L'expression des gènes cibles dans le modelage osseux vertébral de la truite arc-en-ciel d'élevage: effets de différents apports en phosphore

Thèse

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Résumé

Les rejets phosphorés issus des productions aquacoles sont connus pour être, en partie, responsables de l'eutrophisation des eaux douces. En raison du manque de précision des outils actuellement disponibles pour l'estimation des besoins en phosphore (P) chez la truite arc-en-ciel (*Oncorhynchus mykiss*), il est encore distribué en excès dans la majorité des élevages. Par ailleurs, si l'on sait que le P joue un rôle prépondérant dans la formation du tissu osseux, des questions subsistent quant à son rôle sur l'apparition d'anomalies vertébrales. Pour suivre les réponses à court et à long terme d'une déficience en P sur l'organisme, des truites arc-en-ciel triploïdes ont été soumises à deux régimes alimentaires (riche ou carencé en P). Nos résultats indiquent, dans un premier temps, que dès la semaine 2, les poissons nourris avec une diète déficiente montrent des statuts en P, dans les écailles et les carcasses, inférieurs à ceux des poissons nourris avec une diète suffisante en P. À l'inverse, la minéralisation des vertèbres semble être préservée jusqu'à la semaine 4. Pourtant, à la semaine 5, les radiographies révèlent qu'environ 50% des individus déficients présentent des malformations vertébrales. Pour comprendre l'impact de cette déficience au niveau moléculaire, un séquençage haut-débit (RNA-seq Illumina) nous a permis de construire le premier transcriptome spécifique des vertèbres de truite arc-en-ciel. L'analyse fonctionnelle de ce transcriptome révèle une impressionnante conservation des gènes clefs impliqués dans la régulation du tissu osseux chez les vertébrés. Ainsi, à l'aide de cette référence, il a été possible de quantifier l'expression des gènes dans les vertèbres d'individus déficients déformés ou normaux, après 27 semaines d'expérience. Ces résultats, combinés à des observations histologiques et le suivi des indicateurs du statut en P, indiquent qu'une déficience a pour conséquence une diminution du degré de minéralisation de la vertèbre (sans affecter la matrice ou sa résorbtion ostéoclastique), possiblement dû à l'activité de protéines Gla impliquées dans le dépôt et la croissance des cristaux d'hydroxyapatite. Les connaissances recueillies seront primordiales pour la sélection de lignées de moindre impact pour l'environnement, ainsi que pour la formulation de diètes adaptées aux besoins nutritionnels réels des truites arc-en-ciel d'élevage.

Abstract

Dietary phosphorus (P) outputs from fish production can contribute to freshwater eutrophication. Due to the inaccuracy of the common tools used for estimating P requirement for rainbow trout (*Oncorhynchus mykiss*), P is still provided in excess in fish diets. Furthermore, we know that P plays a crucial role in regulating normal bone turnover; yet, questions have arisen regarding its role in the prevalence of vertebral deformities. To monitor both the short and long term response of the organism to a P-deficiency, rainbow trout were fed either a P-sufficient or a P-deficient diet over a 27 weeks period. Our results show that P-deficient fish displayed lower mineral status in scales and carcasses than P-sufficient fish as early as the second week of the experiment. Although during the first four weeks of feeding P-deficient diet, the mineralization status of the fish vertebrae was not significantly impaired,, at week 5, x-rays revealed that around 50% of P-deficient fish displayed abnormal vertebrae phenotypes. To understand the molecular mechanisms behind the appearance of vertebral malformations, we built the first transcriptome specific to bone tissue of rainbow trout. Sequencing was based on Illumina HiSeq-2000 technology. Functional analysis of this transcriptome revealed a remarkable conservation of key genes involved in bone regulation in vertebrates. Based on this reference, we could compare the gene expression in the vertebrae between deformed and normal fish at week 27. These results, combined with histological observations and common indicators of P status, suggest that P-deficiency in trout led to a reduced degree of mineralization of the vertebrae (without affecting matrix of resorption activity). The reduced degree of mineralization is possibly linked to higher expression of genes coding for Gla proteins, involved in the regulation of hydroxyapatite crystal fixation and growth. From an aquaculture perspective, these results might serve as a solid baseline for better management of P in production, opening to the possibility to strain selection as well as providing new tools for dietary-P level optimization for rainbow trout production.

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ALP	Alkaline phosphatase
APC	Adenomatous polyposis coli
ATP	Adenosine triphosphate
BGLAP	Osteocalcin
BMC	Bone mineral content
BMP	Bone morphogenetic protein
BMPR	Bone morphogenetic protein receptor
BMU	Basic multicellular unit
Ca	Calcium
CEBPB	CCAAT/Enhancer binding protein (C/EBP) B
CGrP	Calcitonin gene-related peptide
c-Myc	Proto-oncogène c-Myc
COL	Collage type XI alpha 1
COL11a1	Collagen type XI alpha 1
COL12a1	Collagen type X2 alpha 1
COMP	Cartilage oligomeric matrix protein
CREBBP	CREB binding protein
CT	Calcitonin
CTR	Calcitonin receptor
CTSK	Cathepsin K
CV	Coefficient of variation
d°	Day-degree
DAPI	4',6'-diamidino-2-phénylindole
DCHS1	Dachsous Cadherin-Related 1
DKK	Dickkopf-related proteins
DLX5	Distal-less homeobox 5
DM	Dry matter
DNA	Deoxyribonucleic acid
DVL	Dishelved protein

ECM	Extracellular matrix
ERK	Extracellular signal-regulated kinase
ESTs	Expressed sequence tags
FAT4	FAT atypical cadherin 4
FBN2	Fibrillin
FC	Fold change
FGF	Fibroblast growth factor
FGFR4	Fibroblast growth factor receptor 4
FLNA	Filamin A, alpha
FST	Follistatin
FTIM	Fourier transform infrared microspectroscopic
FZDs	Frizzled receptor
GCG2	Glucagon-2
GPC1	Glypican 1
GSK3β	Glycogen synthase kinase 3
HA	Hydroxyapatite
HAPLN1	Hyaluronan and proteoglycan linked protein 1
HIVEP3	Human immunodeficient virus type 1 enhancer binding protein 3
HSCs	Hematopoietic stem cells
HSPG2	Heparan sulfate proteoglycan 2
IHH	Indian hedgehog
IKKs	Protein kinases
IL-1	Interleukin-1
JNK	c-Jun N-terminal kinase
JUNB	Jun B proto-oncogene
JUND	Jun D proto-oncogene
K	Condition factor
LEF1	Lymphoid enhancer-binding factor 1
LRPs	Lipoprotein receptor-related proteins receptor
M-CSF	Macrophage colony stimulating factor

M3K1	Mitogen-activated protein kinase kinase kinase 1
MCP-1	Monocyte chemoattractant protein-1
MEF2C	Myocyte-specific enhancer factor 2C
MEK1	Mitogen-activated protein kinase kinase 1
MEKK1	Mitogen-activated protein kinase kinase kinase 1
MGP	Matrix Gla protein
MKKs	Matrix-activated protein kinases
MLK3	Mitogen-activated protein kinase kinase kinase 11
MMPs	Matrix metalloproteases
MSCs	Mesenchymal stem cells
MtPi	Mitochondrial Pi Carrier
MYCB2	Myc binding protein 2
MYCPP	C-myc promoter-binding protein
MYO10	Unconventional myosin-X
NaPi-II	Sodium-phosphate co-transporter
NF1	Neurofibromin 1
NFIX	Nuclear factor 1/X (CCAAT-binding transcription factor)
NGS	Next-Generation Sequencing
NIK	Mitogen-activated protein kinase kinase kinase kinase 4
NKFkB	Nuclear factor-kappa B
NKX3.2	Homeobox protein Nkx3.2
NOG	Nogging
OPG	Osteoprotegerin
OPN	Osteopontin
OSX	Osterix
P	Phosphorus
p38	P38 mitogen-activated protein kinase
PHEX	Phosphate-regulating neutral endopeptidase, X-linked
Pi	Phosphate
PPi	Pyrophosphate
PTH	Parathyroid hormone

PTHR	Parathyroid hormone receptors
PTHrP	Parathyroid hormone-related protein
RANK	Receptor activator of nuclear factor κ B
RANKL	Receptor activator of nuclear factor kappa-B ligand
RNA	Ribonucleic acid
RT-qPCR	Reverse transcription polymerase chain reaction
RUNX2	Runt-related transcription factor 2
SFrP	Secreted frizzled-related proteins
SHH	Sonic hedgehog
SOS1	Son of sevenless homolog 1
SOST	Sclerostin
SOX9	Sex determining region Y-Box 9
SPARC	Osteonectin
SPP1	Osteopontin
SRCAP	Snf2-related CREBBP activator protein
STC	Stanniocalcin
TAK1	Mitogen-activated protein kinase kinase kinase 7
TCF	T-cell-Specific transcription factor
TGFR	Transforming growth factor beta receptor
TGFβ	Transforming growth factor beta
TMEFF1	Tomoregulin-1
TNR5	CD40 molecule TNF receptor family member 5
TRAF6	TNF receptor-associated factor 6, E3 ubiquitin protein ligase
TRAP	Tartrate-resistant acid phosphatase
TWIST2	Twist family BHLH transcription factor 2
USP9X	Probable ubiquitin carboxyl-terminal hydrolase FAF-X
VDR	Vitamin D receptor
VEGF	Vascular endothelial growth factor
VGFR1	Vascular endothelial growth factor receptor 1
WGD	Whole genome duplication
WNT	Proto-oncogene Wnt

μCT	Micro-computed tomography

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« *La vie ne réside pas dans les molécules, mais dans les liens qui les unissent* »

Dr. L. Pauling

Avant-propos

Cette thèse se compose de cinq chapitres incluant une revue de littérature (chapitre 1), une discussion et conclusion générale (chapitre 5) et trois articles originaux (chapitre 2 à 4).

Le premier article présenté dans cette thèse (chapitre 2) intitulé: Responses of different body compartments to acute dietary phosphorus deficiency in juvenile triploid rainbow trout (*Oncorhynchus mykiss*, Walbaum), a été publié dans **Journal of Applied Ichtyology** et j'en suis le premier auteur. Le Dr. M.-H. Deschamps ainsi que l'étudiante à la maîtrise N. Poirier Stewart et la professionnelle de recherche É. Proulx ont participé à l'échantillonnage des poissons, ont participé aux acidifications et à l'obtention des valeurs en cendres. É. Proulx a conduit les analyses de teneur en P sur Dionex. Tous les co-auteurs ont participé activement à la rédaction et à la correction du papier.
Auteurs : Le Luyer J., Deschamps M.-H., Proulx É., Poirier Stewart N., Robert C., Vandenberg G. W.

Le second article présenté dans cette thèse (chapitre 3) intitulé: Establishment of a comprehensive reference transcriptome for vertebral bone tissue to study the impacts of nutritional phosphorus deficiency in rainbow trout (*Oncorhynchus mykiss*, Walbaum), a été publié dans le journal **Marine Genomics** et j'en suis le premier auteur. Le Dr. M.-H. Deschamps ainsi que l'étudiante à la maîtrise N. Poirier Stewart et la professionnelle de recherche É. Proulx ont participé à l'échantillonnage des poissons. Le Dr. C. Robert, le Dr. A. Droit et l'étudiant au doctorat C. Joly Beauparlant m'ont appuyé pour l'analyse des données de RNA-seq. Tous les co-auteurs ont participé activement à la rédaction et à la correction du papier.
Auteurs : Le Luyer J., Deschamps M.-H., Proulx É., Poirier Stewart N., Joly Beauparlant C., Droit A., Robert C., Vandenberg G. W.

Le troisième article présenté dans cette these (chapitre 4) intitulé: RNA-seq transcriptome analysis of pronounced biconcave vertebrae: a common abnormality in rainbow trout (*Oncorhynchus mykiss*, Walbaum) fed a low-phosphorus diet, a été soumis au **Journal of Next-Generation Sequencing and Applications** et j'en suis le premier auteur. Le Dr. M.-

H. Deschamps, l'étudiante à la maîtrise N. Poirier Stewart et la professionnelle de recherche É. Proulx ont participé à l'échantillonnage et ont mené les analyses morphométriques et histologiques. L'équipe du Dr. J.-Y. Sire a réalisé des mesures de μCTs. Tous les co-auteurs ont participé activement à la rédaction et à la correction du papier.

Auteurs : Le Luyer J., Deschamps M.-H., Proulx É., Poirier Stewart N., Droit A., Sire J.-Y., Robert C., G. W. Vandenberg

1 Chapter 1: Literature review

1.1 Fish production

1.1.1 Aquaculture in Canada

Aquaculture is one of the fastest growing animal production industries in the world and symbolically overtook fisheries as the major supplier of aquatic products. To date, grass carp (*Ctenopharyngodon idella*), silver carp (*Hypophthalmichthys molitrix*) and tilapia (*Oreochromis niloticus*) represent the largest quantity of finfish product globally reaching, in 2012, 5.0 Mt, 4.2 Mt and 3.8 Mt, respectively [1]. However, Atlantic salmon *(Salmo salar*) remains the most valuable production totalling around 2.1 Mt for up to US$10.1 billion in 2012 [1] far beyond rainbow trout (*Oncorhynchus mykiss*). Yet, over the recent past, the global production of rainbow trout has increased 1.55-fold in volume (+55.7% from 549 kt in 2002 to 855 kt in 2012) and 2.43-fold in value (+143% from US$1.4 to US$3.6 billion in 2012) making it also one of the most valuable farmed finfish species [1, 2]. In Canada, salmon farming is by far the most important finfish production with a total volume of 101 kt valued at US$691 million in 2010, whereas trout production level is barely maintaining at 7 kt per year for a global value of US$35 million. If trout production in Quebec accounted for only 19% of the total Canadian trout production in 2010 [2], it still remains the most important finfish production sectors at the provincial level. In Quebec, trout aquaculture is mainly based on freshwater production with final product directed primarily to re-stocking (75%), but also for human consumption and recreational fishing. Trout production in Quebec represents 140 companies and more importantly, provides full-time employment in rural areas. Although its production yield is relatively low compared to other Canadian provinces such as New Brunswick, British Columbia or Nova Scotia, Quebec displays a tremendous potential regarding the technical knowledge and the natural resources available. Nevertheless, correctly developping the sector will require a strong support from multiple stakeholders and a focus on sustainable development through the reduction of the negative impact of production on receiving water bodies.

1.1.2 General problematic and management strategies for aquaculture farming

The rapid expansion of aquaculture industry worldwide over the past decades has raised concerns about its impacts on surrounding ecosystems. For instance, interactions between farmed and wild populations have been particularly studied with regard to competition for resources as well as transmission of diseases and genetic material (interbreeding) by mature and highly competitive farmed fish [3–6]. From a productivity point of view, sexual maturation is also detrimental mainly because of lower flesh quality and lower yield [3, 4]. In order to minimize both ecologic and economic impacts of farmed fish maturation, one management strategy is to produce sterile fish. Sterilisation by triploidisation (three sets of chromosomes) appears to be the only commercially-acceptable method [9], and has been widely studied particularly in salmonids [10]. However, triploid males are still hormonally competent and can display gonadal development [11]. To date, most of the global trout production is thus based on all-female triploids. The use of sterile fish is particularly relevant in the province of Quebec where rainbow trout has been imported and could significantly compete and/or hybridize with native trout populations. Another issue facing the growing aquaculture industry is the global impact of agricultural effluents and soil erosion on surface water [12] despite the implementation of management regulations to all agricultural sectors, including aquaculture, aiming to reduce environmental pollution and water contamination. Indeed, special environmental concerns with regard to fish farming excessive output water contamination strongly affected the expansion of Quebec freshwater aquaculture, partially explaining the stagnation of provincial rainbow trout production. As in some other agriculture systems, discharge of phosphorus (P) in particular has been a recurring problem in fish production over the past two decades.

1.1.3 Phosphorus uses and problematic in aquaculture

Phosphorus is limiting for primary production in freshwater environments. Phosphorus emanating from fish production arises uniquely from the feed. With regard to fish production, we know that P from faeces or uneaten feed is mainly liberated as soluble element directly available for algal growth [13, 14]. Bureau and Cho [15] demonstrated, for example, that P status in fish increased with increasing P dietary levels, and thus resulted in increasing P renal excretion. These wastes are known to negatively affect water quality and

to lead to eutrophication in freshwater ecosystems [16, 17]. Indeed, an abnormal net augmentation of P level causes rapid algal growth and the rapid accumulation of organic matter in streams and lakes bottoms. The resulting increased presence of heterotroph bacteria consuming these algae might induce severe oxygen deprivation in the water, leading to hypoxia and/or anoxia with fatal consequences for a wide range of aquatic species. Almost 40 years ago, field studies had already demonstrated that high P concentrations affect species diversity in a closed environment, as illustrated by the lake Memphremago case in the Quebec-Vermont region [18]. Consequently, to prevent the negative impact of P excess in watershed, several technologies have been investigated, involving capture or removal of uneaten feed and faeces, treatment of effluents or optimisation of P retention. Yet, to date, up-stream control of P provided to fish (using low-P diets) appears to be the most promising and tangible approach to minimize P output [19].

The dynamics of dietary P levels are, however, particularly challenging since P intake and bioavailability can significantly vary with the diet composition, the level of digestible energy, P origin, P chemical form/solubility and the fish species. For instance, studies reported that P originating from bone in fishmeal was moderately available to fish [19]. However, phosphorus hydrolyzed from animal-by-product is fairly digestible for the fish [20] whilst P from plant seeds is bound to phytate, which significantly reduced its availability. Yet, due to increased global demand and limited availability of marine-derived feedstuffs, several alternatives have been developed to increase the amount of plant-derived proteins in aquafeeds. The use of technologies to increase P availability from the sources has been explored [21–23]. For instance, Carter and Sajjadi [24] showed that Atlantic salmon fed low fishmeal (4.5%) and 60% soy protein concentrate, benefited from phytase (at least 1000 FTU/kg or 250 FTU/kg when pre-treated before making the diets) for final growth, mineral availability and fish body composition. However, in addition to the other anti-nutritional factors present in plant-derived feedstuff, determining optimum levels of phytase remains particularly challenging since it might be strongly dependent on temperature, plant ingredients or associated treatments during feed processing or may vary between species. Finally, independently of the source of P, providing P in optimum levels

relies primarily on a precise measurement of fish minimal P requirement, which still remains to be better defined.

1.2 Phosphorus

1.2.1 Phosphorus requirement

Phosphorous requirements for rainbow trout have been estimated between 0.54% and 0.61% total P in the dry matter basis (DM) [25, 26] with a digestible P to digestible protein ratio ranging from 0.19 to 0.27 MJ-1 [25, 27]. To date, total P levels in commercial diets available for salmonids range from 1.0% to 1.4% (DM) in accordance with Canadian fish feed legislation (set to a minimum of 1% and a maximum of 2.5% total P, DM). Over the past decades, a large number of studies have helped refine this requirement for several fish species. Yet, these studies also revealed that P requirement calculation is significantly affected by fish size, growth rate and life stage [26–33], species [25], fish digestive tract anatomy (e.g. fish with or without acid secreting stomach) [34], as well as feeding procedures [35] and rearing systems [36]. Comparing results from these studies is thus complex and further challenged by the use of a multiplicity of indicators to assess fish response to low-P diets. By order of occurrence, common indicators include somatic growth, P status (in ash or P) in vertebrae, whole-body and scale, non-faecal P excretion, plasma P concentration and retention and, more recently, genes expression in the kidneys and the intestines [31, 36, 37]. A meta-analysis conducted on up to 64 studies, reported that using a weight gain indicator could lead to significantly lower (up to 50%) estimation of P requirement compared to an estimation on whole-body P concentration [36]. Consequently, the weight gain would not be an efficient indicator for early detection of certain deficiency symptoms, even when total body P reserves are strongly depleted [38]. To date there is no consensus as to which indicator should be used, and basing minimum requirement calculation on certain P status assessment could be misleading. Indeed, analysis reported that minimal available P requirement could be estimated at 2.8%, 4.7% and 5.2% P available (DM), when calculated from the levels of P in the plasma, in the whole-body and in vertebrae, respectively [36]. Whilst P plasma levels could appear as the most early and stringent indicator of P depletion in rainbow trout, it is nonetheless largely dependent on

the time of sampling, the dietary status and tissues mobilization [36]. Basing minimum P requirement calculation on P plasma concentration could thus yield very dissimilar results.

With the development of molecular biology and transcriptome-wide tools, specific genes expression has also been observed to be influenced by P-dietary levels in rainbow trout [33, 37, 39, 40]. For instance, the expression of sodium-phosphate co-transporter (*NaPi-II),* vitamin D receptor (*VDR*) as well as 25-hydroxyvitamin D-24-hyroxylase (*cyp24*) were affected by P levels in the diet in rainbow trout kidney whilst *NaPi-II* expression was also affected in the small intestines and the pyloric caeca [33]. However, Sugiura et al. [37] showed that, in 1.5 years old rainbow trout, calculation of minimal P requirement using gene expression assessment was relatively variable within treatment and time of sampling, ranging from 0.33% to 0.71% (DM total P) according to mitochondrial Pi carrier (*mtPi*) and intestinal *NaPi* and renal *NaPi* cotransportors expression, respectively. Consequently, if the use of gene expression gives precious information of how Pi absorption is regulated, its utility for detecting clinical symptoms and for assessing minimum P requirements gave mitigated results due partially to its transient expression. From a production perspective, it is thus crucial to detect early P depletion in fish before the rise of P deficiency epidemics. Indeed, deficiency in early developmental stages can cause long-latency diseases such as the severe deformities reported in Atlantic salmon when transferred to seawater [41]. Reliable indicators for the detection of early effects of P-deficiency in fish are thus needed for timely remedial or for preventing action to be taken.

1.2.2 Mineral homeostasis

Accurately estimating P-requirements, to ensure fish health without arming the environment, requires a better understanding of P function and regulation, and mineral homeostasis in fish. All vertebrates, including teleosts, have to maintain a tight balance between intra and extracellular minerals levels. Minerals can be divided into two groups based on the quantity required in the diet: macro-minerals, such as sodium, chloride, potassium and P, and micro-minerals. P plays a large range of crucial biological functions in fish and intervenes in energy transfer (ATP), genetic material composition (DNA, RNA) and structural support and protection provided by membranes (phospholipids) and bones

[42]. As mentioned earlier, P concentration is limiting in water environment whilst calcium (Ca) is largely available, hence the hypothesis that P might be the driving force regulating mineral homeostasis in fish [43]. Vielma and Lall [44] have shown that absorption through the intestines, conservation in the kidneys and storage in the bones mainly control P homeostasis in Atlantic salmon. Like in mammals, rainbow trout P absorption mostly occurs in intestinal and renal components while also possibly in the skin and gills, through both paracellular passive diffusion and transcellular active transport [45]. Numerous studies have focused on identifying genes involved in P absorption and reabsorption in intestines, pyloric caeca and kidneys in rainbow trout [33, 39, 40]. These studies showed that regulation of Pi absorption, or at least of the NaPi-II action on Pi absorption, might vary between the intestines and pyloric caeca. Indeed, *NaPi-II* gene expression in the intestines increased from day 2 in fish under low-P diet, indicating that the intestines may be the first responsive tissue, yet the increase was relatively low after 20 days (fold change, FC = 3). In contrast, *NaPi-II* expression in pyloric caeca was higher in low-P fed fish from day 5 and strongly increased until day 20 (FC = 10.1) [33]. Identifying P-responsive genes in trout are relevant for further understanding of mammal physiology since P-homeostasis among monogastrics species display common characteristics [46]. However, differences in the digestive and the kidney systems (lack of vili and loop of Henle, respectively, in fish) suggest differences in Pi homeostasis among vertebrates. Furthermore, the exact proportion of P that fish uptake directly from the water through the gills is still to be quantified, but it could provide explanation for the differences observed between flow-through and recirculating systems in minimal P requirement calculation [36].

Another source of debate is about the ability of fish to mobilize their P reserve from the endoskeleton to supply other body compartments. For instance, studies based on the endoskeleton mineralization under varying level of Ca in the water reported evidence that Ca deprivation did not trigger acute bone resorption nor mineral redistribution in fish with acellular bone, except under drastic conditions [for review see 47]. However, in fish, P rather than Ca is considering as the main actor influencing mineral deposition/resorption [43, 47], hence these studies might not be conclusive on the role of bones in mineral homeostasis. Furthermore, beside the endoskeleton, fish have several other sources of P

such as scales [31, 48, 49] or dentition [50] that might be more readily available. Indeed, previous studies demonstrated that scales were preferentially resorbed under low P-diets [31, 48, 51]. However, is it true that, under very extreme conditions such as migration or sexual maturation (inducing elevated mineral requirement), salmonids can effectively mobilize P (and Ca) reserves from distinct skeletal compartments [52–54]. Similarly, rib shape has been identified as first signs of P deficiency in fish [48, 55]. Yet, P-deficiency might be limited to scales and ribs without affecting other skeletal compartments or dramatically affecting fish well being, at least during the initial period of deficiency. However, altered vertebral structure and associated defaults in fish performance are responsible for significantly reduced yield and are thus considered as major threats to aquaculture productivity. For this reason, this project focuses on specific vertebral responses to P deficiency.

1.3 Bone tissue

1.3.1 Bone components and vertebrae

The skeleton is involved in body scaffolding, muscle anchoring, organs and soft tissues protection (vertebrae, scales) as well as food acquisition and processing (teeth and tooth-like organs), along with primordial physiological roles [56, 57]. Bones of teleost fish, like those of mammals, are constituted of an extracellular matrix (osteoid or cartilage matrix) containing collagen fibers and other non-collagenous proteins that will eventually mineralize during fish development. As mentioned above, P absorbed in the organism is accumulated in several soft tissues (heart, liver, kidney, muscle and blood) but mostly in the skeletal tissue [58]. Indeed, the mineralized portion of the skeleton constitutes up to 85% of the total body P in fish [45, 59]. Bone mineralization or cartilage calcification results from the precipitation of Ca and P into crystals of hydroxyapatite (HA; Ca5(PO4)3(OH)) and its deposition on the matrix. Why and how minerals precipitate is not fully understood, yet the combination of a favourable environment (pH, $Ca \cdot P$ saturation) and the removal of mineralization inhibitors (such as pyrophosphate, PPi) are known to be key factors of crystal formation [60]. A recent study on Atlantic salmon vertebrae revealed that mineralization is initiated with accretion of HA on the surface of the collagen matrix in

the notochord sheath [61]. After nucleation, the HA crystals fix themselves to the organic matrix, yet the mechanisms controlling their growth and expansion remain to be defined. However, it is known that matrix proteins, such as the most common non-collagenous proteins including bone Gla protein (osteocalcin, BGLAP), matrix Gla protein (MGP), osteopontin (OPN or SPP1) and osteonectin (SPARC), partially control the mineral deposition/expansion process [62, 63] together with the structure provided by the orientation and assembling of the collagen fibers. Mineral fixation to the matrix needs to be strong, as indicated by the relative stability of the mineral phase; yet, it must also allow the mobilization of minerals for later need by the organism. Mineral status is tightly correlated with the specific physical and physiological roles of each organ [60]. Consequently, it is not surprising that differences are observable regarding the fine composition (proportions of organic matter, minerals and water, and Ca:P ratio) of the different mineralized body parts. Interestingly, several descriptive studies have shown that vertebrae display differences in size or mineral status along the vertebral column depending on their role in the fish physiology [53, 64].

From an evolutionary perspective, teleost bones display the singularity to be divided in two different types: cellular and acellular. Typically, cellular bone (containing cells, osteocytes), resembling mammallian bone morphology, is present in some species belonging to lower order of teleost fish such as clupeiformes, scopeliformes and salmoniformes, including rainbow trout [43, 47, 65]. However, even if phylogenetic differentiation is clear, some exceptions occur within the Protacanthopterygii, Salmonidae and Thunnini families [66]. Inversely, acellular bone is characterized by the absence of osteocytes, where osteoblasts recede from the mineralization front and are not embedded in the matrix [67]. From a physiological perspective now, bone formation can follow two distinct patterns: intramembranous or endochondral ossification [68]. Intramembranous ossification is the process by which bone is produced without prior formation of a cartilage template. During endochondral ossification, on the contrary, mesenchymal cell condense and become cartilage-producing cells serving as template for later bone deposition. In contrast with mammals, fish vertebrae differentiate directly from the notochord sheath [69], except for a very localized section called the basalia, and thus are mainly formed through

intramembranous ossification (Figure 1.1). The basalias are cartilage-specific tissue responsible for the production of haemal and ventral arches in fish [70] and display a typical endochondral ossification [71]. According to the different compartments of the vertebrae, structures consist on either mineralized tissue from notochordal sheath or direct ossification. Development of the vertebrae and bone formation in Atlantic salmon has been well described [69]. Briefly, at approximately 680 day-degrees (d°), Atlantic salmon notochord first differentiates into chordocentra next to the dorsal fin to form complete acellular mineralized rings separated by non-mineralized intervertebral spaces. Collagen fibers are oriented perpendicularly to the cranio-caudal axis. Later, at 800 d°, a second layer of collagen, parallel to the cranio-caudal axis, is formed on vertebral and intervertebral spaces. In the thin outside layer of chordacentrum, osteoblasts deposit osteoid with collagen fibers oriented perpendicularly to the cranio-caudal axis. At this stage, the vertebrae acquire their amphicoel form. Direct deposition on new bone tissue is undertaken on the lateral sides of the vertebrae, at approximately 1000 d°. In this bone portion, collagen fibers appear as not identically oriented (woven bone) and in lower density than in the others layers, resulting in a vertebra with both compact and cancellous (or trabecular) bone tissue. A total of approximately 64 vertebrae interconnected by intervertebral tissue form the vertebral column in rainbow trout [53].

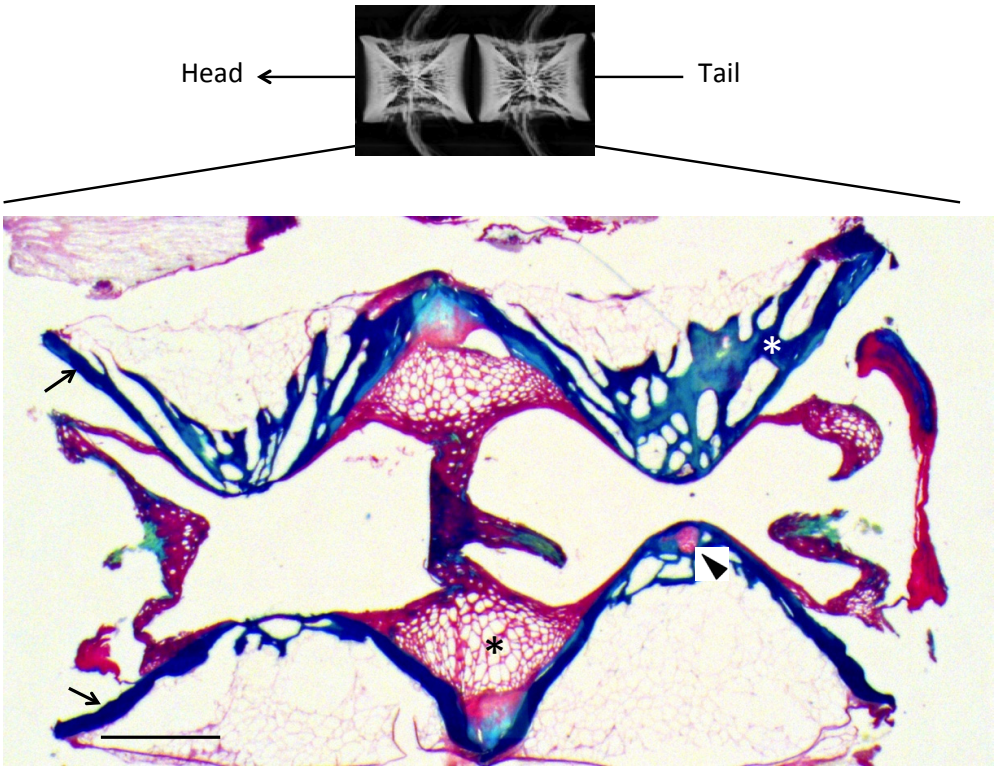

Figure 1.1: Longitudinal section of normal vertebrae of rainbow trout, visualised by Masson's trichrome staining. Normal vertebrae bodies are jointed by notochordal tissue (red) characterized by large cells (black asterisk). Bone tissue (blue) consisted of trabecular bone (white asterisk) and a thinner layer of compact bone (black arrows). Basalias (black arrowhead), composed of chondrocytes cells, are responsible for the production of both hemal and dorsal arches. Bar = 1 mm.

1.3.2 Bone deformities with emphasis on P-induced vertebral deformities

The occurrence of deformities is a subject of importance that has been studied for several decades in various species of interest related to fish physiology. Indeed, we know that deformities might *in fine* affect fish growth or decrease their immunological capacities to face diseases or parasitological events, hence significantly affecting productivity. Deformities affect almost all mineralized tissues in fish although, here, we will focus on the vertebral deformities, a common problem in fish, especially salmonids, farming [64]. From a pathology point of view, there are a plethora of causes inducing the occurrence of spinal

deformities. Indeed, environmental factors such as photoperiod [72, 73], water temperature [74–77], genetics [78–81], ploidy [7, 82–85] or rearing systems [86, 87] are major factors of deformity prevalence in farmed fish. Feed composition, such as vitamin levels, particularly vitamin C, D and K [88–93], is also a common source of deformity occurrence. Among other nutritional factors, the role of minerals, such as P, and of micronutrients levels have been particularly studied and remain, to date, one of the most explored factor of deformities occurrence [58, 94]. Studies on vertebrae body require an integrated approach to overview its complexity. Such approach, proposed by Ytteborg et al. [70], incorporates several levels of techniques ranging from a simple visual examination to molecular tools. A description of those tools with an emphasis of the observation of vertebrae under P-deficiency will be detailed below.

1.3.2.1 Visual examination

Rapid and frequent sampling/examination allows the detection of defaults in fish performance. Defects in growth, feed intake, feed conversion or higher condition factor are common indicators of abnormal fish development under P-deficiency [36, 58, 94]. Indeed, fish exposed to a P-deficiency period commonly display higher condition factor (K) resulting from a reduced growth, particularly correlated with fast-growing fish rather than large fish (for review see [94]). Yet, P deficiency in juveniles can induce long-term consequences appearing in specific conditions such as transfer from fresh to salt-water in salmonids [41]. Defaults in fish performance might also reflect more serious complications such as the apparition of severe malformations [95]. For instance, Fjelldal et al. [96] reported the common skeletal deformities observed in cultured Atlantic salmon such as abnormal jaws curvature, shorter operculum or default in the vertebral column. Vertebral deformities assessed by visual examination or palpation includes fish showing non-standard body shape (such as scoliosis, kyphosis and lordosis) [58, 97]. However, those observations are often associated with late and severe diseases and early detection should preferentially rely on more sensitive observations.

