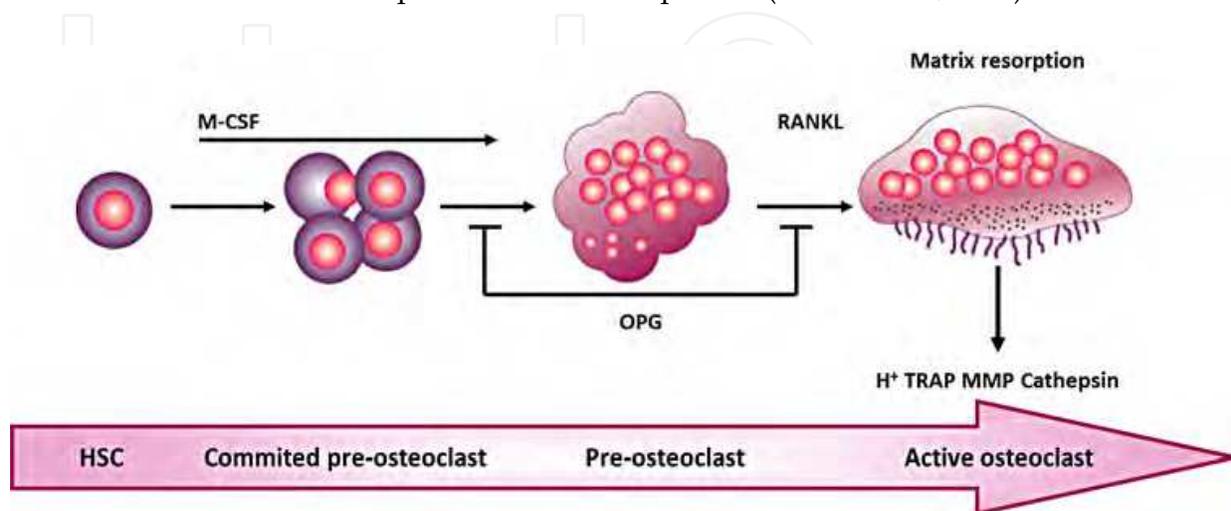


Fig. 5. Osteoclast differentiation, maturation and key factors involved. Fully mature osteoclasts are able to dissolve bone. See text for details.

## 2.2 Matrix mineralization

Skeletal formation and growth occurs as a result of mineralization of ECM. A time lag where collagen synthesis decreases and mineralization increase appears to be required for allowing modifications of the osteoid so that it is able to support mineralization and hydroxyapatite ( $\text{Ca}_{10}[\text{PO}_4]_6[\text{OH}]_2$ ) formation (Hernandez et al., 2000). Mineralization of both bone and cartilage occurs by deposition of inorganic hydroxyapatite crystals in the ECM. This process has not yet been described in teleosts. In mammals, the initiating step of hydroxyapatite formation occurs in ECM vesicles secreted from mature osteoblasts (Anderson et al., 2005, 1996, 1995). These vesicles create an environment where deposition of minerals (mainly  $\text{Ca}^{2+}$  and  $\text{P}_i$ ) occurs and hydroxyapatite is produced, a process involving proteins like Annexins and Alp (Balcerzak et al., 2003; Kirsch et al., 2005). The attachment of the vesicles to bone is not well understood, but Alp and Annexin in the vesicle membrane are reported to anchor to collagen fibrils (Wu et al., 1991). Vesicle formation is followed by the linking of hydroxyapatite crystals to ECM components (Balcerzak et al., 2003) using the  $\text{Ca}^{2+}$  and hydroxyapatite binding properties of Osteonectin, Osteopontin, Osteocalcin and Bone sialoprotein (Hoffmann et al., 1996; Pinto et al., 2001; Furie et al., 1991). Hypertrophic chondrocytes are also capable of initiating calcification processes by releasing similar matrix vesicles as osteoblasts and it has been suggested that hypertrophic chondrocytes may participate actively in bone formation (Anderson et al., 1975; Kirsch et al., 1997). Moreover, hypertrophic chondrocytes from both mammals and teleosts express genes like osteocalcin, osteonectin and alp (Ytteborg et al., 2010b; Ishizeki et al., 1996; Lian et al., 1993). Cancedda et al. (1992) showed that hypertrophic chondrocytes from chicken can be induced to obtain a strictly osteoblastic phenotype *in vitro*. These findings are supported by Yasui et al. (1997)

who suggested that hypertrophic chondrocytes are able to trans-differentiate into osteoblasts and produce bone through a process called trans-chondroid ossification. More than 10 different forms of cartilage and several other tissues with histological characteristics between bone and cartilage have so far been identified in fish (Huysseune et al., 1986; Huysseune et al., 1990). This makes bone studies in Atlantic salmon more complicated, as strict lines between cell types and distinct borders between tissue structures are difficult to define. However, intermediate tissue is instructive due to the many molecular pathways and cellular adaptations during pathological development and normal growth.

### 3. Pipeline for studying vertebral development

Bone deformities in Atlantic salmon are a complex problem, which may have diverse causes, acting either one by one or in combination, hence, a number of different tools are important to establish in order to cover different mechanisms involved in their development. The pipeline for studying bone development in teleosts is shown in figure 6. In vertebrates, both bone and cartilaginous structures coexist during development of the vertebral column and both tissues are built up mainly of the organic ECM. Cartilage and bone cellular activity largely depends on the interaction with ECM components. ECM components regulate cell growth and differentiation by interacting with growth factors and enzymes, provide the tissue with mechanical strength and resilience and constitute the template for mineralization during development of the vertebral column. The composition and structure of molecules in the ECM are shown to play pivotal roles in bone formation and changes therein may result in deformities in the spine of both mammals and teleosts (Pedersen et al., 2010). Radiography, or the use of X-rays for analysis, is the preferred method for fish skeletal deformity diagnostics. X-rays have enough energy to penetrate soft tissues, but not bone and other hard substances. Moreover radiography thus allows the creation of a negative image of the skeletal structures of the fish, which allows the evaluation of calcification level and for identification of pathology in the bones, without cutting into or even killing the fish. However, fish radiography has its limits and it is difficult to diagnose fish before the deformity has developed. More sensitive techniques are therefore necessary. So far *in vivo* trials with Atlantic salmon using different temperatures and light regimes, water speed for studying the effect of training and feeding trials using custom-made feeds for studying mineral and vitamin components has been applied for deformity studies. In addition to radiography and measuring rate of development and growth, essential minerals have been followed from uptake and secretion in the intestines using quantitative real time PCR, to incorporation in the bone matrix using mineral analysis and Fourier Transform InfraRed (FT-IR), histological staining techniques and screening techniques such as microarray. Important pathways for cellular differentiation of bone and cartilage have been followed using gene expressional tools, like quantitative real time PCR, *in situ* hybridization and immunohistochemistry. In a recent publication, (Ytteborg et al., 2010b), it was shown by using molecular markers and gene transcription techniques, that fish susceptible for developing vertebral fusions could be detected already at 2g size. Atlantic salmon *in vitro* based systems are also developed, where cellular differentiation and lineage determination can be studied in more controlled environments (Ytteborg et al, 2010a). Combining radiography, histological staining techniques and molecular tools has led to a more complete understanding of how normal and pathological bone formation in Atlantic salmon progress and opens up for prospective advanced functional studies in

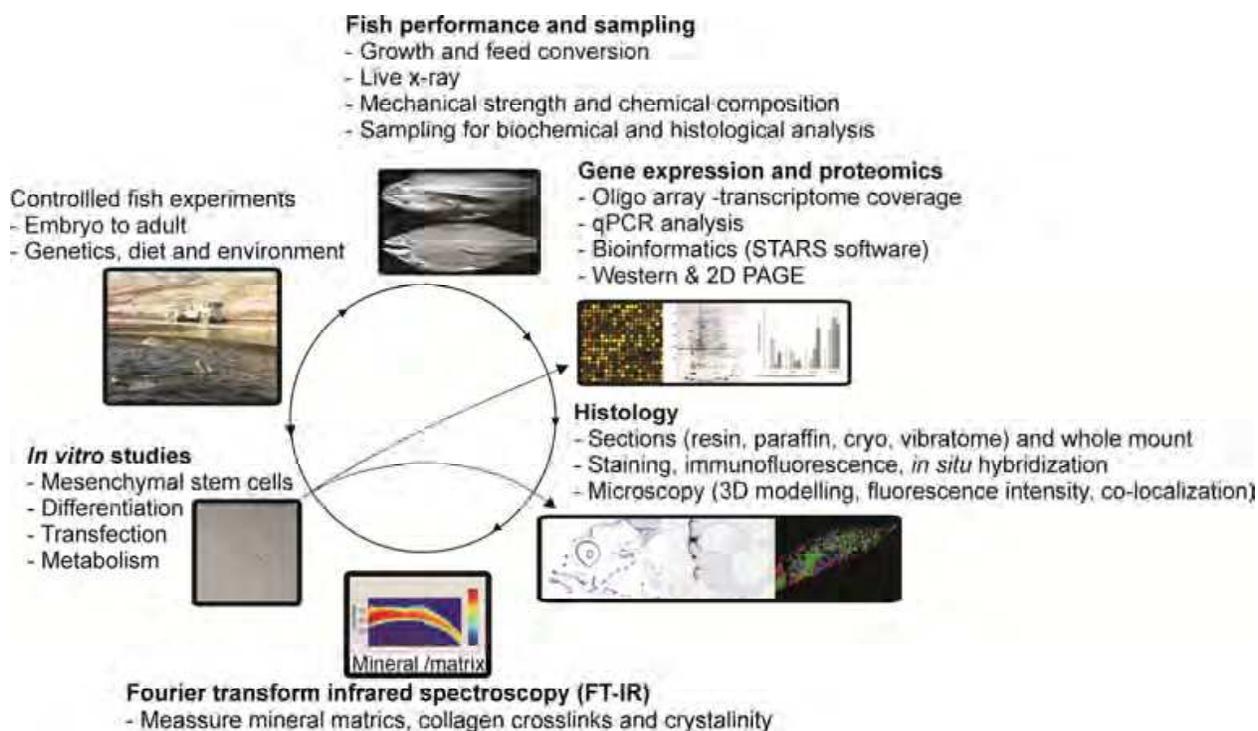


Fig. 6. Pipeline for studying vertebral development.

commercial teleosts species. Importantly, management control of deformities and health in general demands precise tools and knowledge to depict any problem as early as possible in the production line. The reliable correlation between defined skeletal markers and the risk of developing vertebral deformities has indicated that these genes can be developed as prognostic markers and further be used to investigate how the progression of skeletogenesis is modulated in response to other stimuli.

#### 4. The teleost vertebra

The vertebral column is the defining feature of all vertebrates, composed of an alternating pattern of vertebral bodies (centra) and intervertebral regions. While centra give support and strength to the organism, intervertebral regions provide flexibility. The segmented pattern of the spine is established during embryogenesis when the precursors of the vertebrae, the somites, are formed (review in Brand-Saberi et al., 2000). The mature Atlantic salmon vertebra consists of approximately 58 vertebral bodies with neural and heamal arches protruding from the top and bottom of the centrum, respectively (Kacem et al., 1998). Grotmol and co-workers (Grotmol et al., 2006, 2005, 2003) have previously described the early development of the Atlantic salmon vertebrae in details. However, few studies have defined the nutritional needs or described the functions needed to keep continuous growth, remodelling and homeostasis in the mature vertebrae. An overview of the Atlantic salmon vertebra features is shown in figure 7.