1.3.2.2 Radiographic analysis and mineralized structure

Radiographic analyses allow the detection of discreet (or internal) deformities associated with abnormal vertebral structures in cultured fish [96, 98–102]. Discreet vertebral deformities represent vertebral body showing no-standard structure. Based on x-rays, Witten et al. [103] compiled a list of 20 types of vertebral deformities observed in Atlantic salmon and reported that some of these deformities were primarily observed in fish under mineral deficiency. Indeed, previous work on salmonids reported that fish with lower levels of dietary P displayed increased internal malformations along the vertebral column compared to P-sufficient fish [30, 41, 104–106]. Based on Witten et al. [103] classification, Deschamps et al. [100] reported that malformations in P-deficient fish spread all along the column and mostly consisted in namely: homogenous compression, compression and reduced intervertebral space, compression without x-structure and pronounced biconcave phenotypes. Similarly, Poirier Stewart et al. [104] observed that the main deformities phenotypes in P-deficient rainbow trout were pronounced biconcave and homogenous compression, wilst they also noted a high representation of small and widely spaced vertebrae (Figure 1.2). Furthermore, the latter work provided a new tool for rapid and subjective discrimination of those common deformities by measuring angles, length and height of the vertebrae. Interestingly, the prevalence of deformities observed in fish might differ depending on the body regionalisation in accordance with previous observations reporting the variability in size and mineralization status of the vertebrae along the vertebral column [46, 65]. For instance in farmed rainbow trout, the majority of vertebrae affected by a deformity were localized in the post-cranial (V1-7) and caudal regions (V50-60), plus some within the middle region [98, 99]. Interestingly, vertebrae with altered structure, leading in some severe cases to fusion, might either be a starting point for a spread severe malformation or remain contained to these vertebrae bodies [107]. Besides, the pronounced biconcave phenotype, characterized by transparent vertebral body endplates, vertebral body with vertical apple core shape and an x-shaped centrum of the vertebrae displaying more acute angles [104], is considered as a transient phenotype toward a more severe compression type. Surprisingly, however, under specific conditions, pronounced biconcave vertebrae might return to a normal phenotype [104], hence the importance to understand the mechanisms behind the vertebra body structure regulation.

Radiographs are thus a potent and non-invasive tool for quick detection of vertebral deformities, yet they have their limits and only give an idea of the condition of the mineralized structure of the vertebrae.

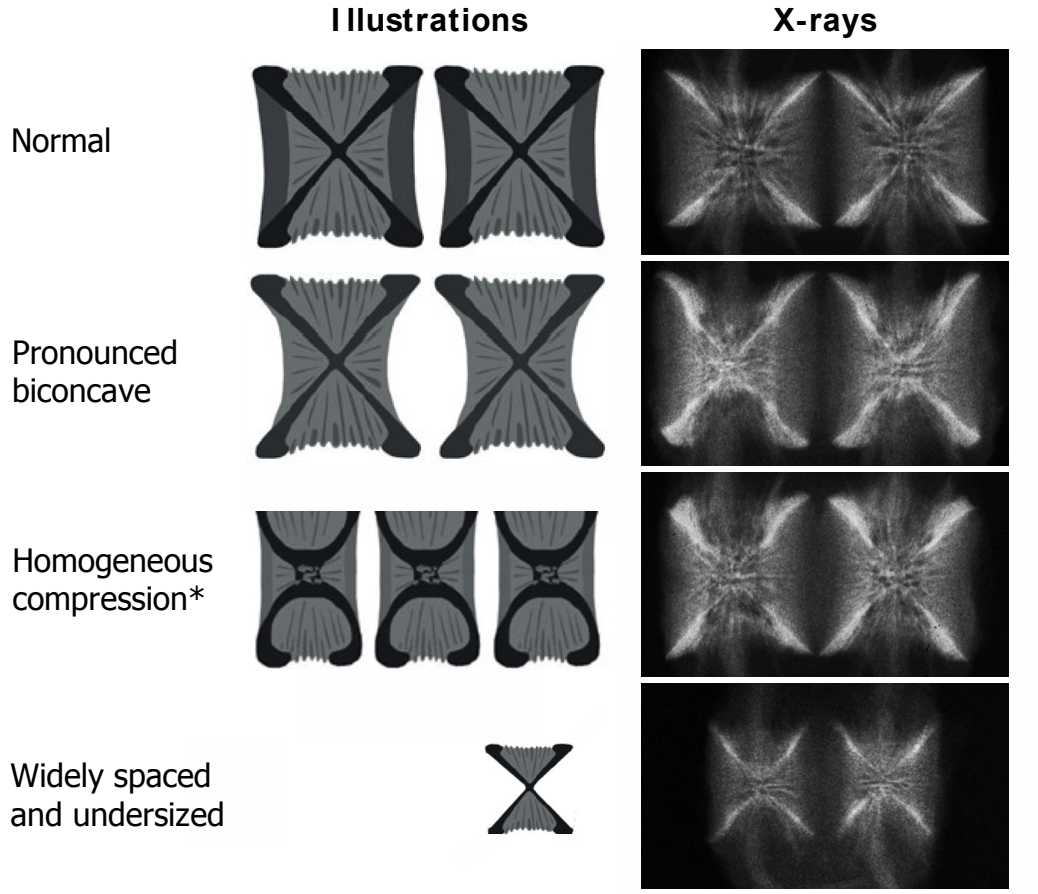

Figure 1.2: Illustrations and X-rays of the most reported vertebrae phenotypes associated with mineral deficiency in teleost. Illustrations were adapted from Witten et al. [103] and Poirier Stewart et al. [104]. * Despite the absence of endplate binding, associated x-rays correspond to the homogenous compression phenotype (Witten personal communication).

To explore in more deepth how the bone mineral structure is affected by low-P diets, several other tools are available. As mentioned above, P deficiency commonly results in a lower bone mineral content (BMC), expressed as ash or P content, of the mineralized tissue including scales and vertebrae [36, 108]. In production situations, mineralization default and lower BMC were also associated with abnormal vertebral morphology [90, 91, 98, 106], hence the use of BMC as early indicator of abnormal vertebrae development. However, BMC at late stages of deformity development like in already fused vertebrae, for

instance, might be similar to normal vertebrae BMC [82]. Thus, due to the complexity of mineral deposition in the vertebrae, understanding the processes involved in the mineralization of the extracellular matrix (ECM) should not be limited to the monitoring of BMC content. Several histological-based techniques such as Von Kossa staining or Fourier transform infrared microspectroscopic (FTIM) have allowed a sensitive description of the mineral deposit conformation and composition (Ca:P ratio). For instance, Boskey et al. [109] used FTIM to interpret the role of BGLAP in the regulation of HA crystals expansion in BGLAP-deficient mice. Furthermore, Deschamps et al. [110] developed a model based on transverse ground section (125 μm) imaging to reveal a bone compactness of the vertebral profile. The bone compactness index was particularly useful in assessing resorption events in the trabecular bone region [110]. Finally, advances in imaging such as micro-computed tomography (μCT) imaging also allowed reconstruction of a 3D structure of the vertebrae to quantify total mineral deposit in teleost [111]. To our knowledge, with the exception of BMC, other tools for fine description of the mineral structure have not been employed to assess the impact of low-P diets on vertebral development in fish but would certainly assist in further understanding the process of vertebral deformity appearance.

1.3.2.3 Biomechanics

Skeleton, and particularly vertebrae, serves as support for surrounding muscles to allow efficient locomotion. However, vertebral biomechanical behaviour may vary depending on a wide range of factors, hence modulating the risk of developing abnormal phenotypes. For instance, Fjelldal et al [112] reported that vertebrae from fish subject to a pinealectomy displayed significant lower bone stiffness. Similarly, underyearling smolts had a significantly lower stiffness of the vertebrae than the yearling smolts and wild post-smolts sampled after their first summer at sea [113]. Consistently with previous observations, P-deficient fish had a lower P status and lower BMC of mineralized tissue, also correlated with a lower stiffness of the vertebrae [111, 113, 114]. These observations echo the lower P status of the mineralized skeleton components as well as the delayed ossification observed in fish subject to P deficiency, together with their higher rate of deformities [30, 31, 48, 105]. Less-mineralized vertebrae are more flexible and thus may be more deformable under

compressive forces [103]. Indeed, a higher bone fracture rate was observed in fish deficient in vitamin C but not in P-deficient fish, suggesting that bone in P-deficient fish are softer, limber and more bendable [90]. Finally, as developed in the previous section, the extracellular matrix conformation strongly participates in the regulation of the mineral structure and needs to be further described.

1.3.2.4 Histology and immunohistology

X-rays and mineral structure monitoring are limited to the mineralized portion of the vertebrae and fail in determining if P-induced malformations either result from a lower bone deposition and/or a higher resorption activity. To fill this gap, histological staining is commonly used to assess cellular recruitment and tissue distribution within the vertebral and intervertebral regions. For instance, Masson's trichrome staining was used to monitor tissue distribution in fused vertebrae of Atlantic salmon and highlighted the presence of ectopic mineralized cartilage replacing the notochord of the intervertebral space [107]. Remodelling of the notochord of fused vertebrae was further described by using immunohistochemistry (based on specific antibodies) to detect distribution of aggrecan, perlecan and substance P as well as DAPI and Nucleic Acid Staining to localize cell nuclei, in the intervertebral space [115]. Consistently, authors further demonstrated that the notochord in fused/hyperdense vertebrae was mineralized by the action of metaplastic notochordal cells [115]. Similarly, Fontagné et al. [30] used common alizarin red and alcacian blue staining for identification of calcified bone and cartilage tissues, respectively, in the entire rainbow trout fry. The authors thus observed that fry subjected to P deficiency displayed delayed ossification. Additionally, cellular distribution and activity have been monitored in teleost skeleton using histological techniques [116, 117]. However, to our knowledge, no work to date reported the histological observation based and tissue distribution and cellular activity of the common vertebrae deformities observed in P deficient fish.

1.3.2.5 Molecular studies

All the previous detailed tools give precious information allowing a refined characterisation and detection of vertebral abnormalities. Yet, by only detecting abnormalities when they

are already established, they fail in preventing the initial negative impacts of P-deficiency in fish. Molecular changes are known as the first sign of cellular metabolism response to stimuli and have therefore been particularly studied to possibly address the shortage of adequate "warning indicators". For instance, the quantitative polymerase chain reaction (qPCR) has been largely used over the past years, together with *in situ* hybridization techniques, to quantify and localize gene expression for specific responsive-genes. For instance, Ytteborg et al [77] reported 20 temperature-responsive genes involved in bone mineralization, matrix formation, chondrocyte differentiation and bone resorption, correlated with higher risk to develop vertebral deformities in Atlantic salmon. Similarly, Darias et al. [118] showed that inadequate level of ascorbic acid in the diet induced appearance of vertebral deformities in sea bass (*Dicentrarchus labrax*) and impaired the expression of genes related to skeletogenesis and mineralization, such as the bone morphogenetic protein 4 (*BMP4*) and *BGLAP*. The latter led to the development of new technological tools such as micro-arrays, allowing quantifying thousands candidate genes. For instance, Ferrareso et al. [119] developed a DNA micro-array chip to study differential gene expression between control and sea bass showing jaw deformities, also allowing the selection of specific traits for particular strain selection. Similarly, microarray analysis was employed by Wargelius et al. [73] who identified the COL11a1 as an actor of vertebral plasticity potentially involved in the higher risk of vertebral deformity in salmon. To our knowledge however, while micro-arrays were used to detect intestinal-response to a P-deficiency [39, 40], quantitative gene expression was limited to a few markers of cellular activity in the study of vertebral response to P-deficiency. The lack of such data may partially be explained by the relatively limited genomic data available for rainbow trout bone-specific genes so far.

More recently, the development of next-generation sequencing (NGS) technologies has considerably expanded the possibilities to work on non-model species and to use genomics without prior references. Although implementing NGS tools in species with highly polymorphic and complex genomes remains a challenging task, the rapid evolution of bioinformatics for large dataset analyses provides novel opportunities to correctly assemble the transcriptome of non-model species [120, 121]. Recently, the first

transcriptomes for vertebrae and gill arches of sea bream (*Sparus aurata*) have been sequenced using 454 Roche technology [122]. However, in contrast with basal teleost such as trout and other salmonids, bones of advanced teleost lack osteocytes (acellular bone). Development and regulation of bone tissue might, therefore, differ considerably [47, 122]. Moreover, according to Vieira et al. [122], the limited depth of 454-sequencing was an issue for the detection of genes expressed at very low abundance. Indeed, genes known to control P and calcium homeostasis and/or bone mineralization (such as matrix proteins produced by cells specific to bone tissue) may show relative low expression; this demonstrates the need for tissue-specific experiments and high depth sequencing. To our knowledge, NGS technologies have been used for a broad range of biological studies in teleost, including rainbow trout, but these studies were mostly based on the response of soft tissue or of the whole organism without specific consideration to the skeleton [123–126].

The vertebral column being a complex tissue with remarkable cell diversity under tight-hormonal regulation, an intimate understanding of its response to P-deficiency remains challenging. Yet, such work, together with the use of common tools for mineralized structure description, histology and visual monitoring, would deeply benefit from a novel transcriptomic approach based on NGS technologies.

1.4 Cellular activity in bone formation and remodelling

Normal bone turnover is under a balanced control of bone formation and bone resorption [43, 47]. It is relevant to mention, however, that fish vertebrae are under constant growth throughout life, hence their putative lower need for bone remodelling in comparison to mammals [70]. Similarly to vertebrates, teleost skeletal structure is mainly under the control of four living cells types: osteoblasts, osteocytes, osteoclasts and chondrocytes.

1.4.1 Osteoblasts and osteocytes

Osteoblasts, derived from mesenchymal stem cells (MSCs), represent up to 6% of the total bone cells and are considered as the main actors of bone deposition [127]. Osteoblasts achieve secretion of the ECM throughout the life of the organism and play a significant role in its mineralization through production of HA in extracellular vesicles derived from

osteoblast membranes [127–129]. However, recent work indicates that the role of osteoblasts was not limited to bone formation but also includes the regulation of osteoclastogenesis and of hematopoietic stem cells (HSCs) as well as a role in endocrine functions [127]. For instance, the production of circulatory BGLAP by osteoblasts also interferes with the whole organism energy metabolism by stimulating production of insulin in pancreatic islet and increasing insulin sensitivity in the adipose tissue [57, 127, 130]. Furthermore, proliferative osteoblast progenitors and mature osteoblasts express alkaline phosphatase (ALP) and collagen fibers such as COL1a1. Due to the relative simplicity to monitor ALP by either gene expression or histological methods and its specificity to osteoblast cell-lines, ALP is usually used as a marker for osteoblast detection and bone formation monitoring in mammals [131] as well as in teleosts [116]. According to Willems and al. [132], these cells could also have an important role in the mineralization of chordal centrae since conditional ablation of osteoblasts in medaka (*Oryzias latipes*) displayed a continuous mineralized notochordal sheath without intervertebral spaces. Fjelldal et al. [72] reported a lower ALP activity in vertebrae of Atlantic salmon fed low dietary-P levels, suggesting lower osteoblastic activity and lower bone formation. Finally, mature osteoblasts may enter apoptosis, become bone-lining cells (preventing bone remodelling) or become osteocytes (Figure 1.3).

Osteocytes are osteoblasts embedded in the matrix that they produce and represent by far, the more abundant cells in bone tissue, constituting up to 90% of the total cell number [127, 133]. Franz-Odendaal et al. [133] described different stages of differentiation from osteoblasts to osteocytes shedding light on the different stages/functions of osteocytes and their role in both bone formation and resorption. Indeed, work has shown that, at first, osteocytes display a large Golgi apparatus system suggesting a higher capacity of collagen production whilst, in later stages (when osteoid is fully mineralized), they undergo a reduction of both endoplasmatic reticula and Golgi apparatus, indicating their functional shift to bone resorption (for review see [127]). Similarly, a switch of expression from osteoblast specific markers to osteocytes-specific markers accompanies osteocyte-differentiation [127, 134]. In addition, osteocytes might play a role in the regulation of bone resorption by inducing osteoclast differentiation [135, 136] and promoting the formation of

multinucleated osteoclasts in fish [47]. Moreover, osteocyte-dentritric extensions create a web connection, comparable to neuronal networks, which might be incredibly efficient for signal transfer and physiological response. For instance, studies in mammals have shown that osteocyte activity is particularly responsive to mechanical load [137–139]. As mentioned earlier, vertebrae of P-deficient fish are more limber and more subject to compression forces, suggesting that osteocyte activity might also be strongly affected by a P-deficiency; no such information is available to date.

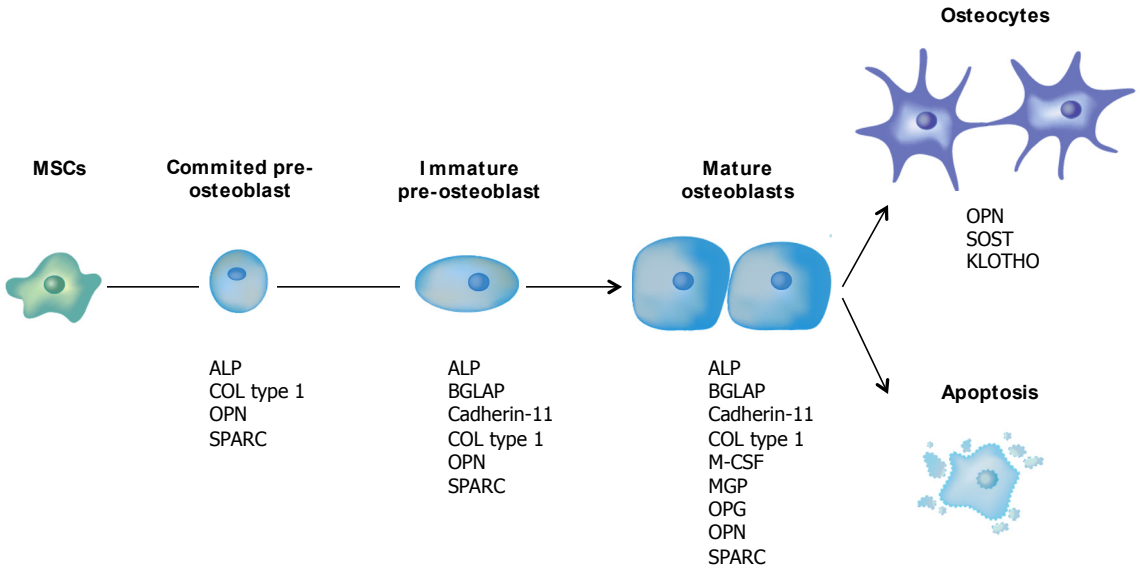

Figure 1.3: Schematic representation of osteoblastogenesis leading to bone matrix production. Gene symbol: ALP- Alkaline phosphatase; BGLAP – Osteocalcin; COL -. Collagen; MMPs – matrix metalloproteases; M-CSF – Macrophage colony stimulating factor; MGP – Matrix Gla protein; OPG – Osteoprotegerin; OPN – Osteopontin; PHEX - Phosphate-regulating neutral endopeptidase, X-linked; SOST – Sclerostin; SPARC - Osteonectin

1.4.2 Osteoclasts and bone resorption

As in mammals, bone resorption in teleost is an extraordinarily energy-demanding process that mainly results from osteoclast activity or osteoclasy [47, 140, 141]. Osteoclasts derive from monocyte/macrophage HSCs (Figure 1.4). However, since teleosts lack proper hematopoietic bone narrow, the equivalent tissue being located in the head kidney, the exact origin of fish osteoclasts is still unknown [43]. According to numerous studies focusing on osteoclasts, it has been shown that this cell type can display different morphologies and activities. For a long time, activated osteoclasts have been associated

with multinucleated cells with ruffled borders. Further observations have demonstrated that multinucleated osteoclasts are indeed associated with deeper bone resorption while mononucleated osteoclasts without ruffled borders exist and induce smooth, superficial resorption [47, 142–144]. Witten and Huysseune [39] suggested a link between acellular bone and the predominance of mononucleated osteoclasts in contrast to cellular bone and multinucleated osteoclasts. Indeed, in basal teleost such as salmonids, both mono- and multinucleated active osteoclasts have been observed [143] and intense resorption was observed during specific physiological conditions [39]. However, in advanced teleosts having acellular bone (e.g. sea bream), mononucleated osteoclasts seem to be predominant if not exclusive, suggesting a poor ability of rapid bone turnover [145]. The activity of osteoclasts in vertebrae results in the woven aspect of the trabecular bone. In mammals, active osteoclasts secrete cathepsin K (CTSK) and tartrate-resistant acid phosphatase (TRAP), acidifying the subcellular space locally to degrade bone matrix proteins and dissolve minerals [146]. The CTSK is considered as the main cathepsin involved in osteoclastic bone resorption [147]. Indeed, CTSK is a potent collagenase, able to cleave type 1 collagen, and further reported that a mutation of *CTSK* gene coding region induces bone diseases (such as pycnodysostosi) in mammals [147]. Both TRAP and CTSK are also found in various fish species and commonly used as markers of active osteoclast [144, 148]. Similarly, in Haddock (*Melanogrammus aeglefinus***)**, Roy and Lall [149] showed that higher recruitment of osteoclasts in P-deficient fish is responsible for higher bone resorption and results in lower vertebral mineralization. Inversely, Fjelldal et al. [72], showed a decrease in bone TRAP activity in low-P fed Atlantic salmon. Consequently, new insights are necessary to assess P impact on osteoclast activity.

Additionally, two osteoclast-independent patterns of resorption are also observable in teleosts. A first pattern consists of a halastic mineral loss, which refers to the demineralization process resulting of a diffusion-only component of mineral loss [47, 56]. The resorption under the halastic process would thus serve as a fine-tuning component for homeostasis regulation in teleosts [52, 54]. The second pattern is the osteocytic osteolysis, which in some cases, might act as osteoclasts to increase the size of the “bone capsule” in which they are trapped resorbing both the matrix and the mineralized structure [150, 151].

Interestingly, previous studies linked both halastatic and osteocytic resorption with Ca (and possibly P) higher requirement in salmon undertaking migration and consequently experiencing severe starvation prior reproduction [52–54].

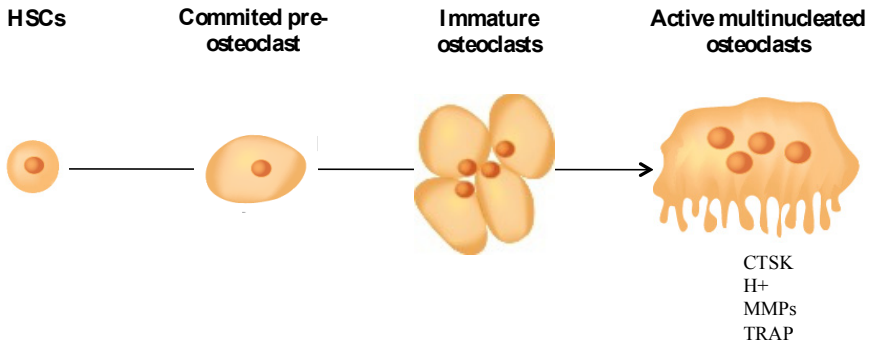

Figure 1.4: Schematic representation of osteoclastogenesis leading to bone resorption. Gene symbols: CTSK – Cathepsin K; MMPs – matrix metalloproteases; TRAP - Tartrate-resistant acid phosphatase

1.4.3 Osteoclast-osteoblast communication

Bone formation is a much slower process than resorption [152] hence the need for a tight connection to precisely orchestrate bone cell activity and prevent important bone loss. If osteoblasts and osteoclasts display antagonist effects, they are in reality, highly orchestrated in a basic multicellular unit (BMU). As mentioned above, osteoblast functions also include modulation of osteoclast differentiation either by cell-to-cell contact, formation of gap junctions or through transmission of paracrine factors [135, 153]. In addition, actors produced by osteoblasts have been identified as putative osteoclast-product recruiters, such as monocyte chemoattractant protein-1 (MCP-1), stimulating osteoclast differentiation [153]. Similarly, osteoblastic resorption induces the liberation of factors stored in bone matrix or cellular products (also called clastokines) promoting osteoblast differentiation and maturation as well as osteoblast recruitment [154, 155].

1.4.4 Chondrocytes, cartilage matrix and endochondral bone formation

As described above, under normal conditions, teleost bone displays a wide spectrum of intermediate skeletal tissues (from cartilage-like to bone-like tissues), which is complex to study [43, 156]. In normal shaped teleost vertebrae, more-or-less mineralized healthy

cartilage is observed in the basalias. Ectopic cartilage related to a metaplastic shift of bone and co-transcription of chondrocytic and osteogenic markers have also been reported in deformed vertebrae [115, 157, 158]. Understanding the processes underlying chondrocyte differentiation, proliferation, hypertrophy and maturation is thus essential to study bone metabolism in fish vertebrae. The chondrocytes also derived from MSCs are responsible for cartilage specific matrix production. The mesenchymal cells condense (involving high mitotic activity and lower cell movement) to differentiate in chondroblasts (chondrocytes progenitors) and finally mature chondrocytes producing the cartilage-specific matrix [43, 156, 159]. Mature chondrocytes produce typical COL2a1 cartilage fibers and will enter in a process of hypertrophisation. Hypertrophic chondrocytes, producing high level of vascular endothelial growth factor (VEGF), will then enter in apoptosis (Figure 1.5). The VEGF gradient of expression is mandatory for normal directional cartilage growth, invasion of blood vessels (angiogenesis) regulated by osteoclastic activity and finally penetrance of active osteoblasts [154, 160]. In teleosts, metaplastic shift from notochordal tissue to typical chondrocytic expression patterns have been associated with development of fused vertebrae [157, 158]. Furthermore, Ytteborg et al. [115] found a higher representation of chondrocytes hypertrophisation in Atlantic salmon vertebrae more likely to develop deformities, while the terminal step of hypertrophy differentiation, allowing final calcification of the cartilage, seemed to be impaired.

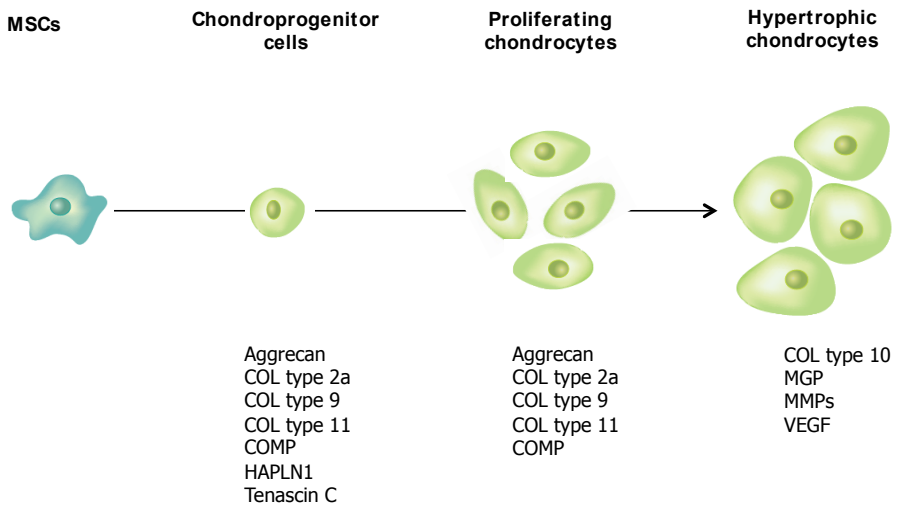

Figure 1.5: Schematic representation of chondrogenesis. Gene symbols represent the main marker of cells activity and ECM components expressed by chondrocytes. Gene symbol: COL – collagen; COMP – Cartilage oligomeric matrix protein; HAPLN1- Hyaluronan and proteoglycan linked protein 1; MGP – Matrix Gla protein; VEGF - Vascular endothelial growth factor.

1.5 Hormonal and micro-nutrient regulation

The activity of bone remodelling cells, such as osteoblasts and osteoclasts, is also known to be under hormonal regulation in mammals [131]. Several hormones either stimulating bone formation such as melatonin [112] or bone resorption such as prolactin, somatolactin [161] or cortisol [162, 163], are particularly well conserved among vertebrates. Additionally, in mammals and in birds, steroids hormones (testosterone, estrogen) have been shown to play a part in bone formation and resorption [114]. Yet, their function (e.g. estradiol) might differ from mammals or between fish species, putatively due to the biochemical properties of their receptors [164]. Similarly, several hormones also play a role in regulating body levels of P and Ca as well as their role in skeletal tissue regulation. Here we will only focus on the most studied calciotropic hormones: the parathyroid hormone-related protein, the vitamins D_3 and K, and their counterparts, stanniocalcin and the calcitonin.

1.5.1 Parathyroid hormone-related protein

Parathyroid hormone (PTH), produced by the parathyroid gland in mammals, is responsible for increased osteoclast resorption. High P serum levels also increase PTH activity, hence the importance of PTH for mineral homeostasis [165–167]. Since teleosts are devoid of a parathyroid gland, PTH has been largely ignored. However, some studies have suggested the presence and the biological action of PTH and PTH-like compounds (a potential ancestor of PTH hormones) in pufferfish (*Fugu ruditapes* and *Tetraodon fluviatilis*, [168]) as well as in zebrafish (*Danio rerio*, [169]). For instance, Suzuki et al. [170] reported that PTH1 activates mature osteoclast differentiation, hence inducing remobilization of minerals, such as P, from scales in goldfish (*Carassius auratus*). Records of PTH in other teleost remain scarce, however several studies reported the presence of parathyroid hormone-related protein (PTHrP) in sea bream [49], pufferfish [171], flounder (*Platichthys flesus*, [172]) and Atlantic salmon [61]. The PTHrP has a wide range of action in teleosts.

In bone, similarly to action of PTH in mammals, PTHrP stimulates osteoclastic bone resorption by activating expression of the receptor activator of nuclear factor kappa-B ligand (RANKL) by osteoblasts but also increases osteocytic resorption [49, 163, 173]. Furthermore, previous *in vitro* cartilage cell culture studies demonstrated that PTHrP has a positive effect of the differentiation of chondrocytes while inhibiting their entrance in a hypertrophic process [174]. In addition, PTHrP also affects osteoblast activity and can inhibit bone mineralization in sea bream [175]. It is interesting to note that the wide tissue distribution of *PTHrP* transcripts suggests both autocrine and paracrine effects [171, 176, 177]. There are three distinct receptors reported within the PTH and PTHrP family, namely PTH1R, PTH2R and PTH3R [71, 175, 178]. As in mammals, PTH and PTHrP in teleosts bind to PTH1R whilst PTH3R, structurally related to PTH1R and specific to teleost, preferentially binds to PTHrP [175]. Finally, PTH2R showed species-specific binding preferences (either PTH and PTHrP or none of them) and might not be directly related to bone metabolism [175]. Interestingly, changes in PTHrP activity as well as in *PTHrP* and *PTH1R* expression in gills and the pituitary gland in vitamin D-deficient sea bream, suggest a relation between both hormones [179].

1.5.2 Vitamins D_3 and K

Vitamin D originates either from plant (vitamin D_2) or animal source (vitamin D_3) both containing calcitriol, the biologically most-active vitamin D metabolite [45]. In mammals, vitamin D_3 and calcitriol are known to maintain serum mineral levels as well as to activate bone resorption by osteoclasts [180]. However, conflicting responses to vitamin D on P dynamics have been observed in fish, suggesting differences between teleost and mammals as well as between fish with cellular or acellular bones. In Atlantic salmon, vitamin D_3 serum levels were affected by varying P levels in the diet and continuous light [72], suggesting that the effects of low P diet together with continuous lighting might be transmitted through the vitamin D_3 system. However, incubation of rainbow trout intestine in a calcitriol-rich solution did not affect net Pi transport [72, 181]. Similarly, low (11.2 IU VD3/g diet) and high (from 42 IU VD3/g upward) vitamin D_3 levels in the diet induced vertebral deformities in sea bass while juveniles; Atlantic salmon seemed more resistant to extremely high doses (up to 2280 IU VD3/g diet) and did not present any significant higher

deformity rate [91, 182]. Vitamin K is also known as a central actor of bone tissue regulation, mainly through the large amount of vitamin K-dependent proteins expressed in bone [183]. Indeed, vitamin K regulates the activity of both BGLAP and MGP thereby regulating tissue mineralization [184, 185]. In Haddock, Roy et al. [89] reported that vitamin K deficiency led to more flexible vertebrae and to a higher risk of developing vertebral malformations, mainly due to a default of mineralization. Yet, previous studies reported that the co-administration of vitamin D_3 and K increased the bone mineral density, to higher levels than when vitamin K was administered alone [185]. Consequently, both hormones might interact to regulate mineral homeostasis, particularly P absorption and excretion, and thus regulate bone turnover. Further studies are required to elucidate these mechanism.

1.5.3 Stanniocalcin

Stanniocalcin (STC) was first identified in teleost and is known as one of the main hormone regulating mineral body levels as well as Ca and Pi transport. In fish, STC inhibits Ca^{2+} absorption through the gills while increasing phosphate reabsorption in the kidney [186, 187]. As mentioned earlier, studies in mammals widely focused on Ca. Yet, we could hypothesis that STC would also regulate P retention in teleost. In the green spotted pufferfish (*Tetraodon nigroviridis*), two STC-coding genes, namely *STC1* and *STC2,* are highly-expressed in the corpuscles of Stannius, yet both gene expression were also reported in a large variety of tissues, including chondrocytes and osteoblasts, but not in osteoclasts, suggesting both endocrine and autocrine/paracrine fashions [188, 189]. Regarding the role of STC in bone, studies in mammals reported that overexpression of *STC1* in transgenic mice led to decreased bone production and increased cartilage matrix production, as well as suppressed osteoclast activity [189–191]. However, in specific rat calvaria cells, expression of *STC1* induces osteoblast differentiation, suggesting that the response to STC might be dependent on the stage of development [192]. Intriguingly, the action of fish STC in mammal osteoclasts has a PTH-like effect, while it does not in fish-osteoclasts (see in [191]). Consequently, the precise role of STC in fish remains not fully understood and further studies focusing on specific role of STC regarding homeostasis and bone turnover regulation are required.

1.5.4 Calcitonin

Salmon calcitonin (CT) has been largely used to treat pathological bone loss in humans, such as osteoporosis, due to its major role in inhibiting mature osteoclast differentiation, preventing osteoblast apoptosis and thus increasing P deposition and BMC [193–197]. In teleosts, such as *Fugu* [198] and salmonid species [199, 200], several CT isoforms have been characterized. Similarly to PTHrP, CT has a wide range of tissue expression in fish but is mainly expressed in pituitary and ultimobranchial gland, hence its potential role as a paracrine hormone [198, 201]. As in mammals, Suzuki et al. [164] showed that CT decreased osteoclastic activity in cultured scales from both seawater (nibbler fish, *Girella punctata*) and freshwater (goldfish) fish in a concentration dependent fashion. Interestingly however, it might exist some differences in tissue distribution of CT as well as CT levels with regard to environmental adaptation. Indeed, CT has been detected in the gills of the euryhaline pink salmon (*Oncorhynchus gorbusha*), but not in the similar tissue of the freshwater rainbow trout [202–204]. Confounding effects have also been reported with experiments in fish, hence the need to further define the role of CT in bone homeostasis [205]. As in mammals, CT binds to the CT receptor (CTR) in fish [201]; yet, CTR in mefugu (*Takifugu obscurus*) also binds to other members of the calcitonin gene-related peptide (CGrP) suggesting putative different role for CTR in teleost [201]. To our knowledge, no CTR receptor expressed in bone has been reported for rainbow trout to date.