##### 4.1 The intervertebral regions

The notochord is found in embryos of all chordates, being well conserved between species as the forerunner of the spinal column. However, whereas only remnants of the notochord

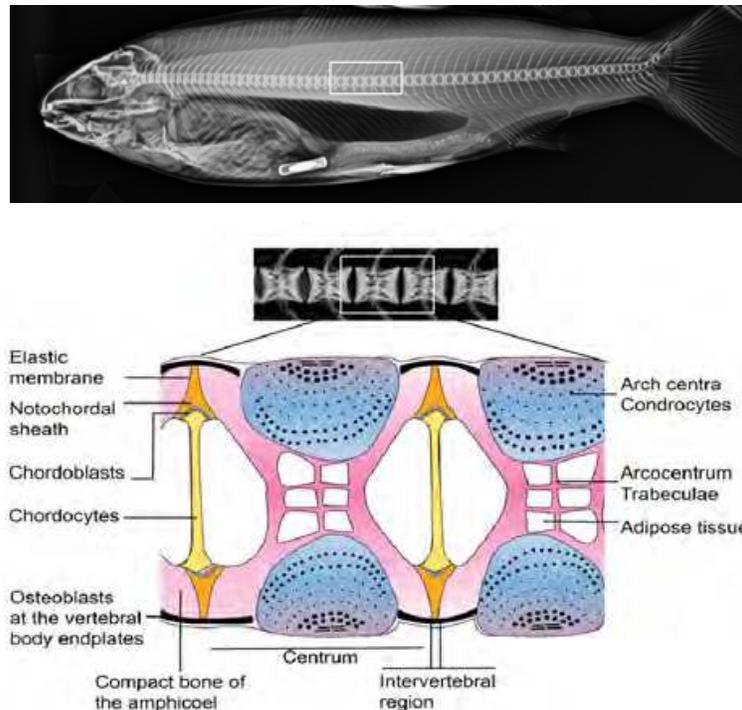


Fig. 7. Overview of the Atlantic salmon vertebra.

exist in the mammalian intervertebral disc (IVD) between adjacent vertebrae (Walmsley et al., 2009), the notochord persists throughout all life stages and throughout the entire length of the fully developed vertebral column in many teleosts, including Atlantic salmon. The morphology and function of the notochord of the mature vertebrae has not been thoroughly described. However, the layers and cell types found at early larval stages persist throughout all life stages in the salmonides. Hence, the mature notochord of Atlantic salmon consists of a core of chordocytes, a layer of chordoblasts, an acellular fibrous sheath and an outer elastic membrane (Grotmol et al., 2006). The chordoblasts continue to divide throughout life in accordance with sustained notochordal growth (Grotmol et al., 2006) and mature into chordocytes, containing large fluid filled vacuoles (Adams et al., 1990; Glickman et al., 2003; Nordvik et al., 2005). The chordoblasts also produce the basal membrane and ECM components of the notochordal sheath, which in both mammals and teleosts like Atlantic salmon, has been shown to consist of mainly Col2 fibrils (Domowicz et al., 1995; Linsenma et al., 1973; Sandell et al., 1994). In mammals, the remnants of the notochord, the chordoblasts and their subsequent matrix, develop into the intervertebral discs (IVDs), which separate the vertebral bodies. The *annulus fibrosus* (AF) surrounding the *nucleus pulposus* (NP) of mammalian discs consists of overlapping collagen and elastin fibrils, forming transversing bands crossing the joint in opposite directions, hence, stabilizing and supporting the intervertebral regions. The NP consists of a fluid filled matrix which distributes the hydraulic pressure in all directions within each disc under compressive loads. Similarly in teleosts, the helical geometry shift between adjacent collagen lamella in the acellular notochordal sheath restricts expansion of the vacuolated chordocytes (Grotmol et al., 2005; Grotmol et al., 2006; Koehl et al., 2000). The elastic membrane surrounding the notochordal sheath has a thickened structure in the intervertebral regions, further contributing to increased strength in these regions. At more mature stages, the notochordal sheath consists of folded structures (Ytteborg et al., 2010d), which may be the consequence of compressions

of the notochordal sheath upon formation and mineralization of the centra. As the cross-helical architecture of parallel Col2 fibrils probably is important for flexural stiffness of the larval body during development (Grotmol et al., 2006) the folded pattern may contribute to increased flexibility and normal functioning of the mature spinal column.

In addition to its structural role, the notochord secretes factors to surrounding tissues and contributes to vertebral patterning during embryogenesis (Cleaver et al., 2001; Fleming et al., 2004). The role of the notochord in patterning of the somites is known from several studies from chicken, mouse and zebrafish, in which secretion of Sonic hedgehog (Shh) from the notochord appears to be essential both for somite survival during the early somitogenesis and for induction of the sclerotome during later somitogenesis (review in Monsoro-Burq et al., 2005). In vertebrate species with limited growth, such as humans, the notochord ceases its regulating role for vertebral development as part of the normal ontogeny, followed by the transformation of notochordal tissue into cartilage (Hunter et al., 2003; Oegema et al., 2002). In Atlantic salmon, however, the notochord should fulfil its regulating role for vertebral body differentiation throughout life, since salmon and other fish species do not stop growing. Immunohistochemistry with the proteoglycan component Perlecan has revealed that this protein is abundantly present in the notochordal sheath of Atlantic salmon (Ytteborg et al., 2010d). Perlecan has structural roles in mammalian cartilage and IVD (Sivan et al., 2006) and is important for proper establishment of basement membranes in different vertebrates including teleosts (Parsons et al., 2002; Aviezer et al., 1994). An interesting aspect of Perlecan is its link to nutritional transportation over the notochordal sheath. Parsons et al. (2002) have previously suggested similarities between the structural role of the teleost notochordal sheath and the mammalian glomerular kidney membrane (GBM). GBM is an important part of the filtration machinery in the kidneys and involved in hydrostatic pressure maintenance (Timpl et al., 1996). The heparan sulfate chains of perlecan have further been shown to play important roles in glomerular filtration (Morita et al., 2005) and to be involved in diffusion of nutrients during tooth development in mice (Ida-Yonemochi et al., 2005). The mammalian IVD basically relies on diffusion for nutrient supplies and removal of waste products. As no evidences for vascularization of the Atlantic salmon notochord exists today, it seems likely that a similar transportation system must apply for the vacuolated chordocytes in the notochord core.