More work is thus needed to fully assess the hormonal bone tissue regulation in fish, especially in fish experiencing P deficiency. Consequently, further studies correlating serum hormone levels with bone tissue monitoring and further downstream regulation of gene expression would be particularly valuable to explore the effect of P on bone tissue.

1.6 Transcriptional regulation of bone

Although P is known to be a primordial component and regulator of bone and cell activity in fish, few studies have focused on specific bone-derived gene response to different P-dietary levels. However, studies have reported specific bone responsive genes found to be relevant for skeletal development, maintenance and cell differentiation. Interestingly, the

major metabolic pathways regulating bone cell activity seem to be highly-conserved in vertebrates with a strong homology between teleosts and humans [122, 158, 206, 207]. In this section we focus briefly on describing these metabolic pathways with special emphasis on P regulation and vertebral deformity appearance.

1.6.1 Osteoblasts and osteocytes regulation

As mentioned earlier, altered mineralization under P-deficiency could arise from a default in osteoblast number and/or function responsible for matrix production and mineralization. In Atlantic salmon with a higher risk of developing vertebral deformities, Ytteborg et al. [77] reported that maturation of osteoblasts seemed restrained. Various metabolic pathways might be involved in the regulation of osteoblast and osteocyte cells fate and activity.

Among these pathways, the TGFβ/BMP signalling has been largely documented. Briefly, both BMPs and TGFs bind to their respective heteromeric complex of type I and II serine/threonine kinase receptors (Figure 1.6). This binding will activate a cascade of SMAD (1, 4, 5 and 8) that will ultimately enter the nucleus and directly act as transcription factors [208–211]. BMP signalling has been identified in mammals as playing a crucial role *in vivo* in the induction of bone formation but also *in vitro* in activating osteoblast differentiation and proliferation [212–215]. For instance, BMP2-induced signalling pathway stimulates the expression of three distinct genes coding for transcription factors such as Runt-related transcription factor 2 (RUNX2), Osterix (OSX) and distal-less homeobox 5 (DLX5), all key actors of bone production, MSCs differentiation and osteoblast maturation [216, 217]. For instance, *RUNX2*, a "master gene" in the regulation of bone tissue, has a critical function of stimulating osteoblast differentiation as well as controlling bone matrix deposition rate by differentiated osteoblasts [218–220]. Indeed, null-mutation mice for *RUNX2* led to animals with entire cartilaginous skeletons, complete absence of osteoblasts and delayed chondrocyte maturation [152]. In the teleost, the *BMP2* gene has been cloned in sea bream where it displays similar functions than in mammals [221]. In addition to BMP2, mutation in *BMP1* has been shown to be responsible for increased bone mineral density and higher fracture susceptibility in zebrafish [222]. Similarly, *BMP4* expression pattern is known to control the commitment of stem cells

towards the osteoblastic fate whilst a deregulation of its expression is associated with the appearance of skeletal deformities in sea bass, resulting from the impairment of final osteoblast maturation [118, 223]. Besides, the TGFβ pathway is also involved in osteoblast regulation and chondrocyte differentiation [224, 225] whilst being also a pivotal actor in the orchestration of osteoclast-osteoblast communication. Indeed, TGFβ stimulates the differentiation of monocytes to osteoclast precursors, whilst the liberation of sequestered TGFβ in the matrix during resorption will indirectly inhibit osteoclast differentiation by increasing osteoprotegerin (OPG) and decreasing the RANKL production by osteoblasts [226]. In teleosts, both TGFβ and TGFβ receptors have also been observed, notably in goldfish [227], carp [228], sea bream [229], plaice (*Pleuronectes platessa*; [230]) and rainbow trout [231–233] where they assume similar functions.

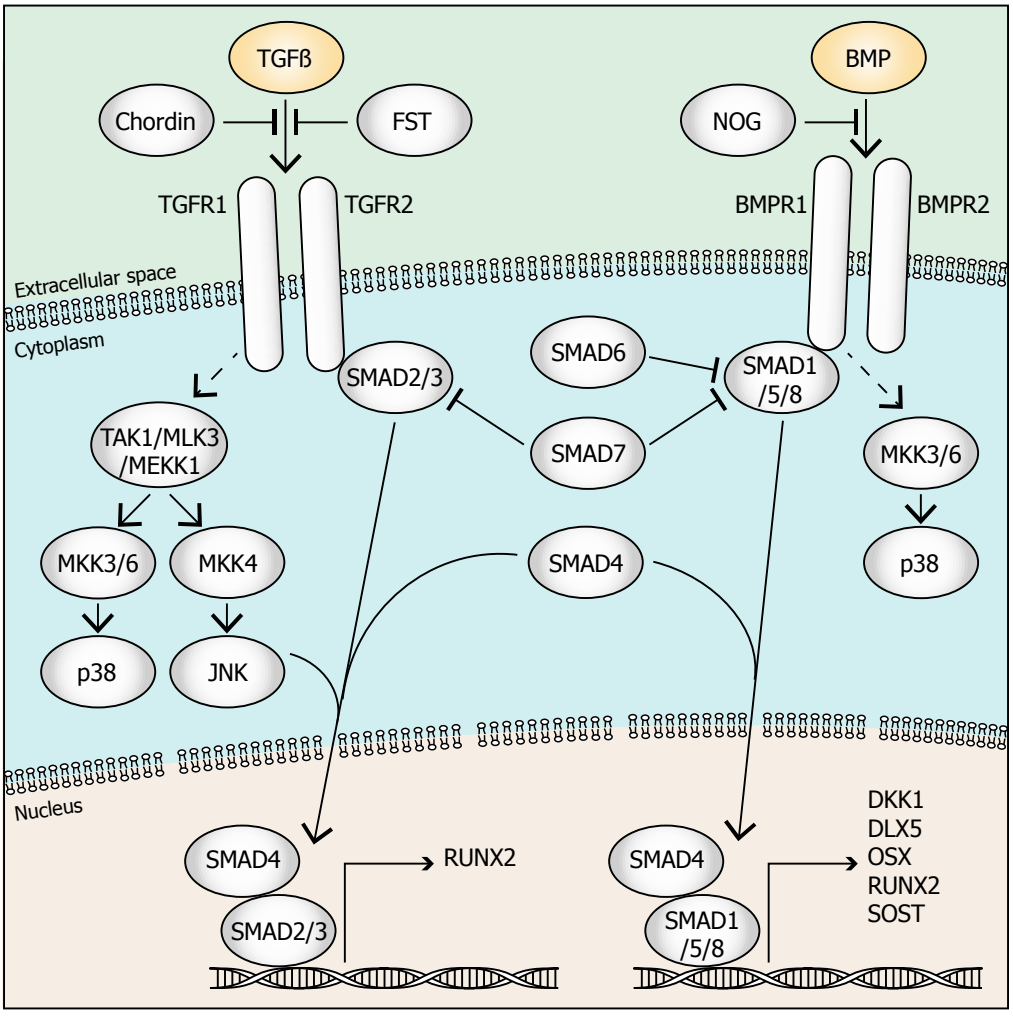

Figure 1.6: Schema of the simplified BMP/TGFβ pathway. Following binding, signal transduction of BMP is undertaken by proteins of the SMAD family. Indeed, BMP receptor I and II form an heterodimer-complex that will phosphorylate SMAD 1, 5 and 8 proteins. Upon activation, all of the activated SMADs can complex with SMAD4, conferring a DNA binding property, and then enter the nucleus where they may directly act as transcription factors. Smad 1 and 5 are preferentially involved in BMP signalling in osteoblast and regulate target genes expression such as Dickkopf-Related Proteins (DKK1), Distal-less homeobox 5 (DLX5), Osterix (OSX), Runt-related transcription factor (RUNX2) and Sclerostin (SOST). Similarly to BMP, binding of TGFβ to TGF-Receptor 1 (TGFR1) and TGFR2 will activate a SMAD cascade (SMAD2 and 3) and finally regulating expression of RUNX2 transcription factor. Although TGFβ can also activate TGF-beta activated kinase 1 (TAK1) and kinases such as Mitogen-activated protein kinase kinase kinase 11 (MLK3), Mitogen-activated protein kinase kinase kinase 1 (MEKK1), Mitogen-activated protein kinase kinase kinase 3, 4 and 6 (MKK3/4/6), that will ultimately activate Nuclear factor-kappa B (NFκB) and p38 and cross talk with RANK pathway. Finally, inhibitors of both BMP such as Noggin (NOG) and TGFβ, such as Follistatin (FST) and Chordin, have been identified.

Among the pathways involved in the control of osteoblasts, the Wnt/β-catenin pathway (or Wnt canonical pathway) is also a major calcium-dependant signalling pathway enabling osteoblast differentiation [234, 235] and activation as well as preventing apoptosis of osteocytes in mammals [138] (Figure 1.7). Briefly, Proto-oncogene Wnt (WNT) binds to low-density lipoprotein receptor-related proteins (LRP5 and 6) and frizzled (FZDs) membrane receptors and finally prevents β-catenin degradation while allowing their translocation into the nucleus. Once in the nucleus, the β-catenins will co-activate specific transcription factors such as lymphoid enhancer-binding factor 1 (LEF1) and T-cell–specific (TCF) transcription factors [235, 236]. A particular interest for TCF/LEF complex is justified by its role in the regulation of the *RUNX2* and *OSX* transcription factors expression [218–220, 237]. In teleosts, Wnt signalling was found to play a role in tissue development [238] and fin regeneration [239] while deregulation of Wnt was associated with craniofacial deformity occurrence [240]. As mentioned earlier, P-induced deformities might arise from default of mineralization also observed by a lower BMC. Interestingly, LRP5 polymorphism in mammals was correlated with the low total bone mineral density acquired in childhood, increasing the risk of developing osteoporosis in adult [241].

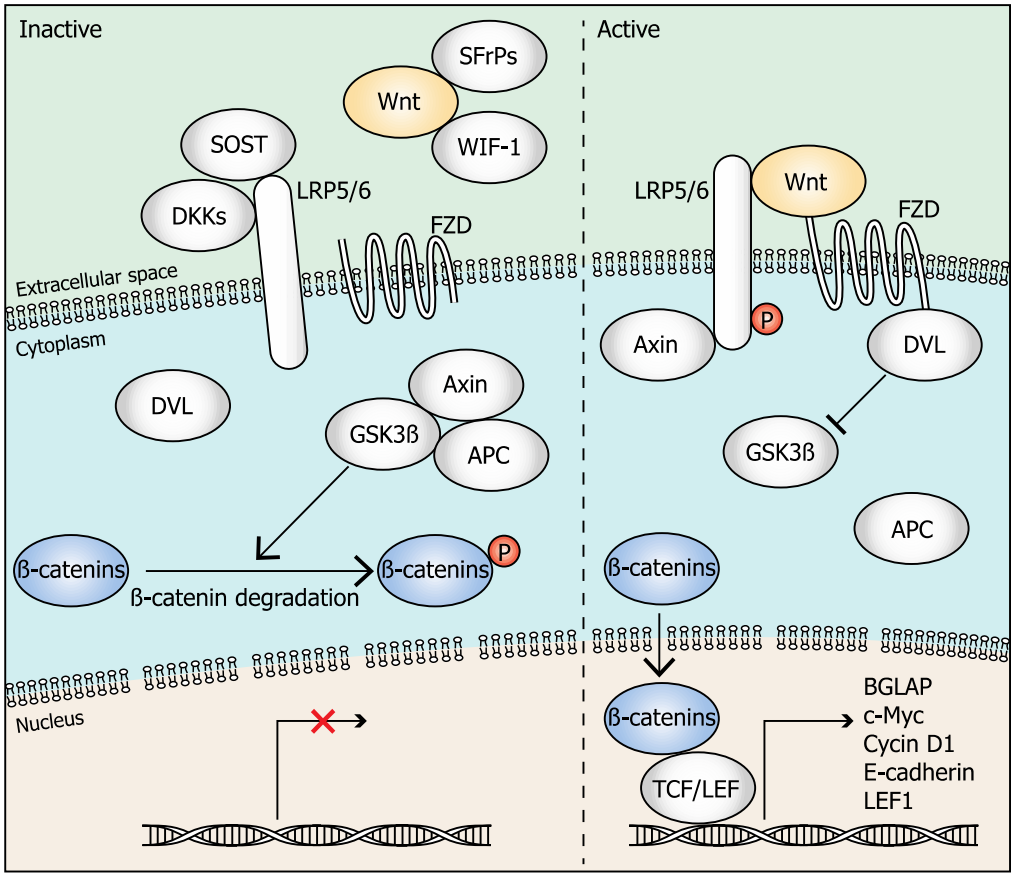

Figure 1.7: Schema of the Wnt/β-catenin canonical pathway. Extracellular binding to Frizzled-lipoprotein-receptor related protein (FZD-LRP5/6) receptor complex phosphorylates the receptor complex and recruits Axin and the Dishelved (DVL) proteins. Recruitment inhibits the formation of the complex Axin-Adenomatous polyposis coli (APC)-Glycogen synthase kinase 3 (GSKβ3) and the phosphorylation of β-catenins. Free β-catenins might then enter the nucleus to activate transcription factors such as T-Cell–Specific (TCF) and Lymphoid enhancer-binding factor 1 (LEF1) and regulate expression of target genes such as Osteocalcin (BGLAP), Proto-oncogene (c-Myc), Cyclin D1, E-cadherin and LEF1. Besides, Wnt binding may be strongly inhibited by soluble antagonists such as Sclerostin 1 (SOST1), Dickkopf-Related Proteins (DKKs), Wnt inhibitory factor 1 (WIF-1) and Secreted frizzled-related proteins (SFrPs).

As developed in this section, TGFβ/BMP and Wnt/β-catenin pathways, and their related genes, are the main metabolic actors regulating osteoblast differentiation, proliferation and maturation. Consequently, monitoring these pathways should be considered as privileged targets to assess the impact of a low-P diet on the appearance of default of mineralization status and deformities. To date however, it is not fully understood

if default of mineralization rather results from a higher matrix resorption. TGFβ/BMP, by regulating osteoblast maturation, also regulates osteocytes fate, which are involved in the non-osteoclastic resorption. Bone cells such as osteoblasts and osteocytes might also have a significant role in regulating osteoclasts activity through the metabolic pathways detailed above [136].

1.6.2 Osteoclasts and bone resorption

Higher resorption activity from osteoclasts has been correlated with higher risk of deformities in fish. For instance, a combination of low-P content and continuous light induced lower BMC as well as a higher TRAP activity in Atlantic salmon [72]. Interestingly, P deficiency in rat (*Rattus norvegicus*) led to increased number, size and possibly activity of osteoclasts and also increased number of osteoblasts. As mentioned earlier, orchestration of osteoblasts and osteoclasts activity is necessary to balance bone apposition and resorption [153]. While signalling through the M-CSF is responsible for osteoclast progenitor proliferation [136], the discovery of RANK/OPG/RANKL pathway (Figure 1.8) lifted the veil on the mechanisms by which mature osteoblasts and osteocytes regulate osteoclasts differentiation and activation [146]. RANKL is expressed in pre-osteoblasts/stromal cells and osteocytes and is known to be a major stimulator of osteoclasts differentiation in mammals [146] as well as in fish [143, 144]. RANKL has thus been largely associated with bone pathologies. For instance, misexpression of RANKL in mammals is associated with osteopetrosis due to a lack of osteoclastic activity [242]. On the contrary, in a medaka osteoporosis model, To et al. [144] observed an altered mineralization structure resulting from a RANKL-induced higher activity of osteoclasts. Besides, OPG, a decoy receptor of RANKL produced by the osteoblasts, is a key actor of the control of osteoclastogenesis by preventing osteoclast maturation, stimulating apoptosis of mature osteoclasts and thus limiting excessive bone turnover [135, 144, 242–245]. In mice, it has been observed than overexpression of OPG can lead to osteopetrosis while inhibition of OPG expression results in higher bone remodelling and osteoporosis [243, 246]. Interestingly, a negative correlation has been observed between serum OPG, MMP2 levels and both serum Ca and bone mineral density in women suffering osteoporosis.

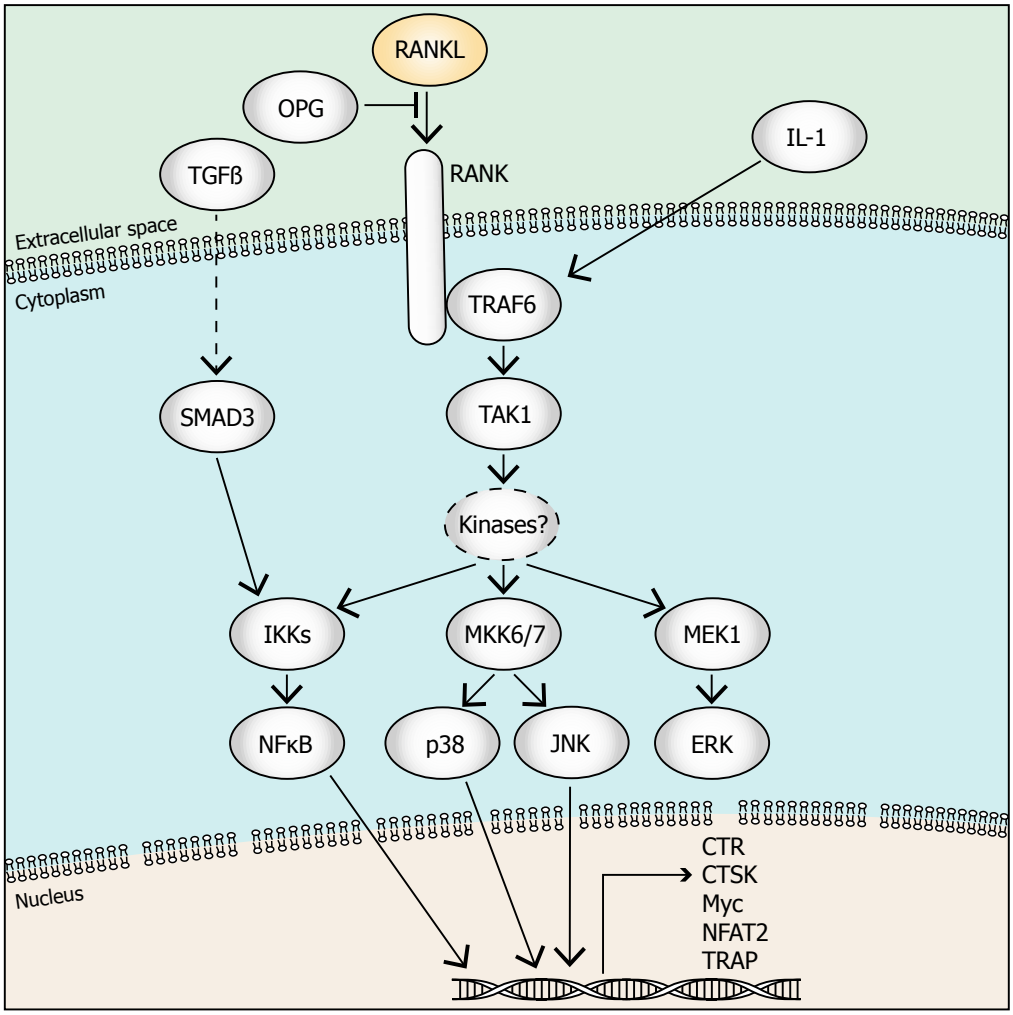

Figure 1.8: RANKL/RANK signalling in osteoclasts. Upon binding of RANKL, RANK receptor will then bind the cytoplasmic TNF receptor-associated factor 6, E3 ubiquitin protein ligase (TRAF6). TRAF6 through binding to with TGF-beta activated kinase 1 (TAK1) will activate serine/threonine kinases as well as possible others kinase that have yet to be determined. RANK cascade through kinases phosphorylation recruits IKK complex that finally liberates Nuclear factor-kappa B (NFκB). Similarly, TAK1 through Mitogen-activated protein kinases (MKK6 and 7) cascade will regulate c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38) and ultimately target genes expressions such as calcitonin receptor (CTR), Cathepsin K (CTSK), Myc, Nuclear factor of activated-cells (NFAT2) and Tartrate-resistant acid phosphatase (TRAP). All those transcription factors are involved in osteoclast differentiation. Beside, RANK cascade through Mitogen-activated kinase kinase 1 (MEK1) also targets Extracellular signal-regulated kinase (ERK) involved in osteoclast survival and block apoptosis. The binding of RANK to its receptor might also be inhibited by Osteoprotegerin (OPG). Besides, TGFβ signalling, through the recruitement of IKK complex is able to liberate and activate NFκB signalling. Finally, Interleukin 1 (IL-1) RANKL/RNAK-independent pathway

is able to recruit TRAF6 and activate kinase casacade leading to osteoclast differentiation and activation.

Matrix metalloproteases are proteins highly expressed in bone and cartilage that are able to cleave all types of extracellular matrix, even at neutral pH [247, 248]. For instance, the matrix metalloproteinase MMP14 is responsible for collagen type I degradation and necessary for chondrocytes hypertrophisation while MMP13 degrades cartilage matrix (aggrecan) and plays a central role in cartilage vascularisation [247, 249]. Similarly, MMP3 and 7 are involved in the proteolytic cleavage of RANKL from the membrane of T-cells, which allows the protein to be soluble hence to stimulate osteoclasts differentiation [135, 247]. Interestingly, besides the role of MMPs in cleavage of collagen fibers, a previous study observed the involvement of MMPs in conversion of osteoblasts to osteocytes in an osteoblast cell line MC3T3-E1 allowing osteocytical matrix resorption [250]. Wargelius et al [251] found up-regulation of *MMP13* in compressed vertebrae with chondrogenic tissue in the intervertebral space, suggesting higher ECM remodelling rate. On the contrary, *MMP13* were consistently down-regulated whether *MMP9* was down-regulated or up-regulated in 2 g and 15 g Atlantic salmon fry, under hyperthermic treatment [77]. Similarly, Fjelldal et al. [72] showed lower *MMP13* expression in fish with lower mineral content and lower stiffness resulting from the combination of low-P content and continuous light treatment. These results suggest that expression levels of *MMP13* is inherent to the state of development, being lower in early compression phenotypes (such as pronounced biconcave vertebra) than in severe compressed phenotype, possibly traducing a transitional need for ECM remodelling during the deformity phenotype aggravation.

Pathways regulating RANK/RANKL and osteoclast differentiation and activity are the main actors responsible for deep bone resorption and remodelling, enabling P mobilization and decreasing BMC to a pathological level. Yet, tissue remodelling also comprises replacement of one tissue by one another, like the metaplastic shift from notochordal tissue to cartilage specific tissue observed in fused Atlantic salmon vertebrae [157, 158]. Similarly, P-deficiency also affects cartilage tissue and chondrocytes cells fate and activity.

1.6.3 Chondrocytes and cartilage matrix

In poultry, pathologies such as tibial dyschondroplasia have helped understand the negative impact of P-deficiency on chondrocyte maturation and hypertrophisation [252]. Like osteoblasts, chondrocytes originate from MSCs and thus share numerous key events during their differentiation and regulation. The commonly-studied actors of this fine orchestration, such as SOX transcription factors and Hedgehog family member proteins might cross-talk with several other signalling pathways, including the Fibroblast growth factor (FGF) and BMP pathways, to promote chondrogenic differentiation and modulate cartilaginous matrix production [253–256]. For instance, the association of SOX9, FGF23 and RUNX2 regulates *MMP13* expression and is involved in the pathological degenerescence of joint cartilage (osteoarthritis). In zebrafish, *SOX9* ortholog genes are involved in the development of craniofacial and pectoral fin development and mutants for *SOX9*a or *SOX9*b ortholog genes led to failure in chondrocyte stacking or proper differentiation to reach sufficient number [257]. SOX9 is considered as a marker of chondrocyte precursor and was particularly highly expressed in totally fused vertebra of Atlantic salmon compared to normal [258] suggesting default in chondrocyte maturation in deformed vertebrae. Besides, *SOX9* expression is regulated by both Sonic hedgehog (SHH) and Indian hedgehog (IHH) proteins [259] and was found down-regulated in deformed Atlantic salmon vertebra [258]. Interestingly, while IHH and SHH promotes chondrocyte proliferation and differentiation [260], IHH might also regulate *PTHrP* expression and thus affect normal chondrocyte maturation.

Interestingly, IHH/PTHrP signalling is affected by an upstream fibroblast growth factor (FGF) pathway, especially by the high expression of gene coding for FGF receptor 3 (FGFR) which leads to a dwarfism phenotype [261]. Indeed, FGF signalling, through *FGF8* and *FGF3*, is required for the early development of craniofacial cartilage in zebrafish [262]. Furthermore, FGF signalling pathway has also important features in bone development and P metabolism. In zebrafish, 22 orthologs genes coding for FGFs have been identified as well as six paralogue genes [263]. The FGF-receptors (*FGFR*s) are widely expressed in the skeleton, and mutations of FGFRs have all been associated with skeletal diseases in human. With regard to P-deficiency impact on skeleton, monitoring of *FGF23* expression is

particularly relevant. Indeed, FGF23 is a hypophosphatemic factor regulating serum P levels and P reabsorption by the kidney in a vitamin D-dependent fashion [264, 265]. In mammals, the *FGF23* gene is mainly expressed in bone [264, 266, 267], yet, orthologs of *FGF23* gene were only identified in the corpuscles of Stannius in teleost [268]. The FGF23 could be a potential substrate for PHEX, a transmembrane endopeptidase associated to X-linked hypophosphatemia disorder [269] that might also negatively affect bone mineralization [270]. To be specific, the binding of FGF23 to its receptor (FGFR1) requires the presence of the cofactor KLOTHO [271]. In fish, orthologs of KLOTHO were identified in the vertebral transcriptome of sea bream [122], whilst PHEX has been reported in zebrafish [272] but not in gills or vertebrae of sea bream [122].

1.6.4 Extracellular matrix components

1.6.4.1 Collagens

In addition to intrinsic regulation, some extrinsic signals are also involved in the regulation of cellular fate and functioning. As in other vertebrates, vertebral bone tissues (compact, trabecular bone as well as maturing cartilage of the basalia) are composed of collagen fibers and non-collagenous proteins binding together the extracellular matrix (ECM) with minerals. Collagenous proteins are the most abundant proteins present in bone, constituting up to 95% of the organic matrix. Several types of collagen fibrils are found in bone such as type 1 collagen (COL1a1), the most represented collagenous protein and currently used as an early marker of bone formation [273]. Dietary-P levels are known to affect the ECM composition (including the bone mineralization and the amount or types of collagen fibers) in bone and cartilage and may thus alter the mechanical strength and supporting role of the vertebrae [105, 106]. In turn, a poor mechanical strength can influence cell differentiation, proliferation and regulation [274, 275] hence resulting in metaplastic shifts as observed during vertebral fusions [276]. Modifications in the expression of some genes coding for collagenous proteins are linked to such vertebral abnormalities. For instance, low expression of collagen type XI alpha 1 gene (*COL11a1*) has been associated with fused vertebrae, which leads to default of mineralization and fibril thickness in Atlantic salmon [73]. Similarly, ectopic expression of *COL2a1* gene in the intervertebral space of Atlantic salmon is associated with a fused vertebral phenotype [66, 67, 111].

1.6.4.2 Non-collagenous matrix proteins

Besides collagen fibers, non-collagenous proteins, such as osteonectin, BGLAP and osteopontin also regulate bone mineralization and structure and bone cells activity [277]. As they are produced by osteoblasts undergoing different maturation stages, they could also play a role in the appearance of vertebral deformities under P-deficient conditions [62, 278].

Osteonectin (SPARC) is a major non-collagenous protein found in the bone matrix. SPARC is expressed by multiple cell types including osteoblasts, hypertrophic chondrocytes as well as osteocytes [279] and has demonstrated important functions in the regulation of bone remodelling. For instance, SPARC-null mice mutants demonstrated a decrease in bone formation and in osteoblasts, osteoblastic precursors and osteoclasts number as well as a decrease in osteoblast survival [280]. Mice lacking SPARC protein would eventually display an osteopenia phenotype [280]. However, the role of SPARC is not limited to cell proliferation and differentiation but also include cell shape and adhesive properties, cell migration, ECM organization, and angiogenesis [281]. The SPARC conformation is modified when binding to Ca^{+} ions, its susceptibility to bind collagen fibers thus being altered [62]. Numerous isoforms are found in salmonids and studies on osteonectin-deficient sea bream suggest high structural conservation of important function domain within vertebrates [282]. Whilst no direct impact of P-deficiency had been correlated with differences in *SPARC* expression, Ytteborg et al. [258] reported the up-regulation of *SPARC* in fused vertebrae of Atlantic salmon.

The BGLAP is an osteoblast-specific protein found in bone and its expression is regulated by DLX5 and RUNX2 transcription factors [68, 283]. The true role of BGLAP in bone mineralization has been a source of debate for long-time. Indeed, the earliest studies reported that BGLAP had no effect on the mineralization status of bone whilst more recent findings revealed its involvement in the maturation of the HA crystals [109, 284]. To date it is largely accepted that BGLAP play a central role in bone mineralization through regulation of HA crystals growth [62] and that it might thus be involved in the response of fish to low P-dietary levels in fish. For instance, Totland et al. [111] reported that *BGLAP*

expression was up-regulated in fish with higher BMC and higher mechanical strength. On the contrary, Ytteborg et al. [258] found that *BGLAP* was down-regulated in the totally fused Atlantic salmon vertebrae. Furthermore, secreted BGLAP is known to control the organism glycaemia through stimulation of insulin production and insulin sensitivity in the adipose tissue [57, 130]. Although BGLAP might assume similar role in fish and mammals [285, 286], cloning studies from Thamamongood and al. [273] revealed that it has low degree of identity in goldfish compared to frog (*Xenopus laevis)*, mouse (*Mus musculus*) and human.

Osteopontin is considered as a marker of osteoblast activity and an early marker of mineralization, even if it is also expressed to a lesser degree in osteocytes [62]. In mammals, OPN is known to be involved in tissue mineralization and in the regulation of mineral proliferation [62]. Indeed, phosphorylated OPN contributes to mineralization processes through inhibition of ectopic calcium deposition, whilst the dephosphorylated form does not present this effect (see for review [287]). Interestingly, OPN might also inhibit osteoclasts differentiation [287]. Fonsesca et al. [288] identified an osteopontin-like protein expressed in an osteoblast-like cell line Vsa16 from sea bream and potentially displaying a similar function. Osteopontin-like proteins were also as reported in the vertebrae of Atlantic salmon [258], and rainbow trout [289].

Here we developed a description and further hypothesis of the regulation of bone tissue under P-deficiency at different biological levels ranging from hormonal regulation and cells activity to gene expression. However, much of the work, particularly the transcriptomic data, is based on mammals; hence the need to refine those concepts and to adapt the observations to teleost species.

1.7 Rainbow trout for genomic studies

The official name for rainbow trout has been subject to intense debates over the past 30 years and resulted in more than 30 nominally described species [290] to finally reach a consensus under the appellation of *Oncorhynchus mykiss* (Walbaum, 1792). The rainbow trout is one of the 68 species belonging to the Salmonidae family. Some members of the

family are anadromous fish, with individuals living primarily in salt water and able to migrate long distances to spawn in coastal streams, and others living permanently in freshwater lakes. Native to the North-American Pacific coast, from Alaska to Mexico, rainbow trout has been widely introduced elsewhere in the world, mainly for production purposes. Its economic importance has made the rainbow trout one of the most studied biological models with regard to nutrition, comparative immunology, ecology as well as evolutionary and quantitative genetic traits [291]. Various physiological advantages of the species (large number of eggs, easy to incubate, to handle and to microinject) and the large amount of genomic data available, have dramatically simplified transgenesis and have largely contributed to biotechnological improvement [291].

The rainbow trout genome size has been previously estimated around 2.4 Gbp for 58 to 64 chromosomes [293, 294]. Rainbow trout is a tetraploid-derivative fish as a consequence of a whole genome duplication (WGD) event that occurred between 25 and 100 million years ago [295, 296]. Salmonid WGD is a relatively recent compared to the same phenomenon that occurred in ray-finned fish over 300 million years ago [297]. Similarly, microsatellite analysis indicates that the entire genome has not the same tetraploidisation/re-diploidisation status [298]. Indeed, tag assembly from BAC library allowed Palti et al. [295] to estimate that approximately two-third of the genome is still duplicated in Swanson clonal line of rainbow trout. This duplication tends to increase paralogue genes, complicating studies reporting the quantitative expression related to bone metabolism. Recently, Berthelot et al. [299] sequenced the first draft of the rainbow trout genome from a single homozygous, doubled haploid, Y male of the Swanson river (Alaska). Authors annotated a total of 46,585 genes and 69,676 transcripts with an average size of 4.8kb.

In addition to genome sequencing, several whole-transcriptomes for rainbow trout have been published thanks to the rapid development of NGS technologies [124–126, 300–303]. Thus in December 2014, 288,810 expressed sequence tags (ESTs) were available for rainbow trout in the public domain of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov). However, as previously mentioned, these data are

derived from studies analysing most tissues of the body (brain, pituitary gland, liver and muscle) with the exception of bones. Thus, despite deep sequencing efforts over the past years, little is known about the bone specific transcriptome in rainbow trout. Nevertheless, the key actors regulating bone tissue metabolism might have been particularly well conserved among vertebrates.

Salmonids, such as rainbow trout, are an interesting model for bone tissue studies owing to their physiological characteristics (relatively long lifetime, large body size, cellular bone type), the relative simplicity of their rearing conditions, and the issues regarding some particularities of bone skeleton development and regulation specific to teleosts. For example, while the notochord may be poorly conserved in humans making it difficult to study, it persists throughout salmonids lifetime [43]. This is of particular interest as cells derived from the notochord are known to be involved in spinal column formation in humans [304]. Therefore, although much caution should be taken when characterizing bone tissue ortholog genes, further work on fish bone tissue could add crucial information to the understanding of skeletal regulation and function in other vertebrates [206, 305].

1.8 Hypothesis and objectives

Increasing demand and constraints for aquaculture products have strongly influenced practices and motivated research. Among the main research focuses, limiting P output from freshwater farms is a major objective of the Quebec government who set the target to 4.2 kg of P released per day per tonnes of fish produced by 2015. To date however, to limit negative impact of a P-deficiency for animal health, the Canadian Feed Act prohibits the use of less than 1% total P (DM) in the diet for salmonids, despite P requirements being significantly lower, particularly for large fish. Thus, successful reduction of P output requires a better comprehension of the mechanisms associated with P-induced deformities in fish structural. In addition to preventing and/or reversing the appearance of bone deformities, this knowledge would strongly help refine P optimum levels in the diet, through the development of new indicators and tools for bone tissue monitoring.

The hypothesis for this study was that P-deficiency results in lower P status in fish, which, through a complex and cross-talking network of genes expressed in the vertebrae, leads to the development of bone deformities in rainbow trout. The use of transcriptome-wide tools will thus be particularly valuable to assess early changes in gene expression in a non-model species. These tools must nonetheless be used in combination with common and other innovative tools for a comprehensive evaluation of the overall response of fish bone to P-deficiency.

Based on a pipeline of analysis for the study of bone tissue proposed by Ytteborg et al. [70], this project aimed to explore the fundamental processes involved in the appearance of deformities through an integrative approach. Ultimately, increasing the amount of molecular data for rainbow trout, as well as highlighting the genes involved in the response of vertebrae to P-deficiency, will considerably benefit further studies on rainbow trout. The overall target was divided in three steps. The first objective was to characterize the early response of fish subject to a P-deficiency, through the monitoring of P-status in various body compartments and X-rays imagery. The second objective consisted in building a reference transcriptome for RNA-sequencing technologies and allowing further quantitative studies. Finally, based on this reference, the last objective was to determine the genes involved in the apparition of vertebral deformities under P-deficiency.

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2 Chapter 2: Responses of different body compartments to acute dietary phosphorus deficiency in juvenile triploid rainbow trout (*Oncorhynchus mykiss*, Walbaum)

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2.1 Résumé

La truite arc-en-ciel (*Oncorhynchus mykiss*) est une des espèces les plus produites à l'échelle mondiale ainsi qu'une des les plus étudiées, notamment d'un point de vue nutritionnel et physiologique. On sait ainsi que les rejets chargés en phosphore (P) issus des élevages peuvent avoir inpact environnemental négatif sur les écosystèmes. Pour cette raison, des restrictions draconiennes ont été mises en place dans de nombreuses régions de la province Québécoise avec l'objectif de réduire la quantité de ces rejets dans les eaux douces. Cependant, des inquiétudes sont apparues quant aux liens entre un développement anormal du squelette, sa minéralisation, et une déficience en P, notamment sur des poissons juvéniles à croissance rapide. Malgré l'abondance des d'études entreprises pour comprendre la dynamique d'une déficience en P et son impact sur la minéralisation des tissus osseux, la réponse physiologique à court terme des poissons nourris avec de faibles niveaux de P dans la diète reste peu décrite. Pour parer à ce manque, des poisons d'un poids initial de 60,8 ± 1,6g ont été nourris avec deux diètes pratiques (suffisante et déficiente, contenant respectivement 0,45% et 0,29% de P disponible) sur une période de cinq semaines. Les taux de cendres et de P dans les écailles, les vertèbres et les carcasses, ont été utilisés pour déterminer le statut en minéral dans les différents tissus. La perte relative en minéral et les coefficients de variation ont également été calculés pour déterminer la réponse spécifique et estimer la variabilité de chaque indicateur. Après quatre semaines d'expérience, aucun effet significatif n'a été observé sur les performances de croissance, la mortalité ou la prise alimentaire. Cependant, dès la deuxième semaine et jusqu'à la fin de l'étude, la quantité en cendres et le taux de P dans les écailles et dans les carcasses étaient significativement inferieurs ($P < 0,05$) chez les poissons soumis à une diète déficiente. À la semaine 4, la réduction de minéralisation atteignait 19,3% et 18,4% respectivement pour le taux de P et la quantité de cendres chez les poissons déficients. De la même façon, les pertes dans les carcasses atteignaient 15,1% et 12,8% respectivement pour le taux de P et la quantité de cendres. Fait intéressant, l'absence le perte minérale significative dans les vertèbres n'a pas empêché de voir une émergence significativement plus importante de malformations vertébrales chez les poissons soumis à une diète déficiente (45,6% ± 11,0%)

comparés aux poissons suffisants (1,5 ± 2,1%). La plus grande majorité de ces phénotypes anormaux observés étaient des vertèbres biconcaves (35,5%) et de petits corps vertébraux largement espacés (9,3%). Les écailles en contre partie, ont montré une variabilité interindividuelle plus importante alors qu'un tel effet n'a pas été observé dans les carcasses ou les vertèbres. Cette étude fournit donc une première comparaison des différents indicateurs couramment utilisés pour déterminer les taux en minéral ainsi qu'une première approche pour évaluer la variabilité de réponse de ces indicateurs chez des poissons nourris avec des faibles niveaux de P dans la diète.

Mot-clefs: Phosphore – Minéralisation – Truite arc-en-ciel – Écailles – Vertèbres - Carcasses

2.2 Abstract

Rainbow trout (*Oncorhynchus mykiss*) is an important production species as well as one of the most studied fish models, particularly regarding nutritional physiology. Due to negative environmental impacts linked with rainbow trout farm effluents, significant restrictions have been established in numerous regions to reduce dietary phosphorus (P) outputs. However, questions have arisen regarding the link between abnormal skeletal development and mineralization and insufficient dietary P availability during rapid fish growth (juvenile fish). Despite significant work to understand the dynamics of P-deficiency and the resulting impact on tissue mineralization, the extent of the early responses in rainbow trout fed low-P diets is not well described. The aim of this study was to explore the early-responses of scales, vertebrae and carcass P and ash in rainbow trout fed low-P versus sufficient-P controls. Two practical diets (sufficient: 0.45% available P and deficient: 0.29% available P) were fed over a five-week period to triploid rainbow trout (initial mass 60.8 ± 1.6g). Ash and P contents were used to assess mineral status of the different tissues. The relative loss of mineral and coefficient of variation were also calculated to compare the relative response and the inter-individual variability. After four weeks of P deprivation, no detectable effects were observed on growth performance, mortality or feed intake. However, as early as the second week onward, ash and P levels in scales and carcasses were significantly lower ($P < 0.05$) in fish fed a P-deficient diet compared to those fed a P-sufficient diet. At week 4, the reduction in mineralization reached 19.3 and 18.4% for P and ash contents in scales, respectively. By the same week, for P and ash contents in carcasses reached 15.1 and 12.8%, respectively. Interestingly, the absence of significant mineral loss in vertebrae did not prevent the emergence of a high incidence of vertebral deformities in P-deficient fish (45.6 ± 11.0%) when compared to P-sufficient fish (1.5 ± 2.1%). The main vertebral deformities observed in P-deficient trout were specific to impede bone matrix mineralization such as pronounced biconcave (35.5%) and undersized and widely spaced vertebral bodies (9.3%). For the scales compartment (ash and P status) and mineral parameter, the coefficients of variation showed a higher inter-individual variability in P-deficient versus P-sufficient fish, while no such effect was observed in carcass or vertebrae

compartment. This study provides a useful comparison of various commonly-used indicators of bone mineral status as well as an approach to assess variability of fish response under low-dietary P level.

Keywords: Phosphorus – Mineralization - Rainbow trout – Scales – Vertebrae - Carcass

2.3 Introduction

Rainbow trout is an important production species as well as one of the most studied biological models with regard to numerous physiological aspects, including nutrition. In freshwater systems, phosphorus (P) is a limiting element for primary production; indeed excessive P output from fish production can contribute to eutrophication in freshwater receiving bodies [1]. Over the past decade in Quebec, significant restrictions of P from fish farm effluents have been legislated by environmental agencies to limit the impact of P output on receiving watersheds [2]. One of the most promising and tangible approaches to minimize P in effluent is to provide P at an optimum dietary level, meeting but not exceeding requirements in fish [3].

To date, a large number of studies have help refine the P requirements for rainbow trout according their life stage, growth rate or size [4–11]. To assess these P requirements, many different indicators have been used, such as P excretion (urinary or faecal), weight gain and growth, bone mineralization status (scales, vertebrae or whole-body) or, less commonly, gene expression [12–15]. Requirements for P based on responses of mineralized tissues are generally higher in fish [15], compared to weight gain, growth or plasma P. Irrespective of the response variable measured, high variations regarding the analyzed indicators still complicates the estimation of the response to P-deficiency [13, 15]. Defining the inter-individual variability of P status in bone tissue may help to understand the extent and limits of the response.

In fish, P absorption is mainly undertaken through the intestine, while P reabsorption is carried out by the kidney [16]. Phosphorus serves vital biological functions such as energy transfer (ATP) or genetic material composition (DNA, RNA). Phosphorus is also a main component of mineralized tissues and as such, 85% of the total body P [17, 18] are being stored in bones under the form of hydroxyapatite [Ca5(PO4)3(OH)] crystals. The amount of P in bone (and resulting bone condition such as strength, mineralization or deformities) are tightly balanced by cellular processes such as bone apposition (osteoblasts)

and bone resorption either diffuse (halastasy) or control by osteoclast and osteocyte (in cellular bone fish such as salmonids) cells [19, 20]. These processes are impacted by various factors such as rearing or environmental conditions [21–24], genetics [25–27], life stage/fish size [28, 29] or nutrient deficiency [30, 32]. The factors affecting bone tissue mineralization and the processes involved in bone formation and resorption/remodeling in larva and juvenile fish have been recently reviewed [33, 34].

Extensive work has reported that prolonged low dietary P level leads to a lower and/or delayed bone mineralization and in severe cases, vertebral deformities in fish [10, 15, 32, 35–40]. Dietary P deficiency at early stages of development may also trigger skeletal abnormalities appearance at later growth stages [39]. As bone defects are an increasingly preoccupation for the aquaculture industry, it thus appears crucial to identify early P-deficiency in mineralized tissues as soon as possible and provide fish with optimal dietary P levels. The aim of this study was to explore the early-response in low-P fed compare to sufficient-P fed fish, regarding to their scales, vertebrae and carcass P and ash contents, as well as their relative intrinsic variability.

2.4 Materials and methods

2.4.1 Husbandry

All-female triploid (produced by hyperbaric pressure according to standard protocol) rainbow trout (60.8 ± 1.6 g, mean ± SEM; n = 1,680) were transferred from a local fish farm (St-Alexis-des-Monts Inc. Canada) to experimental facilities at the 'Laboratoire de Recherche des Sciences Aquatiques (LARSA)' at Université Laval in Québec (Canada). Fish were housed in 12 green circular fiberglass tanks (2000 L; density of 8.5 to 10.1 kg/m3 from week 0 to 5, 6 replicate tanks by diet, n = 140 fish per tank), dispersed in four different units, for five weeks. Prior the beginning of the study, an acclimation period of two weeks was provided during which fish were fed with a commercial feed (Corey Optimum 3 mm) in accordance with the feed manufacturer's table. From week 0, fish were fed at 80% satiety with two experimental diets (so-called P-sufficient and P-deficient diets, containing 0.92 and 0.50% total P, respectively; 6 replicate tanks per diet). Trout were

starved two days prior to all sampling events. Diet compositions (Table 2.1) were adapted from an open formulation previously developed at Guelph University and successfully tested in our laboratory [11, 41, 42].

All fish were supplied with 80% recirculated freshwater (40 L/min; water renewal ~20%) and held at 12 ± 2ºC, near oxygen saturation (10.0 mg/L) and at constant long photoperiod (18L: 6D). Suspended solids were filtered using sand filter (200 L) and ammonia and nitrite were removed using a trickling biofilter (250 L). Levels of ammonia and nitrite were monitored twice weekly to assess biofilter performance. The experiments were conducted in compliance to the guidelines of the Canadian Council on Animal Care (1984) and supervised by the Animal Protection Committee of Université Laval.

Table 2.1: Ingredients and proximate composition of the P sufficient and P deficient diets

	Diets	
Ingredients (g/kg)*	**P-deficient**	**P-sufficient**
Herring meal [a]	75	75
Blood meal [a]	100	100
Feather meal [b]	100	100
Wheat meal [c]	205	180
Soybean meal [c]	80	80
Wheat gluten [d]	70	70
Corn gluten meal [c]	170	170
Fish oil [a]	180	180
$CaHPO_4$ [e]	0	25
Lysine (Biolys) [d]	0.012	0.012
DL-methionin[f]	0.002	0.002
**Vitamin-mineral premix [f]	0.006	0.006
Ethoxiquin (antioxydant) [f]	150 ppm	150 ppm
Proximate composition (%, dry basis)		
Dry matter	90.45	90.18
Crude protein	49.71	49.93
Crude lipid	20.79	21.39
Gross energy (E. MJ/kg)	24.93	24.43
Ashes	3.77	5.41
Total phosphorus (P)	0.50	0.92
Total calcium (Ca)	0.75	1.78
Total magnesium (Mg)	0.22	0.26
Total zinc (Zc, mg/kg)	184	180
Total copper (Cu, mg/kg)	16	16
Digestible dry matter***	62.31 ± 1.74	60.03 ± 0.74
Digestible protein***	42.52 ± 0.96	42.51 ± 0.43
Digestible lipids***	17.54 ± 0.67	16.71 ± 0.90
Digestible energy***	18.94 ± 0.48	17.95 ± 0.31
Digestible P (Dg. P)***	0.29 ± 0.03	0.45 ± 0.03
Dg. P/Dg. E (g/MJ)***	0.15 ± 0.02	0.25 ± 0.02

Values are indicated as mean ± SD

***Canadian supplier: a SANIMAX Marketing Limited. Guelph, ON; b Floradale feed mill limited: Floradale, ON; c Meunerie Gérard Soucy Inc.: Ste-Croix, QC; dADM Alliance Nutrition Canada, St-Hyacinthe, QC; e Laboratoire Mat Inc., Québec, QC; f Corey Feed Mills Ltd., Fredericton, NB.**

**** Supplied the following: (to provide mg/kg except when noted): retinyl acetate 2500 IU; cholecalciferol 2400 IU; tocopheryl acetate, 50; menadione, 10; thiamine, 1; riboflavin. 4; pyridoxine, 3; Ca-pantothenate. 20; vitamin B-12, 0.01; niacin, 10; biotin, 0.15; folic acid, 1; choline, 1000; inositol, 300; magnesium carbonate, 1.24 g/kg; calcium carbonate, 2.15 g; potassium chloride, 0.90 g/kg; sodium chloride, 0.40 g/kg; potassium iodide, 0.4; copper sulphate, 30; cobalt sulphate, 0.2; ferric sulphate, 0.20**

g/kg; manganese sulphate, 30; zinc sulphate, 40; dibasic calcium phosphate, 5 g/kg; sodium fluoride, 10.

***** The digestibility study was conducted the week following the experiments in triplicate tanks (N=3).**

2.4.2 Vertebral deformities

To monitor the occurrence of deformities, X-rays were performed on a random sample of 60 fish prior to the beginning of the study and on all fish at week 5 (n = 1,198). The radiographs were performed using a MINXRAY HF80+ device (Mini X-ray Inc., Northbrock, USA) on the caudal region in a latero-lateral view according to Witten and Hall [42] and with the following settings: 60 kV, 15 mA, 2 sec exposure, beam source distance 40 cm. Radiographs were developed using clinical processing equipment (CAN-Med, Canada) for conventional radiographs (Merry X-Ray 3-7-90 Type S-2 Developer 10G and 3-7-90 Fixer 2 Pak, Canada) according to Witten et al. [43]. Vertebral abnormalities were assessed directly on developed Kodak film (Industrex MX-125 Film Ready Pack. 8X10 inc., Lot 366018, Kodak Industry, France). As post-cranial and ural vertebrae can develop abnormalities irrespective to their nutritional condition [42, 44], we focused on caudal vertebrae (V31-44) using the regionalization proposed by Kacem et al. [45]. Fish were associated with a vertebral phenotype by visual examination according to the classification for salmon from Witten et al. [43].

2.4.3 Growth performance and tissue sampling

From week 0 to week 4, 10 fish per tank (60 fish per week per treatment) were anesthetized in diluted MS-222 bath (75 mg/L, Syndel International Inc., Vancouver, BC, Canada), measured (fork length ± 1 mm), weighed (± 0.1 g) and sacrificed. Scales were scraped from tail to head and four caudal vertebrae (V31-32, V43-44) were collected and kept in 70% ethanol solution. Remaining carcasses (comprising muscle, viscera and remaining bone tissues) were stored in -20°C. Growth performance calculations included the feed intake (FI = total amount of food given to tank / number of fish) and the Fulton's condition factor (K = [final weight (cm) / final length3 (g)] x 100) assessment.

2.4.4 Mineralization status

Carcasses were freeze-dried and mineralized tissues (scales and vertebrae) were dehydrated in a graded series of ethanol (70°, 90°, 100°; 24 h/bath), and delipidated in acetone (2 baths of 24 h), then in trichloroethylene (2 baths of 24 h). Diets and tissues (carcasses, vertebrae and scales) were analyzed for dry matter (drying in a vacuum oven for 18 h at 105 °C) and ash (incinerating in a muffle furnace for 18 h at 550 °C) to the nearest 0.1 mg according to AOAC guidelines (1990). Phosphorus content was determined by ion chromatography (ICS-3000, Dionex Corporation, Sunnyvale, CA, USA) following ash digestion in nitric acid and filtering (Whatman paper No. 1, rinsed 3 times in 100 ml volumetric flask). Separation was carried out on an IonPac AS 17 column (Dionex) with an IonPac AG 17 guard column. For sample loading, a 50 µl sample loop (PEEK) was used. Multi-step gradient concentrations ranging from 9 to 30 mM allowed a complete separation of phosphorus anions in aqueous media at 16 min with a constant flow rate of 1.0 ml/min. Tissue (carcass, scales and vertebrae) ash and P contents were calculated on a dry matter basis: [ash (or P) (%) = ash (or P) content / dry sample weight (g)]. For each parameter (ash and P contents) and sampling week (0 to 4), the impact of P deficiency on a given tissue was assessed using the relative difference between P-deficient and P-sufficient trout: Loss (%) = (value P-sufficient – value P-deficient) / value P-sufficient x 100. To assess the inter-individual variability of response, coefficients of variation for each week and diet was calculated: CV (%) = (standard deviation / mean value) x 100.

2.4.5 Statistical analysis

To assess dietary effects on the incidence of deformities at week 5, means were analyzed with non-parametrical Wilcoxon-Mann-Whitney (or test U).

The results were analyzed using Linear Mixed-Effect Models with diet and time and diet x time as fixed effects and tank within unit as random effect. Adjusted means from replicates tanks (n = 6) were tested for normal distribution (Shapiro-Wilk, skewness and kurtosis tests) and homoscedasticity (Levene's tests). Two-way analysis ANOVAs, were performed for each indicator (ash and P content in scales, carcasses and vertebrae). Post-

hoc multiple comparisons (Tukey HSD tests) were conducted when differences or interactions were found to be significant at $P < 0.05$.

Finally, data on the CV were analyzed using the same linear mixed-effect model with diet, time and diet x time as fixed effects and unit within tank within unit as a random effect. As the mean CVs per replicate tank (n = 6) were not normally distributed, a permutation procedure was used to approximate the distribution of the F-statistics under the null hypothesis of no effect. The number of permutations was set to 1000. When the interaction or main effects were significant, the same permutation procedure was used for the multiple post-hoc group comparisons. In order to control the family-wise error rate, *P* values were adjusted using the Holm-Bonferroni method. Analyses were conducted in R statistical software version 3.0.2 [46], with lawstat [47], moments [48], multcomp [49] and nlme [50] packages.

2.5 Results

In this study, no detectable effect of diet was observed on final body weight, fork length, feed intake or condition factor. Weight, length and feed intake increased significantly with time and similarly for dietary treatments, indicating a significant time-dependent effect. Mortality was very low (< 0.01%) and not correlated to treatment (Table 2.2).

Table 2.2: Weight, length, condition factor (K), feed intake, deformities and mortality in fish fed P-sufficient and P-deficient diets.

	P-sufficient diet	P-deficient diet
Week	**Mean ± SD**	**Mean ± SD**
Fork length (cm)		
0	17.7 ± 1.1 [a]	17.5 ± 0.9 [a]
1	17.9 ± 1.0 [a]	18.2 ± 1.6 [a]
2	18.8 ± 1.0 [b]	18.7 ± 1.2 [b]
3	19.2 ± 1.1 [b]	19.2 ± 1.0 [b]
4	19.9 ± 1.1 [c]	19.9 ± 0.9 [c]
Wet weight (g)		
0	61.1 ± 9.9 [a]	59.6 ± 8.8 [a]
1	62.1 ± 9.5 [a]	65.6 ± 10.2 [a]
2	72.1 ± 10.3 [b]	73.2 ± 12.0 [b]
3	80.1 ± 12.3 [c]	82.1 ± 12.0 [c]
4	91.1 ± 15.3 [d]	92.9 ± 11.8 [d]
Condition factor (K)*		
0	1.1 ± 0.1	1.1 ± 0.1
1	1.1 ± 0.1	1.1 ± 0.2
2	1.1 ± 0.1	1.1 ± 0.1
3	1.1 ± 0.1	1.2 ± 0.1
4	1.2 ± 0.1	1.2 ± 0.1
Feed intake (g/fish)**		
1	5.31 ± 0.17 [a]	5.41 ± 0.29 [a]
2	7.94 ± 0.68 [b]	8.15 ± 0.51 [b]
3	9.24 ± 0.83 [c]	9.56 ± 0.60 [c]
4	11.18 ± 1.08 [d]	10.72 ± 0.72 [d]
Vertebral deformities (%)***		
5	1.5 ± 2.1 [a]	45.6 ± 11.0 [b]
Mortality (%)		
0 to 5	0	< 0.01

Values (for feed intake condition factor, weight and length) are means of six tanks by treatment. Adjusted means from linear mixed-effect models (with tank within unit random effect) were analyzed with two-way ANOVAs (diet and time). For mean within each parameter analyzed, values not sharing identical letters were significantly different (P < 0.05).

*** K = [final weight (cm) / final length3 (g)] x 100**

**** Feed intake= total amount of food given to tank / number of fish**

***** Vertebral deformities assessment was performed at termination of the study (week 5, N=1,198) and means were analyzed with non-parametrical test U**

No external signs of deformity were observed during the five week of experiment. The occurrence of vertebral deformities prior the beginning of experiments was 3.3% (n =

60). By the end of the experiment (week 5), the incidence of vertebral deformities was significantly higher in P-deficient (45.6 ± 11.0%) than in P-sufficient fish (1.5 ± 2.1%) ($P < 0.05$). Within the P-deficient deformed fish, main vertebral phenotypes were pronounced biconcave (type 11; [43]), undersized and widely spaced vertebral body (type 10; [43]), with an observed frequency of 35.9 and 9.3%, respectively. All other abnormal phenotypes observed at week 0 and week 5 were represented in low proportion (< 0.1%). Furthermore, the X-rays indicate that for the two most represented vertebral malformation types (type 10 and 11) in P-deficient fish, all caudal vertebrae were affected, while other types were affecting isolated vertebrae and was not spread in the caudal region.

Interestingly, the effect of the low-P diet on the mineral status of vertebrae was negligible. Vertebra P content significantly increased gradually with time but no effect of the diet was found ($P = 0.08$) (Table 2.2). Likewise, ash content in vertebrae increased gradually with time. Taking all values together, the ANOVA also found a significant effect of the diet ($P = 0.04$). However, multiple comparison tests at each sampling time failed to reveal differences between diets, indicating that the negative effect of the low P-diet on vertebra ash content was minimal. A significant interaction of time and diet was found to affect P and ash status of scales and carcasses. While mineralization of scales and carcasses (either ash or P contents) was kept constant in P-sufficient fish, it gradually decreased in P-deficient trout. Significantly lower P and ash contents were observed from week 2 onwards (Table 2.3) for both compartments. The relative loss in scales and carcasses of P-deficient fish increased with time to reach P levels of 19.3 and 15.1% and ash content of 18.4 and 12.8%, respectively after four weeks of treatment.

Table 2.3: Relative impact on ash and P content in scales, vertebrae and carcass for fish fed P-sufficient and P-deficient diets.

	P-sufficient diet	P-deficient diet	
Week	**Mean ± SD**	**Mean ± SD**	**Loss (%)****
Vertebrae ash (% dry weight)*			
0	51.04 ± 5.58 [a]	51.67 ± 4.43 [a]	n.c
1	60.13 ± 4.28 [c]	57.99 ± 3.09 [c]	3.5
2	55.05 ± 1.63 [ab]	52.02 ± 1.68 [ab]	5.5
3	55.80 ± 1.34 [bc]	52.36 ±1.37 [bc]	6.2
4	56.53 ± 1.58 [bc]	53.01 ± 3.09 [bc]	6.2
Vertebrae P (% dry weight)*			
0	9.08 ± 0.51 [ab]	9.36 ± 0.73 [ab]	n.c
1	8.74 ± 0.42 [a]	8.50 ± 0.27 [a]	2.7
2	9.43 ± 0.29 [bc]	8.97 ± 0.38 [bc]	4.8
3	9.98 ± 0.38 [c]	9.60 ± 0.32 [c]	3.8
4	9.91 ± 0.26 [c]	9.53 ± 0.45 [c]	3.8
Scales ash (% dry weight)*			
0	32.00 ± 1.98 [d]	32.04 ± 2.27 [d]	n.c
1	31.51 ± 1.00 [cd]	29.42 ± 0.96 [bc]	6.6
2	31.55 ± 0.83 [cd]	27.97 ± 0.84 [b]	11.3
3	31.90 ± 1.13 [cd]	27.92 ± 0.85 [b]	12.5
4	31.03 ± 1.31 [cd]	25.31 ± 1.93 [a]	18.4
Scales P (% dry weight)*			
0	5.72 ± 0.38 [cd]	5.77 ± 0.53 [cd]	n.c
1	5.75 ± 0.20 [cd]	5.29 ± 0.16 [bc]	8.0
2	5.76 ± 0.14 [cd]	5.07 ± 0.17 [ab]	12.0
3	5.85 ± 0.27 [d]	5.02 ± 0.16 [ab]	14.3
4	5.91 ± 0.18 [d]	4.77 ± 0.24 [a]	19.3
Carcass ash (% dry weight)*			
0	7.09 ± 0.08 [b]	7.16 ± 0.06 [bc]	n.c
1	7.22 ± 0.19 [bc]	7.00 ± 0.13 [b]	3.0
2	7.22 ± 0.19 [bc]	6.42 ± 0.13 [a]	11.1
3	7.26 ± 0.07 [bc]	6.41 ± 0.13 [a]	11.7
4	7.38 ± 0.16 [c]	6.44 ± 0.22 [a]	12.8
Carcass P (% dry weight)*			
0	1.21 ± 0.02 [d]	1.24 ± 0.03 [de]	n.c
1	1.24 ± 0.04 [de]	1.20 ± 0.04 [cd]	3.0
2	1.29 ± 0.04 [e]	1.13 ± 0.03 [bc]	12.2
3	1.23 ± 0.02 [de]	1.08 ± 0.06 [ab]	12.0
4	1.21 ± 0.04 [d]	1.03 ± 0.06 [a]	15.1

Values are means of six tanks by treatment. Adjusted means from linear mixed-effect models (with tank within unit random effect) were analyzed with two-way ANOVAs

(diet and time). For mean within each parameter analyzed, values not sharing identical letters were significantly different (P < 0.05).

*** Ash (or P) (%) = ash (or P) content / dry sample weight (g)**

****Values are expressed in percentage of loss for P and ash content in P-deficient fish compared to P-sufficient fish at each time point: Loss (%) = [(value P-sufficient – value P-deficient) / value P-sufficient] x 100. Percentages of loss were only use in a descriptive since no statistical tests were conducted on these values. n.c = not calculated**

The coefficient of variation (CV) offers useful properties of having no units, being standardized and allows comparison of inter-individual variability of a given parameter between diets and/or tissue (Table 2.4). No significant effects of diet were observed in P and ash vertebrae CVs ($P > 0.05$). At week 0, CVs in both vertebrae P and ash content were high and overall significant effect of time was observed; however, no such significance were confirmed with multiple comparison. In contrast, a significant interaction of time and diet effects was found to impact ash and P CVs in scales (Table 2.4). At week 4, CVs in scales were significantly higher in P-deficient (10.4 ± 1.9% and 11.9 ± 1.6%, for P and ash, respectively) versus P-sufficient fish (5.8 ± 1.3% and 5.9 ± 1.6%, for P and ash, respectively). No significant effects of time ($P = 0.06$) were noticeable in carcasses ash. Diet has a significant impact ($P = 0.03$); however, multiple comparison tests failed to reveal differences between diets, indicating that the effect of the low P-diet on carcass ash CV was minimal. In contrast, variability in carcass P content was significantly different over the five week of experiment. Indeed, for both diets, CVs of P content in carcass was higher at week 4 than at week 2.

Table 2.4: Coefficient of variation (CV) of ash and P content in carcasses, scales and vertebrae of fish fed deficient or sufficient P diets.

Values are means of six tanks by treatment. Adjusted means from linear mixed-model effect (diet and time and diet x time as fixed effects, and tank within unit as random effect) were analyzed with a permutation procedure (n = 1000 permutations). Multiple

	P-sufficient diet	P-deficient diet
Week	**CV (%)***	**CV (%)***
Vertebrae ash		
0	12.3 ± 7.1	8.4 ± 3.0
1	5.8 ± 2.2	10.2 ± 9.3
2	3.3 ± 1.4	5.6 ± 0.6
3	4.1 ± 1.5	5.4 ± 0.9
4	4.1 ± 1.5	5.0 ± 1.3
Vertebrae P		
0	15.7 ± 9.3	13.8 ± 9.4
1	8.5 ± 5.2	5.8 ± 1.8
2	4.5 ± 1.5	7.1 ± 1.9
3	6.3 ± 3.5	5.5 ± 1.4
4	5.0 ± 1.2	6.3 ± 1.0
Scales ash		
0	6.4 ± 1.3 [ab]	7.2 ± 1.8 [ab]
1	6.0 ± 0.9 [ab]	6.4 ± 2.0 [ab]
2	6.2 ± 1.4 [ab]	8.4 ± 1.9 [b]
3	5.3 ± 1.2 [a]	8.1 ± 1.9 [b]
4	5.9 ± 1.6 [ab]	11.9 ± 1.6 [c]
Scales P		
0	8.1 ± 2.3 [ab]	7.6 ± 2.9 [ab]
1	6.8 ± 1.0 [ab]	8.1 ± 2.4 [ab]
2	6.3 ± 1.3 [ab]	9.7 ± 1.4 [b]
3	6.2 ± 1.7 [a]	8.7 ± 2.1 [ab]
4	5.8 ± 1.3 [a]	10.4 ± 1.9 [b]
Carcass ash		
0	5.9 ± 1.0	5.7 ± 1.8
1	4.7 ± 1.2	5.9 ± 1.1
2	4.8 ± 1.2	5.3 ± 0.9
3	5.9 ± 1.0	6.1 ± 1.8
4	5.8 ± 0.7	6.9 ± 1.6
Carcass P		
0	7.8 ± 4.0 [ab]	7.1 ± 2.1 [ab]
1	5.3 ± 2.0 [ab]	7.9 ± 3.9 [ab]
2	6.3 ± 2.3 [a]	6,6 ± 1.2 [a]
3	8.7 ± 1.3 [ab]	9.5 ± 2.6 [ab]
4	8.6 ± 2.7 [b]	11.0 ± 2.2 [b]

comparisons were analyzed using the same permutation procedure for multiple post-hoc tests and P were corrected with Holm-Bonferroni method. For mean within each parameter analyzed, values not sharing identical letters were significantly different (P < 0.05).

*** Coefficient of variation (CV) = (standard deviation /mean) x 10**

2.6 Discussion

In order to align our efforts with the current commercial aquaculture production context, we focused on triploid fish and the formulation of practical diets. In this study, a P-sufficient diet (0.92% total P; 0.45% available P) was formulated to meet the P requirement (0.54-0.61% total P) for rainbow trout as recommended by the National Research Council [4, 51]. Moreover, this P sufficient diet also displayed a digestible P to digestible protein ratio (0.25 g/MJ) in the range of recommended values (0.19-0.27 g/MJ) for this species [5, 51]. Inversely, the level of dietary P in the deficient diet (0.5% total P; 0.29% available P) was lower than the minimum P requirement for juvenile rainbow trout [11, 41, 42].

Our results confirm that for four weeks, juvenile rainbow trout under P deprivation are able to maintain normal growth and continue to feed normally. Indeed, similar and increasing feed intakes throughout the experiment in both P-sufficient and P-deficient fed trout insured that all groups were fed equally. Moreover, no difference in growth and/or mortality was observed. These findings are similar to previous studies on Atlantic salmon (*Salmo salar*) and rainbow trout [10, 52, 53] where final body weight, length and condition factor were unaffected by early P-deficiency. However, adverse effects are generally reported in larger fish and after a longer period of P deficiency. For instance, rainbow trout fed 0.2% available P were significantly shorter than rainbow trout fed 0.4% available P after 16 weeks of trial [11]. As mentioned by Kaushik [54], growth during a P deprivation period can be maintained as long as the body still contains enough P reserves that can be mobilized to fulfill soft tissues requirements.

One of the major findings of the current study was that scales and whole carcasses were more sensitive to P deprivation than vertebrae (in terms of ash and P contents) or other common growth performance indices such as feed intake and mortality. Our results

showed that fish fed a P-deficient diet displayed significantly lower P and ash contents in carcasses from week 2 onwards which is in agreement with previous studies [10, 16, 28]. As observed in carcasses, P and ash contents in scales were also significantly lower in fed fish a P-deficient diet as early as the second week of experiment. After four weeks, the relative percentage of loss ash and P content in scales was 18.4 and 19.3%, respectively, with carcasses reaching 12.8 and 15.1%, for ash and P content in carcasses, respectively. Interestingly, vertebrae response was low regarding the percentages of loss (6.3 and 4.0%, for ash and P contents, respectively) at week 4, and was not significantly affected by diet. These results are in accordance with previous studies showing that P requirements for growth are lower than requirements for normal whole-body P concentration or tissue mineralization [15, 28, 54, 55]. Previous results from Skonberg et al. [6] also showed similar responses of whole-body and skin (including scales) in fingerling rainbow trout under P deprivation, however no comparison with vertebrae was reported. Nwanna et al. [56] observed that in common carp (*Cyrprinus carpio*) P requirement based on whole-body P content was higher than estimations from bones (scales and central vertebrae) P content (9.1 and 14,7%, total P, respectively). Since in our study, carcasses included mineralized tissues such as scales, vertebrae, skull, ribs and otoliths, the response of the P status in the carcasses might be correlated with mineralized bone tissue rather than P in flesh [28]. As already observed in previous studies, mobilization of P to maintain normal growth will be preferred over tissue mineralization under P deprivation in fish [28]. However, our results suggest that all bone compartments do not respond similarly and that scale mineralization might be mobilized preferentially, to benefit the vertebrae. Whereas, both scales and carcasses were similarly responsive to P deprivation, an assessment of the mineralization status in scales presents the advantage of being non-intrusive and may be particularly useful for early-P deficiency detection in rainbow trout. However, the utility of scales might be limited to short-term response and further studies will have to prove the utility of scales as a long-term response indicator.