#### 4.2 The centra

The Atlantic salmon spinal column is formed directly in bone, in contrast to the formation of the vertebrae of avian and mammalian species, which are first formed in cartilage (Arratia et al., 2001; Smith et al., 2009). At early stages, the precursors for the osteoblasts are situated on the external elastic membrane only interrupted by the neural and haemal arch cartilages. The segmentation process leading to formation of vertebral and intervertebral regions starts with the formation of the chordacentra, where matrix in the outer half of the notochordal sheath becomes mineralized (Fleming et al., 2004; Arratia et al., 2001; Laerm et al., 1976; Grotmol et al., 2003). Osteoblasts at the vertebral growth zones and osteoblasts lining the trabeculae are involved in intramembraneous ossification. Denser osteoblast populations are located along the cranial and caudal rims of each vertebral body, leading to the biconoid hour-glass shaped vertebra. *In situ* hybridization has confirmed transcription of osteogenic marker genes like *runx2*, *col1a*, *osteocalcin* and *osteonectin* in these populations at mature stages in Atlantic salmon ontogeny, confirming their active involvement in osteoid

production throughout life (Krossøy et al., 2009; Ytteborg et al., 2010b and c), shown in figure 8. In the arch centra of Atlantic salmon, *in situ* hybridization have identified sub-populations of chondrocytes corresponding to the resting, proliferating and hypertrophic chondrocytes described in mammals (Ytteborg et al., 2010b; Hunziker et al., 1994). Chondrogenic marker genes, like *col2a*, *col10a*, *sox9* and *mef2c*, are characteristic for specific maturation zones and have been used to characterize the maturation process in the arches of Atlantic salmon (Ytteborg et al., 2010b). TRAP secreting osteoclasts has further been identified at the ossifying borders of the salmon arch centra, marking the ossification front during endochondral ossification (Witten et al., 2009, Helland et al., 2006; Ytteborg et al., 2010c). In the vertebrae of Atlantic salmon, multinucleated osteoclasts have also been identified in the trabeculae but not in the compact bone of the amphicoel (Witten et al., 2009). As the vertebra grow through the activity of osteoblasts located along the distal ridges, the trabeculae becomes more branched and filled with adipose tissue. After finishing shaping the scaffold for the vertebral bodies, the Atlantic salmon vertebrae continue to grow throughout life (Nordvik et al., 2005). Compared to mammals, where bone is constantly remodeled, the shape and constant growth of the salmon vertebrae have indicated that the need for bone remodeling is scarce. During stressful or unfavorable conditions or during periods of rapid growth, the mammalian skeleton is used as a mineral reservoir, where minerals are released through the activity from the osteoclasts. In Atlantic salmon however, such reservoirs are mostly found in the scales. Experiments have shown that long-term

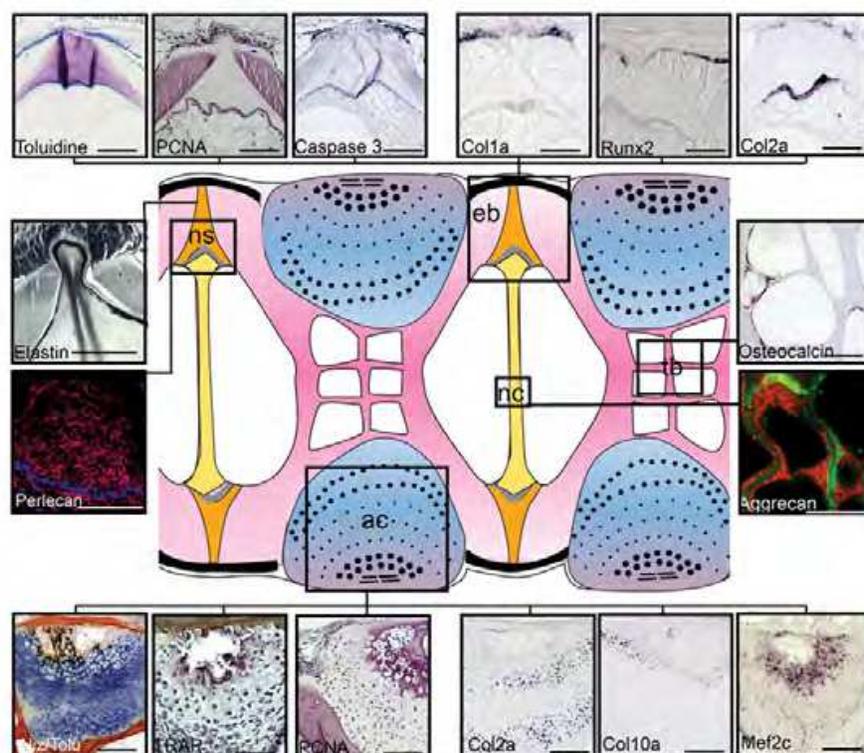


Fig. 8. Overview of histological, immunohistochemical and molecular findings in non-deformed vertebrae. Vertebral endbones (top): Toluidine, PCNA, Caspase 3, *col1a*, *runx2*, *col2a* Elastin in elastic membrane (left), Perlecan in notochordal sheath (left). *Osteocalcin* in trabeculae (right). Aggrecan in chordocytes (right) Arch centra (bottom): Alizarin red/Toluidine blue, TRAP, PCNA, *col2a*, *col10a*, *mef2c*. Trabeculae, tb; Notochordal sheath, ns; Notochord, nc; End bone, eb; Arch centra, ac. Scale bare = 100  $\mu$ m.

stressful conditions rather manifests in salmon as overall improper bone formation and cellular disturbances rather than increased bone resorption. This has been shown through x-ray visualization of lower radiodensity (e.g. in “ghost” and “hyperdense” vertebrae), development of “soft” bone phenotype, transcriptional analysis (e.g. reduced transcription of genes involved in production and mineralization of ECM) and immunohistochemistry showing disturbed cell cycling (e.g. using PCNA antibodies) in vertebrae not yet possessing skeletal malformations. However, these disturbances might further develop into vertebral deformities at later stages.