During this study, the absence of any noticeable effect of P-deficiency on the mineralization of the vertebrae did not prevent the emergence of vertebral anomalies. Indeed, a higher incidence of vertebral abnormalities was observed in low-P fed fish (45.6

± 11.0%) at week 5, compared to those fed P-sufficient diets (1.5 ± 2.1%). The main types of malformations were pronounced biconcave (type 11) and undersized and widely spaced vertebral body (type 10) as described by Witten et al. [43]. Such abnormalities are generally specific to impeded mineralization [41, 43]. It is well documented in fish that P-deficiency can lead to under-mineralization and in some severe cases to bone deformities [28, 32, 36, 57]. The cellular processes leading to under-mineralization of the endoskeleton could be the result of an impeded mineralization of the new bone matrix by osteoblast and/or higher bone resorption processes by osteocytes and osteoclasts [58] or by halastasic mineral diffusion [19]. Since osteocytes have not been reported in dermoskeleton, osteoclasts in scales might have a major role in bone resorption [59, 60]. It is still under debate if advance teleosts having acellular bone (i.e., lacking of osteocytes) can draw actively on bone reserves [58]. However, in early teleost as salmonids, bone tissues are known to be mineral reservoirs, at least under specific physiological conditions such as starvation, migration and reproduction [45, 61]. As we aimed to evaluate the short-term response to P deficiency in term of ash and P contents, these considerations are beyond the scope of our study and will thus not be discussed further. In any cases, our results demonstrate that internal vertebral abnormalities appear early following induction of P deficiency. These internal abnormalities could eventually result in external deformities if they are aggravated during further growth [44, 62], highlighting the importance to detect P deficiency as early as possible. In this regard, other indicators not measured in our study, such as gene expression [7, 9, 12–14, 63] may be even more informative of early responses to P deficiency in the endoskeleton.

The aim of the current study was also to highlight the inter-individual variability of response within the same dietary treatment. No significant effect of diet was observed in the CV both vertebrae and carcass P and ash content. A relative high variability was observed at week 0 in vertebrae (P and ash content). Since average amount of ash vertebrae material at this sampling time was very low (6 ± 0.2 mg) and no such variability was noticed for other compartments at the same week, one could assume that this particular variability might mainly reflect the limit of detection for this technique. In contrast, after four weeks of experiment, the CVs in scales were significantly higher in P-deficient versus

P-sufficient fish. These observations are in accordance with results from previous work, showing that the coefficient of variation for ash in P-deficient rainbow trout ranged from 9 to 15% while it only ranged from 3.5 to 9% in fish fed a control diet [28]. Interestingly, variability in vertebrae P content was also observed in smolting Atlantic salmon and was not correlated to any variability in the non-mineral (protein) vertebrae content, suggesting individual variations in the rate of mineral deposition on the matrix [36]. The inter-individual variability in mineral deposition may result from a large range of factors, such as those related to tank hierarchy and individual access to food or stress responses in relation to aggressive behaviour of conspecifics [64–66]. In our study, low fish densities as well as adequate dispersion of the rations were used to reduce dominance effects and no difference in feed intake was observed between P-deficient fish and P-sufficient fish. Moreover, triploid fish are known to display less aggressive behaviour than their diploids counterparts [67]. Besides, genetic background has been frequently mentioned as a significant factor affecting the response variability in fish [33, 34, 40]. Genetic variations have already been linked with feed utilization [68–69], mineral absorption [70] or spinal deformities occurrences [25–27, 71]. In an applied context, further studies focusing on genetic variation related to plasticity of response to P deprivation might be highly relevant to help reduce P output and ensure animal welfare.

2.7 Conclusion

The current study was an attempt to further define the short-term responses of carcass, scale and vertebral compartments of rainbow trout fed a practical low-P diet. According to the different tissue compartments analyzed herein, both scales and carcasses appeared as a useful tool for early-detection of P deficiency in trout. Furthermore, the scale compartment offers a non-invasive approach to facilitate the monitoring of P status. Phosphorus deficiency had no noticeable effect on P and ash level in vertebrae but induced higher incidence of vertebral deformities after a five week trial. Furthermore, we highlighted the inter-individual variability of the response in low P-fed fish. In light of this variability, further studies looking at the genetic variations related to phenotypic plasticity in fish fed low-P diet might be of particular interest for fish strain selection and industry sustainability.

2.8 Acknowledgements

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3 Chapter 3: Establishment of a comprehensive reference transcriptome for vertebral bone tissue to study the impacts of nutritional phosphorus deficiency in rainbow trout (*Oncorhynchus mykiss*, Walbaum)

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3.1 Résumé

La réduction du phosphore (P) dans la diète, est une approche communément utilisée pour limiter les rejets en P dans les effluents d'élevage. Néanmoins, une réduction importante pourrait conduire à une déficience, potentiellement impacter la condition du tissu osseux et induire l'apparition de malformations vertébrales chez les poissons. À ce jour, aucune étude de séquençage à haut-débit ne s'est appliquée à l'étude du squelette vertébral chez les salmonidés et les changements au moléculaire restent ainsi peu caractérisés. Cette étude a donc pour objectif de fournir le premier transcriptome du tissu vertébral chez la truite arc-en-ciel (*Oncorhynchus mykiss*) et ainsi permettre la réalisation d'études quantitatives et fonctionnelles ultérieures. Les poissons, d'un poids initial de 60,8 ± 1,6 g, ont été nourris pendant 27 semaines en utilisant deux diètes pratiques contenant respectivement 0,29% (déficient) et 0,45% (suffisant) de P disponible. Un séquençage haut-débit à partir d'un pool incluant des poissons soumis à une diète déficiente et suffisante en P ainsi que des individus ayant développés des malformations vertébrales, a été réalisé en utilisant la technologie HiSeq2000 Illumina (100 paires de bases et brins pairés). Ainsi, plus de 140 millions de reads « nettoyés » ont pu être assemblés en *de novo* avec Trinity pour aboutir à 679869 transcrits d'une taille moyenne de 542,5 pb. Au total, 340747 séquences ont pu être associées avec des marqueurs de séquence exprimée (ESTs) déjà référencés pour la truite arc-en-ciel. De plus, 141909 et 117564 séquences ont respectivement pu être annotées grâce à l'appui des banques de données protéiques Nr et Uniprot. De manière intéressante, nous avons observé que la majorité des acteurs clefs dans la régulation du tissu osseux chez les mammifères étaient également présents chez la truite arc-en-ciel.

Mot-clefs: Transcriptome - RNA-sequencing - Assemblage *de novo* - Os et cartilage - Phosphore - *Oncorhynchus mykiss*

3.2 Abstract

Reducing dietary phosphorus (P) is a common approach to reduce effluent P outputs. The potential resulting P- deficiency is known to negatively impact fish bone condition and might result in vertebral deformities. To date, no large-scale study involving deep sequencing of the bone transcriptome has been conducted in salmonids and vertebral molecular changes remain poorly described. This study aims to provide the first comprehensive vertebral transcriptome for rainbow trout (*Oncorhynchus mykiss*) to allow functional and quantitative expression studies. Fish weighing 60.8 ± 1.6 g, were fed for 27 weeks using two practical diets having 0.29% (deficient) and 0.45% (sufficient) available phosphorus (P), respectively. Deep sequencing was conducted using HiSeq2000 Illumina 100 paired-end technology from pooled P-deficient and P-sufficient fish and individuals displaying vertebral deformities. Over 140 million trimmed paired-end reads were assembled *de novo* with Trinity and resulted in 679,869 transcripts with a mean length of 542.5 bp. From these sequences, 340,747 matched with referenced ESTs from rainbow trout. Furthermore, 141,909 and 117,564 sequences were function- ally annotated against Nr and Uniprot databases, respectively. Interestingly, we observed putative homologue sequences for most of the key components involved in bone formation and turnover in mammals.

Keywords: Transcriptome - RNA-sequencing - *de novo* assembly - Bone and cartilage - Phosphorus - *Oncorhynchus mykiss*

3.3 Introduction

Concerns about freshwater pollution from fish farming have led to the development of high-performance low-phosphorus (P) diets, resulting in a reduced nutrient output [1]. However, questions have been raised regarding the impact of insufficient P during rapid fish growth on the occurrence of vertebral deformities [2–5]. In most cases, skeletal abnormalities in early stages are not visually detectable [6] and may be reversed to a certain degree, hence the importance of understanding initial underlying biological mechanisms. To date, transcriptomic data are still lacking regarding specific bone response to P deficiency. Here, we used RNA-sequencing technology, which appeared suitable to generate transcriptomic information on non-model species [7, 8]. This study aims to provide a more comprehensive reference transcriptome for the bone tissue in trout as well as to annotate and highlight sequences that have biological functions involved in P regulation. It is intended as a first step permitting quantitative genomic studies on the skeletal tissue in relation to P requirements.

3.4 Data description

3.4.1 Husbandry

All-female triploid rainbow trout (*Oncorhynchus mykiss*) were transferred (N = 1680, initial mass 60.8 ± 1.6 g; St-Alexis-des-Monts Inc., Canada) to the experimental facilities of the Laboratoire de recherche des sciences aquatiques (LARSA), Université Laval (Québec, Canada). Fish were initially acclimated for two weeks and fed a commercial feed (Corey Optimum 3 mm) in accordance to the manufacturer's tables. Thereafter, fish were fed with practical diets, either P-deficient (D, available P: 0.29%) or P-sufficient (S, available P: 0.45%), already tested in our laboratory [5, 9–11]. Animal rearing, P status and vertebrae monitoring were assessed according to the published methods (see for details [9, 11]). Experiments took place in compliance with the guidelines of the Canadian Council on Animal Care (2005) and supervised by the Animal Protection Committee of the Université Laval.

3.4.2 Sampling and tissue collection

Fish sampling was performed to maximize the representation of fish sizes, diets and vertebral phenotypes. Initially (week 0) and for P-deficient and P-sufficient diets (week 4), fish were randomly sampled. Two individuals displaying the two most common types of P-related vertebral deformities at week 27 were also added [11]. Three caudal vertebrae (V35–36–37) with ligaments and intervertebral tissues were collected from each fish. Hemal and neural arches were removed and the remaining body and flesh re- moved by rinsing and brushing with PBS (1X). Samples were immediately placed in liquid nitrogen then stored at −80 °C.

3.4.3 RNA extraction and cDNA library preparation

Vertebral samples from each individual were first crushed in liquid nitrogen. Total cellular RNA was extracted using TRIzol Reagent (Life Technologies) according to the manufacturer's recommendations. Total extracted RNA was subjected to DNAse treated (ArcturusPicoPure RNA isolation kit, Life Technologies) and RNA integrity and purity were assessed using a Bioanalyzer 2100 (Agilent Technologies). RNA was quantified using ND-1000 spectrophotometer (NanoDrop Technologies Inc.). RNA samples from weeks 0 and 4 were pooled (3 fish per pool) according to sampling time and diet, while fish sampled at week 27 were processed separately. Libraries were created using TruSeq Sample Prep Kit v2 (Illumina, USA) following the manufacturer's instruction. Resulting libraries were quantified using a Bioanalyzer 2100 (Agilent Technologies). Samples were multiplexed (6 samples per lane) and sequenced at McGill genomic platform (Montréal, Canada) with HiSeq2000 sequencer and a 100 paired-end (PE) technology.

3.4.4 Bioinformatics and transcriptome assembly

Reads from HiSeq2000 Illumina were processed with Trimmomatic v0.30 [12] to remove low quality (trailing: 20, lowest quality: 30) and short reads (b 60 bp). Trimming also included removal of Illumina adapters together with the most common contamination vectors from UniVec database (https://www.ncbi.nlm.nih.gov/tools/vecscreen/univec/). The combined high quality reads (pools/samples) were *de novo* assembled using the Trinity assembler [13].

3.4.5 Functional annotation

To compare our transcriptome with already-published data, we used a Blast algorithm [14] against both nucleotide and protein databases. Blastn searches were performed against rainbow trout data retrieved from the National Center for Biotechnology Information (NCBI), Siganae (National Institute for Agricultural Research, INRA), the NAGRP Aquaculture Genome Projects (http://www.animalgenome.org /aquaculture/salmonids/rainbowtrout/EST_WV.html), as well as trout ESTs data from Castaño Sánchez et al. [15]. To highlight the lack of data for rainbow trout, we chose a high threshold evalue <1e-4 for blastn searches [16]; in contrast, to evaluate the representation of the reference databases in our experiment we choose a more stringent threshold (evalue <1e-10). Transcripts from our bone transcriptome that did not match under this threshold (evalue <1e-4) with already-referenced data were considered as "novel sequences".

Blastx searches (threshold evalue <1e-8) were performed on Uniprot-Swissprot and NCBI non-redundant nucleotide databases (Nr). Since Uniprot-Swissprot is better annotated, the former has been privileged for subsequent downstream sequences analysis. For non-matched sequences against Uniprot database, an open reading frame (ORFs) detection has been done with getorf algorithm from EMBOSS 6.5.7 [17]. The getorf parameters focus on theoretical ORFs, between two STOP codons to counterpart possible bias in 5'-UTR regions, with a minimum length of 100 amino acids. Furthermore, to highlight the main biological processes, cellular localization and molecular function, a gene ontology (GO) association was conducted with GoMiner software based on unique Uniprot entries [18]. All figures have been carried out with R 2.15.2 software [19] using ggplot2 [20] packages.

3.5 Results and discussion

Sequencing yielded 185,369,129 reads for each end. Trimming de- creased the amount of reads to 141,986,373. Assembly for Illumina 100PE reads led to 679,869 transcripts for a mean length of 542 bp (Table 3.1). This Transcriptome Shotgun Assembly (TSA) project

has been deposited at DDBJ/EMBL/GenBank under the accession GBTD00000000. The version described in this paper is the first version, GBTD01000000.

Table 3.1: Summary of RNA-seq sequencing and assembly results

Sequencing	
Number of reads	185,369,129
Number of reads after trimming	141,986,373
Mean reads length (bp)	96.7
Maximum reads length (bp)	100
Minimum reads length (bp)	60
Mean base quality	36.5
***De novo* assembly**	
K-mers length (bp)	25
Number of transcripts	679,869
Number of large transcripts (>500bp)	146,287
Number of large transcripts (>1000bp)	95,343
Number of components	521,483
Mean transcripts length (bp)	542.5
Maximum transcripts length	15,880
Minimum transcripts length (bp)	100
N50 transcripts length (bp)	1,768

Blastn of the 679,869 transcripts against subset ESTs database resulted in 340,737 matches, with an average length of 903.6 bp (threshold evalue <1e-4). Consequently, despite deep sequencing efforts already published for rainbow trout [15, 21–23], 339,132 transcripts (49.9%) remained non-referenced or matched with evalue >1e-4 (average length of 179.6 bp). Non-matched sequences against rainbow trout ESTs databases are referenced as novel sequences in the following paragraphs. Interestingly, 90.9, 92.7, 89.0 and 91.0% of the rainbow trout sequences available in Siganae, NCBI, NGRAP database and from [24], respectively, found homologies (evalue <1e-10) with sequences in our study.

For functional annotation we compared our transcripts against protein sequence databases (Nr and Uniprot). Blastx on Nr was executed in order to extract the species distribution of top-hit matches. From the 679,869 transcripts, a total of 141,909 found

homology (threshold evalue <1 e-8) with referenced sequences. Top-hit species distribution (against Nr database) results were comparable to other published data for different tissues in rainbow trout [15, 25]. Indeed, most of the sequences were matched preferentially with teleost species, and particularly with cichlids (*Oreochromis niloticus*, 15.2%; *Haplochromis burtoni*, 6.4%; *Pundamilia nyererei*, 5.6% and *Maylandia zebra*, 4.8%, Figure 3.1A). Sequences matching the model fish species zebrafish were the most abundant (16.4%); yet, best-hit representation of salmonids was relatively low with 15.2% and 3.1%, for Atlantic salmon and rainbow trout, respectively.

In parallel, best-hits for each Uniprot blastx matches (threshold evalue <1e-8) were selected for further functionally annotation steps since Uniprot provides better annotation than Nr. Blast search against Uniprot resulted in 117,564 transcripts (17.6%), related to referenced protein sequences (i.e. 25,679 unique Uniprot entries). Most of the transcripts matching with a homolog Uniprot sequence were transcripts larger than 1,500 bp. Best-hit distribution from Uniprot largely differed from Nr results but as expected (for review see [26], we observed a strong homology with higher vertebrates with 36.4% and 20.4%, for human and mouse (*Mus musculus*), respectively. This association was even stronger than for other teleost species such as zebrafish (12.8%), most probably explained by the higher quantity of data for human or mouse available on Uniprot. Furthermore, a total of 497 and 454 sequences displayed a top match with rainbow trout and Atlantic salmon, respectively, indicating that biological information on Uniprot database regarding those species is limited. In parallel, from the 339,132 novel sequences, 11,417 found a match (evalue < 1e-8) against Uniprot database, corresponding to 6,337 unique Uniprot entries. Consequently, functional annotation revealed 327,715 novel sequences yet to be annotated. Average lengths were 1,869 and 265 bp for mapped and non-mapped transcripts, respectively. As suggested by Liu et al. [27] the non-matching transcripts might correspond to UTR region, non-coding RNA and/or possible novel sequences for this species. Indeed, only 5.4% of the non-matching sequences against Uniprot displayed theoretical ORFs superior to 100 amino acids. Some of the sequences displaying theoretical ORFs among the non-matching sequences might be too different from known sequences to allow finding homologies.

In this study, we used the GO associations in order to compare our results with other transcriptome on the basis of a standardised vocabulary. The GO association resulted in 11,202 associations with 10,427, 10,210 and 9,697 matches, representing cellular component, molecular functions and biological processes, respectively. The GO associations revealed that bone transcriptome resembles other GO analyses carried out on distinct tissues from trout and zebrafish. The most common associations were binding (76.6%), and catalytic activity (39.2%) for molecular function, cellular processes (76.6%), metabolic processes (58.3%) and regulation of biological processes (44.7%) for biological processes, and cell (88.2%), cell part (88.2%), organelle (59.4%) and membrane (43.9%) for cellular components (Figure 3.1B).

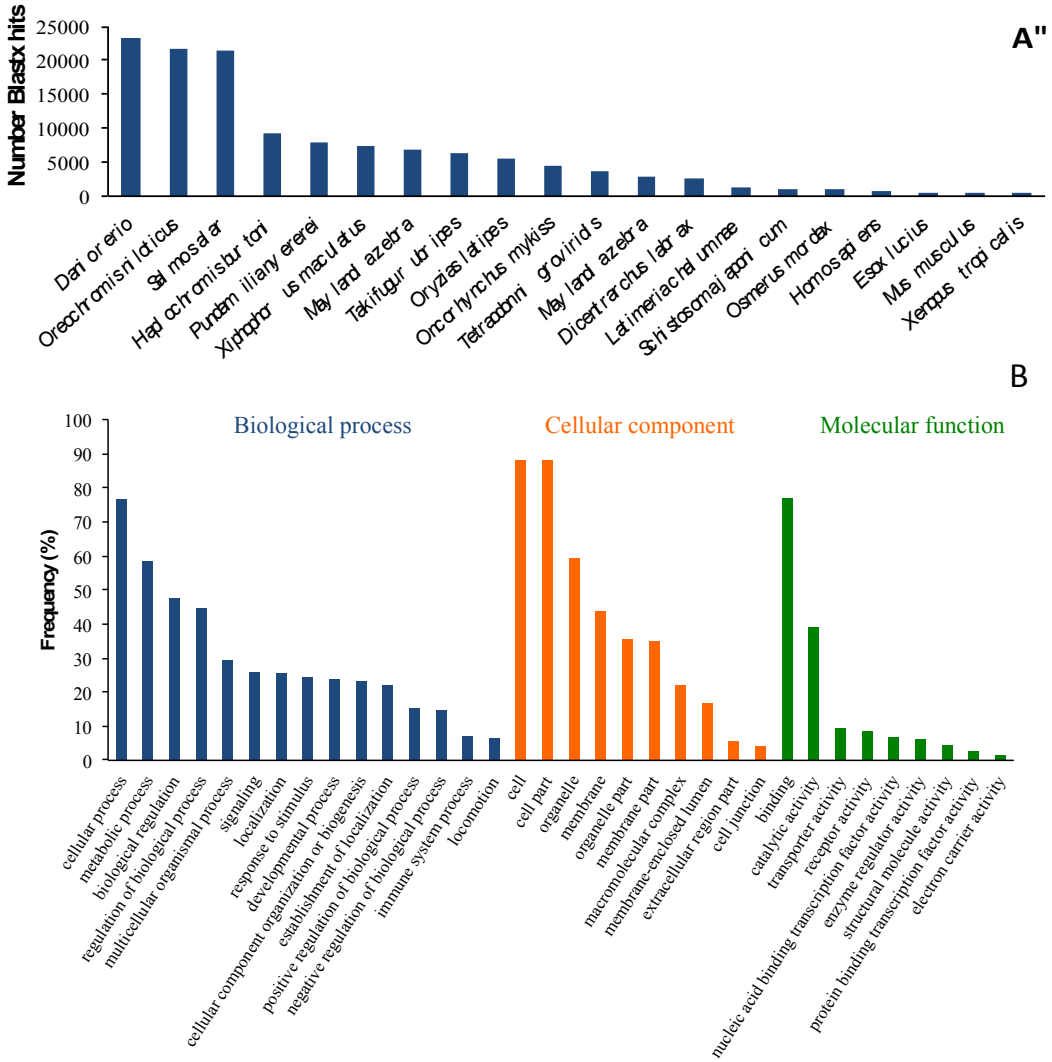
A"
Number Blastx hits
25000
20000
15000
10000
5000
0
Dani orerio
Orechromisriloticus
Salmosalar
Hapl ochromisbur toni
Puntiam iliarryerei
Xiphophor usmaculatus
May landi azebra
Takifugur ubripes
Oryzias latipes
Oncorhynchus mykiss
Tetraodonni groviridis
May landi azebra
Dicentrarchus labrax
Latimeriachal umnae
Schistosomaj aponi cum
Osmerus mordax
Homosapiens
Esox lucius
Mus musculus
Xenopus tropicalis
B
Biological process
Cellular component
Molecular function
Frequency (%)
100
90
80
70
60
50
40
30
20
10
0
cellular process
metabolic process
biological regulation
regulation of biological process
multicellular organismal process
signaling
localization
response to stimulus
developmental process
cellular component organization or biogenesis
establishment of localization
positive regulation of biological process
negative regulation of biological process
immune system process
locomotion
cell
cell part
organelle
membrane
organelle part
membrane part
macromolecular complex
membrane-enclosed lumen
extracellular region part
cell junction
binding
catalytic activity
transporter activity
receptor activity
nucleic acid binding transcription factor activity
enzyme regulator activity
structural molecule activity
protein binding transcription factor activity
electron carrier activity

Figure 3.1: Functional annotation of the transcripts. A) Best hits against the non-redundant protein database (Nr, NCBI). From the 659,869 transcripts, 141,909 (20.9%) were matching on the Nr database (evalue <10e−8). B) Gene Ontology (GO) association of the transcripts matching on the Uniprot database. Assignment from GoMiner software was limited to the second level GO representing main molecular, cellular component and biological processes.

From the transcriptome analysis, relevant putative homologues for bone metabolism with emphasis on P-metabolism are summarized in Figure 3.2 and the sequences are provided in Annexe 1.

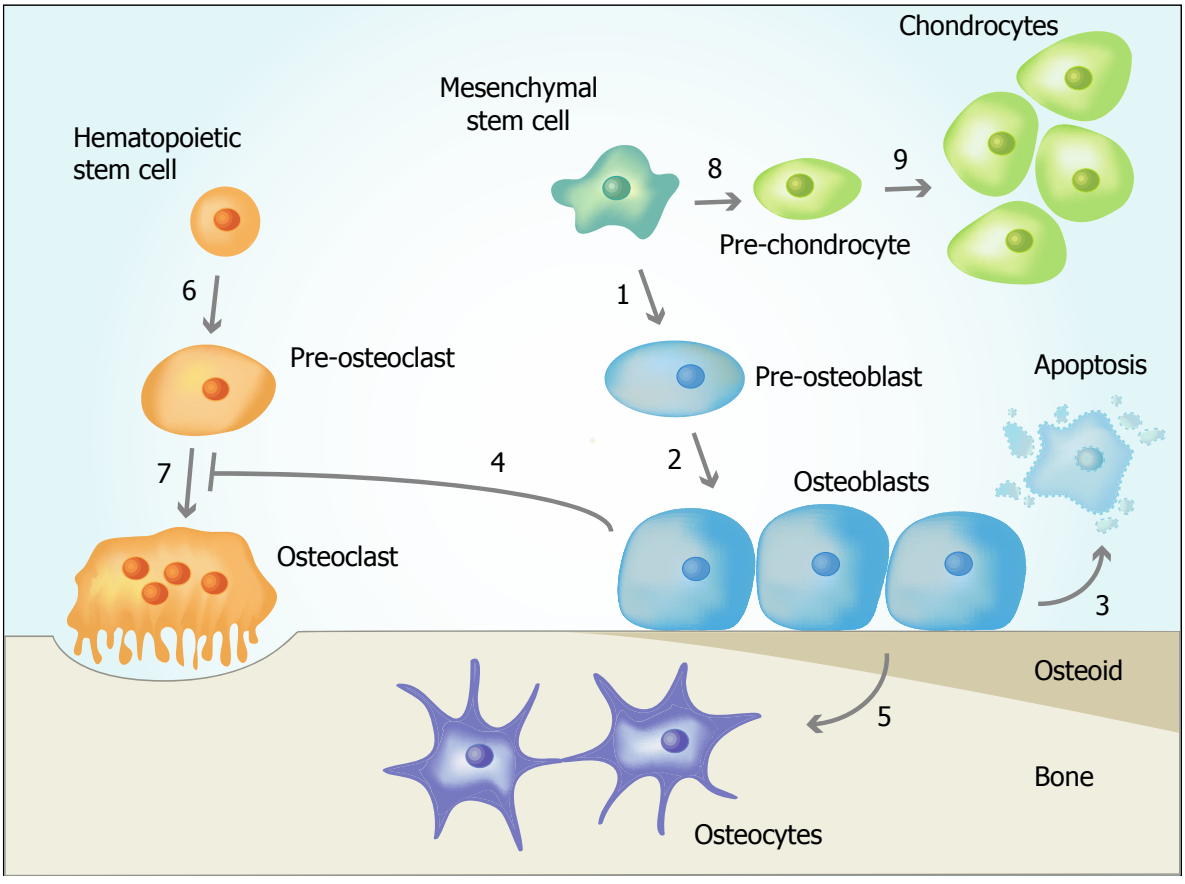

Figure 3.2: Schematic representation of the main actors involved in the regulation of bone and cartilage tissues. The actors mentioned here are based on relevant actors referenced in mammals. 1) Osteoblast differentiation: Msh homeobox 2 (MSX2), Signal transducer and activator of transcription 1, 91 kDa (STAT1), transcription factor Sox (SOX) 8, Runt-Related Transcription Factor 2 (RUNX2), β-catenin,

Insulin-like growth factor (IGF)-Binding Protein 10, Twist Homolog 2, jun D proto-oncogene, C-Fos, Homeobox A10, Osterix; 2) Osteoblast activation and function: Distal-less homeobox 3 and 5, Activating transcription factor 4 (ATF4), Alkaline phosphatase, RUNX22; 3) Osteoclast apoptosis: β-catenin, MSX2, lymphoid enhancer-binding factor 1; 4) Regulation of osteoclast maturation by osteoblasts: osteoprotegerin (OPG); 5) Osteocyte differentiation and function: KLOTHO, Fibroblast growth factor (FGF) 23, phosphate regulating endopeptidase homologue, X-linked 6) Osteoclast differentiation: Transcription factor PU.1, colony stimulating factor 1 receptor, RANK, RANKL, OPG, tumor necrosis factor (TNF)-α, TNF receptor-associated factor 6, Interleukin (Il)-1 β precursor, Il-1 receptor accessory protein, Transforming growth factor (TGF)-β1; 7) Osteoclast differentiation and function: Matrix metalloproteases 9 and 13, Cathepsin K (CTSK), Tartrate-resistant acid phosphatase (TRAP), Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1, Parathyroid hormone 1 receptor (PTH1R), microphthalmia-associated transcription factor, Estrogen receptor α/β; 8) Chondrocyte differentiation: TGFβ1, 2 and 3, wingless-type MMTV integration site family, member 7A, GLI family zinc finger (GLI) 3, Bone morphogenetic protein 2 and 4, FGF2, 4 and 8, SOX5, 6 and 9, IGF1 and IGF1-receptor, FGF-receptor (FGFR) 2; and 9) Chondrocyte hypertrophisation, activation and function: GLI2 and 3, Indian hedgehog protein, parathyroid hormone 1 receptor, BMP2 and 7, FGF18, FGFR1 and 3, STAT1, RUNX2, vascular endothelial growth factor A, ATF4, β-catenin, MSX2, myocyte enhancer factor 2C and Osterix.

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4 Chapter 4: RNA-seq transcriptome analysis of pronounced biconcave vertebrae: a common abnormality in rainbow trout (*Oncorhynchus mykiss*, Walbaum) fed a low-phosphorus diet.

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4.1 Résumé

La prévalence des malformations osseuses, particulièrement celles ayant pour origine une déficience alimentaire en minéral, est un problème récurrent en aquaculture. Des truites arc-en-ciel (*Oncorhynchus mykiss*) juvéniles et triploides ont été nourries avec une diète faible en phosphore (P) pendant 27 semaines (de 60 à 630 g). Au terme de l'étude, 14,2% des poissons avaient développé un phénotype anormal caractérisé par des vertèbres biconcaves (phénotype déformé) alors que 13,5% n'avait jamais développé de malformations au cours des 27 semaines d'experience (phénotype normal). Cette étude visait à identifier les changements moléculaires et décrire les mécanismes physiologiques impliqués dans l'apparition de ces phénotypes. À l'aide d'un séquençage haut-débit (RNA-sequencing) et grâce à la disponibilité du transcriptome de référence pour le tissu osseux vertébral chez la truite arc-en-ciel, nous avons ainsi quantifié l'expression des gènes dans les vertèbres des phénotypes normaux et déformés. Au total, 1289 gènes étaient différentiellement exprimés. Parmi eux, nos observations ont reporté la surexpression de *BGLAP* et *MGP,* deux Gla protéines impliquées dans les processus de minéralisation ainsi que de *NOG*, un inhibiteur de la voie métablique des BMPs. À l'inverse, L'expression de *COL11a1* était significativement réduite chez les poissons déformés. Par ailleurs, à travers l'analyse du statut en P, du suivi des performances de croissance, des analyses biomécaniques ainsi que l'observation de coupes histologiques et de sections de vertèbres par μCTs, les résultats suggèrent que ces gènes pourraient être des acteurs centraux impliqués dans la réduction du degré de minéralisation, un facteur déterminant pour l'apparition de malformations. Ainsi, ces résultats viennent compléter notre compréhension des mécanismes impliqués dans l'apparition de malformations vertébrales chez des poisons déficients en P et fournissent par ailleurs de nouveaux outils qui permettraient d'améliorer la gestion du P en aquaculture.

Mot-clefs: RNA-sequencing - Malformations vertébrales - Truite arc-en-ciel - Phosphore

4.2 Abstract

The prevalence of bone deformities, particularly linked with mineral deficiency, is an important issue for fish production. Juvenile triploid rainbow trout (*Oncorhynchus mykiss*) were fed a low-phosphorus (P) diet for 27 weeks (60 to 630 g body mass). At study termination, 24.9% of the fish fed the low-P diet displayed homogeneous biconcave vertebrae (deformed vertebrae phenotype), while 5.5% displayed normal vertebral phenotypes for the entire experiment. The aim of our study was to characterize the deformed phenotype and identify the putative genes involved in the appearance of P deficiency-induced deformities. Both P status and biomechanical measurements showed that deformed vertebrae were significantly less mineralized (55.0 ± 0.4 and 59.4 ± 0.5, % ash DM, for deformed and normal vertebrae, respectively) resulting in a lower stiffness (80.3 ± 9.0 and 140.2 ± 6.3 N/mm, for deformed and normal phenotypes, respectively). The bone profiles based on μCT observations showed no difference in the osteoclastic resorption while no difference in matrix production was observed between deformed (total bone area 5442.0 ± 110.1 μm^2) and normal vertebrae (total bone area 5931.2 ± 249.8 μm^2) in this study. Consequently, the lower P content rather results from a reduced degree of mineralization in the deformed phenotype. Finally, we quantified differential gene expression between deformed vertebrae (pronounced biconcave) and normal phenotype by employing deep RNA-sequencing and mapping against a reference bone transcriptome for rainbow trout. In total, 1289 genes were differentially expressed. Among them, in deformed fish we observed that *BGLAP*, *MGP* and *NOG*, an inhibitor of BMP signalling pathway, were up-regulated while *COL11a1* was down-regulated. These genes are central actors involved in the reduced degree of mineralization triggering vertebral deformities. These results will further the understanding of P deficiency-induced deformities; hence providing new tools for improved P management in production settings.

Keywords: RNA-sequencing - Deformities - Rainbow trout - Phosphorus

4.3 Introduction

Extensive work regarding deformity development in teleost fish have revealed that biotic and abiotic factors affect this condition including genetics, ploidy as well as nutrition [1–3]. Among the main nutrients affecting skeletal development in fish, phosphorus (P) particularly has drawn particular attention [4–6]. Bone tissue mineral (hydroxyapatite) constitutes the main reservoir of P, comprising up to 85% of the total body content [7, 8]. To date, many studies in salmonids have attempted to identify and characterize the response of the fish skeleton to low-P diets. Under extreme conditions such as reproduction, migration or starvation, teleosts mobilize P from scales and bone tissues [9–13]. During a prolonged P deficiency, fish may also display lower growth performance [14], lower mineral status of bone, scales and flesh [3, 15, 16] and, in some severe cases, increased occurrence of specific phenotypes of vertebral deformities [17–20].

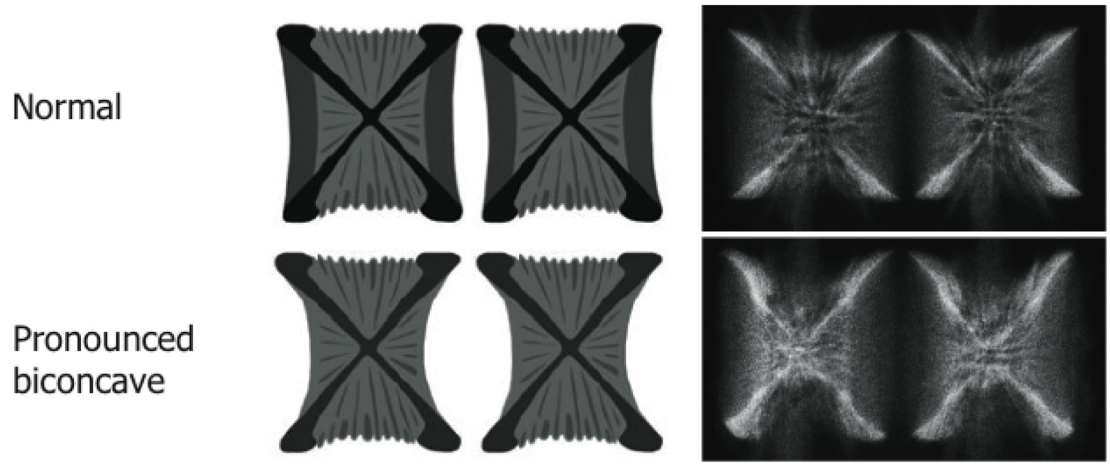
The study of vertebral abnormalities is particularly difficult in regard to the complexity and the role of the axial skeleton. In trout, this system is comprised of 64 vertebrae, composed of several tissues and cell types, interconnected by intervertebral tissue and undergoing compression forces during swimming. As in other vertebrates, under non-pathological conditions, a tight balance in bone tissue is maintained between bone apposition by osteoblasts and bone resorption, resulting in a continuous turnover. In trout and in other species that belong to basal teleostean lineages, osteoblasts are trapped in the matrix and become osteocytes, a condition that defines cellular bone [21]. The osteocytes also play a role in bone apposition and bone resorption around their lacunae [22]. In vertebrae, chondrocytes are also present at the base of neural and hemal arches (basalia). Their progressive endochondral mineralization enables the complete fusion of the arches with the vertebral body during juvenile growth, at least in the caudal portion of the axial skeleton [9]. Thus, the occurrence of vertebral anomalies reflects a disturbance of the homeostatic mechanisms and well-coordinated actions of several cell types.