## 5. Vertebral deformities

Deformities in the spinal column have been observed in a diverse array of vertebrates and a number of causatives have been suggested. Spinal disorders are a major concern for human health and often related to painful conditions (Freemont et al., 2009). Spinal lesions observed in wild animals, such as brown bear, sandtiger shark and smallmouth bass are occasionally found and often reflect environmental problems (Preziosi et al., 2006; Bengtsson et al., 1979; Vandenvyle et al., 1989; Wagner et al., 2005). Deformities in domesticated animals like chicken, broilers, pigs and farmed fish are recognized as a reoccurring problem in intensive production system and represent both ethical and economical challenges (Berg et al., 2006; Hammond et al., 2007; Julian et al., 1998; Reiland et al., 1978; Sullivan et al., 2007). Fish with spinal deformities, such as salmon, trout, cod, halibut, sea bass and sea bream, do not swim efficiently, are less capable of acquiring food, are at a greater risk of predation and are more susceptible to physiological imbalance, in addition to being down-graded at slaughter (Silverstone et al., 2002). Most deformity studies in teleosts have been largely descriptive and primarily performed to reveal factors contributing to increased occurrence of skeletal deformities, e.g. genetics, infections, fast growth, light regimes, vaccination, water current and quality, pollution, malnutrition and elevated temperatures (Berg et al., 2006; Berntssen et al., 2003; Cahu et al., 2003; Divanach et al., 1997; Gjerde et al., 2005; Koumoundouros et al., 2001; Lall et al., 2007; Madsen et al., 2000; Roy et al., 2002; Vagsholm et al., 1998). Spinal deformities in Atlantic salmon have been intensively studied during the past years due to the importance of this specie to the aquaculture industry. Bone deformities in Atlantic salmon are a complex problem, which may have diverse causes that may act alone or in combination. Among these causes of bone deformities, the effect of temperature stress during the early developmental stages is best documented (Ytteborg et al., 2010a,b; Wargelius et al., 2005). Malformations later in life are often related to abnormal nutritional preferences, malnutrition or fast growth. Until recently, the molecular development of spinal deformities in fish has received relatively little attention and few deformities have been explored beyond the level of association with particular causative factors. However, accumulated studies on intensive production regimes and incidence of deformities have been followed by more and more advanced studies on vertebral development and bone biology. Below is the current state of knowledge on cellular mechanisms for pathological bone development. In figure 9, major causatives, radiography and histological staining of normal and deformed salmon is shown.

### 5.1 Cellular mechanisms behind weakened bone structures and development

Conditions that accompany fast growth in farmed animals, e.g. light and feeding regimes, elevated temperatures and breeding, are linked to increased numbers of spinal deformities

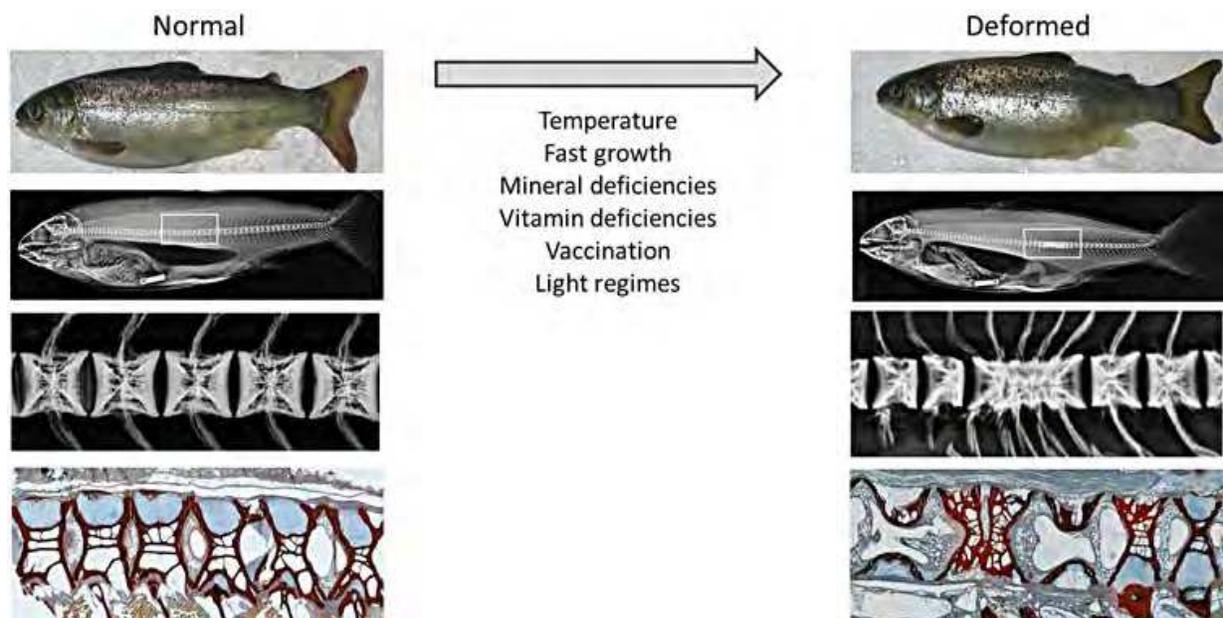


Fig. 9. Normal (left) and deformed (right) Atlantic salmon. From the top: photography of the fish, radiographic image, enlarged radiography, Alizarine red S and Toluidine blue double staining.

(Julian et al., 1998; Reiland et al., 1978; Wargelius et al., 2009). Fast growing Atlantic salmon has been shown to develop soft, low mineralized, bone compared to fish with lower growth rates (Fjelldal et al., 2006) and to have an increased risk of developing vertebral deformities (Fjelldal et al., 2007; Fjelldal et al., 2005). In fast growing Atlantic salmon, elevated muscle mass exercise pressure on under-calcified bone that increases the mechanical pressure, which might trigger formation of intermediate tissues and malformations (Witten et al., 2005). Comparative studies have been performed in commercially farmed chicken, which are the product of long-term selective breeding for high growth rates (Letierrier et al., 1992). Fast growing chicken have weaker bone structures and increased rates of skeletal abnormalities than slower growing broilers, which reduces the bone's ability to adapt to the higher loads induced by the increasing body weight (Rawlinson et al., 2009). In Atlantic salmon, however, high genetic growth rates have not been correlated to increased rates of deformities (Gjerde et al., 2005). To fulfil the requirements for bone mineralization, fast growing animals need to assimilate a higher proportion of the mineral intake (Hernandez et al., 2000). However, knowledge concerning mineral uptake and transportation in the fish intestines is lacking and needs to be studied further. The current change in fish feed production, switching to a vegetable based lipid diet, may further change the intestinal uptake of minerals, vitamins and amino acids (Jutfelt et al., 2007). Achieving predictable production of high-quality fish that perform well later in life therefore requires a high level of control of various factors influencing normal development and growth during early phases of life. Understanding the interactions between dietary mineral levels, n-6/n-3 fatty acid ratios, bioavailability, growth rate, temperature and intestinal uptake is imperative to be able to balance diet composition and use available feed ingredients adequately.