The molecular pathways involved in P-deficiency-induced deformities are far from being well understood. In Atlantic salmon (Salmo salar) presenting vertebral fusions [23, 24], more than 20 actors including transcription factors and genes encoding molecules involved in bone cell activation and differentiation, and extracellular matrix (ECM) formation were detected. Similarly, a large-scale study on the impact of photoperiod on vertebral bone formation was conducted in Atlantic salmon [25] and highlighted the structural role of COL11a1 in the occurrence of vertebral deformities. Recently, characterization of vertebrae transcriptomes in sea bream (Sparus auratus) [26] and rainbow trout [27] suggested that homologs of key genes involved in bone metabolism are conserved in vertebrates. As bone metabolism in fish is complex, transcriptomic-wide analyses may be the best approach to identify the principal affected pathways during P deficiency.

A recent study [20] showed that the most common deformities observed in P-deficient all-female triploid rainbow trout are pronounced biconcave and homogeneously-compressed vertebrae, biconcave vertebrae being considered an early stage of vertebral compression. Most of the fish undergoing prolonged P deficiency develop vertebral abnormalities, however, some individuals are able to maintain a normal phenotype. Given this phenotypic plasticity, it is of interest to better understand the cellular mechanisms underlying the appearance of vertebral abnormalities.

The aim of this study was to identify molecular changes that are involved in the appearance of pronounced biconcave vertebrae (Figure 4.1) when trout are fed a low-P diet. We compared gene expression in normal and biconcave vertebrae from P-deficient all-female triploid rainbow trout using next-generation sequencing (NGS) on the Illumina HiSeq2000 platform. To support the transcriptomic results, in each individual we characterized the P status and the quality of bone tissue based on chemical, histological and biomechanical determinations. We discuss our findings with regard to transcription regulation and the formation, mineralization and resorption of bone tissue.

Figure 4.1: Illustrations (left) and X-rays (right) of the reported vertebral phenotypes in this study (normal and pronounced biconcave, respectively) and associated with mineral deficiency in teleost. Illustrations were modified after Witten et al. [28]. For further details regarding the phenotypes see Poirier Stewart et al. [20].



4.4 Material and methods

4.4.1 Husbandry

All-female triploid rainbow trout (N= 1,680, initial mass 60.8 ± 1.6 g) were transferred from St-Alexis-des-Monts Inc., Canada to experimental facilities at the Laboratoire de recherche des sciences aquatiques (LARSA), Université Laval in Québec City (Canada). An acclimation period of two weeks took place, during which fish were fed a commercial feed (Corey Optimum 3 mm) in accordance with manufacturer's tables. Thereafter, fish (N = 840) were fed with practical P-deficient diet (available P: 0.29%) using either apparent satiation or pair-fed feeding regimes. The P-deficient diet was found to induce severe deficiency in rainbow trout as already reported [16, 19, 20].

The fish were reared for 27 weeks in six circular tanks (2000L) in a recirculating aquaculture system (6 replicates, n = 140/tank, 8.5 to 52.7 kg m-3, 12 ± 0.3°C), with near oxygen saturation and at constant, long (LD, 18:6) photoperiod. During experimental manipulations, fish were anaesthetised in a MS-222 bath (75 mg L-1; Syndel International Inc., Vancouver, BC, Canada). Experiments took place in compliance to the guidelines of

the Canadian Council on Animal Care (1984) and supervised by the Animal Protection Committee of Université Laval.

4.4.2 Deformities assessment and tissue sampling

At week 5, all trout were PIT-tagged with a microchip enabling individual survey (weight, length and condition factor: K = 105 x g/mm3) for each sample. X-rays permitted individual monitoring and was performed at week 5, 15 and 24. X-rays were performed on the trunk-caudal region of all fish at 60 kV (2s, 15 mA, distance of 40 cm). Monitoring of vertebral abnormalities was assessed directly on developed films and abnormalities along the vertebral axis were sorted according to the classification proposed for Atlantic salmon [28]. Fish were associated to a phenotype when all caudal vertebrae (V31-V44) showed a homogenous pattern of abnormalities (see details in [20]). At the termination of the study (week 27), P-deficient fish displaying normal (n=3 fish) and pronounced biconcave (n=3 fish) vertebrae were randomly sampled. This abnormality type was chosen as it was by far the most represented type observed in the current study [20]. Moreover, pronounced biconcave vertebrae are related to early stages of mineral deficiency and might be a transition stage to vertebral compression, which are of major concern for fish growth [28]. For each fish, blood was collected from the caudal aorta, centrifuged, and the plasma stored at -20 C. Scales were scraped from tail to head and were collected in a 70° ethanol solution. Caudal vertebrae were removed for bone histology (V32-33 and V38-39), bone genomics (V35-37), bone structure (V40-42), biomechanics and bone chemistry (V31-32 and V43-44). All of these analyses were conducted on vertebrae from the same individuals.

4.4.3 Inorganic phosphorus in plasma

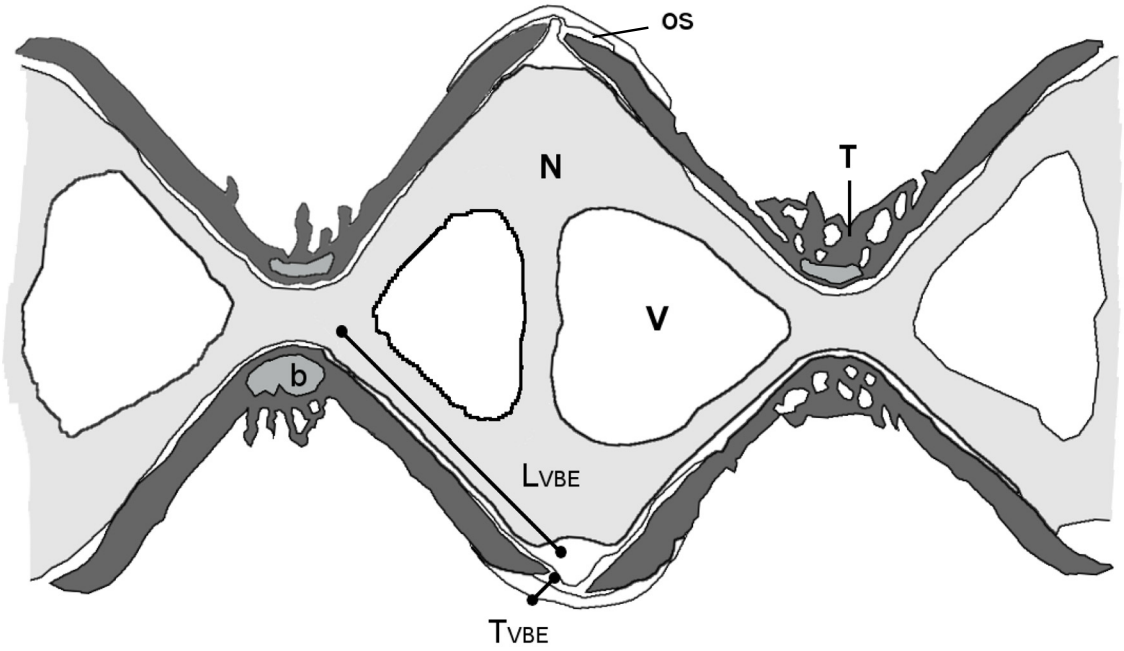
Colorimetric assays (phosphomolybdate; K003912, JAS diagnostics, Inc., USA) were performed to measure inorganic phosphates (Pi = PO_4^{3-}) in individual samples of plasma (150 µl) using an automatic spectrophotometer (Trilogy Multi-purpose diagnostic analyzer system, Drew Scientific, USA).

4.4.4 Bone histology

To quantify the mineralization of basalias, two attached vertebrae (V38-39) were fixed in 4% formaldehyde and decalcified in 25% decal solution for 24h. Vertebrae were then dehydrated in graded series of ethanol prior to being embedded in paraffin. Up to 15 adjacent longitudinal cross-sections (5 μm) from either side of the mid-region of the vertebrae (i.e., where the notochord canal is present at the narrowest) were obtained using a microtome (Microm HM 330, Heidelberg, Germany). The sections were stained using Sirius red techniques [29]. Two other attached vertebrae V33-34 (fixed in cold acetone) were embedded in glycol methacrylate. One longitudinal section was obtained by sanding one side of the block to the mid-region of the vertebrae and by cutting the other side of the block to a thickness of 1.5 mm using a band saw and a cutting guide. The sections were polished before being stained using Von Kossa techniques [30].

Morphometrical results at the vertebral body endplates (VBE) were estimated from the Von Kossa sections. For each individual, tiff images from the longitudinal section (4x magnification) were obtained using a digital camera (EXI Blue Q35618, Q Imaging Inc., BC, Canada) mounted on a microscope (Nikon eclipse E600) and captured with Q-capture Pro 7 software (Q Imaging Inc., BC, Canada). Once transferred to ImageJ v1.40 (U. S. National Institutes of Health, USA, http://rsb.info.nih.gov./ij/) software (with Java, 32 bits), the freehand selection tool was used to manually delineate (graphic tablet Intruos3, model PTZ-631W, Wacom Co. Ltd, China) areas and calculate a number of pixels for the mineralized trabecular area (tb) and total bone area, comprising tb and the un-mineralized osteoid at the apposition front (os), for each vertebral body endplate (VBE) (n=8/individual). The mean proportion of os (%) at the VBE was calculated as follows: os/(os + tb) x 100. The length (LVBE) and thickness (TVBE) of the vertebral body endplates were also measured. LVBE included the total distance from the notochord canal to the apposition front. TVBE was defined as the perpendicular distance from the point of attachment of the notochord tissue to the inner limit of the compact bone layer. A schematic representation including the different vertebral body parts mentioned in this section is proposed in Figure 4.2.

Figure 4.2: Scheme of a longitudinal section of the mid-region of two adjacent vertebrae including basalias and notochordal tissues. With b = basalia; L_{VBE} = length of the vertebral body endplate; N = notochordal tissue; os = osteoid; T = trabecular bone; V = vacuolated tissue; VBE = vertebral body endplates; T_{VBE} = thickness of the vertebral body endplate.



The relative mineralization of the basalias was estimated from digital pictures of Sirius red sections at 200x magnification (N=5 images/basalia/individual section) using a digital camera (Infinity2-3C; Lumenera 7.0.3.23) mounted on a binocular microscope (Olympus BX53) and captured with Image-Pro Plus software (version 7.0.0.591). The basalias were cut and cell nuclei removed using the selection tools of GIMP v2.8.10 software (ww.gimp.org). The number of pixels for two RGB colours (unmineralized collagen = red (222, 333, 136); mineralized collagen = grey (142, 59, 142) were analysed using ImageJ and colour deconvolution Plug-In as published elsewhere [31]. The proportion of mineralized and unmineralized areas of the basalias were calculated as the number of pixels for a specific colour divided by the total number of all defined pixels x 100.

4.4.5 Bone profiling

Bone density and structure of vertebrae V40-42 were estimated using the approach of the modelling of bone area profiles according to previously-published methods [32]. Briefly, total bone area (Tt-B.Ar.) and mean bone area according to the relative distance from the notochordal canal were measured using Bone profiler software [33] and modeled using 12 parameters in four predefined regions of the vertebral body (notochord, transition, middle and periphery). Measurements were performed on 125 μm-thick transverse sections of the mid-region of the vertebrae, in which the notochord canal is the narrowest and bone tissue the largest. Virtual cross-sections were obtained from μCT images (National Museum of Natural History, France).

4.4.6 Biomechanics

Four vertebrae (V31, 32, 43 and 44) were used for mechanical testing prior mineral content analyses. Neural and haemal arches were removed, and the amphicoelus centra were compressed along the cranial–caudal axis at a steady rate of deformation (0.1 mm sec^{-1}) using a texture analyzer (TA-XT2 Texture Analyzer, Stable Micro Systems, UK) and the Texture Expert Exponent 32 acquisition software (TE32 version 5.1.1.0, Texture Technologies, USA). For each vertebra, load (N) and deformation (mm) data were continuously recorded and the stiffness ($g.mm^{-1}$) was calculated according previously-published methods [34].

4.4.7 Mineralization status

The mineral content was determined for scales and vertebrae (V31, 32, 43 and 44). The samples were dehydrated in a graded series of ethanol (70°, 90°, 100°; 24 h/bath), and lipids were extracted in acetone (two baths of 24 h), then in trichloroethylene (two baths of 24 h). Diets and tissues were analyzed for dry matter (drying in a vacuum oven for 18 h at 105°C) and ash (incineration in a muffle furnace for 18 h at 550°C), weighed to the nearest 0.1 mg according to AOAC guidelines (1990). P content was determined by ion chromatography (ICS-3000, Dionex Corporation, Sunnyvale, CA) following ash digestion in nitric acid solution and filtering (Whatman paper No. 1, rinsed 3 times). Separation was carried out on an IonPac AS 17 column (Dionex) with an IonPac AG 17 guard column. For

sample loading, a 50 µl sample loop (PEEK) was used. Multi-step gradient concentrations ranging from 9 to 30 mM allowed for the complete separation of phosphorus anions in aqueous media in 16 min at a constant flow rate of 1.0 $ml.min^{-1}$.

4.4.8 Sequencing

Three connected vertebrae (V35-37, comprised of ligaments and intervertebral tissue) were rapidly dissected, arches removed and the remaining vertebral body quickly cleaned of flesh by rinsing and brushing with PBS (1X) to remove all muscle tissue. The vertebrae from each individual was then immersed in liquid nitrogen, crushed into a fine powder, and total RNA extracted using TRIZOL Reagent (Life Technologies) at a ratio of 1 ml per 100 mg tissue according to manufacturer's recommendations. Total RNA was subjected to DNAse treatment on a column (ArcturusPicoPure RNA isolation kit, Life Technologies), and integrity and purity assessed using a Bioanalyzer 2100 (Agilent Technologies). RNA was also quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc.). For each sample, 1 µg of total RNA was used to create cDNA libraries with TruSeq Sample Prep kit v2 (Illumina, San Diego, CA, USA) following manufacturer's instructions. Resulting libraries were quantified using a Bioanalyzer 2100 (Agilent Technologies). Samples were multiplexed (6 samples per lane) and sequenced at McGill genomic platform with HiSeq2000 sequencer and 100 paired-end (PE) technology.

4.4.9 Differentially expression study and annotation

Reads from HiSeq2000 Illumina were first processed with Trimmomatic v0.30 [35] to remove low quality (trailing: 20, lowest quality: 30) and short reads (<60 bp). Trimming with Trimmomatic also included removal of Illumina adapters together with the most common contamination vectors from UniVec database (https://www.ncbi.nlm.nih.gov/tools/vecscreen/univec/). Read abundance and differentially-expressed genes were estimated using Trinity pipeline published recently for non-model species [36] by mapping reads on a reference transcriptome for rainbow trout bone tissue [27]. Briefly, gene abundance estimations have been assessed individually using RSEM tools [37] and bowtie v1.0.0 [38]. The differentially-expressed gene assessment based on RPKM values, was conducted using both EdgeR v3.8.5 [39] and

DESeq v1.18.0 [40] packages with correction of false discovery rate (FDR) of 5%. Only common genes detected as differentially expressed with both analyses have been used for further analyses and functional annotation. Blast-x searches (cut-off e-value < 10-8) were performed on Uniprot-Swissprot database. Gene ontology (GO) association was realised using GOminer software [41]. To estimate the proportion of long non-coding RNAs (lncRNAs), remaining sequences non-matching against Uniprot database were analyzed using PLEK software [42] (length threshold > 200bp). Visualization of biological functions was conducted using IPA core analysis (Ingenuity Systems, www.ingenuity.com). The Ingenuity platform allows novel protein sequence association with networks, canonical pathways and biological functions relative to human and mouse systems. The genes specifically related to bone metabolism were manually searched by compiling relevant metabolic pathways (Table 3). The analysis pipeline for gene expression quantification is described in Figure 4.3.

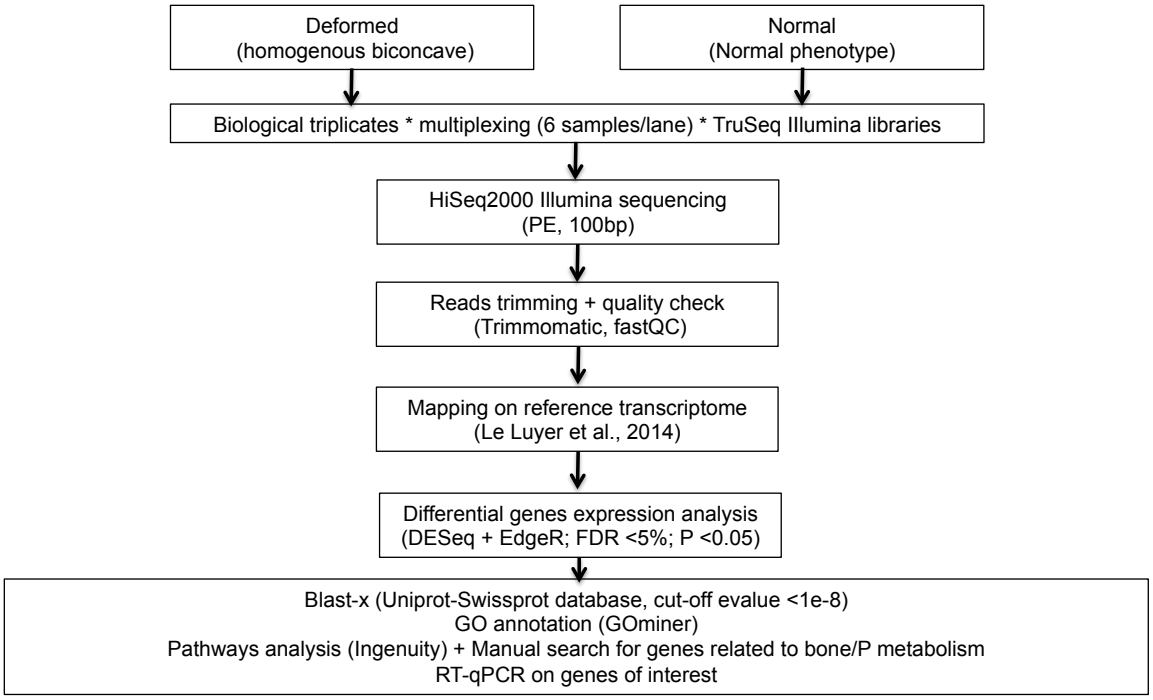


Figure 4.3: Pipeline of bioinformatics and gene expression analyses. PE= paired-ends

4.4.10 RT-qPCR

Here we used RT-qPCR to assess differential gene expression for keys genes involved in each cellular types: MEF2C, TRAP and BGLAP, for chondrocytes, osteoclasts and osteoblasts, respectively. For each sample total RNA was converted into complementary DNA (cDNA) using Omniscript reverse transcriptase kit (QIAgen). Specific primers (Table 4.1) were designed using Primer Quest from DNA Technologies (IDT, Coralville, IA) for Real-Time quantitative PCR (480 Roche lightcycler 2.0, Roche Diagnostics, Canada) and SYBR green I staining [42]. Quality and specificity of amplicons were visually confirmed on 2% gel electrophoresis and by DNA sequencing. A normalization factor for each sample was assessed using GeNorm software [43] based on two housekeeping gene expression levels, β-actin and Heat Shock Protein 90B (HSP90B). Expression of genes assessed by RT-PCR is presented in Figure 4.4.

Table 4.1: Primer sequences, product sizes and annealing temperature of selected genes for RT-qPCR

Gene name	Amplicon (bp)	Sense	Sequence (5'to3')	Length (bp)	Tm
TRAP	162	F	CGGAGAGGTAACCAAGAACAAG	22	60.2
		R	TGTGACCGTCTATTTCGTCATC	22	60.0
Osteocalcin	119	F	TCTCTCGCTCTCCCTCTAACAC	22	60.2
		R	TCTATTCTGCAAGGCTTTTTGG	22	60.6
Mef2c	158	F	ACCCACAAATACACAACACAGG	22	59.7
		R	GTCTGAAATGGAACCCTATTGC	22	59.8
β-actin	133	F	GAAAGACAGCTACGTGGGAGAC	22	60.2
		R	CTCGTTGTAGAAGGTGTGATGC	22	59.8
Heat shock protein 90 B	232	F	CGACTTCATCAAGCTGTTTGTC	22	59.9
		R	CTTGTCGTTGTACTGCTCTTCG	22	60.1

4.4.11 Statistical analysis

The histological results and fish performance are reported as mean ± standard error of the mean (sem). After ensuring normality (Shapiro-Wilk, Skewness and Kurtosis tests) and homoscedasticity (Bartlett test), data were analyzed using one-way ANOVAs. For the length of trabecular bone observed with Von Kossa, data were Log10 transformed. The

results were assumed to be significant at p-values below 0.05. All the tests were conducted with R software version 3.0.2 [45] using Moments package [46].

4.5 Results and discussion

This experiment employed all-female triploid individuals fed with a single P-deficient diet in order to overcome possible factors increasing phenotypic variation such as differences in somatic growth, sex and sexual maturation. After 27 weeks of feeding low-P diets, mortality was similar and negligible (3.2%) in all tanks and no significant difference in growth performance was observed in control and experimental fish. Moreover, no difference in length, weight and condition factor (Table 4.2) were observed between experimental individuals ($p = 0.06$ and 0.09, respectively) (see also [20]) and external visualisation could not detect any defects of conformation (gross deformity).

Here, we focused on pronounced biconcave phenotype because of its high frequency in salmonids prone to mineral deprivation and because it is considered as an initial development stage of more severe compression deformities [20, 28]. Pronounced biconcave vertebrae after 27 weeks were observed in 24.9% (N=113) of the fish. Only 5.5% (N=25) of individuals displayed normal vertebrae (healthy control fish) all along experiments (i.e., from week 9 to week 27). Other fish showing normal phenotype at week 27 (31.1%; N=141) were not considered because they were known to recover from an abnormal phenotype at week 18. Moreover, other types of deformities such as homogeneous compressions (29.4%; N=133), small and widely spaced vertebrae (5.5%; N=25) and other types of deformities (3.5%; N=16) were also observed but were not considered for this study.

The P status of deformed fish displaying pronounced biconcave vertebra was negatively affected as shown by the lower ash content in their scales and vertebra, and the lower plasma Pi concentration (Table 4.2). Our results are consistent with previous observations that showed a significant correlation between low mineral content in scales and higher occurrence of vertebral deformities in rainbow trout fed low-P diets [19]. Whether the altered mineral status resulted from differences in individual P requirements or

from difference in feed intake due to limited food supply inherent to feeding approaches and/or competition between congeners deserves to be further studied. The lower mineralization of biconcave vertebrae resulted in lower biomechanical competence when submitted to a compression force (stiffness, Table 4.2), a finding that is coherent with similar results reported in Atlantic salmon [47, 48]. Lower P content and mechanical strength are recognised as initial indicators of deformity occurrence, particularly for the various compression types [48]. Phenotypically, pronounced biconcave vertebrae differ from normal vertebrae by their apparent "transparent" vertebral body endplates upon X-ray evaluation (Figure 4.1); the shape of the vertebral body appears as a vertical apple core and the X-shaped centrum of the vertebrae display more acute angles [20, 28]. Therefore, our results may suggest that the lower biomechanical competence of the vertebrae promotes the alteration of the vertebral body endplates (apposition site of newly formed bone) as well as in binding the X-structure of the vertebral body (more acute angles). If this hypothesis is true, the mechanisms of vertebral compression would not only affect intervertebral tissues at the vertebral body endplates as suggested by some authors [23, 28], but also the entire structure of the vertebral body.

A recent study from our group reported the first reference transcriptome for rainbow trout vertebral bone and its characterization revealed that key regulators of bone metabolism have been well conserved in vertebrates and are present in trout [27]. Here, we compared the overall gene expression by mapping paired-end reads on the transcriptomes in pronounced biconcave (deformed) vertebra with normal vertebral phenotype after 27 weeks of feeding a P deficient diet (Table 4.3). Using edgeR and DESeq package (FDR < 5%; $P < 0.05$), a total of 1289 genes were found differentially expressed. Of these, 700 were down- and 589 up-regulated in biconcave vertebrae. A blast-x search (cut-off 1e-8) was conducted to identify these sequences in Uniprot-Swissprot database reference and only genes with unique match were conserved. A total of 793 sequences (61.5 % of the total genes) corresponding to 485 down- (61.9%) and 308 up-regulated (38.8%) genes were associated with known proteins. Furthermore, from the 1289 genes DE, 23.1% (N=298) were associated with putative lncRNAs. The unidentified sequences were not retained in

our analyses although we believe that the study of these non-referenced sequences may improve our understanding of the process involved in deformity appearance.

Table 4.2: Growth, condition factors, stiffness, P mineralization status (dry tissues) calculated as ash or P content and results of histological observations by Sirius red and Von Kossa. Values are expressed as means (n=3) ± sem. P = phosphorus; Sc = scales; Vt = vertebrae; Tt.B.Ar = total bone area. Asterisks (*) represent significant difference (P < 0.05).

	Sirius red (basalias)			Von Kossa (trabecular bone)			μCT
Phenotypes	Non mineralized area (%)	Intermediary mineralized area (%)	Mineralized area (%)	Total area (μm^2)	Mineralized area (μm^2)	Osteoid (μm^2)	Tt.B.Ar (%)
Normal	59.4 ± 11.5	32.0 ± 9.6	8.5 ± 1.9	5931.2 ± 249.8	5711.0 ± 233.1	220.1 ± 16.7	39.3 ± 3.2
Deformed	68.6 ± 7.8	17.7 ± 2.0	13.7 ± 6.0	5442.0 ± 110.1	5166.1 ± 99.8	275.9 ± 10.7	43.3 ± 3.6

Phenotypes	Fork length (cm)	Weight (g)	Condition factor (K)	Stiff. (N/mm)	P Vt. (%)	Ash Vt. (%)	P Sc. (%)	Ash Sc. (%)	P plasma (mg/dl)
Normal	35.1 ± 0.6	657.3 ± 33.2	1.5 ± 0.0	140.2 ± 6.3*	9.3 ± 0.3	59.4 ± 0.5*	5.5 ± 0.1	29.8 ± 0.7*	11.5 ± 0.4*
Deformed	32.6 ± 0.8	526.9 ± 49.6	1,6 ± 0.1	80.3 ± 9.0 *	8.8± 0.3	55.0 ± 0.4*	4.4 ± 0.5	26.3 ± 1.1*	7.6 ± 0.7*

Table 4.3: Sequencing results of biconcave (Deformed, Def.) and normal (Norm.) vertebra. DE = Differentially expressed.

Sequencing	Normal	Deformed
Ave. number reads	44.6 M	46.1 M
Ave. number trimmed reads	35.3 M	37.8 M
Ave. mapping (%)*	71.7	71.6
DE analysis	Def. Vs. Norm.	
Up-regulated in Def.	700	
Down-regulated in Def.	589	
Total	1 289	
Match Uniprot	793	

FDR: 5%, triplicates. Unique gene match in Uniprot-Swissprot database (cut-off evalue < 1e-8). Analyses on genes levels expression with both edgeR and DESeq packages.

*** Trimmed reads with at least one reported alignment using RSEM output.**

Gene function analysis using the Ingenuity platform revealed that the top three networks differentially represented in this study were "cellular morphology, embryo development, hair and skin development and function", "organismal development, skeletal and muscular system development and function in cancer" and "hereditary disorder, neurological diseases, organismal injuries and abnormalities". They comprised 30, 28 and 28 genes, respectively. In addition, pathway analysis showed that a wide range of canonical pathways were differentially represented ($p < 0.05$). They included, among others, protein kinase A signalling ($p = 1.2E-4$), actin cytoskeleton signalling (6.5E-4), epithelial adherens junction signalling (8.5E-4), axon guidance signalling (1.7E-3), paxillin signalling (5.4E-3), notch signalling (6.9E-3), inhibition of metalloproteinases (7.8E-3) and FGF signalling (1.9E-2). Enrichment analysis using GOminer highlighted 123 clusters with enrichment > 2 and FDR < 5%. Within these clusters are included relevant biological processes, cellular components and molecular functions involved in bone regulation: "GO:0008147 structural constituent of bone": 23.1; "GO:0003308 negative regulation of Wnt receptor signalling pathway involved in heart development": 15.4; "GO:0051019 mitogen-activated protein

kinase binding": 13.86; "GO:0030520 estrogen receptor signalling pathway": 6.08; and "GO:0051216 cartilage development": 2.1. Integration of functional analysis coupled to manual screening pointed to putative relevant genes involved in bone metabolism regulation (Table 4.4).

To understand the mechanisms involved in the depletion of the mineral status linked to the apparition of biconcave vertebrae in P-deficient fish, we combined RNA-seq experiments with recently developed morphometrical techniques. Our first hypothesis was that the lower mineral content and stiffness of deformed vertebrae resulted from higher osteoclast activity (bone resorbing cells). In human osteoporosis, the loss of bone and mineral is commonly linked to higher bone resorption by osteoclasts [49]. Similarly, RANKL-induced ectopic osteoclast activity led to osteoporotic phenotype in medaka (*Oryzias latipes*), a teleost fish [50]. Mature osteoblasts produce RANKL, which stimulate osteoclast proliferation, differentiation and activation, but they also synthesize osteoprotegerin (OPG), which is the major inhibitor of RANKL/RANK ligation [51]. Here, no differences were observed between both OPG and RANKL gene expression. However, RNA-seq results showed that Tumor Necrosis Factor Receptor Superfamily, Member 5 (*TNR5/CD40*) was up-regulated in the deformed group. The ligation to TNR5 receptor induces RANKL pathway activation and bone resorption [52]. To verify whether the lower mineralization in deformed trout vertebrae resulted from higher bone resorption, we verified Tartrate Resistant Acidic Phosphatase (TRAP) gene expression using RT-PCR. Expression of this marker of osteoclast activity was not significantly affected in deformed fish compared to normal individuals (Figure 4.4). However, in the former, *unconventional Myosin 10* was down-regulated. Myosin 10 is a crucial protein for podosome belt formation allowing osteoclast attachment [53]. These results are consistent with total bone area assessment using μCT that did not indicate significant difference between deformed and normal fish (Table 4.2). Similar bone area profiles are also observed (Figure 4.5): the higher bone area in the middle area suggesting even lower resorption in deformed vertebrae. Therefore, the lower mineral status in fish with deformed vertebrae did not likely result from higher osteoclast activity. On the contrary, it appeared that low levels of unconventional myosin 10 might impair osteoclast activity after 27 weeks of feeding low-P

diet. Thus, our study suggests that impaired vertebral mineralization resulted from either alteration of matrix production and/or mineralization processes.

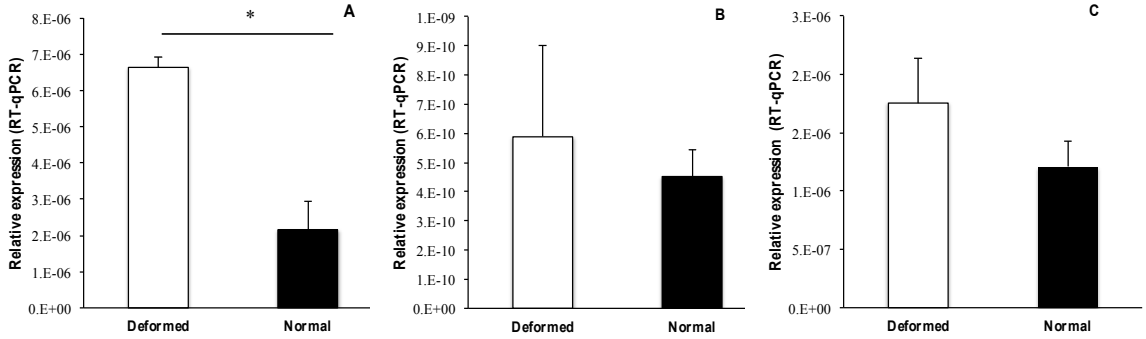


Figure 4.4: RT-PCR results on selected bone related genes. Graphs represent the gene expression analysis performed independently by RT-qPCR with A) BGLAP, B) MEF2C and C) TRAP. Asterisks (*) represent significant difference between groups (ANOVA $P < 0.05$). Data represent means (n=3) ± sem.

The mechanisms by which osteoblasts and osteoclasts control bone formation are not fully understood. However, various metabolic pathways affecting bone and cartilage formation have been identified in mammals and recent whole-transcriptome annotation provided evidence that these pathways were also present in teleosts, which suggests high conservation during osteichthyan evolution [26, 27]. Genome-wide experiments revealed that most of the pathways (e.g. TGFβ/BMP, WNT and ERK/MAPK pathways) are involved, and may putatively cross-talk, during transcriptional regulation of osteoblast differentiation, proliferation and maturation [54–58]. Our results suggest that the pathways involved in the regulation of osteoblast differentiation and proliferation are down-regulated in deformed fish. Indeed, the following genes were down-regulated in deformed vertebrae: co-receptor activators or receptors (TGFβ/BMP: *BMPR2*; WNT: *LRP6* and *MACF1*) and downstream actors (ERK/MAPK: *NIK, MEKK1, SOS1, MYCB2* and *MYCPP*; TGFβ/BMP: *USP9X*). Conversely, inhibitors of ligand binding receptor were up-regulated (TGFβ/BMP: *NOG, TEFF1* and *FBN2*; WNT: *SFRP1*). For instance, *NOG* expression was substantially up-regulated (fold change > 9) in deformed compared to normal fish. In transgenic mice, over-expression of NOG triggered osteopenia, i.e. the abnormally low mineral status of bone tissue [59, 60]. Finally, most of these signal pathway are either directly or indirectly controlled by a master regulator of osteoblast differentiation: RUNX2 [61]. The expression

level of RUNX2 was similar in deformed and normal vertebrae, but conflicting results were obtained regarding RUNX2 regulation. Indeed, in deformed fish we observed a lower expression level of *HIVEP3*, a gene negatively regulating osteoblast differentiation by stimulating degradation of RUNX2 and acting as a damper for signalling pathways such as WNT, TGFβ and BMP2 [62–66]. *TWIST2*, a RUNX2 inhibitor, was up-regulated in deformed fish while activators, such as *STAB2*, were down-regulated [67]. In vitro experiments have shown that osteoblastogenesis is a physiological process that spends several weeks, during which specific actors are expressed at different times/stages [68]. Yet, in deformed fish RUNX2 expression was not significantly affected, a finding that may be consistent with the higher expression of *osteocalcin* (also known as bone Gla protein, BGLAP), which normally occurs several weeks after a peak of RUNX2 [69].

BGLAP expression is a marker of mature osteoblasts. In deformed fish, the higher expression of *BGLAP* is concomitant with the higher expression of *JUND*, an AP-1 transcription factor complex, responsible for binding to *BGLAP* promoter and enhancing *BGLAP* expression in mammalian osteoblasts [70]. In vitro experiments showed that *JUND* expression was relatively low during osteoblast proliferation and then increased during matrix maturation to reach a peak during matrix mineralization [71].

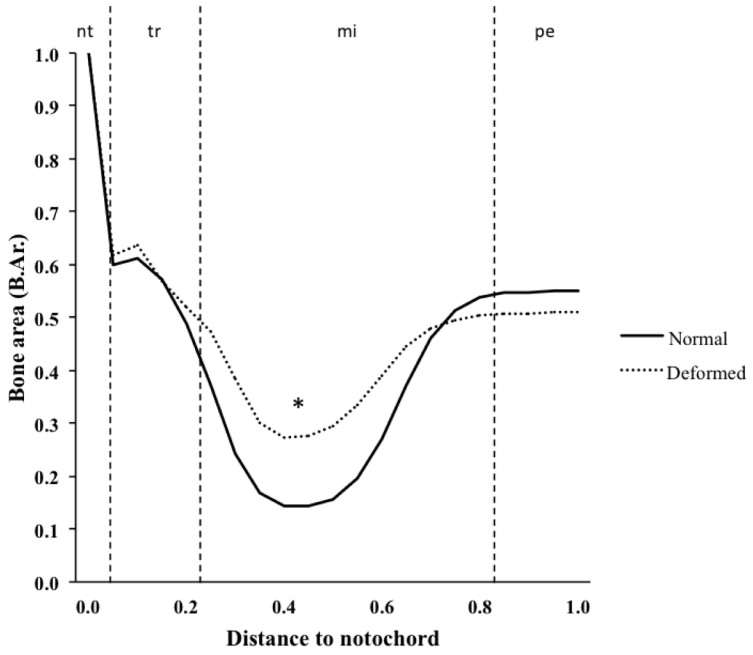


Figure 4.5: Vertebral bone profile for normal and deformed groups. Bone area (expressed as a ratio from 0 to 1) as a function of the relative distance to the notochord. nt = notochord area; tr = transition area; mi = middle area; pe = periphery area. Asterisk (*) shows a slight significant difference for the lost of bone in the middle area between groups (ANOVA P < 0.05; n=3 fish/condition).

Taken together our analyses of biconcave vertebrae of deformed fish suggest that the processes involved in osteoblast differentiation and proliferation are reduced while the high level of *BGLAP* expression indicates the presence of numerous mature osteoblasts. We observed consistently in these deformed fish a low expression of *COL12a1*, a collagen type highly expressed by differentiating osteoblasts but not in mature osteoblasts, in which its expression is reduced [72]. Furthermore, previous studies report that *COL12a1* knockout mice displayed altered vertebral structure with decreased bone strength [73], which is coherent with the lower bone stiffness observed in deformed fish. Interestingly, the same authors reported no difference in *TRAP* expression or in other markers of bone resorption in *COL12a1* -/- mice vs. wild-type control, indicating that decreased bone mass resulted from abnormal bone formation rather than from changes in bone resorption [73]. The expression of *COL11a1*, a gene coding for a structural component of cartilage matrix, trabecular and compact bone as well as notochord tissue was also down-regulated in deformed fish

compared to normal individuals. In mammals, COL11a1 ensures cohesiveness of collagen matrix and its altered expression results in thicker fibers and increased mineralization [74]. In fish, COL11a1 is mostly present in notochord tissue, and is also expressed by osteoblasts in trabecular bone [25]. Interestingly, in Atlantic salmon a long photoperiod resulted in a lower amount of COL11a1 in compact bone of compressed vertebrae [25]. *COL11a1* expression was also reduced in Atlantic salmon subjected to a combination of low P-dietary treatment and long photoperiod [75].

Nevertheless, at the histological level our study demonstrated that both trabecular bone area and the surface of mineralized bone areas were not significantly different ($p = 0.39$ and 0.32, respectively) in pronounced biconcave compared to normal vertebrae (Table 4.2). We conclude that if the capability of osteoblasts to form new osteoid was not differentially affected in P-deficient fish with either biconcave or normal vertebrae, then the observed differences in mineral content might thus rather be related to the alteration of mineralization degree of the bone matrix.

Bone mineralization corresponds to the precipitation of calcium phosphate as hydroxyapatite (HA) crystals within a collagen-rich matrix, although how mineral formation is controlled is not fully understood. The combination of a favourable environment along with removal of mineralization inhibitors are known to be key factors regulating crystal formation [76]. Similarly, the interaction of HA crystals with the organic matrix and their growth and expansion have not been fully elucidated. However, we know that bone matrix, including non-collagenous proteins such as BGLAP, matrix Gla protein (MGP), osteopontin (also known as secreted phosphoprotein 1, SPP1) and SPARC, also control mineral deposition in bone tissue [77, 78]. As said above *BGLAP* expression was higher in biconcave than in normal vertebra with regard to RNA-seq experiment as well as by confirmation with RT-qPCR (Figure 4.4). In mammals *BGLAP* is known to be expressed exclusively by mature osteoblasts and osteocytes [68, 70] and constitutes the major non-collagenous protein in bone tissue. The role of circulating BGLAP is particularly relevant for energy metabolism and glycemia regulation [79]. Interestingly, we observed a substantial up-regulation ($FC > 34$) of *GCG2*, involved in lipid and glycogen hydrolysis and higher calcium intake [80]. However, despite extensive studies, the direct role of

BGLAP in bone tissue is still subject to debate. For instance, BGLAP-deficient mice displayed no detectable impact on bone mineralization using Von Kossa staining [81], but infrared microspectrometry showed its positive role in the maturation of hydroxyapatite crystals [82]. In juvenile Atlantic salmon with high growth rate *BGLAP* expression was negatively affected and the activity of mature osteoblasts and mineralization were reduced [24]. Low *BGLAP* expression could result in a default in mature osteoblast function, hence triggering default in mineralization status. However, by controlling the direction of crystal growth or even inhibiting it, high *BGLAP* expression may also have negative impacts on mineralization [77, 83, 84]. The up-regulation of *BGLAP* is also consistent with a higher expression of *C/EBP-β*, a putative positive regulator of BGLAP, a finding which was also reported in rat [85]. Interestingly, vitamin D is known to be a regulator of *C/EBP-β* expression and has also been involved in regulation of *BGLAP* expression [70, 86]. Similarly, *vitamin D* and expression of its receptor, *VDR* are affected by P-level in the diet [48, 75, 87, 88]. In rainbow trout vitamin D induces *NaPi-II* expression, which is responsible for P absorption and retention [89]. However, conflicting responses of P dynamics to vitamin D treatments have been observed in fish, indicating differences between teleosts and mammals [87, 89]. *MGP*, encoding a protein involved in the regulation of the mineralization process, was up-regulated in deformed fish. MGP is a potent inhibitor of bone and cartilage mineralization [90, 91]. In contrast to BGLAP, *MGP* is expressed in various tissues and is not limited to bone tissue in Atlantic salmon [92]. The expression of *MGP* in deformed fish is negatively correlated to bone ash content and mechanical quality (stiffness) in deformed fish.

The ossification by matrix proteins was related to expression of *DCHS1* and *FAT4*, both being lower in deformed than in normal fish. Interestingly, studies in transgenic mice indicated that the two proteins act as ligand-receptor complexes; Mice in which *DCHS1* and *FAT4* were invalidated show defects in vertebral column development and abnormal patterns of skeletal ossification [93]. The mechanisms by which these proteins play a role in skeletal tissue are not fully understood. However, according to the authors, DCHS1 and FAT4 could play a role in interacting with planar cell polarity effector, a pathway that was significantly impacted in the current study. Taken together, these results highlight various

actors, including matrix proteins that could be involved in the default of degree of mineralization observed in deformed fish and thus putatively triggering the development of deformities.

Finally, under normal conditions, the teleost skeleton displays a wide spectrum of intermediate skeletal tissues (from cartilage-like to bone-like tissues), which are complex to study [94, 95]. In teleost vertebrae, more or less mineralized healthy cartilage as well as ectopic cartilage can be observed, the former being related to a metaplastic shift of bone and co-transcription of chondrocytic and osteogenic markers [23, 24, 96, 97]. During development, the cartilage-type tissue localized in the basalia is replaced by bone-type tissue by a process called endochondral ossification. This process requires chondrocyte hypertrophy and apoptosis, blood vessel invasion and ECM degradation. It was thus important to assess whether molecular activity or overall mineral status might be biased by the high cellular activity within the basalias. Both osteoblasts and chondrocytes differentiate from mesenchymal stem cells and thus share numerous key events involved in their differentiation and regulation [26]. Here, we observed that pathways involved in chondrocyte differentiation were affected in deformed fish (down-regulation of *SHH* and *FGFR4*) but not the expression level of a key mediator of cartilage formation SOX9 [98] and of MEF2C, a marker of chondrogenesis [23, 99], which remained constant. Expression of *MEF2C* was further confirmed by RT-qPCR (Figure 4.4). Furthermore, *NKX3.2*, a transducer of SOX9 signals was up-regulated in deformed fish [67, 100]. Our results also suggest that the invasion of blood vessels in the cartilage matrix, its degradation and replacement by osteoblasts might be negatively impacted in deformed fish, even if a distinctive phenotype is lacking. Indeed, deformed fish displayed impairment of the VEGF signalling pathway as indicated by lower expression of *HSPG2, GPC1* and *VEGFR1*. The VEGF pathway is involved in angiogenesis (invasion of blood vessels) and ossification [101–103]. *HSPG2* -/- mice displayed strong impairment of cartilage formation, ultimately leading to diminished osteoblast replacement and endochondral bone formation [104]. Interestingly, in deformed fish we did not observe differences in the mineralization status of basalia. Consistent with observations of *HSPG2* -/- mice, *MMP10* expression was increased in deformed fish. *MMP10* is expressed in chondrocytes degrading the cartilage

matrix as well as in some, but not all osteoclasts and is also responsible for activation of several other MMPs including MMP9, that is involved in the vascularization process of the growth plate [105–107]. Here however, we observed an over-representation of canonical pathways for the inhibition of MMPs, hence the complexity to interpret the role of MMPs in deformed vertebrae. Thus, our results suggest that basalia deformed vertebrae might experience a subsequent endochondral ossification but with no impact of the calcification of the basalia by the time of sampling. To confirm the gene expression results, we used Sirius red staining coupled with image processing to extract the basalias and quantitatively assess the presence of mature collagen fibers (putatively mineralized) and that of less mature collagen fibers. In both groups, we observed that the vertebral arches were not fully ossified, but no significant differences were observed in their mineralization status (Table 4.2). Furthermore, no obvious presence of ectopic cartilage or remodelling of the notochord was observed in either deformed or normal fish, as previously reported in severe compression type deformities in other salmonids [24, 96, 108].

Table 4.4: Selection of differentially expressed genes putatively related to bone metabolism. The homologies were revealed with blast-x search against Uniprot-Swissprot database (cut-off evalue < 1e-8). Differentially expressed genes were conserved if they display a unique and FDR < 5%.

Gene header	Uniprot Acc.	Gene name	Log2FC	|FC|
Down-regulated genes in deformed vertebra				
comp149051_c0	A4IFW2	Protein tyrosine phosphatase receptor type F	1.64	3.12
comp138459_c1	Q64487	Protein tyrosine phosphatase receptor type D	1.62	3.08
comp145566_c4	Q96JQ0	Dachsous Cadherin-Related 1	1.55	2.93
comp144178_c2	Q6ZRS2	Snf2-related CREBBP activator protein	1.49	2.8
comp146067_c0	P35556	Fibrillin 2	1.42	2.67
comp132718_c0	P45481	CREB binding protein	1.38	2.61
comp149014_c5	Q8BNA6	FAT atypical cadherin 3	1.37	2.59
comp144153_c0	O95644	Nuclear factor of activated T-cells, calcineurin-dependent	1.31	2.49
comp133481_c0	Q9Y3S1	Serine/threonine-protein kinase WNK2	1.31	2.49
comp148107_c0	Q8WYB5	K (Lysine) acetyltranferase 6B	1.24	2.35
comp147565_c0	Q5I598	Methylenetetrahydrofolate (NAD(P)H)	1.22	2.33
comp143062_c0	Q13332	Protein tyrosine phosphatase receptor type S	1.19	2.28
comp126856_c0	P17948	Vascular endothelial growth factor receptor 1	1.17	2.24
comp134803_c0	Q14517	FAT atypical cadherin 1	1.14	2.21
comp143303_c7	Q8VI24	SATB homeobox 2	1.12	2.18
comp145538_c0	Q99715	Collagen type X2 alpha 1	1.12	2.18
comp131699_c0	F1QCC6	Glypican 1	1.1	2.14
comp146160_c1	Q9Z180	SET binding protein 1	1.1	2.14
comp149571_c0	Q9P2D1	Chromodomain helicase DNA binding protein	1.09	2.13
comp140581_c1	Q92794	K (Lysine) acetyltranferase 6A	1.05	2.07
comp128868_c0	O75581	Low density lipoprotein receptor-related protein 6	1.04	2.05
comp142199_c3	O70496	Chloride channel voltage-sesitive 7	1.01	2.01
comp147156_c2	O89026	Roundabout axon guidance receptor homolog 1	0.97	1.96
comp135768_c0	P27658	Collagen type VIII Alpha 1	0.97	1.97
comp149750_c0	Q6V0I7	FAT atypical cadherin 4	0.97	1.96
comp134251_c0	Q8BTM8	Filamin A, alpha	0.97	1.96
comp141372_c4	P98161	Polycystin 1	0.95	1.93
comp144389_c0	A7XYQ1	Jackson circler protein 1	0.94	1.92
comp67973_c1	Q6NSM8	SIK family kinase 3	0.93	1.91
comp135751_c0	Q9UBS9	Sun domain-containing ossification factor	0.93	1.9
comp135751_c0	Q9QYP0	Multiple EGF-like-domain 8	0.91	1.88
comp145151_c0	P26012	Integrin β 8	0.9	1.87

comp136915_c0	Q13233	Mitogen-activated protein kinase kinase kinase 1	0.89	1.85
comp149523_c0	O88491	Nuclear receptor binding SET domain protein 1	0.87	1.83
comp149485_c0	Q9UHF7	Trichorhinophalangeal syndrome I	0.87	1.83
comp139497_c0	P21359	Neurofibromin 1	0.86	1.81
comp149389_c0	O35607	Bone morphogenetic protein receptor, type II	0.85	1.81
comp146007_c0	Q14938	Nuclear factor 1/X (CCAAT-binding transcription factor)	0.85	1.8
comp146819_c2	Q90413	Fibroblast growth factor receptor 4	0.81	1.75
comp146502_c4	P20909	Collagen type XI alpha 1	0.8	1.74
comp149588_c0	A2A884	Human immunodeficient virus type 1 enhancer binding protein 3	0.79	1.73
comp147279_c4	P98160	Heparan sulfate proteoglycan 2	0.78	1.72
comp144268_c0	Q09472	E1A binding protein P300	0.78	1.71
comp149387_c2	Q8IXJ9	Additional sex combs like 1	0.78	1.71
comp148958_c0	Q9ULT8	HECT domain containing E3 ubiquitin protein ligase 1	0.78	1.71
comp149573_c0	P10587	Myosin heavy chain 11 smooth muscle	0.77	1.71
comp149223_c0	P79114	Unconventional myosin-X, role n podosom belt osteoclast	0.77	1.7
comp149584_c1	P35580	Myosin 10	0.75	1.68
comp138258_c6	Q07889	Son of sevenless homolog 1	0.69	1.61
comp147381_c0	Q91YM2	Rho GTPase activating protein 35	0.68	1.6

Up-regulated genes in deformed vertebra

comp146739_c2	Q9YHV3	Noggin-3	-3.19	9.13
comp142082_c0	P57102	Heart and neural crest derivatives expressed 2	-1.84	3.59
comp148652_c4	Q9JK00	Sodium channel voltage gated type III β subunit	-1.63	3.09
comp149418_c1	P05549	Activating Enhancer-Binding Protein 2-Alpha	-1.51	2.84
comp142563_c0	P09238	Matrix metallopeptidase 10	-1.46	2.76
comp88970_c0	P79703	Jun B proto-oncogene	-1.42	2.67
comp144447_c2	Q05826	CCAAT/Enhancer binding protein (C/EBP) B	-1.27	2.42
comp135507_c0	Q920D2	Dihydrofolate reductase	-1.26	2.4
comp146593_c0	P40148	Osteocalcin	-1.13	2.19
comp144817_c0	O75911	Dehydrogenase/Reductase (SDR family member 3)	-0.93	1.9
comp121895_c0	Q800Y2	Matrix Gla protein	-0.86	1.82
comp136344_c0	P52909	Jun D proto-oncogene	-0.85	1.8
comp148539_c0	P70061	NK3 homeobox 2	-0.85	1.81
comp147786_c0	Q92008	Sonic hedgehog	-0.84	1.79
comp143712_c0	P25942	CD40 molecule TNF receptor family member 5	-0.78	1.72
comp149526_c0	Q9NRR1	Cytokine-Like 1	-0.78	1.71
comp141960_c0	P97831	Twist 2	-0.75	1.69
comp98891_c0	O55052	Macrophage migration inhibitory factor	-0.7	1.62
comp148620_c0	P18203	FK506 binding protein 1A, 12kDa	-0.7	1.63

4.6 Conclusion

Interpreting data from RNA-seq experiments, particularly in a complex tissue like vertebra, remains challenging and is limited to the gene expression levels. This approach however, provides an unprecedented overview regarding molecular changes that are involved in the occurrence of vertebral deformities in P-deficient trout. The aim of our study was not to describe the impact of P-deficiency in trout but rather to characterize discrete differences between fish fed low-P diets presenting either deformed or normal vertebral phenotypes. We observed that the initial appearance of malformations in rainbow trout is associated with changes in numerous signalling pathways similar to those described in mammals, confirming their evolutionary conservation. Our results also suggested that after 27 weeks feeding low P diets, deformed vertebrae showed a decrease in osteoblast differentiation and proliferation while they display a higher expression of *BGLAP*, a marker of mature osteoblast activity. Together with histological observations, this suggests that during the weeks prior to sampling, deformed fish were still differentiating osteoblasts and producing matrix. However, higher expression of *MGP* and *BGLAP* as well as lower circulating P significantly impaired mineralization. Furthermore, impaired cohesiveness together with low mineralization reduced vertebrae stiffness. Consequently, reduced vertebral integrity might increase vertebrae susceptibility to deform under normal physical forces. The conserved osteoclast activity together with the production of BGLAP and GCG2 suggests that deformed vertebrae are undergoing significant bone remodelling with high bone resorption process not yet observable in our study. This process may lead to new vertebral phenotypes. As suggested in previous studies [20, 28], pronounced biconcave vertebrae may be a transitory step toward a more dramatic abnormal vertebral phenotype (homogeneous compressed). Similar analysis of vertebral gene expression over time will highlight molecules involved in the occurrence of vertebral deformities. Our study provides consistent evidence of the importance of the matrix proteins and other markers involved in reduced mineralization and mechanical strength of vertebra, recognised as initial indicators of deformity development when feeding low-P diets to fish [48]. Previous family-based studies in fish showed that genetics has an impact on the prevalence of deformities [109–

112]. With the aim of reducing P excretion to promote sustainable freshwater aquaculture production, the indicators revealed in our study may ultimately serve as markers for genetic selection of fish strains displaying normal growth performance with a low prevalence of deformities, when feeding low-P diets.

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5 General discussion and conclusion

In freshwater systems, excessive phosphorous (P) output from animal production, including fish farms may represent a significant risk to the contamination of water under specific conditions. Excess P from feeding fish mainly results from uneaten feed and metabolic wastes, particularly when fed excessive levels of dietary P. To limit the P footprint in receiving water bodies, the province of Quebec implemented new regulation, aiming to reduce P discharge by 2015 to 4.2 kg P/tonne of fish. Several water treatment/management practices have been explored however reducing dietary P levels seems the most practical approach [1]. To date however, the Canadian Feed Act prohibits salmonid diets with less than 1% total P, despite the fact that P requirements have been shown to be significantly lower, particularly in post-juvenile fish [2]. On another hand, the negative impact on bone development, especially in fast-growing juvenile fish, affects limits on the possible reduction of dietary-P. Together, these facts suggest that precise and sensitive indicators to monitor fish response need to be further elaborated.

In this regard, we conducted experiments on juvenile triploid rainbow trout fed deficient or sufficient P-dietary levels. This thesis project aimed to:

1. Describe the first metabolic response to a P-deficiency;
2. Increase the transcriptomic data regarding specific bone tissue in a salmonid species;
3. Characterize the molecular changes involved in the appearance of vertebral deformities in rainbow trout fed P deficient diets.

Therefore, we conducted a fully-integrated study using common histological observations, newly developed quantitative analyses of histological sections, as well as common P status monitoring correlated with cutting-edge sequencing tools for whole-transcriptome characterization of a non-model species.

5.1 Body responsiveness

Our first study (Chapter 2) focused on evaluating P-deficiency on the early response of mineralized tissues (scales and vertebrae) as well as whole-body and plasma. The hypothesis was that the response of fish differs depending of the different body compartments and the measures indicator. The results of this study have demonstrated for the first time that P-deficiency induces lower P status (ash and P content) in both the whole-body and scales, as early as the second week of deficiency induction, without affecting fish growth. However, low dietary P levels did not significantly affect the levels of P in the vertebrae, at least within the first four weeks of experiment, due mainly to the high variability in this compartment. Yet, this study also demonstrated that after five weeks of experiment, up to 50% of the P-deficient fish displayed vertebral deformities. These deformities mainly consisted of pronounced biconcave (35.9%) and undersized and widely spaced vertebral body (9.3%), which is consistent with specific vertebrae phenotypes observed in fish under mineral deficiency. By finely describing the response timeline of these tissue to low-P diets and evaluating their variability, this work serves as a baseline for the development of new metabolic indicators for the physiologic response to low-P diets. Nevertheless, many questions remain pending regarding P metabolism in skeleton and the role of bone in the mineral homeostasis.

Our observations are consistent with previous work reporting that scales might be one of the first body compartments to respond to P-deficiency while, up to a certain point, the integrity of the vertebrae was conserved [3]. Yet, from our experimental design, it could not be ascertained whether P was redirected preferentially to vertebrae from scales, or remobilized from scales to strengthen internal skeletal tissues. Addressing this question would certainly refine the knowledge of mineral homeostasis in this teleost species.

5.2 Reference transcriptome

Over the past years, multiple efforts have been deployed to increase the genomic data available for salmonids, including rainbow trout [4–8], yet, relatively scarce data emerged on the specific bone tissue regulation. With the continuous emergence of novel analytical

resources, the use of NGS is viewed as a powerful tool for transcriptome analysis, allowing for the understanding of the molecular regulators of the bone tissue. By using RNA-seq technology, we first built and annotating reference transcriptome for vertebra tissue, including intervertebral tissue, in rainbow trout (Chapter 3). This study reports the first bone-specific reference for a specific cellular bone tissue in a teleost, and therefore, salmonid species. This led to the production of up to 117,564 annotated transcripts against Uniprot-Swissprot database. With regard to bone metabolism, several pivotal actors known in mammals have also been newly-reported for rainbow trout, supporting the assumption that teleost and mammal bone regulation are particularly comparable [9]. This thesis provides also new insights on the role of bone tissue in the whole organism metabolism regulation (e.g. expression of both *BGLAP* and *GCG2*) in teleosts, in accordance with previous observations in mammals [10]. Thus, contrary to common misconceptions, bones, and especially vertebrae, represent an extremely dynamic tissue whose comprehension could greatly benefit many domains, from animal production to human health.

The abundance of transcripts non-associated with already-referenced sequences traduced the importance of our study in improving database for rainbow trout, and bone tissue particularly. Nonetheless, non-referenced transcripts also open the way to otherwise limited available protein sequences, further moderating the interpretation of transcriptomic studies. Undoubtedly, similar outcomes will definitely profit from the increase of proteomics studies. Furthermore, assembly of transcriptomic sequences from non-model species remains a challenging task, particularly with complex genomes such as those from salmonids, as suggested by the rapid development of analytical resources. The main challenges reside in:

1. The presence of orthologs and paralogs that could not be separated in our study;
2. The relatively short length of P-E reads from HiSeq2000;
3. The high-consuming memory resources of bioinformatic pipelines.

The first publication of the rainbow trout genome [11] together with the novel bioinformatic resources, our assembly undoubtedly enables the realization of many novel studies while leaving room for further improvement.

5.3 Quantitative study

In the light of the observation that P-deficient fish at week 27 may display normal vertebral phenotypes, while others developed pronounced biconcave vertebrae, we then explored the differences in gene expression between normal and deformed fish (Chapter 4). We based our analysis on the reference transcriptome built in Chapter 3, and we were also able to compare our RNA-sequencing data with histological observations and physiological measures. We observed that P-deformed vertebrae displayed a lower ash status and showed lower biomechanical resistance, whereas bone compactness, mineralized trabecular bone and total matrix areas were similar between deformed and normal phenotypes. Similarly, no difference in tissue mineralization was observed between basalias of P-deficient and P-sufficient fish. We thus concluded that major differences were attributable to default in the degree of mineralization rather than a reduction in bone matrix production or an increase in bone resorption. This explanation was supported by the overexpression of both *MGP* and *BGLAP* genes. Nevertheless, we observed that signalling pathways promoting osteoblast differentiation and proliferation were negatively affected by the phenotypes (overexpression of *NOG*, a BMP signalling inhibitor) suggesting that from week 27, bone matrix formation might be impaired. BMP is also considered as a central pathway for stimulating initial commitment of MSCs toward the osteoblastic lineage at the expense of the chondrocytic lineage [12, 13]. As suggested in previous studies, biconcave vertebrae might represent a transient phenotype before the apparition of more severe compressions [14, 15]. By monitoring the evolution of compression phenotypes, studies reported the metaplastic shift of the notochordal tissue and the expression of ectopic cartilage [16, 17]. Thus, we can hypothesize that a reduction of osteoblast differentiation and proliferation by the inactivation of the BMP pathway is the first step in stimulating chondrocyte differentiation. Consequently, both Gla proteins and particularly the BMP canonical pathway associated gene (*NOG*) may be relevant candidates for early detection of vertebral deformities appearance in low-P fed rainbow trout. The development of such indicators

would thus be useful for the aquaculture industry in the formulation of diet with optimum P-levels.

Bone formation being extremely tightly-orchestrated, interpretation of a range of snapshot observations, such as gene expression, remains challenging to replace within a dynamic process of tissue metabolism. Furthermore, due to the complexity of the regulatory systems that control vertebral metabolism and the cell diversity in this tissue, the absence of precise expression localization, by *in situ* hybridisation, further limits our interpretation. With the development of the "omic" era, studies of the different biological levels ranging from epigenome and genome to proteome and "phenome" are relatively affordable; yet, they often remain limited to a particular biological level, due to budget restrictions. While coupling several of these tools to provide a holistic overview of the mechanisms involved, it might also reveal some major discrepancies. For instance, mRNA quantity is not directly linked to protein quantity and/or protein activity, even if both are tightly regulated. A comprehensive characterization of the molecular changes is particularly beneficial but also requires the monitoring of downstream responses as well as the careful description of the final phenotype. Consequently, further studies coupling RNA-seq with other biological levels such as proteomics, would certainly lead to further understanding of the mechanisms involved in appearance of vertebral deformities.

Nutrition and nutrigenomics studies face the challenge to develop a well-balanced and correctly formulated feed taking into account the multiple interactions between nutritional components. For this study, the choice of the experimental diets and of their composition was based on their proved efficiency during preliminary studies in our laboratory [3, 18, 19]. To induce a severe P deficiency, fish oil was reduced in both diets and P, under the form of dicalcium phosphate (CaHPO4), was added to the sufficient diet. Consequently, total P reached 0.92% and 0.50% (DM) in S and D diets, respectively. Several questions arose from this procedure. First, Ca levels were also affected by fish oil reduction, reaching 1.78% and 0.75% in S and D diets, respectively. Data regarding Ca deficiency are relatively scarce in the literature, but fish performance is unlikely to result from Ca deprivation. Indeed, Ca levels in our experimental diets were far above the 0.1%

tested by Fontagné et al. [20] and the critical 0.3% reported for other fish species [21]. Second, according to the literature, we assumed that P was absorbed mainly through the intestine; yet, we could not exclude or assess the putative absorption of soluble P in the water through the gills and further nutrition experiments will definitely benefit from such quantification. Third, we observed that our P-deficient diet did not induced as severe malformations as expected [18]. This could be explained by either the lower P requirement of larger fish and/or the redistribution of minerals among tanks by the recirculating system and their possible absorption. Yet, our experimental design allowed fish to develop constant and stable phenotypes throughout the experiment, which was fruitful for a first characterization of the most common abnormal P-deficiency-induced phenotypes. Nonetheless, further experiment in open-water system should be conducted for a comprehensive understanding of the low-P diet impact in an aquaculture context. Finally, levels of dietary vitamin-premix added were also based on previous study in our laboratory but, due to confidentiality and budget restrictions, they have not been analysed in our experimental diet. For their role in phosphate levels regulation, bone deformities incidence and bone-related proteins (such as Gla proteins) regulation, both vitamin D and K are particularly relevant in this case. For instance, deficiency in vitamin K on haddock particularly resembles observations on P-deficient deformed fish in our study, with symptoms like low mineralization and normal numbers and activity of osteoclasts and osteoblasts [22]. Consequently, the combined impact of low vitamins and low P levels in the diet on the final absorption of mineral and bone health should not be excluded. Yet, the exact role of those vitamins and their interaction during bone regulation and mineral homeostasis remain to be understood [23].

5.4 Perspectives

As reviewed in this document, to date, most of the indicators used to assess the effect of P-deficiency on fish, and particularly the effect on bone tissue, are based on the response of internal compartments such as vertebrae. Data are scarce regarding the response of external skeleton compartments to low-P levels in the diets [2, 3]. Yet, our study provides evidences that scales might be one of the first compartments to respond to a P deficiency, resulting in a reduced mineral status. In the vertebrae, we reported that affected degree of

mineralization might be due to a differential expression of Gla proteins such as BGLAP regulating growth and fixation of HA crystals in the ECM. Consequently, monitoring the transcriptomic response of scales, and mainly the expression of genes coding for Gla proteins or inhibitor of BMP pathway, should be considered as a new indicator for nutrition studies allowing refining P requirement in rainbow trout. Furthermore, both the genomic platform and subsequent data analysis are easily adaptable for such study.

Profound differences in skeleton regulation between teleost and mammals, the relative importance of Ca and P in particular, have led to misinterpretations regarding mineral homeostasis in teleost. Additionally, discrepancies are noticeable between advanced teleost with acellular bone, and mammals and "basal" teleosts with cellular bone, regarding the physiological regulation of bone turnover. Consequently, the role of bone tissue in mineral homeostasis in fish remains far from being fully understood. Together with the publication of the first draft of the rainbow trout genome [11] and our bone transcriptome, further phylogenetic studies and comparative genomics would bring crucial information to the functioning of the endoskeleton turnover and its role in homeostasis in basal teleost.

In parallel, by monitoring the appearance of deformities, we reported that some individuals maintained a normal vertebrae phenotype over the 27-week trial without significant impact on fish performance such as growth or condition factor [9, Chapter 4]. For this study, we strived as much as possible to limit dominancy in tanks by dispersing the feed ration and keeping relatively low fish density and frequently monitoring fish behaviour. However, our experimental design did not include individual monitoring of feed consumption, hence the incapacity to exclude the impact of behaviour and dominancy on vertebrae phenotypes. Yet, these results bring some exciting insights to the possibility of fish strain selection. Previous studies reported that differences in bone compactness were correlated with different fish strain in rainbow trout production [24]. Similarly, non-additive and additive effects of genetics on the incidence of skeletal malformations have been observed in larvae of Chinook salmon (*Oncorhynchus tshawytscha*) and 14-17 month-old Atlantic salmon, respectively [25, 26]. As mentioned within a similar study on

European sea bass populations, heritability of malformation incidence might rather originate from a difference in the susceptibility to external causing factors [27]. Due to the interdependency of the numerous causative factors such as genetics, nutrition or environmental stimuli, temperature and photoperiod, as well as potential multiple-gene regulation of deformities, separating only the genetic effect remains a challenging problem. Consequently, further studies with half- or full-sib families focusing on P-levels in the diet and the prevalence of vertebral deformity are required for further implementation of a new stock management strategy and stock genetic improvment. Nevertheless, this thesis and the candidates genes found to be associated with abnormal vertebral development, together with previously highlighted genes of interest [28, 29], will definitely be a starting point for the development of molecular selection tools and P deficiency monitoring in freshwater rainbow trout.

Finally, after completion of our studies, we observed that in some peculiar cases, fish with deformed vertebrae (pronounced biconcave) could recover a normal phenotype. As largely presented in our study and in previous studies based on radiographical observations [14, 15], pronounced biconcave vertebrae may be a first step toward a more severe compression phenotype and is mainly characterized by its lower degree of mineralization (Chapter 4). To date, such molecular mechanisms remain to be appropriately described. Would such information be transferable to other species and clades? Gorman et al. [9, 30] reviewed the potential of the teleost model for human skeletal development including pathological spinal curvature. From a clinical point of view, decrypting the molecular mechanisms involved in the improvement of low mineralized bone could also have positive repercussions for human medicine, including in treatments such as osteoporosis.

To conclude, this work increased considerably the knowledge of bone response to P-deficiency in rainbow trout. Furthermore, together with the specific knowledge and the implementation of novel quantitative imaging techniques to study bone tissue, this project led to the establishment of a NGS platform at the Université Laval in the Department of Animal Sciences. This platform is efficient and fully operational for the preparation of

NGS samples and libraries as well as for the downstream bioinformatic analyses of large datasets. Undoubtedly, these tools might be adapted to other species as well as numerous areas of related multidisciplinary research efforts related to biological and/or physiological studies.