At the cellular level, a general trade-off between proliferation and differentiation has been suggested as a cause for delayed skeletal development in fast growing species of birds (Arendt et al., 2000; Rawlinson et al., 2009). It has further been suggested that during rapid

growth the time required for bone matrix to be produced and mineralized may be reduced to a critical level (Hernandez et al, 2000); hence development of a soft bone phenotype. This causative relation has been suggested for fast growing under-yearling Atlantic salmon smolt that has a higher incidence of vertebral deformities than slower growing yearling smolt (Fjellidal et al., 2006). Temperature and light regimes are factors shown to speed up developmental rate in Atlantic salmon, but also to delay production of osteoid. It therefore seems that bone remodeling in Atlantic salmon is generally sensitive to elevated growth rates (Ytteborg et al., 2010a). Osteoblasts and chondrocytes are cell types producing large quantities of ECM and may therefore be particularly sensitive to stressful conditions, due to reduced normal protein synthesis (Tsang et al., 2007; Haynes et al., 2004). Quantification of mRNA in vertebrae from fast growing Atlantic salmon has revealed a reduced transcription of important genes encoding structural proteins taking part in the bone matrix and mineralization, e.g. *col1a1*, *osteocalcin* and *osteonectin* (Ytteborg et al., 2010b). Furthermore, generally weaker *in situ* hybridization signals were detected for probes targeting these ECM transcripts in areas where intramembranous ossification takes place. These findings further correlated to an impaired mineralization and supported the assumption that disturbances in bone formation constitute an important part of the mechanisms involved in soft bone formation. These observations are further consistent with an Atlantic salmon osteoblast *in vitro* experiment, where long-term 16°C heat exposed cells showed a decreased transcription of *alp*, *col1a1* and *osteocalcin*. Based on *in vitro* and *in vivo* results it seems that Atlantic salmon osteoblasts may be particularly sensitive to elevated temperatures during the early stages of differentiation.

In mammals and teleosts like Atlantic salmon, elevated temperatures and fast growth may also interrupt the normal chondrocytic differentiation pattern and delay endochondral bone formation, further weakening the bony structures (Tsang et al., 2007). A number of studies have linked skeletal malformations to disturbances in chondrocytic maturation (Kieswetter et al., 1997; Farquharson et al., 2000; Julian et al., 1998). Recent results have suggested that fast growth caused by elevated temperatures leads to an arrest prior to the final maturation of chondrocytes in the Atlantic salmon vertebral arch centra (Ytteborg et al., 2010c). Morphological studies of the arch centra of juvenile Atlantic salmon reared under intensive temperatures have identified chondrocytes with a distorted maturation pattern and an increased zone of hypertrophic chondrocytes (Ytteborg et al., 2010c). In this study, an increased zone of hypertrophic chondrocytes correlated with increased transcription of hypertrophic marker genes such as *col10a1* and *mef2c*. Fast growing chickens are also characterized by disturbed chondrocytic maturation where cartilage do not mature enough to ossify (Julian et al., 2005; Farquharson et al., 2000) and increased mechanical load is associated with an increased hypertrophic zone in the growth plate of rat ulnae along with a suppressed mineralization rate (Robling et al., 2001; Ohashi et al., 2002). Furthermore, mammalian osteoclasts are temperature sensitive and hypothermic conditions may stimulate their activity (Patel et al., 2009). Similar observations have been described in Atlantic salmon where no TRAP activity was observed in the arch centra of fish reared at intensive temperatures. Also transcription of osteoclast associated marker genes, like *Mmps* and *Cathepsin K* was reduced (Ytteborg et al., 2010c). Absence of *Mmps* may cause delays in endochondral ossification and *runx2* deficiency may inhibit *mmp* expression and lead to mild disturbances of chondrocyte differentiation (Inada et al., 1999; Kirsch et al., 1997; Pratap, 2005). Disturbances in chondrocytic maturation and endochondral ossification will

overall weaken the vertebrae, and may be an explanation for wrinkled and shortened ribs observed in Atlantic salmon suffering from P deficiency (reviewed in Sugiura et al 2005).

Overall, both bone and cartilage formation seems disturbed during fast growth and may equally contribute to weakened skeletal structures. In Atlantic salmon, experiments have indicated that during rapid growth, both endochondral and intramembraneous ossification is affected. Moreover, fast growth leading to weakened bone and cartilage structures at juvenile stages increases the risk of developing severe deformities later in ontogeny. This might be a result of local cellular compensation and an effort to restore and strengthen a weakened area in the vertebrae, as described in the next chapter.

## 5.2 Cellular mechanisms behind vertebral deformities

Witten et al. (2009) recently published a survey on commonly observed vertebral malformations in Atlantic salmon which included different grades and combinations of platyspondyly (compressions), ankylosis (fusions), lordosis (v-shaped vertebral column), kyphosis (^-shaped vertebral column) and scoliosis (S-shaped vertebral column). Histological characterization of compressions and fusions have described shape alterations of vertebral body endplates, reduced intervertebral space, transformation of intervertebral notochord tissue into cartilage, mineralization of the intervertebral cartilage and replacement of intervertebral cartilage by bone (Witten et al., 2005; Kvellestad et al., 2000; Witten et al., 2006), independent of the factor inducing the malformation. Changes in transcriptional processes in osteoblasts and chondrocytes from both mammals and teleosts are involved in pathological vertebral formation (Hammond et al., 2007; Breen et al., 1999; Wargelius et al., 2005). The development of vertebral fusions is a dynamic process but recent publications have shown that the underlying cellular and molecular mechanisms may be summarized as four key events (Ytteborg et al., 2010a, b and c). These events are illustrated in figure 10 and described in the text below.

### *I: Disorganization*

The initiation of the fusion process includes disorganization and proliferation of osteoblasts and chordoblasts. Osteoblasts at the growth zones of the vertebral body endplates have a markedly increased cell proliferation rate and the growth zones extend spatially along the rims of fusing vertebral bodies. As the intervertebral space narrows, proliferating chordoblasts and denser packet chordocytes appear. With a progressing pathology, proliferating chordoblasts occupy most of the intervertebral space and vacuolated chordocytes disappear.

### *II: Metaplastic shift*

Proliferating cells at the border between the osteoblast growth zones and the arch centra show a transcriptional shift, where co-transcription of osteogenic (*col1a*, *runx2*, *osteocalcin* and *osteonectin*) and chondrogenic (*col2a*, *mef2c* and *col10a*) marker genes are prominent. The marked border between the osteoblast growth zones and the chondrocytic areas connected to the arches becomes less distinct, as proliferating cells and chondrocytes blend through an intermediate zone. A similar shift is found in the notochord where co-transcription of genes such as *col2a*, *sox9*, *col1a* and *runx2* increase with proliferation of chordoblasts. In the central notochord of developing fusions, hyperdense regions of denser packet chordocytes lacking vacuoles appear as the number of proliferating cell increase.

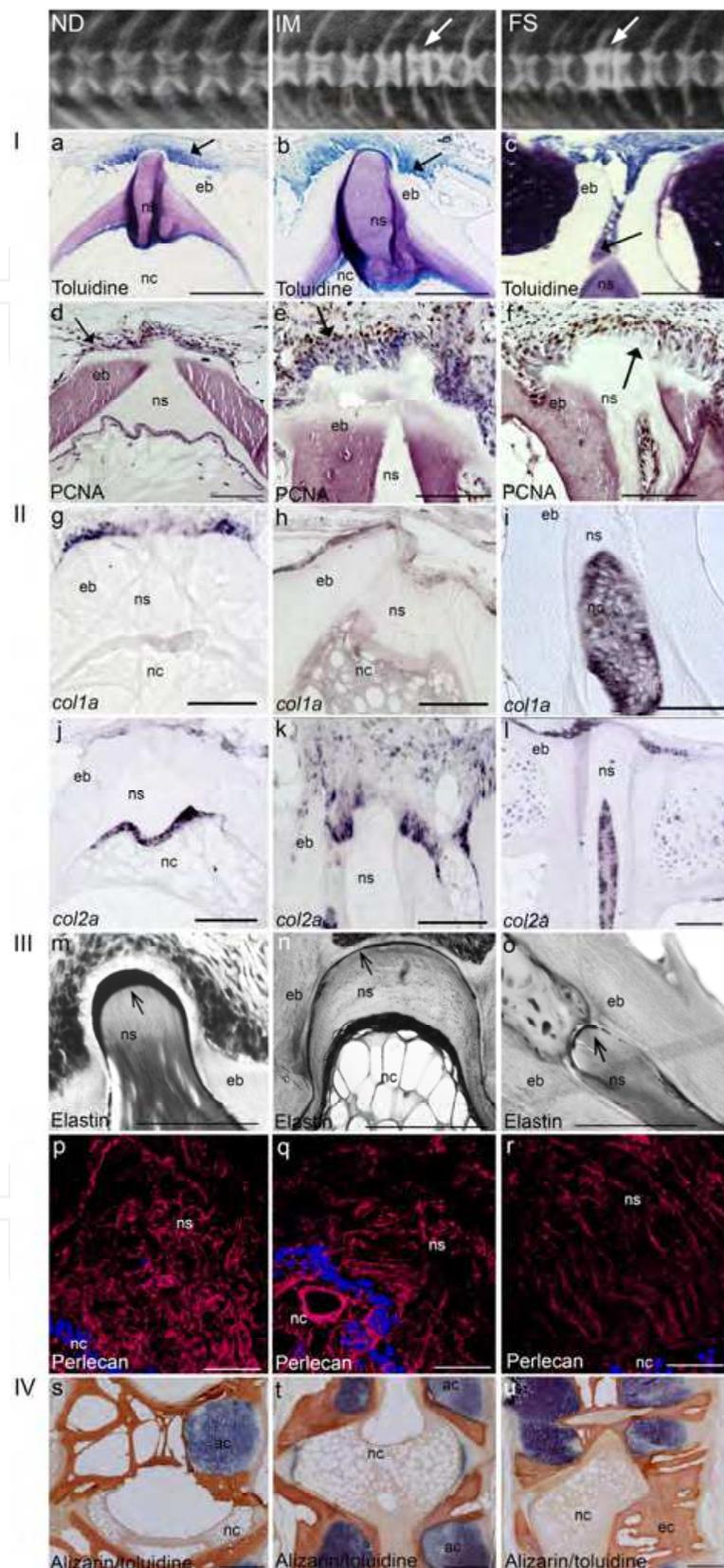


Fig. 10. Major findings during the development of vertebral fusions in Atlantic salmon. I Disorganization, II Metaplastic shift, III Loss of notochordal sheath integrity, and IV Ectopic bone formation. See text for details. Scale bar = 100  $\mu\text{m}$  (a-l), 50  $\mu\text{m}$  (m-r), 200  $\mu\text{m}$  (s-u).

### *III: Loss of notochordal sheath integrity*

The elastic membrane surrounding the notochord becomes fragmented and the notochordal sheath loses its integrity. Verhoeff's hematoxylin staining has visualized a thinner elastic membrane surrounding the notochordal sheath of developing vertebral fusions. In the most severe cases, the elastic membrane is fragmented. Furthermore, the highly folded structures in the notochordal sheath are lost during development of spinal fusions.

### *IV: Ectopic bone formation*

Ectopic bone formation in the affected areas gives the vertebral bodies a squared morphology as the arch centra fuse and ossify. Ectopic mineralization of intervertebral regions and arch centra is formed, indicating that the proliferating and metaplastic cells not only differentiate towards osteoblast-like cells, but also complete the differentiation to cells that are capable of producing mineralized matrix. The intervertebral space narrows completely down and the notochord mineralizes.