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Annexes

Annexe 1: Supplementary tables of the functional annotation of the reference transcriptome. 1) Contig name, length, Uniprot-Swissprot accession number and contig sequence (.fasta) of key actors of bone regulation expressed in rainbow trout vertebrae; 2) Reported matches against Uniprot database (evalue < 1e-8)

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Annexe 2: **X-ray-based morphometrics: an approach to diagnose vertebral abnormalities in under-mineralized vertebrae of juvenile triploid all-female rainbow trout (*Oncorhynchus mykiss*) fed with a phosphorus deficient diet.**

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Summary

Extended phosphorus (P) deficiency has been linked to an increase of skeletal deformities in farmed salmonids. To date, X-ray examination still is the most practical way to detect abnormalities that are not externally visible. The aim of this study was to develop a standardized method based on empirical measurements that provides an early radiograph-based detection of deformities. Triploid all-female rainbow trout (60.8 ± 1.6g) were fed with phosphorus (P)-deficient and P-sufficient diets (total P = 0.5% and 0.92%, respectively). Experiments were carried out in 12 circular re-circulating tanks (n = 140/tank; 8.5 to 52.7 kg m^{-3}; 12 ± 0.3°C; 18L: 6D). All fish were x-rayed at week 5, 15 and 24 to monitor vertebral abnormalities. Fish were assigned to a vertebral phenotype (normal, biconcave or compressed) when all vertebrae of the caudal region (V31-44) showed the same type of abnormalities. At week 9, 18 and 27, ten fish per tank were randomly sampled according to their vertebral phenotype. Vertebrae V38-V39 were collected and a second X-ray was performed to confirm the above-mentioned phenotypes. Morphometrics (vertebral length and height; intervertebral distances and vertebral body angles) were measured directly on radiographs. For all parameters measured, comparisons were made between normal P-sufficient fish and different scenarios of vertebral development (normal, restored, stable and aggravated) in P-deficient fish. The two parameters showing the most sensitivity were the median intervertebral distance and dorsal/ventral vertebral endplate angles. When analysed together, these two parameters allow a quantitative distinction of the different vertebral phenotypes. Measuring dorsal/ventral vertebral endplate angles seems a promising approach to detect aberrant vertebral development at early stages as slight changes were observed in P-deficient vertebrae that were assessed as normal by visual examination. Further studies including histological examinations and comparison with other radiological imaging techniques are required to confirm these results.

Keywords: nutrition, phosphorus status, vertebral abnormalities

Introduction

Vertebral abnormalities and malformed fish have been frequently observed in farmed salmonids such as trout (Madsen and Dalsgaard, 1999; Madsen et al., 2000, 2001; Kacem et al., 2004; Deschamps et al., 2008, 2009; Fontagné et al., 2009) and Atlantic salmon (Sadler et al., 2001; Sugiura et al., 2004; Gil-Martens et al., 2005; Fjelldal et al., 2007a,b; Fjelldal and Hansen, 2010; Fjelldal et al., 2012a,b,c). Malformations can cause an increase in mortality, poor growth performance and potentially a downgrading of the harvested product (Sullivan et al., 2007; Aunsmo et al., 2008: Boglione et al., 2013) and raise concerns about animal health and welfare. Skeletal development is a sensitive process (Fjelldal et al., 2012a) that can be affected by many factors such as elevated temperature (Wargelius et al., 2005; Ytteborg et al., 2010a,b; Grini et al., 2011), photoperiod manipulation (Fjelldal et al., 2005, 2012c; Wargelius et al., 2009), inflammation (Kvellestad et al. 2000; Gil-Martens et al., 2012), triploidization (Tillman 1999; Fraser et al., 2013) and nutrient deficiency (Ogino et al. 1978; Hardy and Shearer, 1985; Porn Ngam et al., 1993; Watanabe et al., 1997; Ørnsrud et al., 2002; Lall, 2002; Waagbø, 2010; Moren et al., 2011; Fjelldal et al., 2012b). In fast-growing salmonids, phosphorus (P) deficiency results in poorly mineralized bone triggering the development of vertebral abnormalities (Sugiura et al., 2004). Hence, it is important to detect signs of P-deficiency and this can be achieved by noticing slight morphological changes as soon as possible.

Radiology is a sensitive and commonly used approach to detect vertebral abnormalities (Fjelldal et al., 2006; Gil-Martens et al., 2006; Witten et al., 2005). Witten et al. (2009) proposed a classification of vertebral abnormalities based on radiological examinations of Atlantic salmon. The aim of the current study was to work with this classification system and to develop an empirical method using classical (length, height and intervertebral distance) and novel (angles) measurements that could allow to confirm particular types of deformities in under-mineralized vertebrae from P-deficient trout and to verify if vertebral measurements on radiographs, could distinguish abnormal vertebrae before deformities could be visually accessed.

Materials and methods

Biological material and rearing conditions

Experiments were conducted on all-female juvenile (60.8 ± 1.6g) triploid rainbow trout (*Oncorhynchus mykiss*). Triploidization was induced via hydrostatic pressure shock at the Pisciculture St-Alexis-des-Monts Inc (St-Alexis-des-Monts, QC, Canada). hatchery according to a standard protocol. Fish were fed with two practical diets with two different levels of phosphorus (total P = 0.5% and 0.92% for P-deficient and P-sufficient diets, respectively; P requirements = 0.8%; NRC, 2011) successfully tested in our laboratories (Table 1; Koko et al., 2010; Deschamps et al., in press). The P-sufficient diet was obtained by supplementing the P-deficient basal diet with 2.5% of inorganic phosphorus source (25g $CaHPO_4$ kg^{-1}).Fish were raised in twelve 2 m^3 freshwater recirculating tanks (40L min^{-1}; six replicate tanks per diet; N = 140 fish per tank with stocking density of 8.5 to 52.7 kg m^{-3}) at constant temperature (12 ± 0.3°C) and photoperiod (16L: 8D) for 27 weeks. Fish were fed twice a day according to pair-feeding techniques. All fish were sedated in MS-222 baths (75 mg L^{-1}) and PIT-tagged (Avid, California, USA) on week 5 to allow individual monitoring.

Assessment of vertebral phenotypes and sampling

In toto X-rays of the caudal region of all fish in the latero-lateral view were taken at 5, 15 and 24 weeks according to Witten and Hall (2002). The beam source was placed at 40 cm from the film with the following settings: 60 kV, 15mA, 2 sec exposure. A MINXRAY HF80+ device (Mini X-ray Inc., Northbrock, USA) and Kodak film (Industrex MX-125 Film Ready Pack. 8X10 in., Lot 366018, Kodak Industry, France) were used. Radiographs were developed using clinical processing equipment (CAN-Med, Canada) for conventional radiographs (Merry X-Ray 3-7-90 Type S-2 Developer 10G and 3-7-90 Fixer 2 Pak, Canada) according to Witten et al. (2009).

A classification proposed for Atlantic salmon (Witten et al., 2009) was used to assess vertebral abnormalities. Fish were associated to a vertebral phenotype when all the caudal vertebrae (i.e. V31-44 using the regionalization suggested by Kacem et al., 1998)

were similar by visual examination. Although other vertebral phenotypes were observed (e.g., type 10 small and spaced vertebrae), we only focused our attention on the three most common phenotypes encountered:

- Type 0 = normal vertebrae displaying symmetrical, centrally-located radio-dense X-structure (referring to the X-shaped structure around the notochord sheath formed by the double cone of intramembranous bone). At week 5, 15 and 24, the total number of fish displaying vertebrae of type 0 was of 573, 433 and 340 for P-sufficient fish and of 320, 61 and 164 for P-deficient fish,.

- Type 11 = pronounced biconcave vertebrae: vertebrae with normal X-structure but with seemingly transparent vertebral body endplates. On the radiograph, the overall shape of the vertebral body appears as a vertical apple core. The total number of P-deficient fish displaying vertebrae of type 11 was of 213, 342 and 112 at week 5, 15 and 24, respectively.

- Type 2 = homogeneous compression of vertebral bodies. Vertebrae that appeared compressed in the cranio-caudal axis with or without inward bending of the edges of the vertebral body endplates. The total number of P-deficient fish displaying vertebrae of type 2 was of 1, 0 and 131 for P-deficient fish at week 5, 15 and 24, respectively.

At weeks 9, 18 and 27, ten fish per tank were proportionally and randomly sampled according to the three phenotypes. Fish were sedated in a MS-222 bath (75 mg L^{-1}), measured (fork length, FL, cm) and weighed (body mass, BM, g) before being decapitated and dissected to remove two caudal vertebrae (V38 and 39). Fulton's condition factor (K) was calculated as follow: (BM FL^{-3}) * 100. The remaining attached vertebrae (V38-V39) were fixed in 4% formaldehyde and stored at 4°C until processed.

Sub-sampling of vertebrae

A sub-sampling (N = 99) was performed to retain fish having experienced four main scenarios of vertebral development according to their phenotypes at week 5, 15 and 24.

- Normal scenario (either P-deficient or P-sufficient): vertebrae that showed a normal vertebral structure (Type 0) from week 9 to 27;

- Stable scenario (P-deficient): normal fish at week 9 which developed and maintained a biconcave phenotype (Type11) at week 18 and 27, respectively;

- Restoration scenario (P-deficient): normal fish at week 9 that developed abnormal biconcave vertebrae at week 18 and returned to a normal phenotype at week 27 and,

- Aggravation scenario (P-deficient): fish showing normal vertebrae at week 9, biconcave vertebrae at week 18 and compressed vertebrae at week 27.

Thus, at week 9 (starting point of the different scenarios), all the fish retained had normal X-shaped vertebrae (i.e., non-deformed; N = 15 and N = 7 for P-sufficient and P-deficient, respectively). At week 18, 18 non-deformed fish (N = 15 and N =3 for P-sufficient and P-deficient, respectively) and 17 deformed fish (P-deficient fish having developed the biconcave vertebral phenotype) were retained. At week 27, 27 non-deformed fish (i.e., N = 18 P-sufficient and N = 3 P-deficient fish having maintained a normal phenotype and 6 P-deficient fish having restored their vertebrae) and 15 deformed (N = 7 and N = 8 P-deficient fish having stabilized or aggravated their vertebral phenotype, respectively). Radiographs of the V38-39 samples stored in formaldehyde were performed again (identical settings described above for fish *in toto,* with slight changes for week 9 samples: 50kV and distance from the beam was 30 cm) to ensure that the vertebral phenotypes were maintained between X-rays points (weeks 5, 15 and 24) and sampling points (weeks 9, 18 and 27) as well as to confirm that the biconcave phenotype observed *in toto* was not due to X-ray artefacts caused by the bending of the caudal fin or the angle between the beam source and the sample (Witten et al., 2009).

Morphometrics on X-rayed vertebrae

X-rays of remaining attached vertebrae (V38-39) were placed in a lightbox and photographed (Canon EOS 7D DS126251EF, 100mm F/2.8L MACRO lens). ImageJ software was used to measure each x-rayed vertebra (Fig. 2): length (L, mm), height (H, mm), dorsal peripheral intervertebral distance (DSp, mm), ventral peripheral intervertebral distance (VSp, mm) and median intervertebral distance (MSp, mm). The dorsal vertebral endplate angle (DAngle, degrees) and ventral vertebral endplate angle (VAngle, degrees) were also measured. DVSp and DVAngle are mean values for DSp and VSp and DAngle

and VAngle, respectively. 'DAngle' and 'VAngle' were defined as the dorsal and ventral angles of the 2D characteristic X-shaped structure on x-rays (Fig. 2). They correspond to the angles between the anterior and the posterior vertebral endplates on the neural (dorsal) and haemal (ventral) sides of the vertebral body, respectively.

Statistics

Results are given in terms of mean ± standard deviation. One-way ANOVAs followed by multiple comparisons Tukey's tests were performed for each morphometric and sampling time. The results were assumed to be significant at P values below 0.05. Given the small number of samples, tendencies (p-values below 0.1) were also considered.

Results

Fish length, weight and Fulton's condition factor

Fork length and weight of fish were too variable to detect differences between vertebral phenotypes and/or different diets (Table 2). However, at week 27, the higher Fulton's condition factor in P-deficient fish having the most severe abnormalities (K = 1.6 ± 0.1 in fish with compressed vertebrae) showed that, for an equivalent weight, non-deformed P-sufficient (K = 1.4 ± 0.1) and restored P-deficient trout (K = 1.5 ± 0.1) were longer. Moreover, P-sufficient fish showed a lower K than P-deficient fish in the stable scenario (K = 1.4 ± 0.1 vs. 1.6 ± 0.1 in fish showing biconcave phenotype at week 27).

Vertebral morphometerics

Vertebrae from P-sufficient fish showed a significantly increased length ($L\text{-}W_9$ = 3.0 ± 0.4 mm, $L\text{-}W_{18}$ = 4.0 ± 0.3 mm and $L\text{-}W_{27}$ = 4.7 ± 0.4 mm) when compared to non-deformed P-deficient vertebrae at week 9 ($L\text{-}W_9$ = 2.5 ± 0.2 mm), to P-deficient biconcave vertebrae at week 18 ($L\text{-}W_{18}$ = 3.5 ± 0.3 mm) and to P-deficient compressed vertebrae at week 27 ($L\text{-}W_{27}$ = 4.3 ± 0.3mm). None of the compressed vertebrae (type 2) showed inward bending of the vertebral body endplates (Fig. 1) as observed in Atlantic salmon. Their length and height were not significantly different for other P-deficient vertebrae at week 27. At week 9, non-deformed P-sufficient vertebrae had a greater height ($H\text{-}W_9$ = 3.2 ± 0.4 mm) compared to non-deformed P-deficient vertebrae ($H\text{-}W_9$ = 2.9 ± 0.2 mm). At week 27, compressed vertebrae tended to have a greater height than normal P-sufficient vertebrae (H-

W_{27} = 5.1 ± 0.3 mm vs. 4.7 ± 0.4 mm, $P < 0.1$). At week 18 and 27, abnormal P-deficient vertebrae (either biconcave or compressed) had significantly greater H/L ratio than normal vertebrae (either P-sufficient or P-deficient). Likewise, stable P-deficient vertebrae had a superior H/L ratio than normal P-sufficient vertebrae. Among all vertebrae from week 27, compressed vertebrae had the greatest H/L ratio. No differences were observed between stable and restored P-deficient vertebrae and normal P-deficient vertebrae at week 27. Similarly, non-deformed vertebrae from P-deficient fish at week 9 tended to have a higher H/L ratio than those from normal P-sufficient fish ($P < 0.1$).

The median and intervertebral spaces were significantly affected in P-deficient trout. At week 18 and 27, larger peripheral (i.e. dorsal/ventral) intervertebral space (DVSp) was observed in P-deficient fish having biconcave (DVSp-W_{18} = 0.4 ± 0.1 mm, DVSp-W_{27} = 0.5 ± 0.1 mm) or compressed (DVSp-W_{27} = 0.4 ± 0.1 mm) vertebrae when compared to normal P-sufficient fish (DVSp-W_{18} = 0.2 ± 0.1 mm, DVSp-W_{27} = 0.2 ± 0.1 mm). At week 27, a wider DVSp was also observed in restored P-deficient vertebrae compared to non-deformed P-sufficient vertebrae (DVSp-W_{27} = 0.4 ± 0.1 mm vs. 0.2 ± 0.1 mm). An increase in intervertebral median distance (DVSp) was only observed in fish displaying biconcave vertebrae at week 18 (DVSp-W_{18} = 0.9 ± 0.2 mm) when compared to non-deformed P-sufficient fish (DVSp-W_{18} = 0.6 ± 0.3 mm) and at week 27 (DVSp-W_{27} = 1.2 ± 0.3 mm for fish within the stable scenario) when compared to non-deformed P-deficient fish (DVSp-W_{27} = 0.5 ± 0.1 mm). Non-deformed P-deficient vertebrae at week 9 tended to have wider DVSp than non-deformed P-sufficient vertebrae (DVSp-W_9 = 0.2 ± 0.1 mm vs. 0.1 ± 0.1 mm, $P < 0.1$).

The typical X-shaped structure of the vertebral body was also significantly affected in P-deficient trout demonstrating abnormal development at week 18 and 27. Indeed, abnormal vertebrae demonstrated more acute angles (< 83°) versus normal counterparts (88° < DAngle < 92°). At week 27, restored vertebrae demonstrated intermediate values between normal and abnormal phenotypes. No differences between non-deformed P-deficient or P-sufficient DVAngles were detected although at week 9, non-deformed P-deficient vertebrae tended to have more acute angles than normal P-sufficient vertebrae with an angle between 83° and 88°.

Discussion

As demonstrated previously in our laboratory (Koko et al., 2010; Deschamps et al., in press), we employed a low P-diet (total P = 0.5%) according to juvenile requirements of 0.8% for this species (Ketola and Richmond, 1994; NRC 2011) to induce a severe deficiency and to trigger the appearance of abnormal vertebrae in rainbow trout. Although the calcium content of the diets also varied, it can be actively absorbed and/or excreted in surrounding water, and phosphorus is thus the only limiting element (Lall, 1991; NRC, 2011). As shown in previous studies, fish fed a P-deficient diet can maintain growth performance (length and weight) similar to P-sufficient fish even over a long-term experiment (Baeverfjord et al., 1998; Fontagné et al., 2009; Gil Martens et al., 2012) and when fish present different numbers of deformed vertebrae (Hansen et al., 2010). Condition factor is known to increase in spinal-deformed fish (Hansen et al., 2010). Our findings are thus consistent with previous work as we did not observe any difference in fish length or weight between P-sufficient of P-deficient groups, but the condition factor was found to be highest in the most deformed P-deficient fish (i.e. aggravation scenario at week 27).

The apparition of vertebral abnormalities in fish associated with P-deficient diets has been repeatedly reported in the literature (see review in Sugiura et al., 2004), as well as in fish deficient in other microminerals involved in bone metabolism such as Mg and Zc (Suigura et al., 2000; Lall, 2002; Lim and Klesius, 2003; Helland et al., 2006; Nguyen et al., 2008). Certain types of abnormalities such as pronounced biconcave (type 11) and undersized and widely-spaced vertebral bodies (type 10) seem specific to impede bone matrix mineralisation (Witten et al. 2009). During our study, these two types of deformities were observed in P-deficient groups. However, as the occurrence of type 10 abnormalities was low, it was not further investigated and we focused our attention on type 11 abnormalities. Moreover, we described morphologically different scenarios of vertebral development that are reported and discussed in the literature (Ytteborg et al., 2010a,b, 2012). As the aim of the current study was to develop and evaluate an empirical method to confirm particular types of deformities, the histological and molecular processes behind

these different scenarios of development are beyond the scope of our study and will thus not be discussed.

The proposed method defines, based on measurements, two distinctive types of abnormalities that have been described morphologically by Witten et al. (2009). The suggested method has permitted to demonstrate that visually-assessed phenotypes can differ from those assessed with measurements. Every abnormal vertebra observed in this experiment showed signs of compression. Even though, it was expected for compressed vertebrae, that biconcave vertebrae also showed a higher height: length ratio and severe acute angle below 83º. Considering that compressed vertebrae did not show inward bending of the edges of the vertebral body endplates and could, therefore, be considered as an intermediate stage between Type 1 and Type 2, (respectively, decreased intervertebral space and homogenous compression; Witten et al., 2009), one can argue that signs of compression appears before a vertebrae can be assigned the Type 2 deformity.

Furthermore, the measurements obtained in this study also allowed to confirm and to compare the non-deformed phenotype of P-sufficient and P-deficient vertebrae. Although none of the measured parameters permitted a clear distinction between the two normal groups, we were able to observe three tendencies ($P < 0.1$) in week 9 (Ratio H/L, DVSpace and DVAngles) which suggests that even vertebrae that were categorised as a normal phenotype via X-ray observation, could present signs of under-mineralization or compression when measured. Thus, it seems important to rely on a standardized method of measurements to be able to detect slight changes in vertebral body shape.

As mentioned in various studies, the compression of vertebrae (higher L/H ratio) is generally accompanied by a reduction of the intervertebral distance (lower DVSp; Witten et al., 2005, 2006, 2009; Witten and Huysseune, 2009; Fjelldal et al., 2007a, 2012a,b). Fontagné et al., (2009) found that a severe calcium-deficient diet (available Ca = 0.0 g/100 g dry weight) lead to modifications in the shape and size of vertebrae in rainbow trout fry by reducing vertebral height, length and intervertebral space. Witten et al., (2005) found that compressed vertebrae in “short tailed” Atlantic salmon showed reduced height and length when compared to normally-shaped vertebrae. Ytteborg et al., (2010b) demonstrated that vertebrae of Atlantic salmon reared in a high temperature regime

showed signs of compression in the cranio-caudal axis. However, to our knowledge, none has used vertebral endplate angles to distinguish different types of abnormalities. Fjelldal et al. (2007a) observed the reduction of the angle between the wall of the cone forming the X-structure and the anterior-posterior axis from around 45º to 90º in compressed vertebrae, but no further data from measurements have been published. Interestingly, during the present study, angles seem to be the most effective parameter to detect early signs of deformities related to P deficiency. Even if at first glance, the measure of length: height ratio tends to give the same information, the current study demonstrates that angles are easier to measure with the approach to radiological processing described herein given that the X-shape structure inside the vertebral body appears less blurred than all the other parts (Fig. 2) and thus, allows a more precise distinction between different types of deformities. Different technical reasons can explain why some parts of radiographs appear blurred such as penumbra, edge effect or anode material (Ishisaka et al., 2000) and the density of the bone can also affect the quality and the crispness of the radiography (Oeser et al., 1963). The X-shaped structure is one of the features that is easily identified and slightly affected by blurring problems, which make it a solid starting point for reliable measurements such as angles.

The combination of median intervertebral distance and dorsal/ventral vertebral endplate angles enables to distinguish between normal and abnormal vertebrae as well as between different types of deformities. A right angle (88º < DAngle < 92º) characterises normal vertebrae, acute angles (<83º) are specific to those considered abnormal. As biconcave and compressed abnormal vertebrae considered in this study show comparable DVAngles, the discrimination between them can be made by measuring the median intervertebral distance between two adjacent vertebral bodies. The measure of a wider or a narrower median intervertebral distance will therefore distinguish between biconcave and compressed vertebrae respectively. Considering that fixation can affect the intervertebral region, measurement in this area on fixed material should be used for treatment comparison and combined with other measurements for diagnostic purposes. Surprisingly, angles seem to be sensitive enough to show transitional stage (~85º) meaning whether vertebrae are heading towards an abnormal state or when it is in recovering process. Another interesting point is that the peripheral intervertebral distances seems to react early (week 9) and

therefore may be a good indicator of early P-deficiency. However, this parameter does not allow a distinction between types of abnormalities and did demonstrate robustness, even for vertebrae in the restoration scenario.

Finally, the measurements of different parameters on X-rayed vertebrae allowed a more refined and precise diagnosis of abnormalities. Since similar anomalies have been identified in other studies with different protocols (Witten et al., 2005; Fjelldal et al., 2007; Ytteborg et al., 2010b; Grini et al., 2011; *Fraser* et al., 2013), it is risky to speculate that these measurements detect P-deficiency only. Nevertheless, acute dorsal and ventral vertebral endplate angles seem to be a good indicator of an abnormal vertebral development and could be a useful monitoring tool. The radiological imaging procedure can affect the quality of the X-rays and thus influence the categorization of vertebral abnormalities (Witten et al., 2009). This suggests that the threshold between normal and abnormal vertebrae and among deformed vertebrae could change, even for the same parameter, when compared among different radiological procedures. The same measurements should thus be taken on X-rays coming from different radiological techniques to be able to compare results and find the most reliable parameter. Further efforts should also be conducted to simplify the method and decrease unwanted biases while fixing vertebrae and/or taking radiographs. Indeed, if we want to apply this method routinely, measurements should be made directly on the radiographs taken *in toto*. Moreover, an increase in the distance between the fish and the x-ray source would reduce the edge effect and improve the quality of the radiographs. The confirmation of the observed and measured phenotypes should also be assessed via histological evaluation. This would allow a precise examination of growth zone and other changes in the vertebral body and/or in the intervertebral space due to compression and, thus, a more precise description of the deformities considered.

Conclusions

This study was an attempt to establish a standardised method based on empirical measurements enabling a more refined analysis of vertebral abnormalities related to P-deficiency observed in triploid all-female rainbow trout. We determined that the combination of measured median intervertebral distance and vertebral endplate angles is a procedure that allows a sensitive diagnosis of abnormalities and could lead to an improved

detection and classification of abnormalities when using X-rays. Moreover, this method enabled the detection of subtle changes in vertebrae that would be categorized as normal under visual examination. This method could be a useful tool for researchers and fish farmers to prevent deformities to worsen. However, the limited number of samples employed in this study does not allow the generalisation of our results. Further studies, including histological observations, should be undertaken before one can conclude that this method detects phenotype associated with P-deficiency only. Thus, measuring of the angles seems appears to be a promising approach for detecting aberrant development at earlier stages compared to traditional X-ray analyses.

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Table A.2.1 Ingredients and proximate composition of the P-deficient and P-sufficient diets.

	Diets	
Ingredients (g·kg^{-1})*	**P- deficient**	**P-sufficient**
Herring meal[a]	75	75
Blood meal[a]	100	100
Feather meal[b]	100	100
Wheat meal[c]	205	180
Soybean meal[c]	80	80
Wheat gluten[d]	70	70
Corn gluten meal[c]	170	170
Fish oil[a]	180	180
$CaHPO_4$[e]	0	25
Lysine (Biolys)[d]	0.012	0.012
DL-methionine[f]	0.002	0.002
**Vitamin-mineral premix[f]	0.006	0.006
Ethoxiquin (antioxidant)[f]	150 ppm	150 ppm
Proximate composition (%, dry basis)		
Dry matter	90.45	90.18
Crude protein	49.71	49.93
Crude lipid	20.79	21.39
Gross energy (E.MJ·kg^{-1})	24.93	24.43
Ash	3.77	5.41
Total phosphorus (P)	0.50	0.92
Total calcium (Ca)	0.75	1.78
Total magnesium (Mg)	0.22	0.26
Total zinc (Zc, mg kg^{-1})	184	180
Total copper (Cu, mg kg^{-})	16	16
Digestible dry matter***	62.31 ± 1.74	60.03 ± 0.74
Digestible protein***	42.52 ± 0.96	42.51 ± 0.43
Digestible lipids***	17.54 ± 0.67	16.71 ± 0.90
Digestible energy***	18.94 ± 0.48	17.95 ± 0.31
Digestible P (Dg. P)***	0.29 ± 0.03	0.45 ± 0.03
Dg. P/Dg. E (g MJ^{-1})***	0.15 ± 0.02	0.25 ± 0.02

***Canadian supplier: [a] SANIMAX Marketing Limited. Guelph. ON ;[b]Floradale feed mill limited: Floradale. ON; [c]Meunerie Gérard Soucy Inc.: Ste-Croix. QC; [d]ADM Alliance Nutrition Canada. St-Hyacinthe. QC; [e]Laboratoire Mat Inc., Québec.QC; [f] Corey Feed Mills Ltd., Fredericton. NB.**

**** Supplied the following: (to provide mg kg-1 except as noted): retinyl acetate 2500 IU. cholecalciferol 2400 IU. tocopheryl acetate. 50; menadione.10; thiamin.1;**

riboflavin.4; pyridoxine.3; Ca-pantothenate.20; vitamin B-12.0.01; niacin.10; biotin.0.15; folic acid.1; choline.1000; inositol.300; magnesium carbonate.1.24 g; calcium carbonate.2.15 g; potassium chloride.0.90 g; sodium chloride.0.40 g; potassium iodide.0.4; copper sulfate.30; cobalt sulfate.0.2; ferric sulfate.0.20 g; manganese sulfate.30; zinc sulfate.40; dibasic calcium phosphate.5 g; sodium fluoride. 10.

***** The digestibility study was conducted the week following the experiments in triplicate tanks (N = 3).**

Table A.2.2: Fish parameters and morphometrics taken on X-rays for all phenotypes and sampling points.

Week (W)	**Diet**	**Phenotypes**	**Scenarios**	**n**	**Fork Length** (FL, cm)	**Body Mass** (BM, g)	**Condition factor** (K)	**Vt. Length** (mm)	**Vt. Height** (mm)	**Ratio H/L**	**DVSp** (mm)	**MSp** (mm)	**DVAngle** (°)
9	Sufficient	Non-deformed	Normal	15	23.0	160.0	1.3	3.0 ^{a}	3.2 ^{a}	1.1	0.1	0.7	88.6
	Deficient	Non-deformed	Normal	7	22.2	146.9	1.3	2.5 ^{b}	2.9 ^{b}	1.1	0.2	0.6	85.1
SEMp					*1.4*	*29.2*	*0.1*	*0.4*	*0.3*	*0.1*	*0.1*	*0.3*	*4.1*
18	Sufficient	Non-deformed	Normal	15	29.2	352.3	1.4	4.0 ^{a}	4.03	1.0 ^{a}	0.2 ^{a}	0.6 ^{a}	92.0 ^{a}
	Deficient	Non-deformed	Normal	3	28.9	334.7	1.4	3.7 ^{ab}	3.82	1.0 ^{a}	0.3 ^{ab}	0.9 ^{ab}	88.7 ^{a}
	Deficient	Biconcave	Biconcave	17	28.2	337.3	1.5	3.5 ^{b}	3.93	1.1 ^{b}	0.4 ^{b}	0.9 ^{b}	81.5 ^{b}
SEMp					*1.8*	*65.0*	*0.1*	*0.3*	*0.4*	*0.1*	*0.1*	*0.3*	*3.7*
27	Sufficient	Non-deformed	Normal	18	35.5	652.5	1.4 ^{a}	4.7 ^{a}	4.7	1.0 ^{a}	0.2 ^{a}	1.0 ^{ab}	91.8 ^{a}
	Deficient	Non-deformed	Normal	3	35.1	657.3	1.5 ^{ac}	4.7 ^{ab}	4.8	1.0 ^{ab}	0.3 ^{ab}	0.5 ^{a}	90.7 ^{ab}
	Deficient	Non-deformed	Restoration	6	36.3	705.8	1.5 ^{ab}	4.7 ^{ab}	5.0	1.1 ^{ab}	0.4 ^{b}	0.9 ^{ab}	85.7 ^{bc}
	Deficient	Biconcave	Stable	7	33.8	603.6	1.6 ^{bc}	4.3 ^{ab}	4.8	1.1 ^{b}	0.5 ^{b}	1.2 ^{b}	82.6 ^{c}
	Deficient	Compression	Aggravation	8	34.6	674.0	1.6 ^{c}	4.3 ^{b}	5.1	1.2 ^{c}	0.4 ^{b}	0.8 ^{ab}	80.6 ^{c}
SEMp					*2.2*	*133.2*	*0.1*	*0.4*	*0.4*	*0.1*	*0.1*	*0.3*	*4.1*

For each parameter, superscript letters within a given week indicate significant differences (i.e., one-way ANOVA followed by Tukey's multiple comparison tests, P < 0.05; SEMp = pooled standard error).

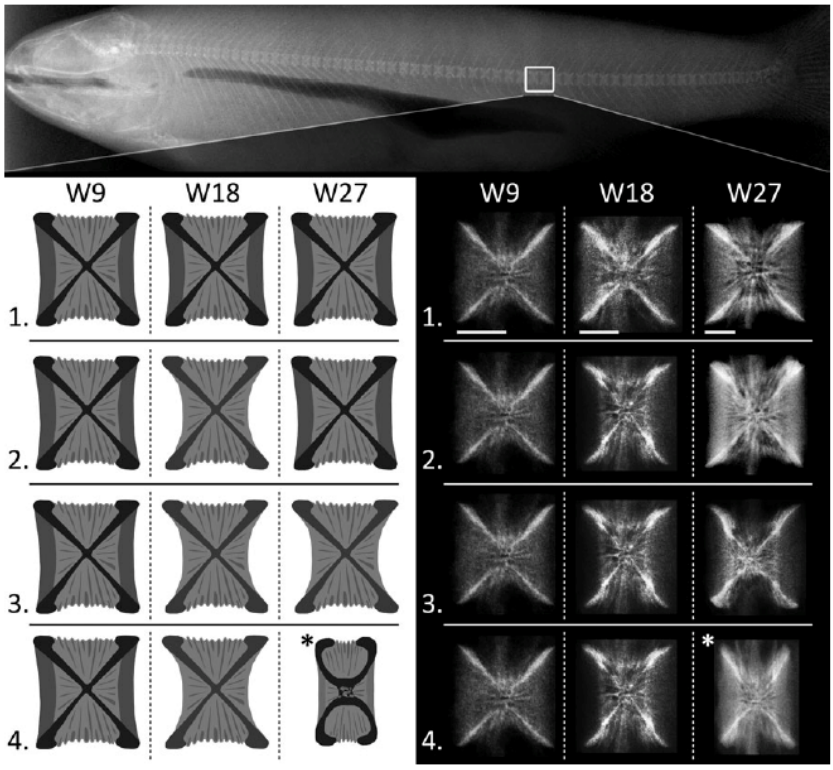

Figure A.2.1. Illustrations modified after Witten et al. 2009 (left) and X-rays observed during this study (right) of four different scenarios of vertebral development between week 9 and week 27: 1. Normal, 2. Restoration, 3. Stable, 4. Aggravation. Black asterisk (*) shows inward bending of the vertebral body endplates that was not observed during this study (white asterisk).

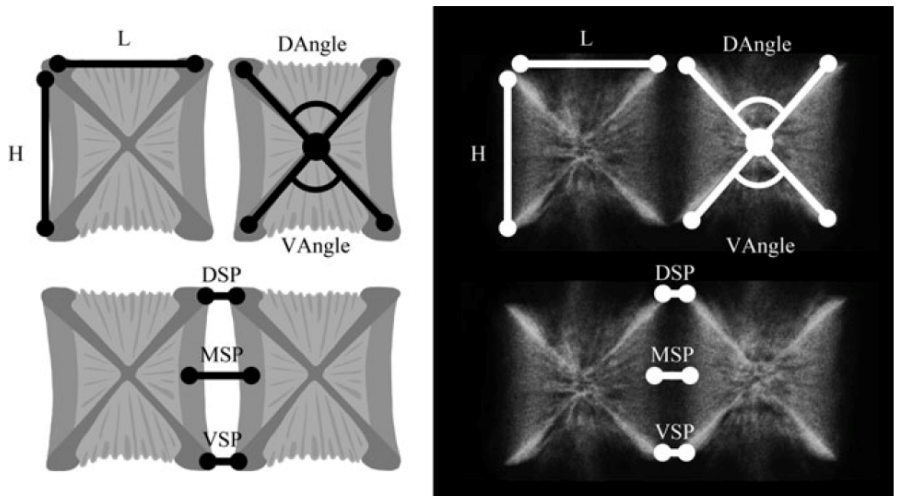

Figure A2.2. Measurements on radiographs using ImageJ software. Illustration modified after Witten et al. 2009 (left) and X-rays observed during this study (right) with normal (i.e. non-deformed) phenotype. L= vertebra length, H= vertebra height, VAngle= ventral vertebral endplate angle, DAngle= dorsal vertebral endplate angle, VSp= ventral peripheral intervertebral distance, DSp= dorsal peripheral intervertebral distance and MSp= median intervertebral distance.