The overall structural and molecular features of bone and cartilage development in vertebral fusions in Atlantic salmon have shown resemblance with similar pathological spinal conditions in mammals (Ytteborg et al., 2010c and d; Gorman et al., 2007; Witten et al., 2006). For example several mammalian studies have suggested that changes in the balance between cell death and cell proliferation is involved in bone and cartilage defects which may lead to malformations (Cockroft et al., 1978; Miura et al., 2004; Breen et al., 1999; Farquharson et al., 2000). Spinal fusions in Atlantic salmon are characterized by changes in ECM components and mineralization of the intervertebral regions (Ytteborg et al., 2010c; Witten et al., 2006). Similarly, intervertebral disc degeneration (IDD) in mammals involves breakdown of ECM components in the AF and calcification of the NP (Takaishi et al., 1997; Kanemoto et al., 1996; Antoniou et al., 1996). Fusion, compression and chondrogenic transformation of skeletal tissue have also been reported from lordosis and kyphosis in sea bass. Histological examinations of both lordosis and hyperdense vertebrae have further indicated cellular plasticity (like metaplastic shifts and trans-differentiation) and development of intermediate tissues as pathological events (Ytteborg et al., 2010c; Helland et al., 2006; Kranenbarg et al., 2006; Witten et al., 2006; Witten et al., 2005). It has previously been suggested that a metaplastic shift is involved in the development of spinal fusions, leading to the formation of chondroid bone which at later stages in the fusion process is replaced by bone. As previously discussed, chondrocytes associated with calcifying cartilage can acquire properties of osteoblasts (Cancedda et al., 1992) and are able to change their phenotype from a primarily cartilage synthesizing cell type to a bone synthesizing cell type (Lian et al., 1993). Co-transcription of chondrogenic and osteogenic marker genes in the arch centra and notochord supports the suggestion of an adaptation through metaplastic shifts during development of vertebral fusions, which may be induced to produce more robust cells that are able to withstand increased mechanical load. A pathway to bone formation through chondrocytes might be possible during development of vertebral fusions and fast growth, which could be similar to trans-chondroid ossification, as described by Yasui et al. (1997). Trans-differentiation and ectopic calcification has also been suggested as pathological pathways in lordotic sea bass where deformations stimulate ectopic bone formation in the intervertebral regions between two affected vertebral bodies and along the rims of the vertebral body endplates (Kranenbarg et al., 2006). Similarly, a shift in the mammalian IVD NP cell population coincides with spinal disorders like intervertebral disc degeneration and changes in the synthesis of matrix molecules differ

with the degree of degeneration (Handa et al., 1997). The mammalian AF is further strengthened through cartilage formation upon elevated mechanical load (Lotz et al., 2002; Prescher et al., 1998). Moreover, breakdown of PG components, like Aggrecan and Perlecan, may lead to reduced hydrostatic pressure, invasion of nerves and blood vessels and loss of transportation of nutrients and waste products in degenerating IVD (Kauppila, 1995; Urban et al., 2003 and 2004; Melrose et al., 2002; Yasuma et al., 1993). Loss of Aggrecan resulting in tissue dehydration, reduces the ability of mammalian IVD to transmit and absorb compressive load (Kanemoto et al., 1996; Urban et al., 1985). Loss of Aggrecan and Perlecan has also been observed in the notochord of Atlantic salmon during development of vertebral fusions (Ytteborg et al. 2010d), which may possibly reduce the hydrostatic pressure and hence the transportation of nutrients and alteration of pH values. Another comparative pathological process to teleost vertebral fusions is the mammalian “Bamboo spine”, describing a condition where vertebral bodies have fused and reshaped through ectopic bone formation (Bakay et al., 1970; Resnick et al., 1983). Witten et al.(2005) have described similar processes in Atlantic salmon. Fusing vertebral bodies may either stabilize as on large vertebral body or continue to develop through neighbouring vertebrae. What kind of cellular actions leading to a stabilized or aggravating fusion remains to be answered. However, it seems that different types of deformities have similar pathways of cellular pathological development, processes involving proliferation, metaplastic shifts, cellular instability and trans-differentiation.

## 6. Conclusion

During the last decade, fish have emerged as suitable animal models for studying bone and cartilage biology and have shown to be a suitable supplement to mammalian systems aiming to uncover the corresponding fundamental cellular and molecular mechanisms of action. In the light of metaplastic shifts during skeletal deformities in Atlantic salmon, a cell culture based system allowing for cellular differentiation and lineage determination studies have been developed. In this particular system, precursor cells are stimulated to myogenic, adipogenic and osteogenic differentiation, and opens up for studies where these cells can be manipulated upon different stimuli to undergo metaplastic shifts. Hence, functional studies can be performed to better characterize the pathology, define particular requirements and minimize the occurrence of bone disorders. Advanced methods and defined molecular markers should enable us to detect the risk of developing deformities early in ontogeny. Similar diagnostics and medications as those existing in the human medicine will not be applicable for farmed animals. However, treatments and diets have shown to be well suited also for teleosts. Exercise and addition of minerals in the feed have already shown positive effects in regards of bone quality and should be further addressed in future research.

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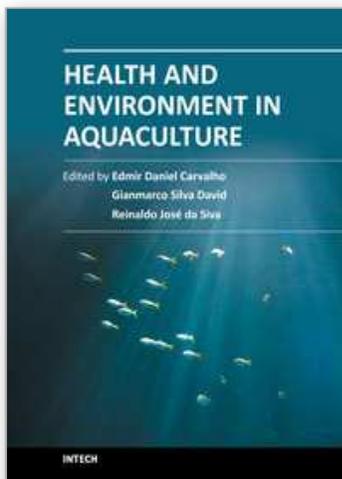
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Aquaculture has been expanding in a fast rate, and further development should rely on the assimilation of scientific knowledge of diverse areas such as molecular and cellular biology, and ecology. Understanding the relation between farmed species and their pathogens and parasites, and this relation to environment is a great challenge. Scientific community is involved in building a model for aquaculture that does not harm ecosystems and provides a reliable source of healthy seafood. This book features contributions from renowned international authors, presenting high quality scientific chapters addressing key issues for effective health management of cultured aquatic animals. Available for open internet access, this book is an effort to reach the broadest diffusion of knowledge useful for both academic and productive sector.

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Unit 405, Office Block, Hotel Equatorial Shanghai  
No.65, Yan An Road (West), Shanghai, 200040, China  
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元  
Phone: +86-21-62489820  
Fax: +86-21-62489821

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